Universidade de Aveiro Departamento de Biologia
2011

Cátia Sofia Oliveira Pereira

Lipid antigen presentation in Fabry disease patients

Apresentação de antigénios lipídicos em doentes de Fabry

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Maria de Fátima Matos Almeida Henriques Macedo, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro e da Doutora Maria Clara Sá Miranda, investigadora principal e directora da UniLiPe.

o júri

presidente	Professor Doutor António José Arsénia Nogueira Professor Associado com Agregação da Universidade de Aveiro
arguente	Professor Doutor Pedro Nuno Simões Rodrigues Professor Associado do Instituto de Ciências Biomédicas Abel Salazar
orientador	Professora Doutora Maria de Fátima Matos Almeida Henriques de Macedo Professor Auxiliar Convidado da Universidade de Aveiro
co-orientador	Doutora Maria Clara Sá Miranda Investigadora do Instituto de Biologia Molecular e Celular

agradecimentos

À minha orientadora, Doutora Fátima Macedo, por estar sempre disponível, por apoiar todas as fases deste trabalho, por tudo aquilo que me ensinou e por ter criado um bom ambiente para a minha aprendizagem.

À Doutora Clara Sá Miranda, pela co-orientação da tese e pela oportunidade de desenvolver este trabalho na Unilipe.

Aos membros da Unilipe e OBF, que se mostraram sempre prontos para esclarecer qualquer dúvida que surgisse.

Aos meus colegas biólogos e biomédicos, que me foram acompanhando neste percurso académico, por todos os momentos em que me proporcionaram na sua companhia.

À Sofia, que passou comigo os melhores e os piores momentos dos últimos cinco anos e me apoiou sempre.

À Carina, pelos agradáveis momentos de conversa.

Aos meus pais, que se esforçaram para que eu pudesse seguir o meu sonho e sempre acreditaram que eu ia atingir os meus objectivos.

Ao meu irmão, por tornar cada dia meu um dia melhor.

À Fundação para a Ciência e a Tecnologia pelo apoio financeiro essencial à realização deste trabalho.

palavras-chave

antigénios lípidicos; CD1; células dendriticas; células T restrictas a CD1; doença de Fabry; doenças de sobrecarga lisossomal;

resumo

O lisossoma é um organelo celular que degrada macromoléculas. As doenças de sobrecarga lisossomal (DSL) surgem quando há acumulação destas no lisossoma devido principalmente a defeitos genéticos em hidrolases lisossomais. A doença de Fabry é uma DSL causada por mutações no gene *GLA* que codifica a hidrolase α-Galactosidase A. O lisossoma é importante no processo de apresentação de antigénios lipídicos às células T. A apresentação de antigénios lipídicos é mediada pelas moléculas CD1 existentes nas células apresentadoras de antigénios, que se associam aos antigénios lipídicos formando complexos que são capazes de activar células T que respondem às moléculas de CD1 (células T restritas a CD1). Existem cinco isoformas de CD1 (a,b,c,d,e), mas somente quatro delas (a,b,c,d) apresentam antigénios. O tráfego intracelular destas quatro isoformas é diferente. Um dos locais em que há associação das moléculas de CD1 aos antigénios lipídicos é o lisossoma, sendo esperado que o processo de apresentação de antigénios lipídicos e consequente activação das células T restritas a CD1 estejam afectados nas DSL. Em vários modelos animais de DSL, incluindo a doença de Fabry, foi observada uma redução na percentagem de um tipo de células restritas a CD1d (células iNKT) e também na capacidade de apresentação de antigénios lipídicos. Em humanos, estudos anteriores não encontraram alterações na percentagem das células iNKT em doentes de Gaucher e de Fabry. No entanto, a capacidade de apresentação de antigénios lipídicos em doentes com DSL não foi ainda analisada. Neste estudo, analisámos as células iNKT e suas subpopulações e a capacidade de apresentação de antigénios lipídicos de doentes de Fabry. Não foram encontradas alterações na percentagem de células iNKT entre doentes e controlos. Em humanos, as células iNKT podem ser divididas em três subpopulações que produzem diferentes tipos de citocinas de acordo com a expressão de CD4 e CD8: as células iNKT que só expressam CD4 ($CD4^+$) produzem citocinas Th1 e Th2, as células iNKT que não expressam nenhuma das moléculas (DN) produzem essencialmente Th1 e as células iNKT que só expressam CD8 produzem principalmente IFN-γ. Neste estudo, detectou-se uma redução na percentagem das células iNKT $CD4^+$ em doentes de Fabry, quando comparados com controlos. Cinco doentes foram testados relativamente à capacidade de apresentação de antigénios lipídicos através de duas moléculas de CD1 (b,d). Não foram encontradas alterações na capacidade de apresentar o antigénio sulfatídio através da molécula CD1b. Curiosamente, a apresentação do antigénio GM1 por CD1b estava reduzida em dois doentes. Dois doentes tinham reduzida capacidade de apresentar o antigénio sulfatídio através de CD1d. Concluindo, há uma alteração nas subpopulações das células iNKT em doentes de Fabry, que pode estar associada a modificações no perfil de citocinas produzido, contribuindo para a patologia da doença. Pela primeira vez foram realizados ensaios de apresentação de antigénios lipídicos em doentes com DSL, mas são necessários mais estudos para tirar mais conclusões.

keywords

CD1; CD1-restricted T cells; dendritic cells; Fabry disease; lipid antigens; lysosomal storage diseases

abstract

The lysosome is a cellular organelle responsible for the degradation of macromolecules. Lysosomal storage diseases (LSDs) arise from the accumulation of undegraded macromolecules in the lysosome, mainly due to genetic defects in the lysosomal hydrolases. Fabry disease is a LSD caused by mutations in the *GLA* gene which codifies the enzyme α-Galactosidase A, a lysosomal hydrolase responsible for globotriaosilceramide degradation. The lysosome is also a key player in the process of lipid antigen presentation to T cells. Lipid antigen presentation is mediated by CD1 molecules existent in antigen presenting cells (APCs), which associate with lipid antigens, forming complexes that are able to activate T cells responsive to CD1 molecules (CD1-restricted T cells). There are five isoforms of CD1 molecules (a,b,c,d,e), but only four of them (a,b,c,d) present lipid antigens to T cells. The four isoforms have different intracellular trafficking patterns. One of the places where association of lipid antigens with CD1 molecules occurs is the lysosome. Therefore, it was hypothesized that the process of lipid antigen presentation and consequent activation of CD1-restricted T cells is impaired in LSDs. Several mouse models of LSDs, including Fabry disease, were already shown to have reduced frequencies of a specific type of CD1d-restricted T cells (iNKT cells) and decreased capacity of lipid antigen presentation. In humans, previous studies have found no alterations in iNKT cell percentage in patients of two different LSDs: Gaucher and Fabry diseases. Studies assessing the efficiency of lipid antigen presentation in LSD patients are missing. In this study, we analysed the iNKT cells and iNKT cell subsets and the capacity of lipid antigen presentation of Fabry disease patients. We found no differences in percentage of iNKT cells between Fabry disease and control subjects. In humans, iNKT cells can be divided in three subsets that produce different types of cytokines according to CD4 and CD8 expression: iNKT cells expressing only CD4 ($CD4^+$) produce Th1 and Th2 cytokines, iNKT cells that do not express both molecules (DN) produce mainly Th1 and iNKT cells that only express CD8 ($CD8^+$) produce mostly IFN-γ. In this study, we found a reduction in iNKT $CD4^+$ subset in Fabry disease patients when compared to control subjects. In lipid antigen presentation assays, five patients were tested for the capacity of their dendritic cells to present lipid antigens by two CD1 molecules: CD1b and d. We found no alterations in patients' capacity to present the lipid antigen sulfatide by CD1b. Interestingly, presentation of the lipid antigen GM1 by CD1b was reduced in two patients. Regarding CD1d molecules, two patients had reduced capacity of presenting sulfatide when compared to control subjects. Concluding, we found an imbalance in the iNKT cell subsets in Fabry disease patients that can be associated with an alteration in cytokine profile which might contribute to disease pathology. Lipid antigen presentation assays were for the first time performed in LSDs patients, but more studies are needed to take further conclusions.

Table of contents

List of abbreviations

α-GalCer	α-Galactosylceramide
α-Gal A	α-Galactosidase A
APC	Antigen presenting cell
DBS	Dried blood spot
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
FBS	Fetal Bovine Serum
Gb3	Globotriaosylceramide
GM-CSF	Granulocyte macrophage colony stimulating factor
iFBS	Inactivated fetal bovine serum
iGb3	Isoglobotriaosylceramide
IPS	*Instituto Português do Sangue*
iNKT	Invariant natural killer T
li	Invariant chain
LSD	Lysosomal storage disease
LTP	Lipid transfer protein
MHC	Major histocompatibility complex
MPS	Mucopolysaccharidosis
MTP	Microsomal triglyceride transfer protein
NKT	Natural killer T
NPC1	Niemann-Pick type C1
NPC2	Niemann-Pick type C2
OPD	O-phenylenediamine dihydrochloride
PBMC	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
TCR	T cell receptor
SD	Standard deviation

1. Introduction

1.1 Lysosomal storage diseases

The lysosome is an acidic organelle that contains several hydrolases involved in the degradation of macromolecules from either intracellular or extracellular sources [1]. Lysosomal storage diseases (LSDs) are a group of inherited metabolic diseases characterized by the accumulation in the lysosome of non-metabolized macromolecules. These substances accumulate mostly due to mutations in the lysosomal hydrolases [1]. However, mutations in proteins involved in lipid transport, vesicular trafficking and biogenesis of lysosomes were also associated with LSDs [1]. The substrate accumulation in the lysosome is progressive and eventually leads to impaired cell and organ function. Consequently, LSDs tend to worse with age, leading often do early death. LSDs individually are considered rare, but there are few studies reporting their prevalence. A study in Australia has found that LSDs frequency in this country was of 1 in 7700 live births [2]. In Netherlands, a frequency of LSDs of 1 in 7143 live births was reported [3]. A study performed in Portugal found higher frequency of LSDs than the other two, with an incidence of 1 in 4000 live births [4].

LSDs can be classified according to the biochemical nature of the stored material into lipidoses (mainly sphingolipidoses), mucopolysaccharidoses (MPSs), glycogenoses, glycoproteinoses, neuronal ceroid lipofuscinosis and mucolipidoses [5].

The first recognized LSD was Pompe disease, which is characterized by the accumulation of glycogen in the lysosome. It was initially described in 1932, prior to the discovery of the lysosome. In 1963, this glycogen storage disease was associated with a defect in the lysosomal enzyme α-glucosidase [6].

Most of the first described LSDs were sphingolipidosis, which include Gaucher, Niemann-Pick, Tay-Sachs, Sandhoff and Fabry diseases [5]. These diseases were initially called lipidosis, due to the high amount of lipids present in the patients' serum. The enzymatic defects that lead to the diverse sphingolipidoses and the main substrates that are accumulated are described in Table 1.

Table 1. Sphingolipidoses: defective enzyme and accumulated substrates.

Disease	Defective enzyme	Accumulated substrates
GM1 gangliosidosis	β-galactosidase	GM1 ganglioside, oligosaccharides and glycoproteins with a terminal β-galactosidic linkage
GM2 gangliosidosis		
• Tay-Sachs disease	β-hexosaminidase A	GM2 ganglioside
• Sandhoff disease	β-hexosaminidase A and B	GM2 ganglioside, GA2 ganglioside, oligosaccharides and glycoproteins with a terminal β-*N*-acetylglucosamine linkage
Fabry disease	α-galactosidase A	Globotriaosylceramide
Metachromatic leukodystrophy	Arylsulfatase A	Sulfatide
Krabbe disease	Galactosylceramidase	Galactocerebroside, psychosine
Gaucher disease	Glucosylceramidase	Glucocerebroside
Niemann-Pick disease types A and B	Sphingomyelinase	Shingomyelin
Farber disease	Ceramidase	Ceramide

The focus of this study is Fabry disease, which is described in the following section.

1.1.1 Fabry disease

Fabry disease is an X-linked recessive LSD which arises due to a mutation in the *GLA* gene. The *GLA* gene encodes the lysosomal enzyme α-galactosidase A (α-Gal A), responsible for the hydrolysis of the terminal galactose of the glycosphingolipid globotriaosylceramide (Gb3). As degradation of this lipid is compromised, it accumulates in the lysosomes of deficient cells. The deposits occur in several cells, but more

prominently in endothelial and perithelial cells, cells from the smooth muscle of the blood vessels, cornea epithelial cells, glomeruli and renal tubes, muscle fibres of the heart and ganglionic cells of the autonomous nervous system [7].

Molecular basis

The gene that codifies for α-Gal A (*GLA* gene) is localized in a region of the long arm of the X chromosome, Xq22. It has a length of 12kb and comprises seven exons [7]. To date, there are more than five hundred described mutations in the *Human Gene Mutation Database*, including missense and nonsense mutations, deletions, insertions, indels, rearrangements and splicing mutations, with the first two alterations being the most common. These mutations are scattered by all seven exons [8].

The mature α-Gal A is a homodimer composed by two monomers with 398 residues each. Its initial size is 429 aminoacids, but this includes a signal sequence that directs the protein to the lysosome and is then removed. Mutations have been described for nearly half of the residues of this enzyme. Interestingly, structural studies have shown that only 10% of Fabry disease causing mutations are located near the active site of the enzyme, while 65% of them occur in buried aminoacid residues [8]. This indicates that in most of the cases, the reduced activity is related to misfolding of the enzyme.

α-Gal A is glycosylated in the Golgi apparatus, a central process for the enzyme function, but also for its stability. There are three residues that can be glycosylated: N139, N192 and N215. Mutational studies in these residues showed that lack of glycosylation in the position 215 led to a reduction of approximately 50% of enzymatic activity [7]. The glycosylation at this site is essential for correct protein folding but also for traffic to the lysosome. Consistent with these findings, hemyzigotes who have the missense mutation N215S present residual α-Gal A activity, mainly associated with cardiac manifestations [7]. Other mutations were found to be related to specific clinical phenotypes. For example, the mutation M42L was reported in a patient that presented only renal manifestations of Fabry disease [9]. The efforts to establish a genotype/phenotype correlation in Fabry disease proceed with studies of sequence conservation, to identify the key residues for protein function [8].

Clinical Manifestations

Clinical onset typically occurs during childhood or adolescence, but in certain disease variations it can be delayed to adulthood. The first symptoms to arise during childhood are usually pain in the extremities (acroparesthesias), fever of unknown origin, reduced sweating (hypohidrosis) and gastrointestinal manifestations [10]. Because these symptoms are not specific, the disease is hard to diagnose in this phase. In late adolescence more particular symptoms appear, namely skin lesions (angiokeratoma) and corneal opacity [10]. Between the third and fifth decade of life, renal and cardiac problems emerge, leading to decreased life expectancy. Cerebrovascular complications such as stroke are also frequent in Fabry disease [10]. Acroparesthesias are one of the most incapacitating symptoms of Fabry disease. This pain can be constant or appear in the form of crises with variable duration. At the cellular level, this symptom is believed to be caused by a small fibre neuropathy, which is also responsible by decreased sensitivity to cold and warm as well as heat pain [10]. Angiokeratomas are a characteristic of Fabry disease, but they can be absent in some disease variants. They arise due to vascular dysfunction and tend to increase in number and size with age [7]. The main gastrointestinal manifestations are diarrhea and abdominal pain. At the cardiac level, Fabry disease patients might present hypertrophic cardiomyopathy, valvular disease, conduction defects and arrhythmia [7, 10]. Other manifestations that have been reported include respiratory problems, some bone deformities, lymphadenopathy, anemia and delayed growth [10].

While some patients present a classical Fabry disease phenotype characterized by angiokeratoma, corneal opacities and acroparesthesias, there are other variants in which these symptoms are absent. In these variants there is usually a significant residual activity of α-Gal A and lower deposition of glycosphingolipids in the tissues [7]. One example is the cardiac variant, which appears later in life and with manifestations confined to cardiac function. Similarly, there are some cases in which only the kidney is affected [7]. The discovery of such variants highlighted the need for inclusion of Fabry disease in the differential diagnosis of patients with cardiac and renal disease of unknown origin.

Fabry disease can also be present in women with only one defective allele (heterozygous), but usually the symptoms appear later and with different degrees of severity. However, there were reported cases of women presenting a phenotype as severe

as that of classically affected males [7]. This variability occurs due to X-chromosome random inactivation [7].

Diagnosis and treatment

The clinical suspicion for Fabry disease usually occurs when angiokeratoma and corneal dystrophy are present [7]. After clinical evaluation, biochemical and genetic tests must be made to confirm the suspicious cases. In men, if the classical phenotype is present, α-Gal A activity is very low or absent, and usually the biochemical diagnosis is sufficient to identify Fabry disease patients. However, in atypical cases, enzymatic activity can reach 35% of the normal values [7]. When low activity of α-Gal A is detected, the molecular diagnosis is performed to identify the specific mutation responsible for the disease. Due to X-chromosome random inactivation, women can present normal values of α-Gal A activity. Therefore, the biochemical diagnosis is not sufficient to exclude the presence of Fabry disease in women. Thus, the molecular diagnosis is essential for the identification of female Fabry disease patients. The molecular diagnosis is done by denaturing high performance liquid chromatography, used as a screening method, followed by gene sequencing to identify the specific mutation [11, 12].

Treatment for Fabry disease is available since 2001, when enzyme replacement therapy (ERT) was introduced. There are two enzymes available: algasidase α (Replagal, Shire HGT) and algasidase β (Fabrazyme, Genzyme). Their primary structure is identical but there are variations in the glycosylation pattern [13]. However, pre-clinical and clinical trials have shown that both enzymes have similar efficiency. ERT was shown to decrease the plasma levels of Gb3 accumulation within three months of treatment [14]. There is an overall improvement in quality of life, with a reduction in pain and a delay in the decline of renal and cardiovascular functions [7].

1.2 Lipid antigen presentation

T cells are classically known by their ability to recognize protein antigens. However, T cells are also able to recognize lipids. As for proteins, lipid antigens cannot be directly recognized by T cells. Therefore, they need to be presented by antigen presenting cells (APCs) such as dendritic cells. At the APC surface, CD1 molecules loaded with lipid antigens interact with the T cell receptor (TCR) of specific T cells. This interaction causes T cell activation.

1.2.1. CD1 molecules

CD1 molecules are composed by a heavy chain, the α chain, and a light chain, β2 microglobulin. The heavy chain is a transmembranar chain with three extracellular domains: the membrane distal α1 and α2 and the membrane proximal α3. The α3 domain is responsible for the non-covalent binding to β2 microglobulin (Figure 1).

