Universidade de Aveiro Departamento de Biologia
2012

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Células T específicas de lípidos em doentes com Mucopolissacaridose VI

Lipid specific T cells in Mucopolysaccharidosis VI patients

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, 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

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agradecimentos

À minha orientadora, Doutora Fátima Macedo, por tudo o que me ensinou e por ter estado sempre disponível em todas as fases destes trabalho.

À Doutora Clara Sá Miranda, pela co-orientação e por ter tornado possível desenvolver este trabalho na UniLiPe.

Ao Daniel, pelo que me ensinou e por me ter acompanhado na fase inicial deste trabalho.

À Cátia e a Ana, por todo o apoio e por se mostrarem disponíveis sempre que precisei.

Aos membros da UniLiPe e OBF, por estarem sempre prontos a esclarecer qualquer dúvida.

Aos meus amigos, que sempre me apoiaram.

Ao meu namorado, por acreditar em mim e me dar na cabeça sempre que preciso.

Aos meus pais, que sempre estiveram ao meu lado e tornaram possível que eu chegasse até aqui.

À minha irmã, pelos bons momentos que fazem cada dia melhor.

À FCT, pelo financiamento que tornou possível a realização deste projecto.

palavras-chave

antigénios lipídicos; CD1; células dendriticas; células T restritas a CD1; doenças lisossomais de sobrecarga; Mucopolissacaridoses; Mucopolissacaridose tipo VI

resumo

Doenças de sobrecarga lisossomal (DSL) são um grupo de doenças metabólicas hereditárias causadas pela acumulação de moléculas não degradadas nos lisossomas, devido sobretudo a defeitos em enzimas lisossomais. Mucopolissacaridoses são DSL caracterizadas pela acumulação de glicosaminoglicanos anteriormente designados mucopolissacarídeos. O foco deste trabalho é a Mucopolissacaridose tipo VI (MPS VI), que resulta da defeciência de uma hidrolase lisossomal (Arylsulfatase B) responsável pela degradação do sulfato de dermatan, o que leva á acumulação desta macromolécula nos doentes. O lisossoma é um organelo importante na apresentação de antigénios lipídicos ás células T. A apresentação de antigénios lipídicos é mediada por moléculas CD1 existentes nas células apresentadoras de antigénios. A ligação do antigénio lipídico ás moléculas CD1 das células apresentadoras leva á activação das células T restritas a CD1 (NKT). Existem cinco isoformas de moléculas CD1 (a, b, c, d, e), mas apenas quatro são capazes de apresentar antigénios (a, b, c, d). Um dos locais na célula onde a associação das moléculas CD1 com os antigénios lipídicos ocorre é o lisossoma, portanto a apresentação de antigénios lipídicos pode estar afectada em doentes com DSL. Células NKT são um grupo heterogéneo de células T que partilham propriedades das células T e das células natural killer . Em humanos existem três subpopulações de células iNKT dependendo da expressão de CD4 e CD8: CD4+ (apenas expressam CD4), CD8+ (apenas expressam CD8) e duplas negativas (DN) que não expressam nenhumas das duas moléculas. Em estudos prévios foi observado em modelos animais de várias DSL uma diminuição na percentagem de células iNKT. Em doentes de Fabry e Gaucher não foram encontradas alteraçoes. O objectivo deste trabalho é estudar os linfócitos incluindo as células iNKT, as células dendríticas (como células apresentadoras de antigénios) e apresentação de antigénios lipídicos em doentes com MPS VI. Não foram encontradas alterações na percentagem de células iNKT assim como nas suas subpopulações entre doentes com MPS VI e indivíduos controlos. Curiosamente encontramos um aumento na percentagem de linfócitos B em doentes com MPS VI quando comparados com indivíduos controlo. Para determinar o fenótipo das células dendríticas três doentes foram analisados, encontramos para alguns destes doentes uma diminuição na expressão das moléculas CD1a, CD11c e HLA-DR (MHC-class II), mas para tirar mas conclusões mais doentes precisam ser analisados. Três doentes com MPS VI foram analisados para testar a capacidade das suas células dendríticas apresentarem antigénios lipídicos pela molécula CD1b. Não foram encontradas alterações na capacidade destes doentes apresentarem o antigénio lipídico GM1 pela molécula CD1b. Pela primeira vez foram realizados ensaios de apresentação de antigénios lipídicos em doentes com MPS.