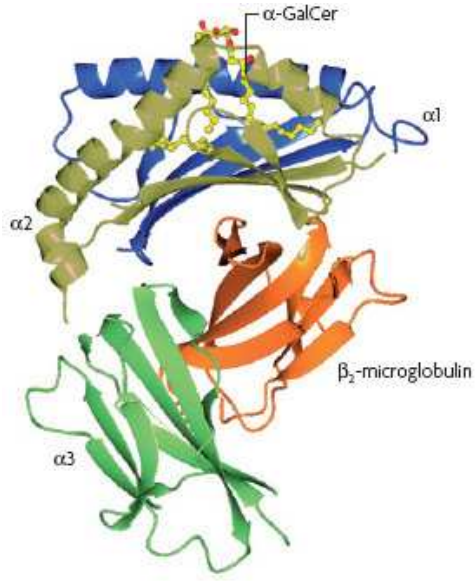

Figure 1. Crystal structure of CD1d associated with α-Galactosylceramide (α-GalCer). From [15].

CD1 expression

In humans there are five isoforms of CD1 α chain (a, b, c, d, e) codified by five different genes located in chromosome 1, while in mice there is only one isoform, CD1d, codified by two genes in chromosome 3 [15]. The β2 microglobulin is codified by a gene in the human chromosome 15 [16]. The CD1 isoforms can be divided in three groups

according to homology of the α1 and α2 domains: group I includes CD1a, b and c; group II is composed by CD1d and group III by CD1e [17]. Group I CD1 molecules are expressed in thymocytes, dendritic cells and Langerhans cells [18]. The expression of CD1c but not CD1a or CD1b molecules on marginal zone B cells and in 50% of peripheral blood B cells was also described [19]. Monocytes lack expression of all group I CD1 molecules. Conversely, CD1d has a broader expression pattern and it is present in most of the cells with hematopoietic origin, with higher levels in dendritic cells, B cells and monocytes. CD1d is also expressed in other cell types, like keratinocytes, hepatocytes, intestinal epithelial cells, hair follicles and Schwann cells [15]. Group I CD1 molecules expression is upregulated by GM-CSF (granulocyte macrophage colony stimulating factor) during *in vitro* dendritic cell differentiation from monocytes [18]. On the contrary, during *in vitro* differentiation there is a reduction in CD1d expression [20]. CD1e is mainly expressed in dendritic cells [21].

Synthesis and Trafficking

To understand lipid antigen presentation it is important to know how CD1 molecules bind to lipids and reach cell surface to interact with lipid specific CD1-restricted T cells. In the endoplasmic reticulum (ER), CD1 proteins are synthesized and its glycosylation is initiated. After this process, CD1 α chain binds in a non-covalent manner to β2 microglobulin [15]. Protein folding is facilitated by the chaperones calreticulin and calnexin and by the thiol oxidoreductase ERp57 [21]. Still in the ER, endogenous lipids bind CD1 molecules and probably prevent their collapse [21]. It is not fully understood how these lipids are loaded onto CD1 molecules, but some studies showed that microsomal triglyceride transfer protein (MTP) plays a key role in this process [22-25]. This protein was previously known for its role in the loading of triglycerides, phospholipids and cholesterol esters onto apolipoprotein B. Initially, MTP was identified as an important protein for activation of CD1d restricted T cells. The gene that codifies MTP was deleted in hepatocytes and silenced in intestinal epithelial cells. This resulted in impaired lipid antigen presentation to a CD1d restricted T cell clone [22]. Later, it was shown *in vitro* that MTP is expressed in APCs, such as mouse liver mononuclear cells and mouse and human B cell lines and is able to directly transfer phospholipids to recombinant CD1d [23]. It was also found that spleen and bone marrow derived dendritic cells in which MTP is absent had

reduced ability to present antigens by CD1d. In a posterior study, monocyte-derived dendritic cells were cultured with an inhibitor of MTP, which led to a substantial decrease in antigen presentation by CD1a, b and c [24], indicating that MTP is important for both group I and II CD1 molecules. More recently, patients with a genetic defect in MTP were studied for immunological alterations. It was found that these patients presented defects in lipid antigen presentation by both group I and group II CD1 molecules, reinforcing the importance of MTP in the process of CD1 molecules maintenance [25].

Properly assembled CD1 molecules move from the ER to the Golgi apparatus, where the glycosylation process is completed. Then, CD1 molecules follow the secretory pathway to the plasma membrane, with exception of CD1e. This isoform does not reach the plasma membrane surface and therefore is not capable of presenting antigens [17]. In immature dendritic cells, CD1e accumulates in the Golgi apparatus after synthesis [26]. After dendritic cell maturation, it migrates to the late endossome and then to the lysosome, where it is cleaved to a more stable soluble form [26]. In the lysosome, CD1e is involved in lipid loading on CD1b [27]. CD1d can be sent to the plasma membrane directly or bind the invariant chain (li) or MHC class II/li complexes and be directed to the lysosome after synthesis [15, 28, 29]. In humans, the presentation of non-self antigens (microbial or environmental) or self-antigens (produced by the organism) by the CD1d molecule occurs by different mechanisms. While the presentation of some non-self antigens seems to need CD1d internalization and lipid lysosomal processing, self antigens appear to be presented even in the absence of CD1d internalization [30]. Furthermore, MHCII/li complex association with CD1d facilitates the non-self antigen presentation, but appears to have no effect on self-reactivity.

In the plasma membrane, CD1 isoforms present some potent antigens (ex.: α-galactosylceramide) that bind directly to CD1 at the cell surface, or are internalized and follow the endocytic recycling pathway. Each CD1 isoform presents different trafficking pathways and distribution patterns (Figure 2). CD1b, CD1c and CD1d internalization occurs by the interaction of tyrosine motifs in their cytoplasmatic tails with the adaptor protein complex AP-2 [15, 31]. This complex allows the internalization of the CD1 molecules by clathrin vesicles. Conversely, CD1a has a short cytoplasmatic tail, without tyrosine motif, so it does not interact with AP-2, being internalized in a clathrin-independent manner [31]. After internalization, CD1b and murine CD1d interact

with another protein complex, AP-3 [15, 18, 31]. This one directs CD1 molecules to the late endossome or lysosome. CD1c and human CD1d do not interact with AP-3, being predominantly localized in the early and late endossomes [15, 18]. CD1a has a distinct distribution pattern, being localized solely in the early endossome [15, 18]. In the endocytic compartments, CD1 molecules are loaded with lipid antigens and then sent again to the plasma membrane, where they are able to stimulate T cells.

Lipid antigens localize in certain organelles according to their biochemical characteristics, as lipid length, saturation and headgroups [32]. Therefore, it was speculated that the variability existent between the pathways followed by each CD1 isoform is essential to allow the encounter with the various types of lipid antigens [33].

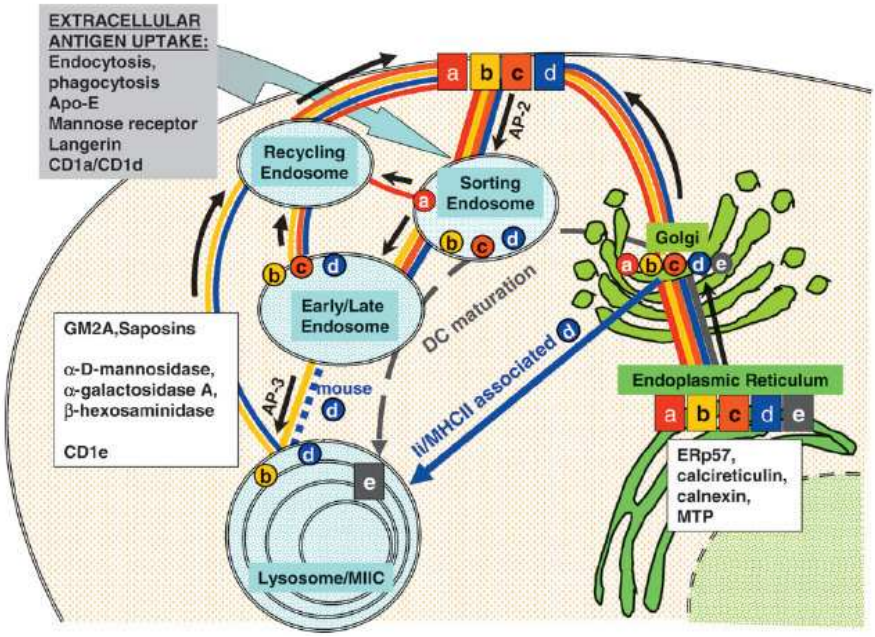

Figure 2. CD1 isoforms present different trafficking routes. Image from [15].

1.2.2. Lipid antigens

Lipid antigens are mainly recognized by T cells [34]. They can be classified according to their origin. If the antigen is produced in the body it is called a self antigen and if it derives from external microbial or environmental sources (i.e. pollens), a non-self antigen. Surprisingly, in contrast to peptide antigens, which are mainly non-self due to negative selection that occurs during development of T cells, lipid specific T cells present a high degree of self reactivity [35, 36]. Lipid antigens can also be divided according to the synthesis place. A lipid antigen that is produced in the same APC that will present it is an

endogenous antigen, whether a lipid synthesised in other cells that is then transferred to an APC is called an exogenous antigen [17]. The first identified and most potent lipid antigen is α-galactosylceramide (α-GalCer), a synthetic glycosphingolipid that has some minor structural modifications from a compound originally isolated from a marine sponge [37]. Several glycosphingolipids of self origin were later identified as lipid antigens, such as GM1, sulfatide and GD3 [17]. Phospholipids can also be antigenic to CD1-restricted T cells. These include phosphatidylinositol, lyso-phosphatidylcholine, phosphatidylcholine and phosphatidylethanolamine. The last two compounds were also found in pollen and were capable of stimulating the proliferation of T cells from allergic patients [38]. Most of the non-self lipid antigens were isolated from mycobacteria and are glycosphingolipids. However, a lipopeptide present in mycobacteria was isolated in complex with CD1a [21].

A lipid antigen can be presented by only one or by several isoforms of CD1 (Table 2). Of all the self lipid antigens, sulfatide is the one with a broader CD1 binding capacity, since it has been identified in complexes with all antigen-presenting CD1 isoforms. Furthermore, sulfatide was shown to be stimulatory for T cells restricted to CD1a, b, c and d [17].

Table 2. Different self lipid antigens presented by specific CD1 isoforms. Adapted from [17].

Self antigens	
Antigen	Restriction
GM1	CD1b
iGb3	CD1d
GD3	CD1d
Phosphatidylcholine	CD1d, b
Sulfatide	CD1a, b, c, d

1.2.3. Lipid antigen loading on CD1 molecules

Exogenous lipids need to reach the APC to bind to CD1. Due to their hydrophobic properties, lipids are transported in association with low density lipoproteins, very low density lipoproteins and high density lipoproteins. When the lipid antigens reach the APC, they are internalized either by lipoprotein interaction with cell surface receptors, insertion in the plasma membrane or internalization of exosomes or apoptotic bodies [39]. The most

common process for lipid acquisition is mediated by lipoproteins. This allows specific delivery of the lipid antigens to the APCs. Some antigens, such as sulfatide, GM1 and α-GalCer do not require internalization by the APC to bind to CD1 and activate T cells [40, 41]. However, other lipid antigens need to be internalized and processed to originate the antigenic form. Lipid processing takes advantage of different enzymes involved in lysosomal glycolipid degradation. Three lysosomal enzymes have already been implicated in this process: α-galactosidase A [42], β-hexosaminidase [43] and α-mannosidase [27].

An important issue is how lipids are extracted from lysosome membranes, digested by hydrolases and loaded in CD1 molecules. Lipid transfer proteins (LTPs), like saposins, GM2 activator protein, Niemann-Pick type 2 (NPC2) protein and CD1e are central in the referred processes [27, 43-46]. Saposin C is capable of extracting lipids from membranes and forming a complex with CD1b, thus indicating that it promotes the lipid loading in CD1b [44]. In fact, APCs which express CD1b but are deficient for saposin C are severely compromised in the ability to present lipid antigens [44]. In addition, the lack of saposins in mice was associated with absence of the CD1d restricted invariant natural killer T (iNKT) cells in the thymus, spleen and liver [43]. Furthermore, thymocytes from saposin deficient mice were not able to stimulate a self-reactive iNKT cell hybridoma [43]. It was later discovered that the most efficient saposin in loading of lipids in CD1d molecules was saposin B [45]. Altogether these data indicate that saposins are essential for lipid antigen presentation through CD1b and CD1d. GM2 activator protein is capable of removing lipids that are already loaded in CD1d and assists in α-GalCer loading in CD1d and [43]. NPC2 is essential for the formation of the CD1d-lipid complex in mice [46]. This protein transports glycolipids to the lysosome, where they are loaded in CD1d. Mice deficient for NPC2 have almost no iNKT cells [46]. It was observed *in vitro* that NPC2 facilitates iGb3 (isoglobotriaosylceramide) binding to CD1d, thus having similar functions to saposins [46]. CD1e is a LTP that has an important function in antigen presentation, helping in lipid processing [27]. This function was discovered by using THP cells transfected with CD1B and CD1E or only CD1B as APCs to activate CD1b restricted T cells [27]. While CD1B+CD1E cells had the ability to present the *Mycobacterium* antigen PIM_6 to CD1b-restricted T cell clones, cells only transfected with CD1B were incapable to present PIM_6 but had no alterations in presentation of other lipid antigens that do not require processing. This indicated that CD1e is a key player in lipid antigen processing. It was further

discovered that CD1e does not have intrinsic enzymatic activity, but it works as a co-factor for some lysosomal hydrolases, such as α-mannosidase [27].

Although LTPs play a major role in lipid loading, other factors facilitate this process. For example, low pH induces conformational changes in CD1 molecules, opening their binding pocket and thus promoting lipid binding [31].

All these processes are essential for CD1-restricted T cells activation.

1.2.4. CD1-restricted T cells

CD1-restricted T cells are activated by the interaction of the TCR-CD3 complex with a lipid antigen bound to one of the isoforms of the CD1 molecule (Figure 3). This interaction initiates a cascade of signals responsible for T cell proliferation, cytokine production and in some cases cytolytic activity. These lipid-specific T cells can be classified according to what CD1 molecule they respond to, i.e. according to CD1 restriction. Some characteristics of the different types of CD1 restricted T cells are presented in Table 3.

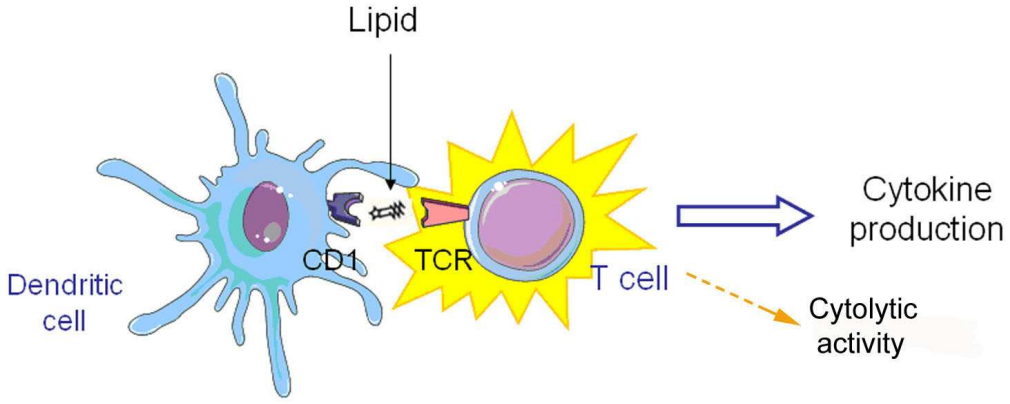

Figure 3. CD1-restricted lipid-specific T cells activation by dendritic cells.

Table 3. Comparison between different types of CD1-restricted T cells. Modified from [47].

	CD1a	CD1b	CD1c	CD1d	
				iNKT	Type II
TCR	αβ and γδ	αβ	αβ and γδ	$V_{\alpha}24J_{\alpha}18$; $V_{\beta}11$	αβ and γδ
CD4/CD8	$CD4^+$, $CD8^+$, DN	$CD4^+$, $CD8^+$, DN	$CD4^+$, $CD8^+$, DN	$CD4^+$, $CD8^+$, DN	CD4+, DN (mouse)
Main lipid antigens	Sulfatide	Sulfatide, GM1, mycolic acid	Sulfatide	iGb3; α-GalCer and analogs	Sulfatide; lysophosphatidylcholine

CD1 restricted T cells can be classified in group I restricted T cells and group II restricted T cells, the natural killer T (NKT) cells. This section will characterize these two different groups.

Group I CD1-restricted T cells

Group I CD1-restricted T cells include T cells restricted to CD1a, CD1b or CD1c. These cells can express a variable repertoire of αβ TCRs [18]. Some studies found that there are also γδ T cells that recognize CD1 [17]. Regarding CD4 and CD8 expression, group I CD1-restricted T cells can be only positive for CD4 ($CD4^+$), CD8 ($CD8^+$) or negative for both (DN) [18]. The effector functions of these cells were mainly analysed in response to mycobacterial antigens. They secrete Th1, Th2 and Th17 cytokines [17]. Recently, a population of CD1a-restricted T cells was shown to produce IL-22 [35]. In addition to cytokine production, both $CD8^+$ and DN CD1 restricted T cells were shown to be efficient cytolytic T cells, as they are able to lyse mycobacterium infected cells [17, 18]. The differences in cytokine production and cytolytic activity suggest that group I CD1-restricted T cells may be divided in distinct functional subsets.

One of the key features of CD1-restricted T cells is self-reactivity, which is related to their ability to recognize self lipid antigens [35]. Although it was known that self-reactive CD1-restricted T cells existed, their abundance was only assessed by the end of 2010 [35, 36]. This is explained by the absence of cell surface markers that allow their identification. However, two recent studies have made some progress in the characterization of group I CD1 restricted T cells. Two different methods were used to assess the frequency of self-reactive group I CD1 restricted T cells. Jong and collaborators assessed self-reactivity by an antigen presentation assay that bypassed MHC restriction and

the requirement for defined antigens, using APCs with low expression of MHC molecules and transfected with CD1 molecules. Then, polyclonal T cells were added to the APCs. When CD1-restricted T cells were present they became activated and produced cytokines that were detected by ELISPOT [35]. In the study of de Lalla and collaborators, screenings of libraries of T cell clones obtained from the peripheral blood of healthy adults and neonates were performed. The results were confirmed using limiting dilution analysis of total adult T cells. Both studies found an unexpected high frequency of self-reactive CD1 restricted T cells. In the peripheral blood of healthy adults CD1a-restricted T cells are the most frequent [35, 36], followed by CD1c, the group II CD1d and finally CD1b-restricted T cells [36]. These subsets seemed to be shaped during life, since the frequencies varied between blood from neonates and adults [36]. Furthermore, CD1-restricted T cells present a naïve phenotype at birth that persists through adulthood, with a high percentage of cells expressing CD45RA [36]. However, there are more CD1 reactive T cells expressing CD45RO in adults' peripheral blood than in neonates' umbilical cord blood [36]. Self-reactive CD1a restricted T cells were further analysed for expression of CD4 and CD8 molecules and $CD8^+$, $CD4^+$ and DN cells were found [35]. Regarding TCR expression, there was no conservation between the different self-reactive CD1a-restricted T cells [35].