keywords

CD1; CD1-restricted T cells; dendritic cells; lipid antigens; lysosomal storage diseases; Mucopolysaccharidoses; Mucopolysaccharidosis type VI

abstract

Lysosomal storage diseases (LSD) are a group of hereditary metabolic disorders caused by accumulation of undegraded molecules in the lysosome, mainly due to the impairment of the function of lysosomal enzymes. Mucopolysaccharidoses are LSDs characterized by the accumulation of glycosaminoglycans previously designated Mucopolysaccharides. The focus of this work is the Mucopolysaccharidosis type VI (MPS VI), which is a disorder caused by a deficiency in a lysosomal hydrolase (Arylsulfatase B) responsible for the dermatan sulfate degradation, that leads to an accumulation of this macromolecule in the patients. Lysosome is an important organelle in the presentation of lipid antigen to T cells. Lipid antigen presentation is mediated by CD1 molecules existent in the antigen presenting cells. The binding of lipid antigens and the presenting cells containing CD1 molecules lead to activation of T cells that respond to those molecules. There are five isoforms of CD1 molecules (a, b, c, d, e), but only four are antigen presenting (a, b, c, d). One of the cell locations where the association of the CD1 molecules and lipid antigens occurs is the lysosome, so that means that antigen presentation could be affected in LSDs patients. NKT cells are a heterogeneous group of T cells that share properties with T cells and natural killer cells. In humans there are three subpopulations depending on the expression of CD4 and CD8 molecules: CD4+ (only express CD4), CD8+ (only express CD8) and double negative (DN) that do not express any of them. In previous studies a decrease in the percentage of iNKT cells were observed in mouse models of several LSDs. However in patients with Fabry and Gaucher diseases no alterations were found. The aim of this work is to study the lymphocytes including the iNKT cells, the dendritic cells (as antigen presenting cells) and the lipid antigen presentation in MPS VI patients. We found no alterations in the percentage of the iNKT cells and in their subsets between MPS VI patients and control subjects. Interestingly we found an increase in the percentage of the B lymphocyte population in MPS VI patients when compared with control subjects. For dendritic cells phenotype three patients were analyzed, we found for some of them a decrease of the expression of CD1a, CD11c and HLA-DR (MHC-class II) however, more patients need to be study before conclusions can be drawn. In lipid antigen presenting assays, three patients were tested for the capacity of their dendritic cells to present lipid antigens by CD1b molecule. We found no alterations in patients' capacity to present the lipid antigen GM1 by CD1b molecule. Studies regarding the lipid antigen presentation were for the first time performed in MPS.

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List of abbreviations

α-GalCer	α-Galactosylceramide
APC	Antigen presenting cell
ASB	Arylsulfatase B
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
GM1	Monosialotetrahexosylganglioside
GM2	Disialotetrahexosylganglioside
GM3	Monosialodihexosylganglioside
GM-CSF	Granulocyte macrophage colony stimulating factor
iNKT	invariant Natural Killer T
LSD	Lysosomal storage disease
LTP	Lipid transfer protein
LTR	Toll-like receptor
MHC	Major histocompatibility complex
MPS	Mucopolysaccharidosis
N_2	Nitrogen
NKT	Natural Killer T
NPC	Niemann-Pick C
PBMC	Peripheral blood mononuclear cells
TCR	T cell receptor

1. Introduction

1.1 Lysosomal storage diseases

The lysosome was discovered in 1955, by Christian De Duve. The lysosome is an acidic organelle, containing many hydrolytic enzymes which are responsible for macromolecule degradation [1].

Lysosomal storage diseases (LSDs) are a group of metabolic disorders caused by a defect in lysosomal proteins, which normally result from a function defect of lysosomal enzymes (that is the case of Mucopolysaccharidoses) however can also be a result of a defect in a transport channel or regulatory protein (Niemann Pick type C disease). The dysfunction of those enzymes results in impaired substrate degradation and subsequently induces the accumulation of biological materials [2, 3]. The accumulation of undigested macromolecules compromises the cell function, which can lead to an organ failure and in some cases early death [4].

LSDs are a group of more than 50 distinct genetic diseases [4]. They are individually rare, but when combined the prevalence estimated is 1 in 8000 births. The prevalence of those rare disorders has been studied in many countries. The frequency of LSDs in Portugal is 1 in 4000 [5], which is a high frequency when compared with other countries. In Australia the frequency is around 1 in 7700 births [6]. In Netherlands the frequency reported is 1 in 7143 births [7], in Italy the prevalence of these disorders is 1 in 8264 births [8]. A recent study shows that the prevalence in Czech Republic is 1 in 8163 births [9].

LSDs are classified according to the biochemical nature of the stored material into: lipidoses (mainly sphingolipidoses), mucopolysaccharidosis, glicogenoses, glycoproteinoses, neuronal ceroidlipofuscinoses and mucolipidoses [2].

Pompe disease was the first recognized LSD, this disease is characterized by the accumulation of glycogen in the lysosome. It was first described in 1932, prior to the discovery of the lysosome [4]. In the year of 1963, this glycogen storage disease was associated with a defect in the lysosomal enzyme α-glucosidase, and so recognized as an LSD [10].

1.1.1 Mucopolysaccharidoses

The Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders caused by deficiency of specific lysosomal enzymes that catalyze the degradation of glycosaminoglycans (GAGs) previously called mucopolysaccharides. GAGs with the exception of hyaluronic acid are degraded products of proteoglycans which exist in the extracellular matrix. The GAGs enter in the lysosome for intracellular digestion. In MPS the undegraded or partially degraded GAGs are stored in lysosomes, this accumulation in various organs of patients, results in several organ dysfunctions, which lead to a multisystemic clinical picture [11, 12].