Group II CD1-restricted T cells: NKT cells

NKT cells are self-reactive CD1d-restricted T cells that express simultaneously NK cells markers and the T cell receptor [48]. According to the TCR properties, NKT cells can be divided in two groups: type I NKT cells, or invariant NKT cells (iNKT), which express a TCR composed of an invariable α chain associated with a limited repertoire of β chains; and type II NKT cells, which have a TCR with variable α and β chains [47]. iNKT cells can be identified by CD1d tetramers loaded with α-GalCer or α-GalCer analogs. On the contrary, type II NKT cells do not recognize α-GalCer and therefore are not stained with this method [47]. Type II iNKT cells recognize antigens such as sulfatide and lysophosphatidylcholine [47]. They have an activated memory phenotype and modulate immune responses [47]. Some functions that were attributed to type II NKT cells include the suppression of autoimmunity and inhibition of tumour rejection [47]. Little information is available regarding type II NKT cells, since they are difficult to identify due to the absence of useful cell surface markers.

Most of the studies in the field of CD1 restricted T cells are centred in iNKT cells, because they can be identified by the use of CD1d tetramers. iNKT cell percentage is very variable among humans, ranging from values of 0.001% to over 3% of peripheral blood mononuclear cells (PBMCs), although usually they vary between 0.01% and 0.1% [49]. Functionally, these cells are involved in the innate and acquired immunity, producing large amounts of cytokines soon after the encounter with antigens and have cytolytic activity [47]. They also activate NK cells, macrophages, neutrophils, B cells and T cells. iNKT cells were also associated with protective and regulatory functions in tumours, infections, transplants and autoimmune diseases [50]. In humans, iNKT cells can be divided in three subsets, according to CD4 and CD8 expression [48]. They can express only CD4 ($CD4^+$) or CD8 ($CD8^+$) or lack both molecules (DN). The cytokine expression profile varies among the different iNKT subsets: $CD4^+$ iNKT cells produce both Th1 and Th2 cytokines (IFN-γ, IL-4, IL-10 e IL-13) whereas DN iNKT cells produce mainly Th1 cytokines like IFN-γ and very low quantities of Th2 cytokines like IL-4 [51-53]. $CD8^+$ iNKT cells produce essentially IFN-γ [53]. The percentage of CD4/CD8 iNKT cell subsets is very variable between individuals. $CD4^+$ and DN iNKT cells are the most common subsets in humans, with $CD4^+$ varying between 17 and 53% and DN between 19-63% of total iNKT cells [54-64]. $CD8^+$ population is rarer, representing 5 to 25% of the iNKT cell population [55, 56, 58, 61, 62]. These frequencies are altered in a variety of conditions. iNKT $CD4^+$ cell percentage was shown to be decreased in HIV infection [61] and after kidney transplants with no rejection [57] and increased in refractory celiac disease [54], during rejection of kidney transplants [57] and during *Mycobacterium tuberculosis* infection [58]. The iNKT $CD4^+$ subset also tends to increase with age [59, 62]. The iNKT DN subset was reduced in hepatocellular carcinoma [55] and during *Mycobacterium leprae* infection [58]. Alterations in the iNKT $CD8^+$ subset included an increase in HIV infection [61] and a decrease in metastatic uveal melanoma [55].

iNKT cell development in the thymus begins with the expression of the invariable TCR. This event directs cells to the iNKT differentiation pathway. The cells are positively selected by the interaction of the TCR with CD1d molecules expressed by thymocytes. In mice, iNKT cell maturation is composed by at least four stages. The first is characterized by a population of CD24-positive, $CD44^{lo}NK1.1^{lo}$ cells. Only after CD24 downregulation cells start to proliferate, maintaining low expression of CD44 and NK1.1. After this stage,

CD44 is upregulated ($CD44^{high}$ $NK1.1^{-}$). At this point some cells downregulate CD4, constituting the DN iNKT cell lineage. The last stage is characterized by the upregulation of NK1.1 and other NK cell markers, which usually occurs after migration to the periphery [65]. In humans, iNKT cell maturation is also completed at the periphery, where cells increase the expression of CD161 (human equivalent to NK1.1). The earliest detectable NKT cell precursors in humans are $CD4^{+}$ $CD161^{lo}$ cells. This indicates that the $CD4^{-}$ population appears at later developmental stages [47]. In humans, the origin of the iNKT $CD4^{+}$ and $CD4^{-}$ subsets at the periphery is different. iNKT $CD4^{+}$ subset is mainly sustained by thymic output and survival at the periphery, while iNKT $CD4^{-}$ subset is maintained essentially due to cell division that occurs in the periphery [66].

iNKT cells are activated when they encounter exogenous or endogenous antigens loaded in CD1d molecules. The activation process may occur by three different pathways, depending on the type of antigen [67]. In a direct pathway, the antigen is initially internalized and loaded in CD1d molecules or directly loaded in the CD1 molecules present at the cell surface. Then, the CD1d-lipid complex present at the surface of the APC interacts with the TCR of the iNKT cell, which causes its activation (Figure 4, direct pathway). Some antigens bind toll-like receptors present at the surface of the APC, starting a cascade of events that promotes the loading of endogenous antigens in CD1d molecules and IL-12 secretion. In this case, there is a double activation of iNKT cells by IL-12 and by CD1d interaction with TCR (Figure 4, direct + indirect pathway). There is still a third pathway in which there is no TCR mediated recognition of the antigen. Instead, the antigen only promotes IL-12 secretion by APCs (Figure 4, indirect pathway).

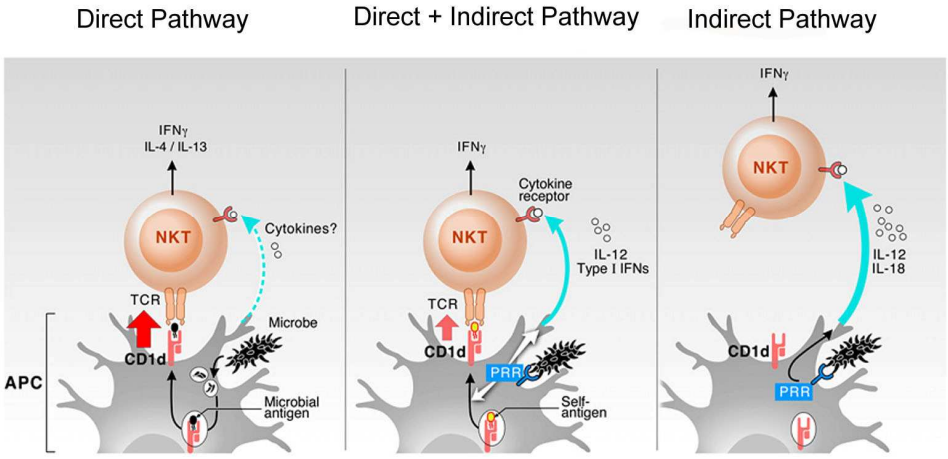

Figure 4. Different pathways leading to iNKT cell activation during infection. Adapted from [67].

1.3 CD1 restricted T cells in lysosomal storage diseases

The lysosome is an important cellular compartment for lipid antigen presentation by CD1 and consequently for CD1 restricted lipid specific T cells. Since lysosome function is impaired in LSDs, lipid antigen presentation can also be defective. A problem in lipid antigen presentation would lead to altered CD1 restricted T cells populations. Most of the studies regarding lipid specific CD1 restricted T cells in LSDs were performed in mice and only iNKT cells were analysed. In mouse models of several LSDs there is a decrease in the percentage of iNKT cells in the thymus and at the periphery. This applies to Sandhoff disease [68-70], GM1 gangliosidosis [69, 71], NPC1 deficiency [69, 72], NPC2 deficiency [46, 71], Tay-Sachs disease [69], multiple sulfatase deficiency [70] and Fabry disease [42, 69, 73] mouse models. In contrast, MPS type I, metachromatic leukodystrophy and Krabbe disease presented no alterations in the percentage of iNKT cells [70]. These results raised the question of whether this reduction in iNKT cells arises from a specific defect that leads to accumulation of a specific lipid or simply due to a non-specific effect of macromolecules accumulation in the lysosome.

In humans, both Gaucher and Fabry disease patients were analysed regarding iNKT cell percentage. In Gaucher disease no alterations were found in iNKT cell percentage when compared to controls. There were also no differences in iNKT cell percentage when patients under ERT or not under ERT were compared [74]. In Fabry disease patients, no alterations in the percentage of iNKT cells were found [73, 75]. Fabry disease patients under ERT and not under ERT were also compared and no differences were detected [75]. Due to high individual variation of iNKT cell percentage, small alterations in iNKT cell percentage might not be detected. Studies that follow the percentage of iNKT cells along ERT at an individual level are missing and can be useful to better understand the effect of ERT in iNKT cell populations. No studies regarding lipid antigen presentation of either CD1d or group I CD1 molecules were performed in LSDs patients.

To investigate the cause of the decrease in the percentage of the iNKT cell population in LSDs, the capacity of APCs from LSDs mouse models to present lipid antigens to iNKT cells was assessed. In the Shandhoff, Fabry, GM1 gangliosidosis, NPC1 deficiency and NPC2 deficiency diseases it was confirmed that the reduction in the number of iNKT cells is associated with deficient lipid antigen presentation [42, 46, 68, 69, 71,

72]. In 2007, it was shown that two types of alterations can occur depending on the specific LSD [71]. In GM1 gangliosidosis disease mouse model, there is reduced exogenous and endogenous antigen presentation to iNKT cells by thymocytes, but not dendritic cells. When fixed thymocytes are used in presentation studies, they show normal presentation of both types of antigens indicating that surface expressed CD1d is functional and that it is forming complexes with the lipid antigen. This suggests that the defect leading to reduced iNKT cell percentage might be related to intracellular alterations, such as the retention of lipid antigens in the storage compartments. A different type of alteration was detected in NPC2 deficient mice. In these animals, there is altered presentation of endogenous and exogenous antigens by thymocytes and dendritic cells and the defect persists even when fixed dendritic cells were used. Furthermore, there is no formation of complexes between CD1d and the lipid antigen. This indicates that there is an extracellular problem, which could arise from the strong association of the accumulated lipids with CD1d, impairing the linkage of the real antigen [71].

Recently, it was shown that dendritic cells from Fabry disease mice (α-Gal A deficient) induced a strong response of iNKT cells [76]. This response disappeared when glycosphingolipid synthesis or recognition was artificially abolished. This suggests that α-Gal A is important for stimulation of iNKT cells by glycosphingolipids. It was then observed that iNKT cells from Fabry disease animals presented signals of chronic stimulation at the periphery: there was increased apoptosis in liver iNKT cells, but not in thymus iNKT cells; the expansion of liver and spleen iNKT cells as measured by $BrdU^+$ cells was reduced; iNKT cells were less responsive to the exogenous antigen α-GalCer, producing lower amounts of IFN-γ than control iNKT cells. Altogether these data indicate that α-Gal A has an important role in the activation of iNKT cells at the peripheral level [76].

2. Aims

Considering that the lysosome is an important compartment for lipid antigen presentation and that alterations in lipid antigen presentation in mouse models of LSD were already found, the main aim of this work was to study the effect of Fabry disease metabolic alterations in lipid antigen presentation and recognition. The more specific aims were:

1. To quantify the subsets of the lipid specific iNKT cells, in Fabry disease patients.
2. To test the capacity of dendritic cells from Fabry disease patients to present lipid antigens by the CD1 isoforms b and d.

3. Methods

All the experimental analyses were performed using peripheral blood samples from Fabry disease patients and control subjects. PBMCs were isolated and used for: 1) flow cytometry analyses, to study the iNKT cell population; 2) dendritic cells generation. For patients under ERT, the analyses of the iNKT cell population were done every four months. For four patients, the first of the five determinations was performed by Dr Fátima Macedo prior to the start of this thesis. Dendritic cells were cultured for 5 days and then analysed by flow cytometry to confirm the success of the differentiation and to assess CD1 cell surface expression, or used in antigen presentation assays. In these assays, dendritic cells were incubated with different concentrations of lipid antigen and a T cell clone restricted for a CD1 molecule. The capacity of antigen presentation was evaluated by the amount of GM-CSF produced by the activated T cells measured by enzyme linked immunosorbent assay (ELISA). Figure 5 represents the experimental design used in this study.

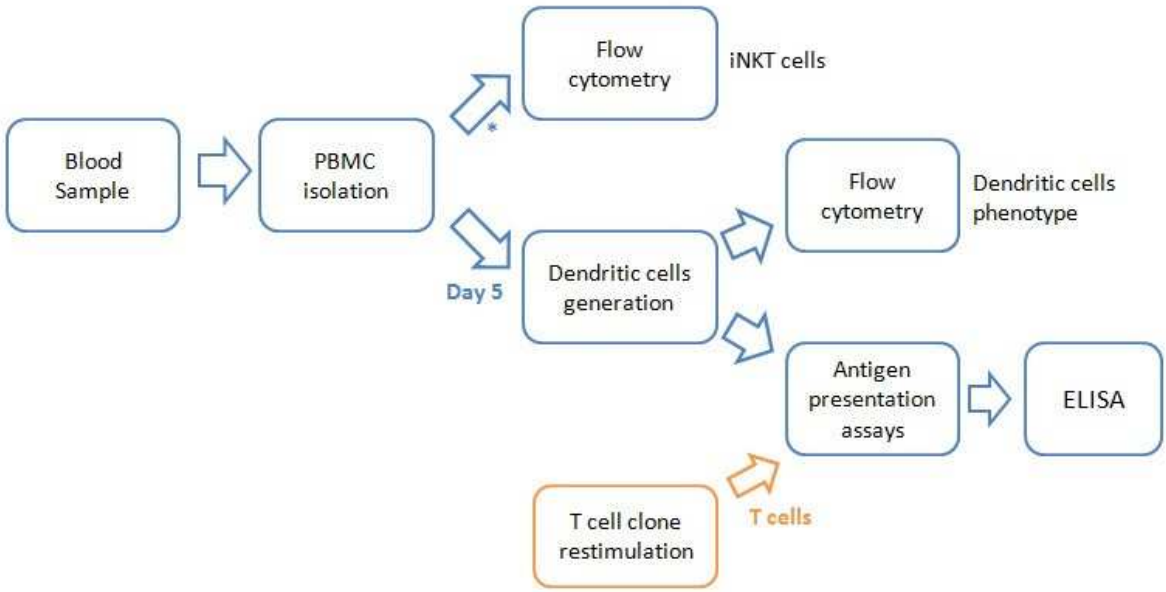

Figure 5. Experimental design of the study. * Every four months for patients under enzyme replacement therapy (ERT).

Before the beginning of the patients' analyses it was necessary to optimize some of the techniques that would be used. These include dendritic cell production, flow cytometry analyses and antigen presentation assays.

Subject selection and sample collection

Seven Fabry disease patients and nine control subjects were analysed in this study. In the Fabry disease group, five were males and two were females. In the control group, there were four males and five females. Control subjects were blood donors at the *Centro Regional de Sangue do Porto – Instituto Português do Sangue* (IPS). Samples were obtained under a protocol between IBMC and IPS. Fabry disease patients were recruited by their physicians, Dr Olga Azevedo Silva from *Centro Hospitalar do Alto Ave* and Dr. Emanuel Correia from *Hospital de São Teotónio*. The biochemical and molecular diagnosis of these patients was done in our laboratory, by Daniel Rodrigues and Paulo Gaspar, under the supervision of Dr Clara Sá Miranda. Briefly, the biochemical diagnosis was done by measurement of α-Gal A activity in dried blood spot (DBS) and genotypic alterations were identified by molecular studies of the *GLA* gene using denaturating high performance liquid chromatography followed by DNA sequencing, as described in [12]. In the beginning of this study, four patients were already under ERT. This treatment consists in the infusion of the α-Gal A enzyme (algasidase α, Replagal, Shire HGT), every other week. Of the other three patients, one started treatment during the course of the study and the other two are not under ERT. The characteristics of the patients included in the study are described in the results section 4.1, on Table 7.

Blood samples were collected in ethylenediamine tetraacetic acid (EDTA) containing tubes. For patients under ERT, the blood withdrawal was done before the enzyme infusion.

Peripheral blood mononuclear cells isolation

Upon sample arrival, PBMCs were isolated using density gradient centrifugation with Histopaque-1077® (Sigma), under sterile conditions. The blood was layered on an equal amount of Histopaque-1077® and centrifuged at 400xg for 30 minutes without brake. After the centrifugation, the PBMCs are located in a ring between the plasma and Histopaque-1077® (Figure 6). PBMCs were then collected and washed once by adding 10 mL of phosphate buffered saline 1x (PBS 1x, see appendix I) and centrifuged at 250xg for 10min. Then, remaining erythrocytes were lysed by incubating cells for 10min with 10mL of ACK lysis solution (see appendix I). Cells were washed once more with 10 mL of PBS

and counted using a microscope counting chamber (Neubauer chamber). After cell count, PBMCs were used for flow cytometry or for dendritic cell differentiation.

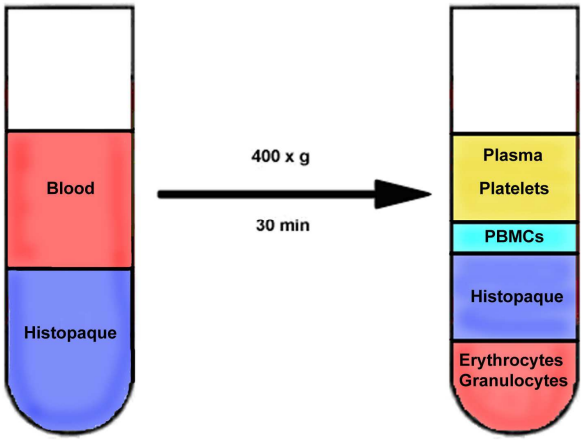

Figure 6. PBMC isolation using Histopaque 1077®.

Flow cytometry

Up to 1 x 10^6 PBMCs or about 0.25x10^6 dendritic cells were stained per well in a round-bottomed 96-well plate. After cell addition, plates were centrifuged at 1200 rpm, for 2 min. For PBMCs, supernatant was rejected and cells were resuspended in 25µL of the antibody/tetramer mix diluted in PBS 0.2% BSA 0.1% NaN_3 (flow cytometry solution, see appendix I). The antibodies listed in Table 4 were used.

Table 4. Antibodies used in PBMCs flow cytometry to identify iNKT cells populations.

Antibody	Clone	Fluorochrome	Brand
Anti-human CD3	SK7	PerCP-Cy5.5	eBioscience
Anti-human CD4	RPA-T4	PE-Cy7	eBioscience
Anti-human CD8	RPA-T8	APC-eFluor 780	eBioscience

APC-eFluor 780 – Allophycocyanin conjugated with eFluor 780; PE-Cy7 – Phycoerythrin conjugated with Cy7; PerCP-Cy5.5 - Peridinin chlorphyll protein conjugated with Cy5.5.

To identify iNKT cells, the CD1d tetramer loaded with PBS57 and labelled with the fluorochrome phycoerythrin (PE), from the National Institute of Health tetramer core facility was used. PBS57 is a recently developed analogue of α-GalCer, that was shown to have indistinguishable activity from α-GalCer, being the CD1d-PBS57 tetramer capable of

effectively detect both mouse and human iNKT cells [77]. An unloaded CD1d tetramer was used as a control to identify unspecific staining.

Dendritic cells needed to be blocked to avoid unspecific staining, so they were resuspended in 50µL of PBS 25% human serum (see appendix I) and incubated for 20 min, on ice. After that, dendritic cells were washed twice by adding 100µL of flow cytometry solution and centrifuged at 1200 rpm, for 2 min. Then, 25µL of the antibody mix were added. The antibodies used for dendritic cell staining are described in Table 5.

Table 5. Antibodies used in dendritic cells flow cytometry to confirm differentiation and to assess CD1 expression.

Antibody	Clone	Fluorochrome	Brand
Anti-human CD1a	HI149	PE	eBioscience
Anti-human CD1b	SN13	FITC	Biolegend
Anti-human CD1c	L161	PerCP	Biolegend
Anti-human CD11c	3.9	PE-Cy7	eBioscience
Anti-human CD80	2D10	APC	Biolegend

APC – Allophycocyanin; FITC – Fluorescein; PE – Phycoerythrin; PE-Cy7 – Phycoerythrin conjugated with Cy7; PerCP - Peridinin chlorphyll protein.

Both PBMCs and dendritic cells were incubated for 20 min, at 4ºC, in the dark. After incubation, PBMCs were washed three times by adding 100µL of flow cytometry solution, centrifuge at 1200rpm for 2 min, reject supernatant and resuspend cells. After the last wash, PBMCs were resuspended in 400µL of PBS 1% formaldehyde (see appendix I) and dendritic cells were washed twice and resuspended in 400µL of PBS 1x. Then, all samples where transferred to FACS tubes (Falcon, BD Bioscience). PBMCs were kept at 4ºC in the dark and acquired in the next day. Dendritic cells were acquired in the same day of the staining.

For PBMCs, up to $1x10^6$ events were acquired in a FACSCanto or FACSAria (BD biosciences), using the FACSDiva software (BD biosciences). In the case of dendritic cells, 20000 events were acquired in FACSCalibur (BD biosciences) using the Cell Quest software. All flow cytometry analyses were done using the FlowJo software (Tree Star).

Dendritic cell differentiation

PBMCs include lymphocytes and monocytes. Dendritic cells can be differentiated *in vitro* from peripheral blood monocytes, so it was necessary to separate the two cell types. This was achieved using immunomagnetic labelling with MACS anti-human CD14 MicroBeads (Miltenyi Biotec). This technique uses magnetic beads bound to an anti-human CD14 antibody that attaches to the monocytes (that are $CD14^+$ cells) (Figure 7).

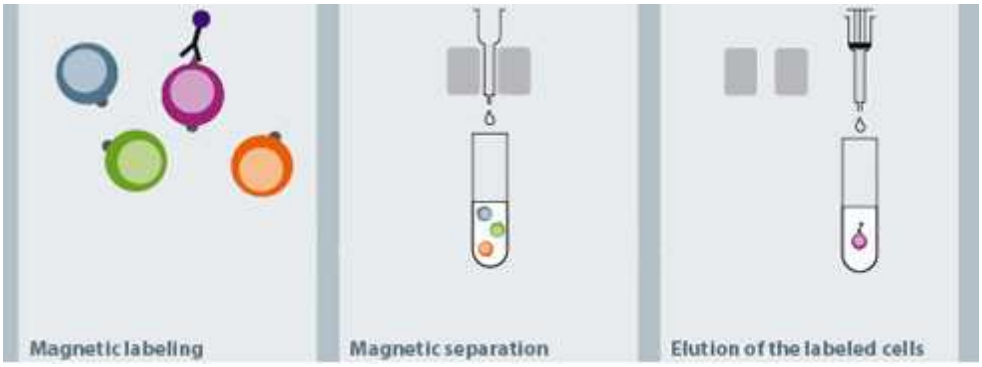

Figure 7. Separation of CD14+ cells by positive selection using MACS beads (Miltenyi Biotech). Figure adapted from http://www.miltenyibiotec.com

PBMCs were washed once with 5mL of 1x PBS 2mM EDTA 0.5% BSA (MACS buffer, see appendix I). Then, they were centrifuged at 1800 rpm for 5 min and the supernatant was rejected. Cells were resuspended in MACS buffer and incubated with the anti-human CD14 magnetic beads, on ice, for 20 min, in the dark, with occasional shaking. This step allows the anti-human CD14 antibodies present in the magnetic beads to bind to the monocytes. The amount of MACS buffer and magnetic beads used depended on the total number of cells in the solution, according to the following proportion: for $100x10^6$ PBMCs, use 475µL of MACS buffer and 62.5µL of anti-human CD14 magnetic beads. After incubation, cells were washed twice with 5mL of MACS buffer. Depending on the number of PBMCs available, MS or LS columns were chosen for isolation, according to the manufacture's instructions. MS columns were used if there were less than $20x10^6$ PBMCs available and LS columns if there were more. The columns were placed in the magnetic support and pre-wetted with MACS buffer. This support keeps the labelled cells from eluting due to the magnetic force existent between the beads and the support. Cells were resuspended in 4mL (always for LS columns) or 0.5mL (always for MS columns) of MACS buffer and applied. The monocytes that bound to the magnetic beads remained in the column, while the non-labelled cells eluted. The column was washed by loading 4mL

or 0.5mL of MACS buffer three times. This step is important to remove from the column cells that are not bound to the magnetic beads, increasing the efficiency of the separation. Finally, the column was removed from the magnetic support and placed in a 15mL falcon tube. 4mL or 1mL of MACS buffer were loaded in the column and then cells were flushed from the column to a 15mL falcon tube, by applying the plunger, supplied with the column. At this step, $CD14^+$ cells are eluted, since the column is no longer in the magnetic support. After the isolation process, cells were counted and plated at $1x10^6$cells/mL in six-well plates, 3mL per well in dendritic cell culture medium (see appendix I), containing 25ng/mL of recombinant human IL-4 (rhIL-4, ImmunoTools) and recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF, ImmunoTools). Cells were incubated at 37°C, 5% CO_2. At day 3 of culture, medium was refreshed by removing 500µL of culture medium per well and by adding 500µL per well of dendritic cell medium with a higher concentration of cytokines (75ng/ml rhIL-4 and 75ng/ml rhGM-CSF). Cells were used for activation assays and flow cytometry at day 5.

T cell clones

T cell clones used for the lipid antigen presentation assays were kindly provided by Prof. Gennaro De Libero from Basel University, Switzerland. T cell clones were established and maintained as described [40, 78, 79]. Briefly, T cell lines were produced by the stimulation of PBMCs with autologous dendritic cells that had been pre-incubated with a mixture of glycolipids [78]. After three weeks, growing T cells were restimulated with heterologous dendritic cells plus the mixture of glycolipids. T cell clones were obtained by limiting dilution and scored for antigen specificity and CD1 restriction using CD1-transfected APCs [40, 78].

Three T cell clones were used, that respond to different lipid antigen-CD1 complexes, as described in Table 6.

Table 6. T cell clones used for antigen presentation assays.

Name	Specificity	Restriction	Reference
GG33A	GM1	CD1b	[78]
DS1C9b	Sulfatide	CD1b	[40]
s33d	Sulfatide	CD1d	*

* Description of this clone was not published yet. Clone from Gennaro De Libero laboratory.

Every 3 weeks T cells need to be restimulated. T cell clones restimulation is done using irradiated PBMCs as feeder cells and the antigen phytohaemagglutinin (PHA) in medium containing recombinant human IL-2 (rhIL-2). PBMCs are isolated following the protocol presented above. Then, PBMCs are resuspended in culture medium (appendix I) at a concentration of $5x10^6$cells/mL and irradiated in a gamma irradiator (Gammacell 1000, Nordion) at 3000 rad. After the irradiation process, cells are washed twice with culture medium and once with PBS. PBMCs are counted and resuspended in T cell culture medium (appendix I) with 2 µg/mL of PHA. The T cell clones to be restimulated are in culture, at a concentration of approximately $1x10^6$cells/mL, 1mL per well. For restimulation, 1mL of irradiated PBMCs is added to each well containing T cells. Two wells have only irradiated PBMCs, to verify the efficacy of the irradiation process. If the irradiation is successful, all irradiated PBMCs should die after approximately 15 days.

Lipid antigen presentation assays

Lipid antigen presentation assays were used to assess the capacity of dendritic cells to present lipid antigens by CD1 molecules. In these *in vitro* assays, dendritic cells are incubated with the lipid antigen and with T cell clones. When the T cell clones recognize the lipid antigen bound to CD1 at the cell surface of the dendritic cell, they start producing cytokines that can be then detected, indicating the efficiency of the lipid antigen presentation.

These *in vitro* assays were performed as previously described [40, 78]. Dendritic cells were collected, counted and resuspended in culture medium (Appendix I) without inactivated fetal bovine serum (iFBS) at a concentration of $0.4x10^6$ cells/mL. This medium does not contain iFBS to facilitate lipid antigen uptake by the dendritic cells. 20000 cells (50µL of the solution of $0.4x10^6$ cells/mL) were added to each well of a 96-well flat-

bottomed plate and incubated at 37°C, 5% CO_2. Then, antigen dilutions were performed. A 5mg/ml stock solution of sulfatide diluted in chlorophorm:methanol (2:1) was prepared. The highest concentration to be tested was of 30µg/ml. However, the final antigen concentration is diluted 4x, since 50µL of the antigen are used in a final volume of 200µL. Therefore, it was necessary to prepare a solution of 120µg/ml. GM1 was initially diluted in a solution of chlorophorm:methanol (1:1) in a concentration of 10mg/ml. The highest concentration to be tested was 50µg/ml. As for sulfatide, GM1 is diluted 4x in the activation volume, so a solution of 200µg/ml was necessary. The lipid antigens were then dried under N_2 and dissolved only in methanol to obtain the needed concentrations, because chlorophorm is toxic for the cells. Then, the antigens were sonicated for 3 min in a water bath sonicator. Eight serial dilutions of 1:3 in duplicates were prepared in 50µl of culture medium without iFBS and added to the wells containing the dendritic cells. The lipid antigen concentrations tested were: 1) for sulfatide, 30; 10; 3.3; 1.1; 0.4; 0.1; 0.04µg/mL and no antigen; 2) for GM1, 50; 16.7; 5.6; 1.8; 0.6; 0.2; 0.0704µg/mL and no antigen. Cells were incubated with the lipid antigens at 37°C, 5% CO_2, for 4h. Meanwhile, the T cell clones were collected, washed once with PBS and counted. 100µl of culture medium with 20% iFBS containing 100000 T cells were added per well. This media has 20% iFBS to obtain a final concentration of 10%iFBS, since 100µl are added in a total final volume of 200µl. The T cell clones, dendritic cells and lipid antigens were incubated for 36 hours at 37°C, 5% CO_2, to allow T cells activation and cytokine production. Then, plates were centrifuged at 1200 rpm for 2 min and 150µL of the supernatant were collected and frozen or used immediately for determination of cytokine concentration by ELISA.

ELISA

Cytokine concentration determination was performed by ELISA. ELISA plates (MaxiSorp®, Nunc) were coated with 50µL per well of purified anti-human GM-CSF (BVD2-23B6, Biolegend,) at 4ºC, over night. The coating solution was then removed and wells were washed once by adding 300µL of washing buffer (PBS, 0.05% Tween 20; appendix I) to each well. Then the wells were blocked by adding 100µL of blocking buffer (PBS, 0.05% Tween 20, 1% BSA; appendix I) and incubated at room temperature for one hour. After this process, wells were washed twice and incubated with 50µL of the sample. In some wells, 50µL of standard rhGM-CSF were added in eight different concentrations

(1:3 serial dilutions starting in 20ng/mL), in duplicates. After one and a half hour, wells were washed three times and 50µL of the biotinilated anti-human GM-CSF antibody (BVD2-21C11, Biolegend) were added to each well. One hour and a half later, wells were washed again with washing buffer three times. For detection of the biotinilated antibody, 70µL of streptavidin conjugated with horseradish peroxidase (Invitrogen) were added to each well. This compound was incubated for one hour at room temperature, in the dark. After washing wells four times, 90µL of SigmaFast™ o-phenylenediamine dihydrochloride (OPD) (Sigma), a substrate for the horseradish peroxidase, were added. The OPD reagent was prepared according to the manufacturer's instructions. The reaction occurred for 30 minutes, at room temperature in the dark. Then 50µL of H_2SO_4 were added to stop the reaction. Absorbance was read at 490nm on an ELISA plate reader (µQuant, Biotek). According to the rhGM-CSF standards, absorbance values were converted to cytokine concentration values.

Statistical analyses

Statistical analyses were made using the GraphPad Prism 5 software. Mean and standard deviation values were calculated and statistical significance was assessed by Mann-Whitney U tests. P-values below 0.05 were considered significant.

4. Results

4.1 Fabry disease patients and control subjects characteristics

Seven Fabry disease patients and nine control subjects were included in this study. The α-Gal A activity at diagnosis, genetic mutation responsible for Fabry disease, sex and age of the patients are presented in Table 7. The range values of normal α-Gal A activity, shown in table 7, were determined in our laboratory by the analysis of: 120 healthy blood donors from IPS for DBS activity; 123 healthy blood donors from IPS for leukocyte activity and 12 healthy subjects for fibroblasts activity. The control group of this study was composed by four males and five females and had a mean age of 44 years old (standard deviation of ± 9). Ages of controls and patients did not differ significantly. In the Fabry disease patients group, four were siblings and six of them had the same mutation. As expected, Fabry disease male patients presented extremely low values of α-Gal A activity. In heterozygous females, due to random inactivation of the X-chromosome, a higher variation of α-Gal A activity among patients and between different tissues of the same patient can be observed [7]. The two female patients studied present different α-Gal A activities: patient number 4 has normal activity in fibroblasts, but reduced activity in DBS and leukocytes; patient number 7 has normal α-Gal A activity in fibroblasts and DBS and presents borderline values concerning leukocytes. Although the biochemical diagnosis for this patient was not conclusive, she presented a clinical phenotype suggestive of Fabry disease. Therefore, a molecular study was performed revealing a newly described mutation, consisting in the deletion of a glutamate in the 358 position.

Four of the patients were already under ERT at the beginning of the study for four months and other three were not. One of these patients started ERT after the analysis of the iNKT cell populations but before lipid antigen presentation assays were performed. Females were not included in the lipid antigen presentation studies.

Table 7. Fabry disease patients and controls subjects characteristics: age, sex, α-Gal A activity at diagnosis and *GLA* mutation.

Patient	Sex	Age	*GLA* Mutation	α-Gal A activity			ERT
				DBS (spot)	Leukocytes (nmol/h/mg)	Fibroblasts (nmol/h/mg)	
1[a]	M	55	F113L	0	1	4	Yes
2[a]	M	52	F113L	0.02	2	4	Yes
3[a]	M	61	F113L	0.01	2	4	Yes
4[a]	F	67	F113L	0.09	3	65	Yes
5	M	40	F113L	0.01	2	4	Yes[b]
6	M	47	F113L	0	3	ND	No
7	F	49	E358del	0.33	27	91	No
			Reference values	0.3-1.2	28-99	44-103	

DBS – dried blood spot; ERT – enzyme replacement therapy; M – male; F – female; ND – not done.

[a] Siblings

[b] Not under ERT when iNKT cell and subset percentage was assessed, but already under ERT when lipid antigen presentation assay was performed.

4.2 Percentage of iNKT cells and iNKT cell subsets in Fabry disease patients

The percentage of iNKT cells is not altered in Fabry disease patients' peripheral blood

The percentage of iNKT cells was shown to be decreased in the thymus, spleen and liver of several mouse models of LSDs, including Fabry disease mice [42, 46, 69-72]. As previously described, the iNKT cell percentage in human peripheral blood is highly variable [47]. In this study, PBMCs were stained with an antibody anti-human CD3 and the human CD1d tetramer loaded with PBS57 and analysed by flow cytometry. After defining the lymphocyte gate in the side scatter and forward scatter plot, T cells ($CD3^+$) were selected and the percentage of CD1d tetramer positive cells within this gate was determined (Figure 8).

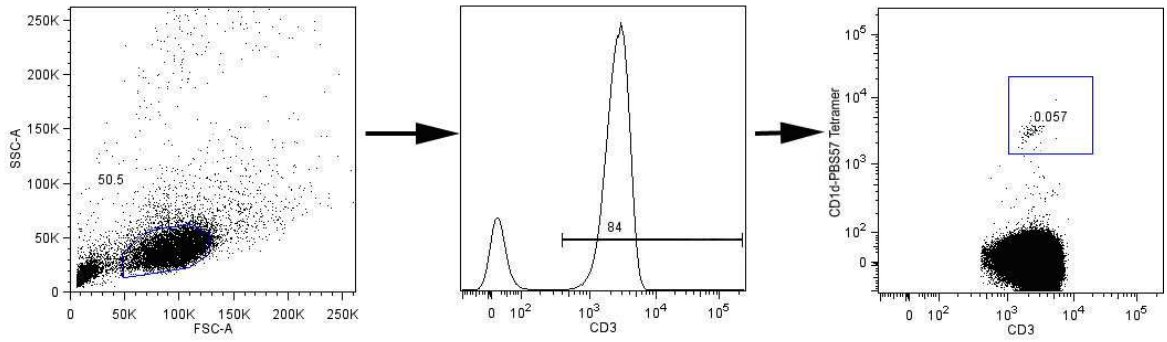

Figure 8. Quantification of iNKT cell percentage using flow cytometry.

Results concerning iNKT cell percentage in Fabry disease patients and control subjects are presented in Figure 9A. Interestingly there was a higher variability in peripheral blood iNKT cell percentage among Fabry disease patients than in control subjects. In the control group, females tended to present higher levels of iNKT cells, as it was previously described [80]. In the Fabry disease group, the two females studied presented low iNKT cell frequencies. Overall, no statistical significant differences were found between the control and Fabry disease patients group in the percentage of iNKT cells.

Four of the analysed patients were under ERT for four months. The comparison between the percentage of iNKT cells of Fabry disease patients under ERT and patients not treated does not reveal significant differences, in accordance to what was previously described [75]. However, due to the high variation of the percentage of iNKT cells in human blood, cross section studies with few patients may not be the best way to elucidate

the effect of ERT. To better assess this effect, the percentage of iNKT cells was determined every four months, during 12 or 16 months. Four patients that had started ERT four months before were included in this longitudinal study. The four patients presented variable values of iNKT cell percentage. In two patients, iNKT cell percentage remained stable during the course of the study (Figure 9B). The other two patients had alterations in the percentage of iNKT cells, but they did not follow a decrease or increase pattern (Figure 9B). In the longitudinal study, sex and age do not appear to influence the effect of ERT in iNKT cell percentage. In conclusion, treatment with α-Gal A for 20 months did not seem to alter the blood iNKT cell percentage in Fabry disease patients.

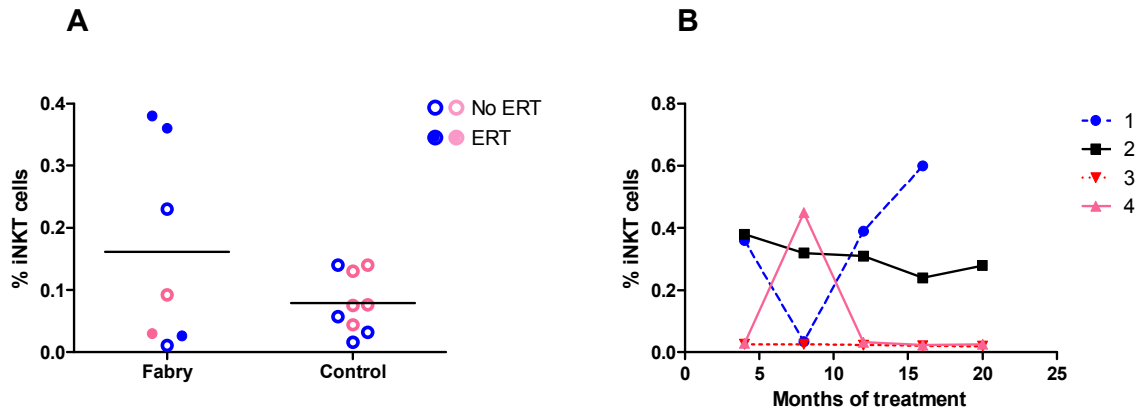

Figure 9. iNKT cell percentage among T lymphocytes ($CD3^+$). iNKT cells were identified in the gate of $CD3^+$ cells as positive for CD1d-PBS57 tetramer. A – Comparison between iNKT cell percentage in Fabry disease patients and control subjects. Horizontal bars represent means. Blue dots represent males and pink dots represent females. For patients that were followed during ERT, the first determination of iNKT cell percentage is represented. B – Variation of iNKT cell percentage in four Fabry disease patients every four months after beginning of ERT.

Alteration in the iNKT cell subsets in Fabry disease patients: decrease in the iNKT $CD4^+$ cell subset

In humans, the iNKT cell population can be divided in three subsets according to the expression of CD8 and CD4 molecules: $CD4^+CD8^-$ ($CD4^+$); $CD4^-CD8^+$ ($CD8^+$) and $CD4^-CD8^-$ (DN). At the functional level, two main subsets can be defined according to cytokine production: iNKT $CD4^+$ and iNKT $CD4^-$. While iNKT $CD4^+$ cells produce both Th1 and Th2 cytokines, iNKT $CD4^-$ cells produce mainly Th1 cytokines [51-53]. The frequencies of iNKT $CD4^+$ cells in Fabry disease patients and control subjects are represented in Figure 10. Age and sex did not seem to influence the percentage of iNKT

cell subsets in both controls and patients. Fabry disease patients had lower percentage of iNKT CD4$^+$ cells in comparison with control subjects ($p<0.05$). Interestingly, one patient, which was not under treatment, presented frequencies of iNKT CD4$^+$ cells even higher than most of the control subjects. In a subsequent analysis of the same patient, after the beginning of ERT, the percentage of these cells remained elevated. This is the same patient that presented the lowest percentages of iNKT cells.

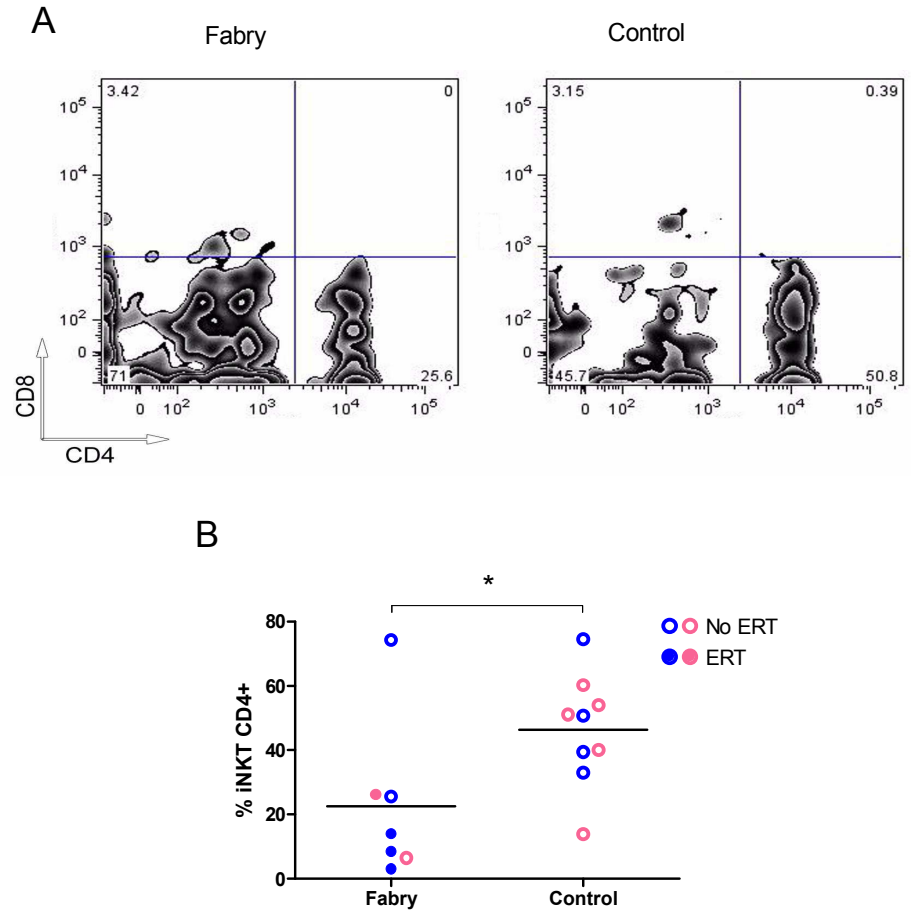

Figure 10. Percentage of iNKT CD4$^+$ subset is reduced in Fabry disease patients. A– Representative example of iNKT CD4/CD8 subsets percentage determined by flow cytometry, showing each subset percentage in a Fabry Disease patient (left) and a control subject (right). B– Frequencies of iNKT CD4$^+$ subset cells in Fabry disease patients and control subjects. Horizontal bars represent means. Blue dots represent males and pink dots represent females. For patients that were followed during ERT, first determination is represented. Statistical significance was calculated by Mann-Whitney U test, $p<0.05$.

It was then assessed whether ERT could reverse the reduction in the percentage of iNKT $CD4^+$ subset in a longitudinal study. As for iNKT cell percentage, four patients were analysed. The results obtained, presented in Figure 11, show that two patients had constant percentages of $CD4^+$ iNKT cells subset while two had variations in the percentage of this subset. Therefore, the percentage of iNKT $CD4^+$ cells in these four patients did not present major alterations along the 20 months of treatment. As for iNKT cell percentage, age and sex did not seem to have an influence in the effect of treatment in the iNKT $CD4^+$ subset.

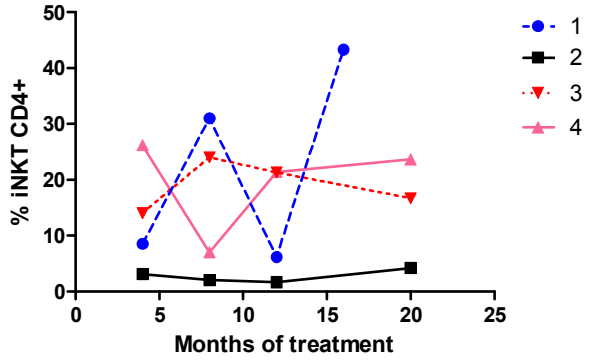

Figure 11. Variation of $CD4^+$ iNKT cell percentage in four Fabry disease patients along 16 or 12 months of ERT. iNKT cells were identified in the gate of $CD3^+$ cells as positive for CD1d-PBS57 tetramer and then analysed according to CD4 and CD8 expression.

The results regarding the other subsets of iNKT cells are presented in Figure 12. Both sex and age did not seem to have an effect on both the iNKT DN and $CD8^+$ subsets. Fabry disease patients tended to have higher percentages of both populations when compared to control subjects (Figure 12A and B). However, these alterations were not statistically significant. When iNKT $CD8^+$ and iNKT DN cells are coupled in a iNKT $CD4^-$ subset, statistically significant differences ($p<0.05$) between control subjects and Fabry disease patients were found (data not shown).

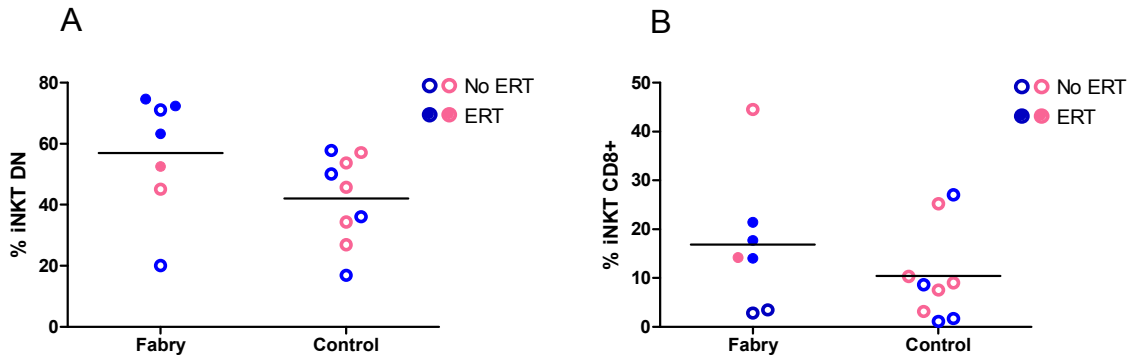

Figure 12. iNKT subsets percentage in control subjects and Fabry disease patients. Horizontal bars represent means. A – iNKT DN cell percentage; B – iNKT CD8$^+$ cell percentage. Blue dots represent males and pink dots represent females. For patients that were followed during ERT, first determination is represented.

The effect of ERT on the iNKT DN and CD8$^+$ subsets was also assessed in the longitudinal study and the results are shown in Figure 13. There was high variation in the percentage of the iNKT DN subset during the time of the study. On the contrary, there seems to be a reduction in the amount of CD8$^+$ iNKT cells. All patients decreased CD8$^+$ iNKT cell percentage after the first 8 months of treatment. Patients' age and sex did not seem to alter the effect of ERT on the iNKT DN and CD8$^+$ subsets.

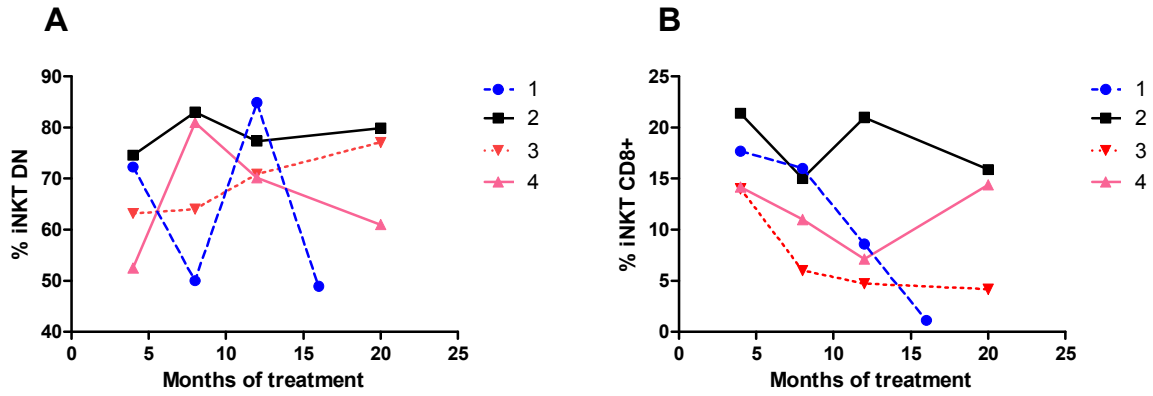

Figure 13. Variation of iNKT DN (A) or CD8$^+$ (B) cell percentage in four Fabry disease patients along 12 ou 16 months of ERT. iNKT cells were identified in the gate of CD3$^+$ cells as positive for CD1d-PBS57 tetramer and then analysed according to CD4 and CD8 expression.

4.3 Dendritic cell CD1 expression

To test Fabry disease patients' capacity to present lipid antigens through CD1 molecules, dendritic cells were generated from Fabry disease patients' monocytes. After five days of culture, dendritic cells were analysed by flow cytometry to confirm the dendritic cell differentiation (CD11c and CD80 expression) and to assess the cell surface expression of Group I CD1 molecules. In all experiments, dendritic cells had a CD80 and a CD11c expression compatible with dendritic cell differentiation (Figure 14).

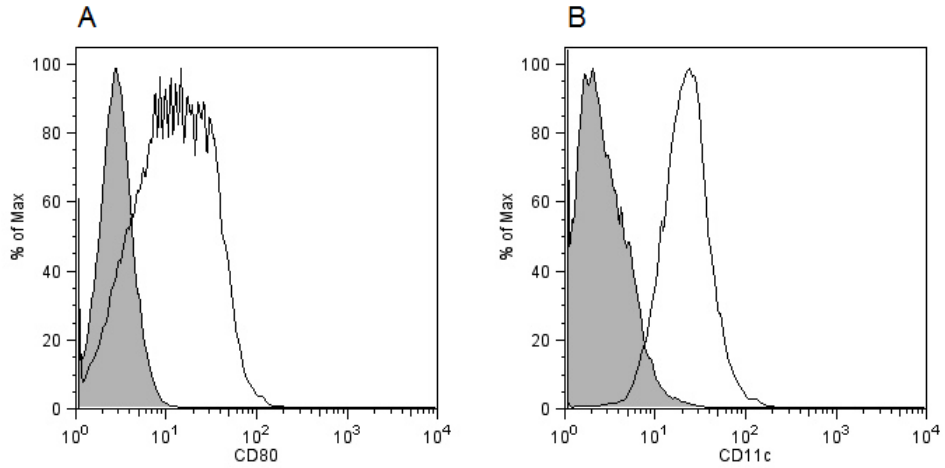

Figure 14. Representative example of monocyte derived dendritic cell CD80 (A) and CD11c (B) cell surface expression. Cells were stained with anti-human CD80 and CD11c and analysed by flow cytometry. Tinted line corresponds to unstained and black line to stained sample.

Five Fabry disease patients' (numbers 1, 2, 3, 5 and 6) and seven control subjects' dendritic cells were tested for cell surface expression of all group I CD1 molecules. In this section are shown representative examples of the results obtained. Complete data of CD1 expression in Fabry disease patients is available in appendix II.

Regarding CD1a, three patients (1, 5 and 6), two of which are under ERT, presented no alteration in dendritic cell surface expression (Figure 15A and appendix II-Figure S1). The other two patients (2 and 3), both under ERT, had a slight increase in the cell surface expression of CD1a (Figure 15B and appendix II-Figure S1) when compared to healthy subjects. The two patients that presented an increase in CD1a expression were tested again approximately two months later and the result was identical (data not shown).

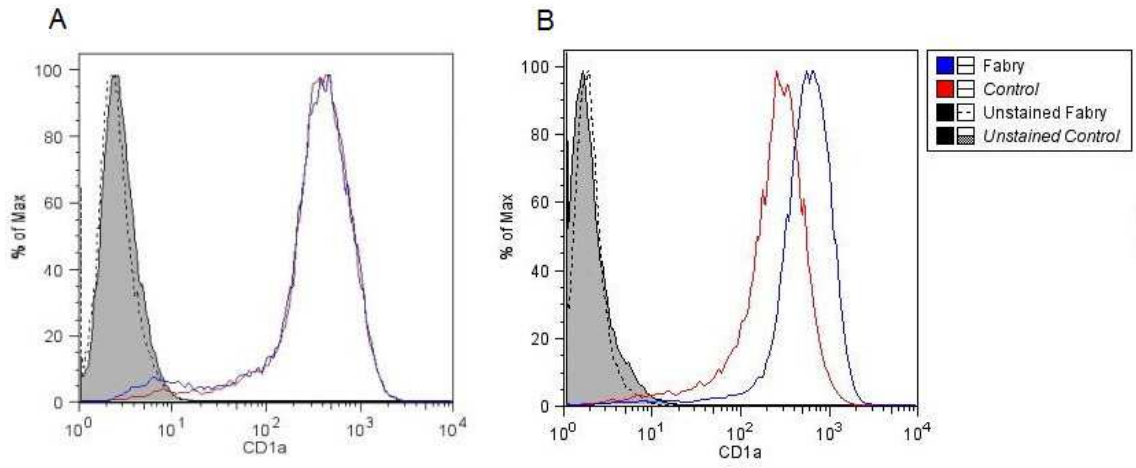

Figure 15. Examples of CD1a expression at the cell surface of dendritic cells from control subjects and Fabry disease patients analysed by flow cytometry. A – No differences between control and patient; B – Higher expression of CD1a in patients. For other patients' data see appendix II.

The results obtained for CD1b were similar to those of CD1a, with the same two patients showing increased CD1b expression and the other three presenting no differences (Figure 16 and appendix II-Figure S2). However, when the two patients with altered expression were tested once more, the difference in CD1b expression decreased or even disappeared (data not shown).

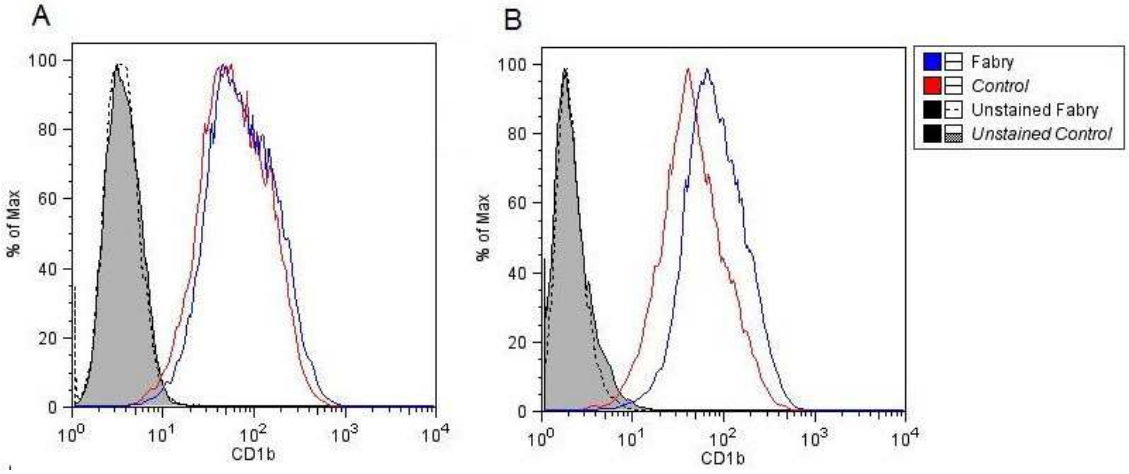

Figure 16. Examples of CD1b expression at the cell surface of dendritic cells from control subjects and Fabry disease patients analysed by flow cytometry. A – No differences between control and patient; B – Higher expression of CD1b in patients. For other patients' data see appendix II.

No differences were found in the dendritic cell surface expression of CD1c. The five patients expressed nearly the same amount of this molecule in comparison with the respective controls, as shown in Figure 17 and appendix II-Figure S3.

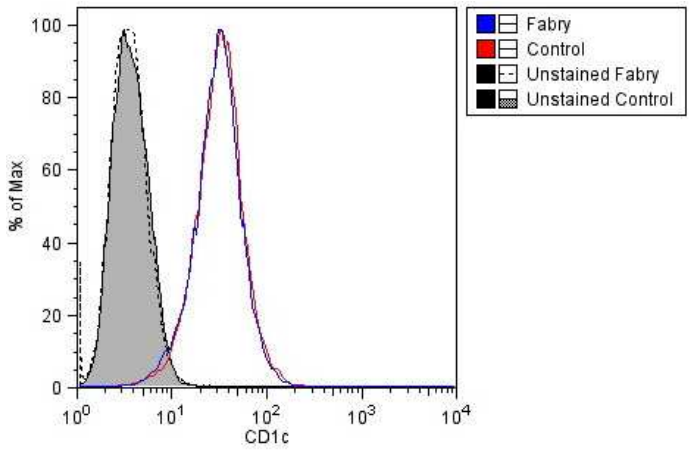

Figure 17. Example of CD1c expression at the cell surface of dendritic cells from control subjects and Fabry disease patients analysed by flow cytometry. No differences are present between patients and controls. For other patients' data see appendix II.

In this study, only Group I CD1 molecules were tested. This is justified by the fact that monocyte derived dendritic cells cultured in media containing FBS do not express CD1d at the cell surface in amounts detectable by flow cytometry [20].

According to these results, there are no major differences in CD1 molecules expression at the dendritic cell surface between control subjects and Fabry disease patients.

4.4 Lipid antigen presentation to T cells

The capacity of Fabry disease patients' dendritic cells to present lipid antigens and activate T cells in comparison to control subjects was assessed using an *in vitro* system of lipid antigen presentation. Briefly, monocyte derived dendritic cells were incubated with different concentrations of lipid antigen for four hours. Then, T cell clones, restricted to one of the CD1 molecules, were added and incubated for 36 hours, to allow activation of the T cell clones and consequent cytokine production. Finally, supernatant was collected and cytokine concentration was determined by ELISA. The amount of cytokine produced is related to the efficiency of the lipid antigen presentation by CD1 molecules. Two CD1 molecules, CD1b and CD1d, were tested. Five patients were tested for CD1b and four for CD1d. At the time of the lipid antigen presentation assays, patient 1 was under ERT for twelve months, patients 2 and 3 were under ERT for twenty months, patient 5 was under ERT for five months and patient 6 was not under ERT.

Lipid antigen presentation by CD1b

Lipid antigen presentation by CD1b was studied using two different self-antigens: sulfatide and GM1. To test the two different antigens, two CD1b-restricted T cell clones were needed: DS1C9b (sulfatide specific) and GG33A (GM1 specific). As shown in Figure 18, Fabry disease patients did not present differences in sulfatide presentation by dendritic cell CD1b.

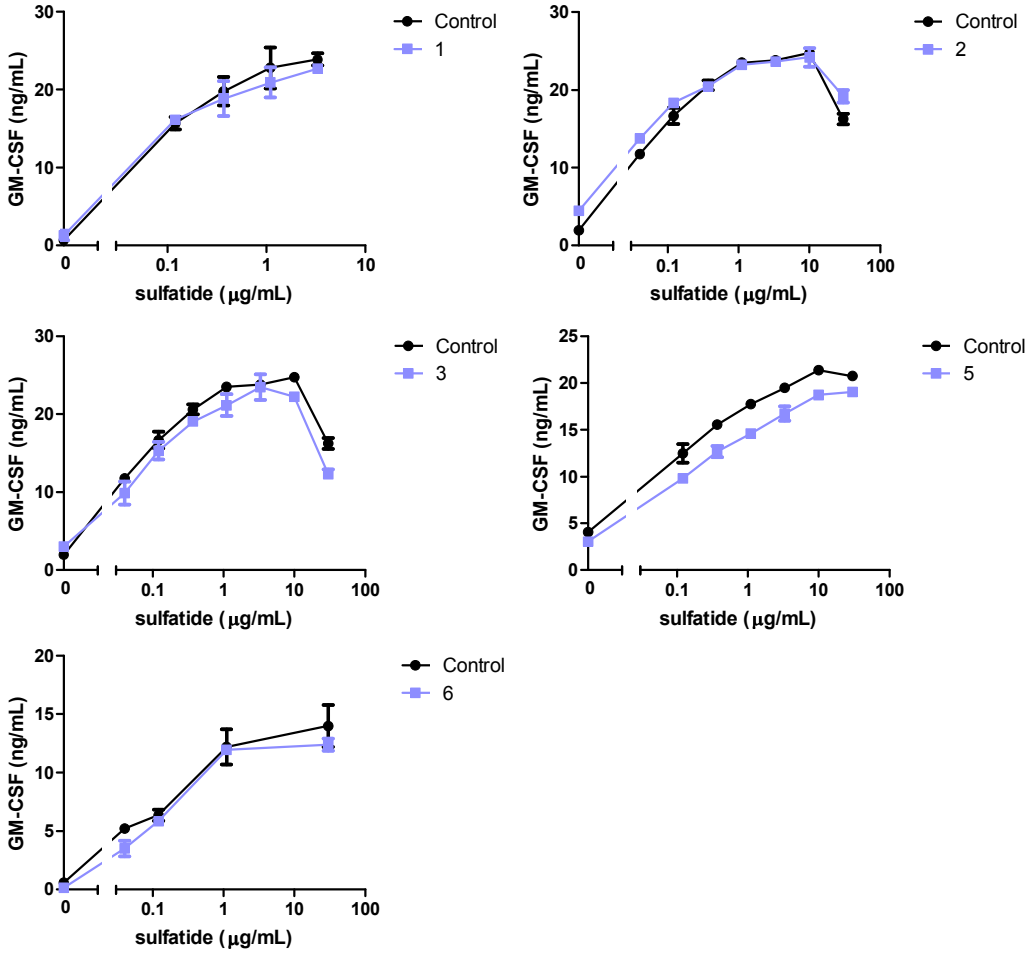

Figure 18. GM-CSF production by T cell clone DS1C9b activated with dendritic cells from a control subject or a Fabry disease patient. Dendritic cells were challenged with sulfatide for 4h and then the T cell clone was added to the culture. Supernatant was collected 36h later and cytokine concentration determined by ELISA. Values represent the Mean ± Standard deviation (SD) of duplicates.

Interestingly, when the presentation of GM1 by CD1b was studied in four patients, two Fabry disease patients showed reduced capacity of presentation (Figure 19). This difference was present already at the lowest concentration of antigen tested. Patient 2 had no alterations in antigen presentation and a forth patient appear to induce a stronger activation of T cells than the control subject. However, this was only verified for higher antigen concentrations and in this assay the control subject presented values of cytokine production below the ones obtained in the other three assays. Therefore, this patient will be again analysed. The alterations observed in GM1 presentation do not seem to be related

with age. Of the two younger patients (5 and 6), one presented a higher and the other a lower capacity of antigen presentation than the control subject.

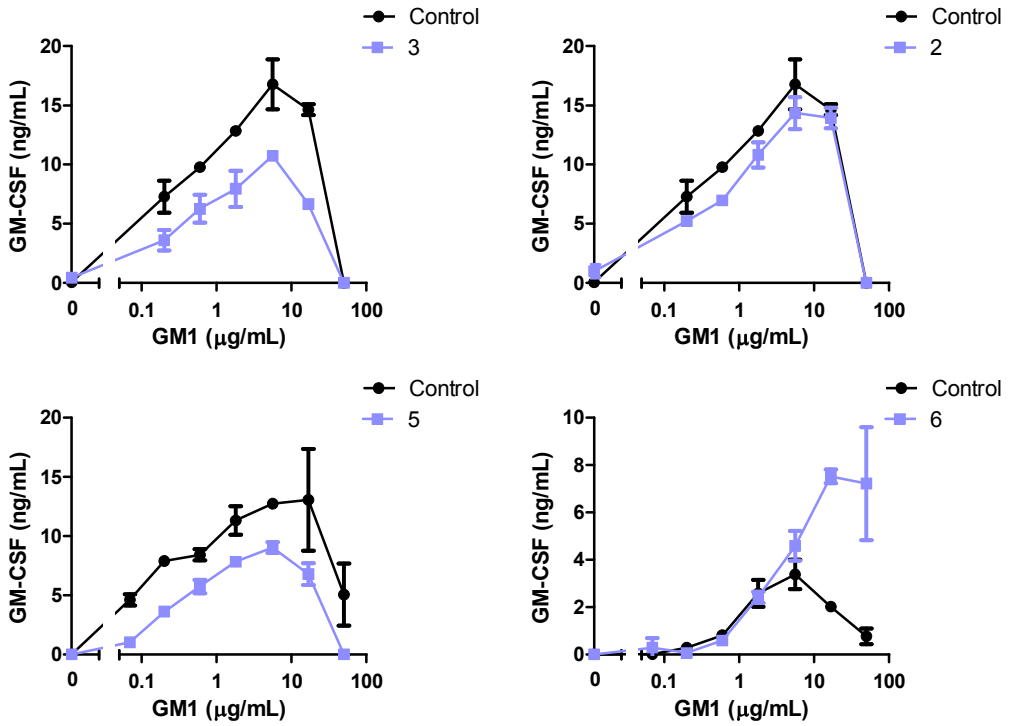

Figure 19. GM-CSF production by T cell clone GG33A activated with dendritic cells from control subjects or Fabry disease patients. Dendritic cells were challenged with GM1 for 4h and then the T cell clone was added to the culture. Supernatant was collected 36h later and cytokine concentration determined by ELISA. Values represent the Mean ± SD of duplicates.

The presence of higher variability in the presentation of the antigen GM1 in comparison to sulfatide by CD1b indicates that the accumulation of Gb3 in dendritic cells might have different influence in the presentation of different lipids by the same CD1 isoform.

Lipid antigen presentation by CD1d

Results of lipid antigen presentation by CD1d are presented in Figure 20. For this CD1 molecule, only the antigen sulfatide was tested. Sulfatide presentation was decreased in two Fabry disease patients and was similar in other two, when compared to normal subjects. The two patients that presented reduced ability to present sulfatide are the younger patients. At the time of the assay, patient 5 was under ERT for only five months

and patient 6 was not under ERT. Patients 2 and 3 were already under ERT for twenty months. Therefore, it is possible that treatment has an effect on lipid antigen presentation. The reduced capacity of CD1d to present sulfatide in the patients 5 and 6 does not appear to relate with the results obtained regarding iNKT cell percentage or the percentage of iNKT cell subsets. Despite the very low iNKT cell percentage of patient 5, patient 6 was one of the subjects with higher percentages of iNKT cells.

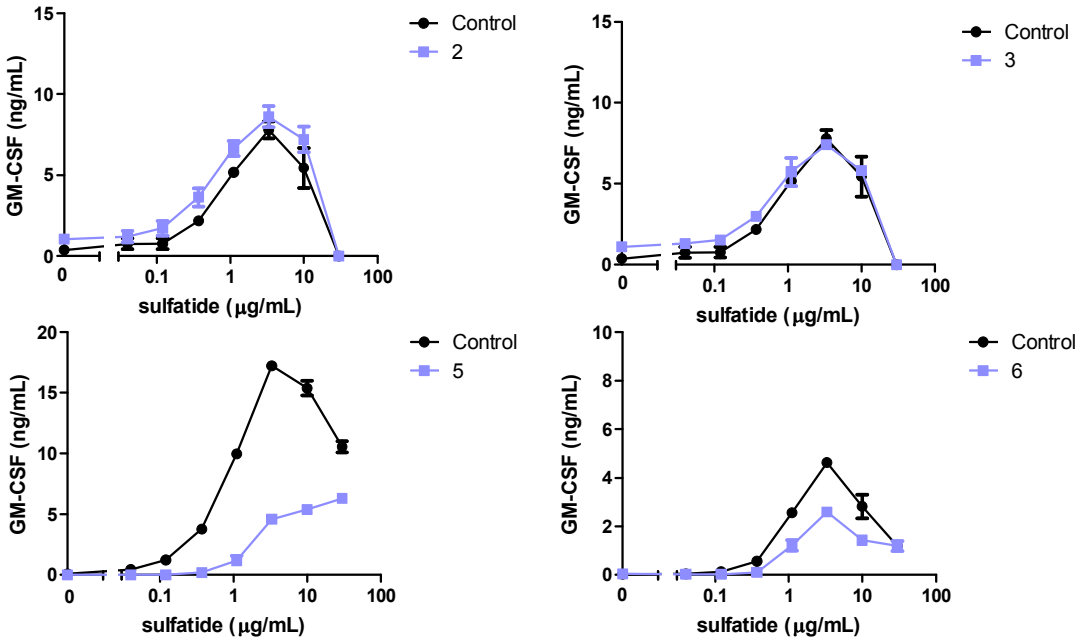

Figure 20. GM-CSF production by T cell clone s33d activated with dendritic cells from control subjects or Fabry disease patients. Dendritic cells were challenged with sulfatide for 4h and then the T cell clone was added to the culture. Supernatant was collected 36h later and cytokine concentration determined by ELISA. Values represent the Mean ± SD of duplicates.

5. Discussion

The lysosome is a key intracellular compartment for lipid antigen presentation and consequently plays an important role in the biology of CD1 restricted lipid specific T cells. Since in LSDs there is accumulation of undegraded macromolecules in the lysosome, it was hypothesized that lipid antigen presentation and thus CD1 restricted lipid specific T cells could be affected in LSDs. This issue was studied mainly by analysing the iNKT cell percentage in mouse models, since these cells are the only CD1 restricted T cells that can be easily detected. It was found that iNKT cells are decreased in the thymus, spleen and liver of various mouse models of LSDs, including in Fabry disease mice [46, 69-71]. Besides the low number of iNKT cells, it was also shown that Sandhoff, GM1 gangliosidosis, NPC1 and NPC2 deficiency mouse models have a decreased capacity to present lipid antigens to iNKT cells [46, 69, 71, 72]. iNKT cell percentage was also assessed in Fabry disease patients, but no alterations were found [73, 75].

In this study, we aimed to investigate the CD1 restricted lipid specific T cells and the process of lipid antigen presentation in Fabry disease patients. For this, we analysed the percentage of iNKT cells and the iNKT cell subsets in Fabry disease patients. Moreover, lipid antigen presentation assays were performed to evaluate the capacity of Fabry disease patients' dendritic cells to present lipid antigens by CD1b and CD1d.

5.1 iNKT cells and their subsets in Fabry disease patients

In accordance with the results previously described, in this study, no differences in iNKT cell percentage between control and Fabry disease patients were found [73, 75]. In the Fabry disease mouse model, it was described that iNKT cells are reduced in the thymus, spleen and liver [42, 69, 73]. This apparent discrepancy can be explained by our preliminary data obtained with the Fabry disease mice model. During the last year, besides the work presented in this thesis, I had the opportunity to collaborate in the study of iNKT cells present in different organs of the Fabry disease mouse model. Our results corroborate the previously described decrease in the iNKT cell percentage in the thymus, spleen and liver. However, we found no differences in the percentage of iNKT cells present in the lymph nodes and blood between Fabry disease and wild-type mice (Macedo et al,

unpublished results). The different effect of the metabolic alterations present in Fabry disease mice on the iNKT cells present in different organs can be explained by: altered traffic of iNKT cells; the difference in the organs lipid content and the presence of different APCs in different organs [50].

It was previously described that age and sex have an influence in iNKT cell percentage. The percentage of iNKT cells decreases with age [59, 62], and females tend to have more iNKT cells [80]. In the control group of our study, females tended to present higher iNKT cell percentages than males. The two Fabry disease females analysed presented low values of iNKT cells. However, due to low number of patients, it is still early to conclude about the effect of age and sex on the iNKT cells in Fabry disease patients.

In humans, iNKT cells can be divided in different subsets according to CD4 and CD8 expression. They can express only CD4 ($CD4^+$), only CD8 ($CD8^+$) or none of the molecules (DN). Regarding the iNKT cell subsets, Fabry disease patients presented lower values of iNKT $CD4^+$ cells and an increase in iNKT $CD4^-$ cells in comparison to control subjects. However, the conventional $CD4^+$ T cells were not decreased (data not shown). This indicates that the defect in the $CD4^+$ subset is specific for iNKT cells. The alterations in the iNKT cell subsets in Fabry disease patients are corroborated by our results with the Fabry disease mouse model. In the Fabry disease mouse model, a reduction in the $CD4^+/CD4^-$ iNKT cell ratio was observed in spleen, liver, lymph nodes and blood but not in the thymus (Macedo et al, unpublished results).

It was described that the iNKT cells tend to decrease and that the iNKT $CD4^+$ subset tends increase with age [59]. In this sample, no age related difference in iNKT $CD4^+$ cells was found. However, a broader age range of Fabry disease patients needs to be analysed to evaluate the effect of age on the $CD4^+$ iNKT cell subset. One of the younger patients tested presented unusually high percentages of the iNKT $CD4^+$ subset. This was the same patient that presented the lowest values of iNKT cells. In fact, it was described by Montoya et al, that the percentage of iNKT cells and the percentage of the iNKT $CD4^+$ subset are inversely correlated [80]. The reduction in the iNKT $CD4^+$ subset is associated with higher values of iNKT cells presented by three of the patients, however, other three patients' still present low levels of iNKT cells with a reduction in the iNKT $CD4^+$ subset.

It is known that iNKT $CD4^+$ cells produce both Th1 and Th2 cytokines, while iNKT $CD4^-$ produce mainly Th1 cytokines. Therefore, it is possible that Fabry disease patients present a bias towards Th1 cytokines. This bias would lead to a pro-inflammatory status that can be related to Fabry disease pathology. It was previously described that the presence of inflammation worsens the prognosis of patients with chronic kidney disease, that can also arise in Fabry disease [81]. Moreover, the inflammation marker C-reactive protein was shown to have a damaging effect on endothelial cells, one of the cell types most affected in Fabry disease [81].

Several factors can be involved in the reduction of the iNKT $CD4^+$ subset. Signalling through the TCR was shown to be important in the CD4-CD8 lineage differentiation of T cells *in vivo*. CD4 differentiation requires a prolonged TCR signal that needs to be present when cells start downregulating CD8. Otherwise, cells will enter the CD8 and not the CD4 lineage [82]. It was shown that iNKT cell development requires stronger signals than conventional T cells through the TCR, and that this signalling also seems to be important for differentiation of the iNKT $CD4^+$ subset [83]. Whereas the iNKT $CD4^+$ subset is mainly supported by thymic output and survival at the periphery with limited cell division, iNKT $CD4^-$ cells expansion occurs mainly in the periphery [66]. Moreover, the factors that are responsible for iNKT cell survival in the periphery vary between the subsets [66]. Some factors are involved in the expression of CD4 and repression of CD8 during the differentiation of CD1d-restricted $CD4^+$ iNKT cells. These include the transcription factors PLZF, GATA3 and ThPOK [84, 85]. Of those, PLZF is the one specific for iNKT cell development [85]. Experiments in PLZF knockout mice showed a selective defect in the iNKT cell development. These cells were reduced by 90-99% in the thymus, spleen and liver while other lymphocyte populations were preserved. Furthermore, the residual population of iNKT cells found in the spleen presented an increase in the proportion of iNKT DN cells, suggesting a role of PLZF in the maintenance of CD4 expression [85]. An alteration in one of these factors could be responsible for the iNKT $CD4^+$ subset reduction. Other conditions in which iNKT $CD4^+$ subset reduction was described in humans include HIV infection, type I diabetes mellitus, long-term rejection after kidney transplant and acute tubular necrosis after kidney transplant [57, 60, 61]. Interestingly, renal pathology is also one of the symptoms of Fabry disease.

ERT for twenty months had apparently no effect in the percentage of iNKT cells, in the four patients tested. These results are in agreement with the ones obtained by Rozenfeld et al, which did not found differences in iNKT cell percentage between Fabry disease patients not under and under treatment [75]. The same was verified for iNKT $CD4^+$ and DN subsets. However, it seems that iNKT $CD8^+$ cells are reduced with the treatment. All patients decreased iNKT $CD8^+$ cell percentage after the first months of treatment. It is necessary to continue the study and to include more patients in order to verify the effect of treatment in this subset.

5.2 Lipid antigen presentation by dendritic cells from Fabry disease patients

Despite the several studies on lipid antigen presentation in LSDs mouse models, herein, for the first time, lipid antigen presentation was analysed in LSDs patients, namely Fabry disease patients. Patients' dendritic cells generated from peripheral blood monocytes were used as APCs. The expression of group I CD1 molecules was analysed by flow cytometry. The Fabry disease patients tested presented no major differences in group I CD1 molecules cell surface expression in comparison to controls. However, two patients had slightly higher levels of CD1b and CD1a dendritic cell surface expression. For CD1b this small increase in CD1b cell surface expression did not have an effect on lipid antigen presentation capacity. CD1d cell surface expression was not detectable by flow cytometry. It was previously described that when dendritic cells are differentiated from monocytes in medium containing FBS, CD1d expression is downregulated [20]. It is possible to detect CD1d surface expression if the FBS is replaced by human serum, but there is downregulation of group I CD1 molecules.

To investigate lipid antigen presentation by Fabry disease dendritic cells, an *in vitro* study was prepared that was based in the co-culture of dendritic cells, lipid antigen and CD1-restricted T cells. The presentation through CD1b and CD1d was evaluated. Regarding CD1b presentation, two antigens were tested. When sulfatide was used, control subjects and patients presented similar levels of T cell activation, indicating that lipid antigen presentation was similar for both groups. However, when the antigen GM1 was used, higher variation was obtained in the results. While a patient promoted T cell responses stronger than the control, two other patients were less efficient in antigen

presentation than controls. Differences obtained with the antigen GM1 but not with sulfatide would suggest that CD1b molecules are functional and that there is some alteration related to the specific lipid that is being presented. This could be related with the different internalization pathways followed by GM1 and sulfatide. Sulfatide is internalized in a clathrin dependent way whether GM1 is internalized by caveolae [21].

Concerning presentation through CD1d, there were two patients that induced weaker T cell activation than controls. The other two patients presented no alterations in comparison to controls. It was expected to find a relationship between the efficacy of presentation by CD1d and iNKT cell percentage. In fact, the patient that presented lowest values of iNKT cells was also the one that had lower capacity of presenting antigens in comparison with the control subject, regarding presentation through CD1d. However, the other patient with reduced lipid antigen presentation had high levels of iNKT cells. While the patients that did not present alteration in antigen presentation through CD1d, patients number 2 and 3, were under ERT for 16 months, patient 5 was only being treated for 4 months and patient 6 was not under treatment (the two patients with reduced lipid antigen presentation capacity). It is possible that treatment is influencing the lipid antigen presentation capacity of the patients' dendritic cells, but it is necessary to analyse more patients. The two patients with lower capacity of antigen presentation in comparison to control subjects were also the younger patients.

Four of the five patients were already under ERT at the beginning of the study. ERT was shown to decrease the plasma levels of Gb3 accumulation within three months of treatment [14]. Therefore, it is possible that the reduction in lipid accumulation due to ERT corrects some defects in the lipid antigen presentation. For this reason, it is important to analyse patients that are not under ERT.

6. Conclusion and Future Perspectives

This study allowed the characterization of iNKT cell CD4/CD8 subsets in Fabry disease patients. A reduction in the iNKT $CD4^+$ subset in Fabry disease patients was described. This result is confirmed by the Fabry disease mouse model, which also presents a reduction in the iNKT $CD4^+$ cells (unpublished results, Macedo et al). Due to the different cytokine production profile of the human iNKT cell subsets, it would be interesting to analyse iNKT cell cytokine production in Fabry disease patients, to verify if there is a bias towards Th1 cytokines, as suggested by the decrease in the iNKT $CD4^+$ subset. The effect of ERT on iNKT cell subsets was also assessed. With the exception of iNKT $CD8^+$ cells, it did not seem to promote recovery from the imbalances initially observed. iNKT $CD8^+$ cells apparently decrease with ERT. To confirm this result it is important to continue the study in these patients and to include new subjects that are still not under ERT. No alteration in iNKT cell percentage in the peripheral blood of Fabry disease patients was found in comparison to controls. Our preliminary results in the Fabry disease mice model show that there are also no alterations in Fabry disease mice blood, despite the reduction in the thymus, spleen and liver. Therefore, it would be important to assess the impact of the metabolic alterations present in the Fabry disease mouse model in the iNKT cells percentage in different organs.

It was possible to conclude that group I CD1 molecules are normally expressed at the cell surface of dendritic cells. It would be interesting to analyse the expression of CD1d at the cell surface of dendritic cells, culturing them with human serum instead of FBS. Regarding lipid antigen presentation by CD1b, two different antigens were tested: sulfatide and GM1. With the antigen sulfatide it was observed that dendritic cells from Fabry disease patients are able to present this antigen by CD1b as well as dendritic cells from control subjects. Interestingly there was more variation when the antigen GM1 was presented by CD1b, which could be related to different internalization pathways of the two antigens. It would be interesting to test more antigens that are presented through CD1b, both self and non-self antigens. However, these assays are dependent on the availability of T cell clones specific for the various antigens. It is also important to test the lipid antigen presentation through the other two CD1 isoforms (CD1a and CD1c) to evaluate if lipid accumulation equally affects all CD1 isoforms. Moreover, it would be interesting to test

APCs other than dendritic cells, since several studies in mice found differences in lipid antigen presentation when splenocytes or thymocytes were used as APCs in lipid antigen presentation assays, in comparison to dendritic cells [69, 71]. Since B cells express CD1c and CD1d and monocytes express CD1d, it is possible to use them in lipid antigen presentation assays to assess if lipid accumulation equally affects APCs capacity to present lipid antigens.

The pathophysiology of LSDs remains largely unknown. Moreover, a relationship between the molecular basis of the disease and the biochemical and clinical features has been hard to establish. With the study of lipid antigen presentation in these diseases, we hope to contribute for the better understanding of the influence of the diverse LSDs metabolic defects in the process of lipid antigen presentation.

7. References

1. Parkinson-Lawrence, E.J., et al., *Lysosomal Storage Disease: Revealing Lysosomal Function and Physiology.* Physiology, 2010. **25**(2): p. 102-115.
2. Meikle, P.J., et al., *Prevalence of lysosomal storage disorders.* JAMA, 1999. **281**(3): p. 249-54.
3. Poorthuis, B.J.H.M., et al., *The frequency of lysosomal storage diseases in The Netherlands.* Human Genetics, 1999. **105**(1): p. 151-156-156.
4. Pinto, R., et al., *Prevalence of lysosomal storage diseases in Portugal.* European Journal of Human Genetics, 2004. **12**(2): p. 87-92.
5. Vellodi, A., *Lysosomal storage disorders.* British Journal of Haematology, 2005. **128**(4): p. 413-431.
6. Hers, H., *alpha-Glucosidase deficiency in generalized glycogenstorage disease (Pompe's disease).* Biochem. J., 1963. **86**: p. 11-16.
7. Desnick, R.J., Y.A. Ioannou, and C.M. Eng, *Alpha-galactosidase A deficiency: Fabry disease*, in *The Metabolic and Molecular Bases of Inherited Disease*. 2001, McGraw-Hill: New York. p. 3733–3774.
8. Garman, S.C., *Structure–function relationships in α-galactosidase A.* Acta Pædiatrica, 2007. **96**: p. 6-16.
9. Rosenthal, D., et al., *A novel alpha-galactosidase a mutant (M42L) identified in a renal variant of Fabry disease.* Am J Kidney Dis, 2004. **44**(5): p. e85-9.
10. Schiffmann, R., *Fabry disease.* Pharmacology & Therapeutics, 2009. **122**(1): p. 65-77.
11. Germain, D.P., *Fabry disease.* Orphanet Journal of Rare Diseases, 2010. **5**(1): p. 30.
12. Gaspar, P., et al., *Frequency of Fabry disease in male and female haemodialysis patients in Spain.* BMC Medical Genetics, 2010. **11**(1): p. 19.
13. Rozenfeld, P.A., *Fabry Disease: Treatment and diagnosis.* IUBMB Life, 2009. **61**(11): p. 1043-1050.
14. van Breemen, M.J., et al., *Reduction of elevated plasma globotriaosylsphingosine in patients with classic Fabry disease following enzyme replacement therapy.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2011. **1812**(1): p. 70-76.
15. Bricard, G. and S. Porcelli, *Antigen presentation by CD1 molecules and the generation of lipid-specific T cell immunity.* Cellular and Molecular Life Sciences, 2007. **64**(14): p. 1824-1840.
16. Strominger, J.L., *An Alternative Path for Antigen Presentation: Group 1 CD1 Proteins.* The Journal of Immunology, 2010. **184**(7): p. 3303-3305.
17. De Libero, G. and L. Mori, *How the immune system detects lipid antigens.* Progress in Lipid Research, 2010. **49**(2): p. 120-127.
18. Cohen, N.R., S. Garg, and M.B. Brenner, *Chapter 1 Antigen Presentation by CD1: Lipids, T Cells, and NKT Cells in Microbial Immunity*, in *Advances in Immunology*, W.A. Frederick, Editor. 2009, Academic Press. p. 1-94.
19. Delia, D., et al., *CD1c but neither CD1a nor CD1b molecules are expressed on normal, activated, and malignant human B cells: identification of a new B-cell subset.* Blood, 1988. **72**(1): p. 241-247.

20. Leslie, D.S., et al., *Serum lipids regulate dendritic cell CD1 expression and function.* Immunology, 2008. **125**(3): p. 289-301.
21. De Libero, G., A. Collmann, and L. Mori, *The cellular and biochemical rules of lipid antigen presentation.* European Journal of Immunology, 2009. **39**(10): p. 2648-2656.
22. Brozovic, S., et al., *CD1d function is regulated by microsomal triglyceride transfer protein.* Nature Medicine, 2004. **10**(5): p. 535-539.
23. Dougan, S.K., *Microsomal triglyceride transfer protein lipidation and control of CD1d on antigen-presenting cells.* Journal of Experimental Medicine, 2005. **202**(4): p. 529-539.
24. Kaser, A., et al., *Microsomal triglyceride transfer protein regulates endogenous and exogenous antigen presentation by group 1 CD1 molecules.* European Journal of Immunology, 2008. **38**(8): p. 2351-2359.
25. Zeissig, S., et al., *Primary deficiency of microsomal triglyceride transfer protein in human abetalipoproteinemia is associated with loss of CD1 function.* The Journal of Clinical Investigation, 2010. **120**(8): p. 2889-2899.
26. Angenieux, C., et al., *The cellular pathway of CD1e in immature and maturing dendritic cells.* Traffic, 2005. **6**(4): p. 286-302.
27. de la Salle, H., *Assistance of Microbial Glycolipid Antigen Processing by CD1e.* Science, 2005. **310**(5752): p. 1321-1324.
28. Jayawardena-Wolf, J., et al., *CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain.* Immunity, 2001. **15**: p. 897–908.
29. Kang, S.J. and P. Cresswell, *Regulation of intracellular trafficking of human CD1d by association with MHC class II molecules.* The EMBO Journal, 2002. **21**: p. 1650–1660.
30. Chen, X., et al., *Distinct Endosomal Trafficking Requirements for Presentation of Autoantigens and Exogenous Lipids by Human CD1d Molecules.* The Journal of Immunology, 2007. **178**(10): p. 6181-6190.
31. Salio, M., J.D. Silk, and V. Cerundolo, *Recent advances in processing and presentation of CD1 bound lipid antigens.* Current Opinion in Immunology, 2010. **22**: p. 81–88.
32. Holthuis, J.C.M. and T.P. Levine, *Lipid traffic: floppy drives and a superhighway.* Nature Reviews Molecular Cell Biology, 2005. **6**(3): p. 209-220.
33. Cernadas, M., et al., *Early Recycling Compartment Trafficking of CD1a Is Essential for Its Intersection and Presentation of Lipid Antigens.* The Journal of Immunology, 2009. **184**(3): p. 1235-1241.
34. Janeway, C., *Immunobiology : the immune system in health and disease*. 6th ed. 2005, New York: Garland Science. xxiii, 823 p.
35. de Jong, A., et al., *CD1a-autoreactive T cells are a normal component of the human alphabeta T cell repertoire.* Nature Immunology, 2010. **11**(12): p. 1102-9.
36. de Lalla, C., et al., *High-frequency and adaptive-like dynamics of human CD1 self-reactive T cells.* European Journal of Immunology, 2011. **41**: p. 602-610.
37. Kobayashi, E., et al., *KRN7000, a novel immunomodulator, and its antitumor activities.* Oncology research, 1995. **7**(10-11): p. 529-34.
38. Agea, E., *Human CD1-restricted T cell recognition of lipids from pollens.* Journal of Experimental Medicine, 2005. **202**(2): p. 295-308.

39. Mori, L. and G. De Libero, *Presentation of lipid antigens to T cells.* Immunology Letters, 2008. **117**(1): p. 1-8.
40. Shamshiev, A., et al., *Presentation of the same glycolipid by different CD1 molecules.* Journal of Experimental Medicine, 2002. **195**(8): p. 1013-21.
41. Shamshiev, A., et al., *The alphabeta T cell response to self-glycolipids shows a novel mechanism of CD1b loading and a requirement for complex oligosaccharides.* Immunity, 2000. **13**(2): p. 255-64.
42. Prigozy, T.I., et al., *Glycolipid Antigen Processing for Presentation by CD1d Molecules.* Science, 2001. **291**: p. 664-667.
43. Zhou, D., *Editing of CD1d-Bound Lipid Antigens by Endosomal Lipid Transfer Proteins.* Science, 2004. **303**(5657): p. 523-527.
44. Winau, F., et al., *Saposin C is required for lipid presentation by human CD1b.* Nature Immunology, 2004. **5**(2): p. 169-174.
45. Yuan, W., et al., *Saposin B is the dominant saposin that facilitates lipid binding to human CD1d molecules.* Proceedings of the National Academy of Sciences, 2007. **104**(13): p. 5551-5556.
46. Schrantz, N., et al., *The Niemann-Pick type C2 protein loads isoglobotrihexosylceramide onto CD1d molecules and contributes to the thymic selection of NKT cells.* Journal of Experimental Medicine, 2007. **204**(4): p. 841-852.
47. Godfrey, D.I., S. Stankovic, and A.G. Baxter, *Raising the NKT cell family.* Nature Immunology, 2010. **11**(3): p. 197-206.
48. Bendelac, A., P.B. Savage, and L. Teyton, *The Biology of NKT Cells.* Annual Review of Immunology, 2007. **25**(1): p. 297-336.
49. Berzins, S.P., M.J. Smyth, and A.G. Baxter, *Presumed guilty: natural killer T cell defects and human disease.* Nature Reviews Immunology, 2011. **11**(2): p. 131-142.
50. Seino, K.i., *Functionally distinct NKT cell subsets and subtypes.* Journal of Experimental Medicine, 2005. **202**(12): p. 1623-1626.
51. Lee, P.T., et al., *Distinct Functional Lineages of Human VÎ±24 Natural Killer T Cells.* The Journal of Experimental Medicine, 2002. **195**(5): p. 637-641.
52. Gumperz, J.E., et al., *Functionally Distinct Subsets of CD1d-restricted Natural Killer T Cells Revealed by CD1d Tetramer Staining.* The Journal of Experimental Medicine, 2002. **195**(5): p. 625-636.
53. Takahashi, T., et al., *Cutting Edge: Analysis of Human V{alpha}24+CD8+ NK T Cells Activated by {alpha}-Galactosylceramide-Pulsed Monocyte-Derived Dendritic Cells.* The Journal of Immunology, 2002. **168**(7): p. 3140-3144.
54. Bernardo, D., *Decreased circulating iNKT cell numbers in refractory coeliac disease.* Clinical Immunology, 2008. **126**(2): p. 172-179.
55. Bricard, G., et al., *Enrichment of Human CD4+ V 24/V 11 Invariant NKT Cells in Intrahepatic Malignant Tumors.* The Journal of Immunology, 2009. **182**(8): p. 5140-5151.
56. Dhodapkar, K.M., et al., *Invariant natural killer T cells are preserved in patients with glioma and exhibit antitumor lytic activity following dendritic cell-mediated expansion.* International Journal of Cancer, 2004. **109**(6): p. 893-899.
57. Galante, N., et al., *Frequency of Vα24Vβ11 NKT cells in peripheral blood of human kidney transplantation recipients.* International Immunopharmacology, 2005. **5**(1): p. 53-58.

58. Im, J.S., et al., *Alteration of the relative levels of iNKT cell subsets is associated with chronic mycobacterial infections.* Clinical Immunology, 2008. **127**(2): p. 214-224.
59. Jing, Y., et al., *Aging is associated with a rapid decline in frequency, alterations in subset composition, and enhanced Th2 response in CD1d-restricted NKT cells from human peripheral blood.* Experimental Gerontology, 2007. **42**(8): p. 719-732.
60. Kis, J., et al., *Reduced CD4+ subset and Th1 bias of the human iNKT cells in Type 1 diabetes mellitus.* Journal of Leukocyte Biology, 2006. **81**(3): p. 654-662.
61. Montoya, C.J., et al., *Invariant NKT cells from HIV-1 or Mycobacterium tuberculosis-infected patients express an activated phenotype.* Clinical Immunology, 2008. **127**(1): p. 1-6.
62. Peralbo, E., et al., *Decreased frequency and proliferative response of invariant Vα24Vβ11 natural killer T (iNKT) cells in healthy elderly.* Biogerontology, 2006. **7**(5-6): p. 483-492.
63. Takahashi, T., et al., *V alpha 24+ natural killer T cells are markedly decreased in atopic dermatitis patients.* Human Immunology, 2003. **64**(6): p. 586-92.
64. Chen, G.B., Z.H. Tang, and X.J. Bai, *Variation and significance of NKT cell and its subset in patients with severe multiple injuries.* Chinese Journal of Traumatology, 2009. **12**(6): p. 323-7.
65. Kronenberg, M. and I. Engel, *On the road: progress in finding the unique pathway of invariant NKT cell differentiation.* Current Opinion in Immunology, 2007. **19**(2): p. 186-193.
66. Baev, D.V., *Distinct homeostatic requirements of CD4+ and CD4- subsets of V 24-invariant natural killer T cells in humans.* Blood, 2004. **104**(13): p. 4150-4156.
67. Brigl, M. and M.B. Brenner, *How invariant natural killer T cells respond to infection by recognizing microbial or endogenous lipid antigens.* Seminars in Immunology, 2010. **22**(2): p. 79-86.
68. Zhou, D., et al., *Lysosomal Glycosphingolipid Recognition by NKT Cells.* Science, 2004. **306**(5702): p. 1786-1789.
69. Gadola, S.D., et al., *Impaired selection of invariant natural killer T cells in diverse mouse models of glycosphingolipid lysosomal storage diseases.* The Journal of Experimental Medicine, 2006. **203**(10): p. 2293-2303.
70. Plati, T., et al., *Development and maturation of invariant NKT cells in the presence of lysosomal engulfment.* European Journal of Immunology, 2009. **39**(10): p. 2748-2754.
71. Schümann, J., et al., *Differential alteration of lipid antigen presentation to NKT cells due to imbalances in lipid metabolism.* European Journal of Immunology, 2007. **37**(6): p. 1431-1441.
72. Sagiv, Y., et al., *Cutting Edge: Impaired Glycosphingolipid Trafficking and NKT Cell Development in Mice Lacking Niemann-Pick Type C1 Protein.* J Immunol, 2006. **177**(1): p. 26-30.
73. Balreira, A., et al., *Anomalies in conventional T and invariant natural killer T-cell populations in Fabry mice but not in Fabry patients.* British Journal of Haematology, 2008. **143**(4): p. 601-604.
74. Balreira, A., et al., *Evidence for a link between sphingolipid metabolism and expression of CD1d and MHC-class II: monocytes from Gaucher disease patients as a model.* British Journal of Haematology, 2005. **129**(5): p. 667-676.

75. Rozenfeld, P., et al., *Leukocyte perturbation associated with Fabry disease.* Journal of Inherited Metabolic Disease, 2009.
76. Darmoise, A., et al., *Lysosomal α-Galactosidase Controls the Generation of Self Lipid Antigens for Natural Killer T Cells.* Immunity, 2010. **33**(2): p. 216-228.
77. Liu, Y., et al., *A modified α-galactosyl ceramide for staining and stimulating natural killer T cells.* Journal of Immunological Methods, 2006. **312**(1-2): p. 34-39.
78. Shamshiev, A., et al., *Self glycolipids as T-cell autoantigens.* European Journal of Immunology, 1999. **29**(5): p. 1667-1675.
79. De Libero, G., *Methods for generation of T cell clones and epithelial cell lines from excised human biopsies or needle aspirates*, in *MHC. A Practical Approach*, N. Fernandez and G. Butcher, Editors. 1997, IRL: Oxford. p. 123-140.
80. Montoya, C.J., et al., *Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11.* Immunology, 2007. **122**(1): p. 1-14.
81. Suliman, M.E. and P. Stenvinkel, *Contribution of inflammation to vascular disease in chronic kidney disease patients.* Saudi J Kidney Dis Transpl, 2008. **19**(3): p. 329-45.
82. Liu, X. and R. Bosselut, *Duration of TCR signaling controls CD4-CD8 lineage differentiation in vivo.* Nature Immunology, 2004. **5**(3): p. 280-288.
83. Becker, A.M., et al., *Invariant NKT Cell Development Requires a Full Complement of Functional CD3 {zeta} Immunoreceptor Tyrosine-Based Activation Motifs.* J Immunol, 2010. **184**(12): p. 6822-6832.
84. Wang, L., et al., *The sequential activity of Gata3 and Thpok is required for the differentiation of CD1d-restricted CD4+ NKT cells.* European Journal of Immunology, 2010. **40**(9): p. 2385-2390.
85. Savage, A.K., et al., *The Transcription Factor PLZF Directs the Effector Program of the NKT Cell Lineage.* Immunity, 2008. **29**(3): p. 391-403.

Appendix I – Composition of the solutions used in the experimental work

ACK lysis solution

For a final volume of 500mL, add 4.15g of NH_4Cl and 0.5g of KCO_3 to H_2O. Adjust pH to 7.2.

Culture media:

- Culture medium:

  500 mL RPMI 1640 (Invitrogen)
  5mL of non essential aminoacids (Invitrogen)
  5mL of kanamycin (Invitrogen)
  5mL of sodium piruvate (Invitrogen)
  50mL (10%) of inactivated fetal bovine serum (Invitrogen)

- Dendritic cell culture medium

  Culture medium plus 25ng/mL of rhGM-CSF (immunoTools) and rhIL-4 (immunoTools).

- T cell culture medium

  500 mL RPMI 1640 (Invitrogen)
  5mL of non essential aminoacids (Invitrogen)
  5mL of kanamycin (Invitrogen)
  5mL of sodium piruvate (Invitrogen)
  25mL of human serum (Invitrogen)
  500µL of rhIL-2 (stock 10000U/mL) (Invitrogen)

Flow cytometry solution (PBS 0.2% BSA 0.1%NaN_3)

For 100mL of solution add 0.2g of bovine serum albumin (BSA) and 0.1g of NaN_3 to PBS 1x.

MACS buffer

Add 5mL of PBS, 2 mM EDTA, 5% BSA to 45mL of PBS 2 mM EDTA.

- PBS, 2 mM EDTA

  2mL of EDTA 0.5 M

50mL of PBS 10x
448mL of H_2O

- PBS, 5% BSA, 2 mM EDTA

  For a final volume of 50mL add 2.5g of BSA to PBS, 2 mM EDTA.

PBS 10x

For a total volume of 1000mL, add:

80g of NaCl
2g of KCl
6.09g of Na_2HPO4
2g of KH_2PO4

Adjust pH to 7.3

PBS 1x

For 500mL, add 50mL of PBS 10x to 450mL of H_2O.

PBS 1% formaldehyde

For a final volume of 4 mL add 1mL of 16% formaldehyde to 3mL of PBS 1x.

PBS 25% HS

For a final volume of 1mL add 250µL of human serum to 750µL of PBS 1x

ELISA solutions:

- Washing buffer (PBS 0.05% Tween20)

  For 100mL, add 50µL of Tween20 to PBS 1x.

- Blocking buffer (PBS 0.05% Tween20, 1% BSA)

  For a final volume of 100mL, add 50µL of Tween20 and 1g of BSA to PBS 1x.

Appendix II – CD1 molecules expression at the surface of dendritic cells

CD1a:

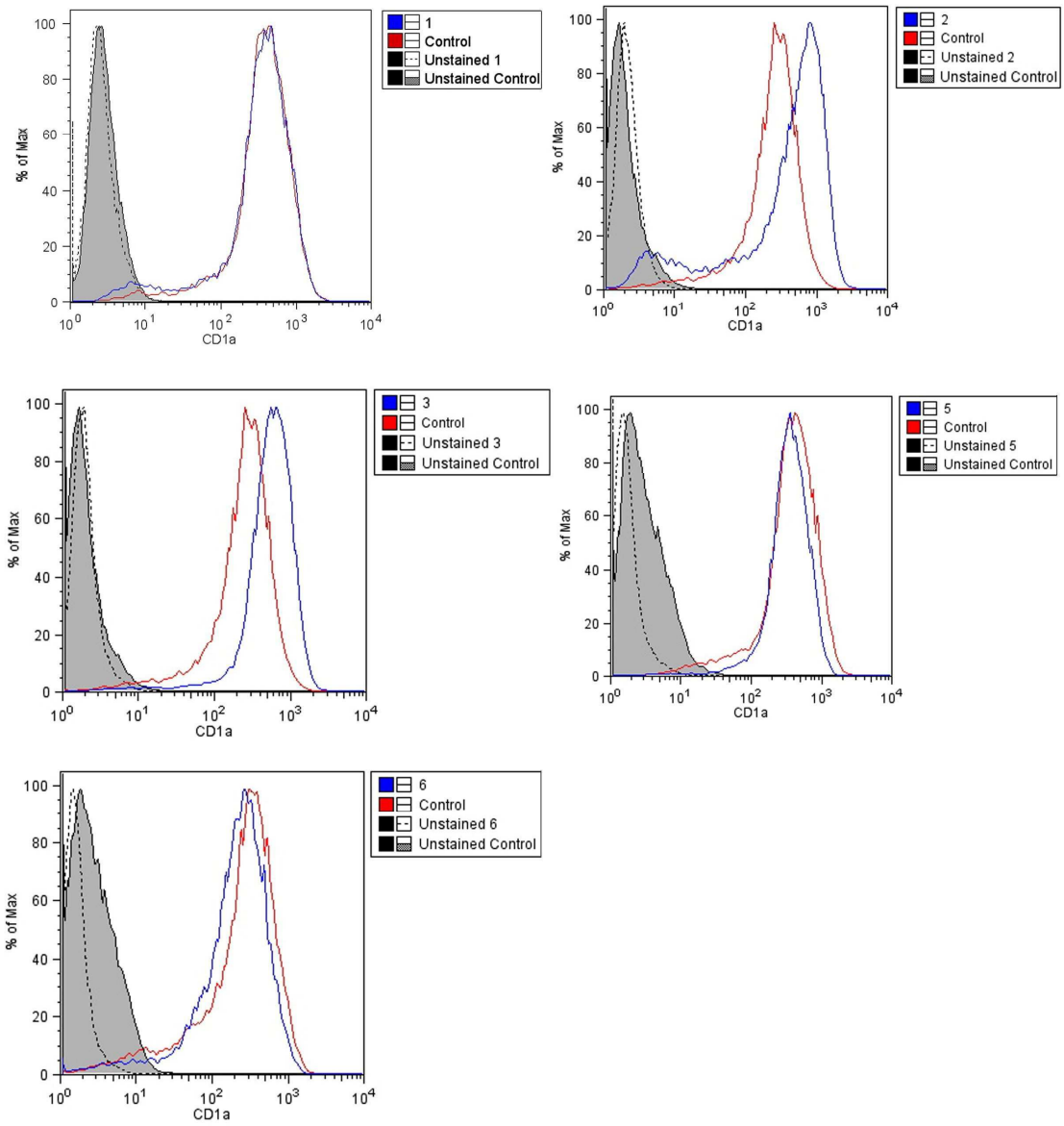

Figure S1. Histogram plots of CD1a expression at the cell surface of DC's from control subjects and FD patients. Dendritic cells were differentiated from monocytes and flow cytometry analysis of CD1 surface expression was performed at day 5 of culture.

CD1b:

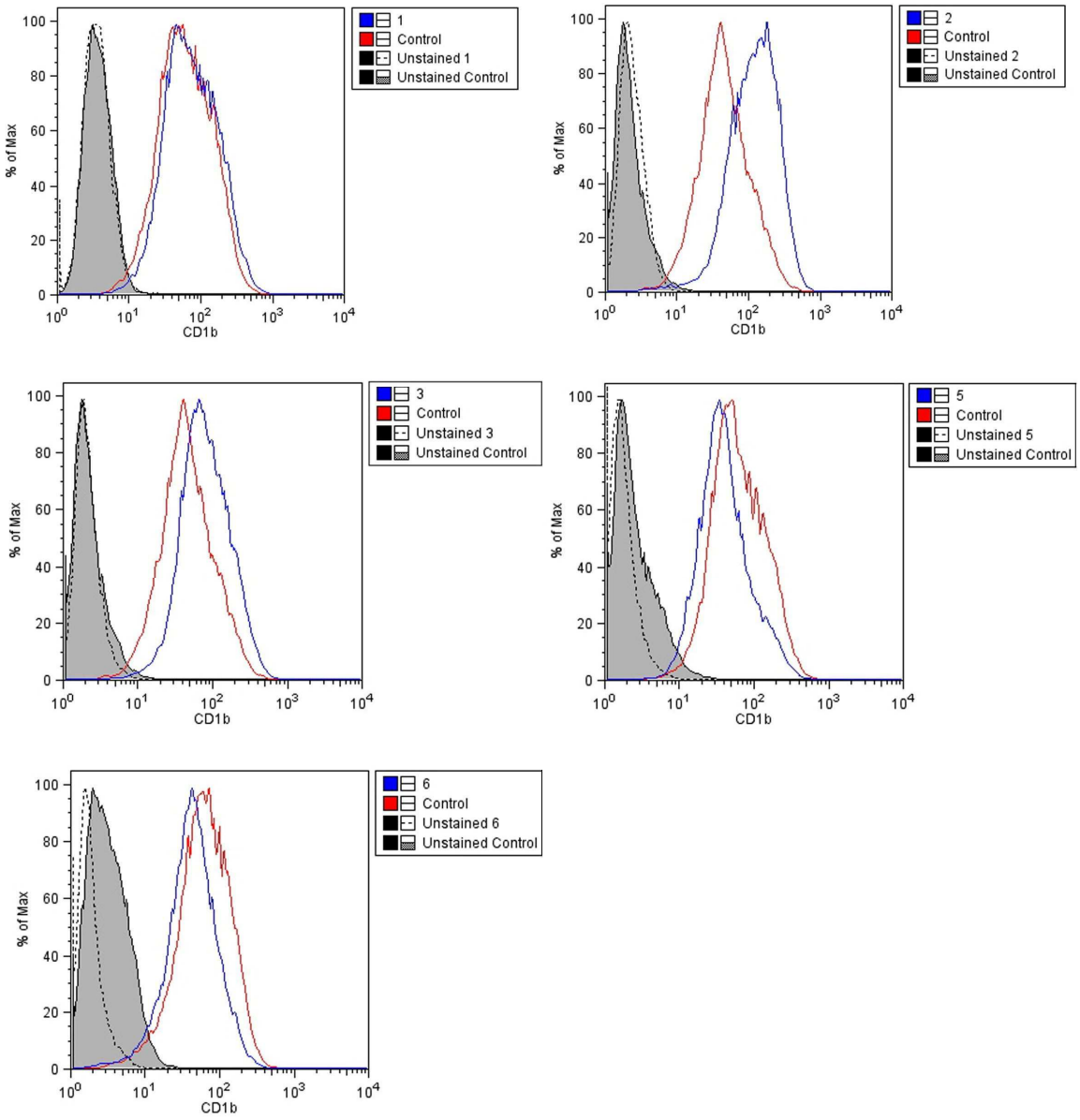

Figure S2. Histogram plots of CD1b expression at the cell surface of DC's from control subjects and FD patients. Dendritic cells were differentiated from monocytes and flow cytometry analysis of CD1 surface expression was performed at day 5 of culture.

CD1c:

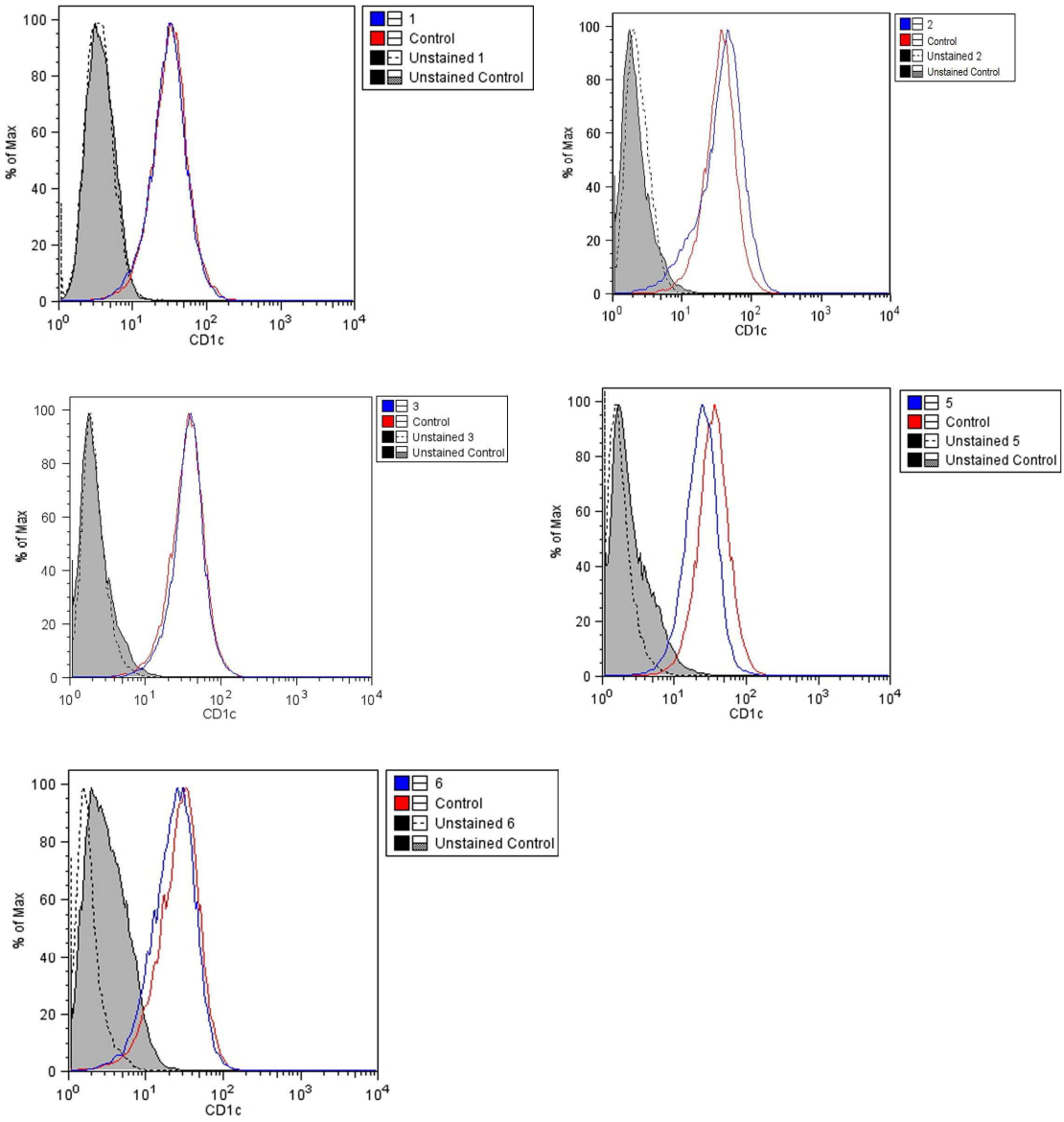

Figure S3. Histogram plots of CD1c expression at the cell surface of DC's from control subjects and FD patients. Dendritic cells were differentiated from monocytes and flow cytometry analysis of CD1 surface expression was performed at day 5 of culture.