Lysosomal degradation of GAGs has different pathways depending on the molecule to be degraded: dermatan sulfate, heparan sulfate, keratan sulfate and chondroitin sulfate. This process requires ten different enzymes: 4 exoglycosidases, 5 sulfatases and 1 monhydrolytic transferase [12].

Patients with MPS have GAGs degradation impaired, although the presentation of GAGs to lysosome for degradation occurs normally which cause a continuous storage that gives rise to an enlargement of the lysosomes. The increase of the size and impaired function of lysosomes could compromise other cellular organelles [12].

The different types of MPS share many clinical features in variable degrees. Those include a chronic and progressive course, multisystem involvement, organomegaly, dysostosis multiplex and facial dysmorphia. Hearing, vision, airway, cardiovascular function and joint mobility may be affected. Profound mental retardation is a characteristic of MPS I, the severe form of MPS II and all subtypes of the MPS III, in other MPS normal intellect may be retained [11].

The enzymatic defects that lead to the diverse MPS types and the main substrates accumulated are described in Table 1.

Table 1. Mucopolysaccharidoses: defective enzyme and accumulated substrates.

Disease	Subtype	Enzyme deficiency	Accumulated GAG
MPS I	Hurler	α-L-iduronidase	Dermatan and heparan sulfate
	Hurler-Scheie	α-L-iduronidase	Dermatan and heparan sulfate
	Scheie	α-L-iduronidase	Dermatan and heparan sulfate
MPS II (Hunter)		Irudonate sulfatase	Dermatan and heparan sulfate
MPS III	Sanfilippo A	Heparan-N-sulfatase	Heparan Sulfate
	Sanfilippo B	α-N-Acetyl-glucosaminidase	Heparan Sulfate
	Sanfilippo C	Acetyl-CoA:α-glucosaminide acetyltransferase	Heparan Sulfate
	Sanfilippo D	N-acetylglucosamine 6-Sulfatase	Heparan Sulfate
MPS IV	Morquio A	Galactose 6-sulfatase	Keratan and chondroitin sulfate
	Morquio B	β-galactosidase	Keratan sulfate
MPS V		This designation is no longer used	
MPS VI (Maroteaux-Lamy)		Arylsulfatase B N-acetylgalactosamine 4-sulfatase	Dermatan sulfate
MPS VII (Sly)		β-glucuronidase	Dermatan, keratan and chondroitin sulfate
MPS VIII		This designation is no longer used	
MPS IX		Hyaluronidase	Hyaluronan

The focus of this study is Mucopolysaccharidosis type VI, which is described in the following section.

1.1.1.1 Mucopolysaccharidosis VI

Mucopolysaccharidosis type VI (MPS VI) is an autosomal recessive MPS, which results from a deficiency of arylsulfatase B also called N-acetylgalactosamine 4-sulfatase. MPS VI is also known as Maroteaux-Lamy syndrome named in 1963 after Dr. Pierre Maroteaux and Dr. Maurice Lamy that first described this disease [13]. Arylsulfatase B (ASB) is a lysosomal enzyme that removes the sulfate group from the dermatan sulfate GAG [12], the degradation pathway of dermatan sulfate are represented in Figure 1. Reduced or absent activity of the ASB lead to an incomplete degradation and cellular accumulation of the dermatan sultafe, causing cell injury [14].

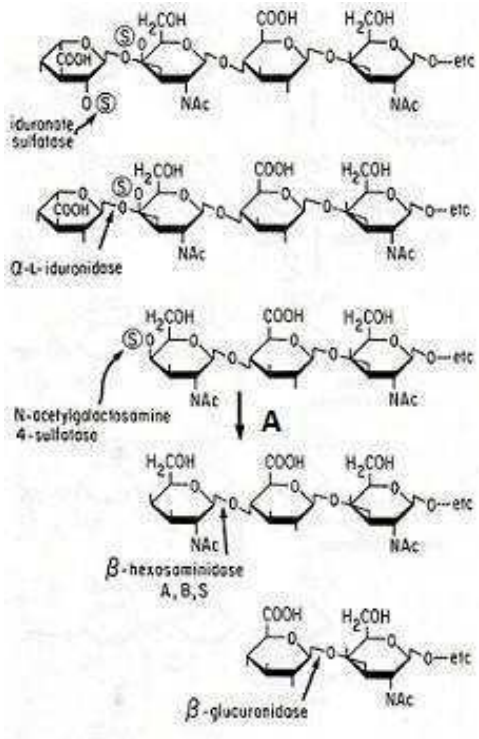

Figure 1. Degradation pathway of dermatan sulfate, (A) represents the step that causes the MPS VI deficiency. Image adapted from [11].

The estimated prevalence of MPS VI in north Portugal is 1 in 238 095 live births [5], in Australia is 1 in 248 372 live births, but in north countries like Sweden or Norway the prevalence is really low comparing with other counties, is 1 in 1 505 160 live births in Sweden and 1 in 1 455 813 live births in Norway [14].

Molecular basis

MPS VI is an autosomal recessive disorder resulting from the mutations in the gene encoding the ASB enzyme (*ARSB*), which is located in chromosome 5 contain 8 exons and span about 206 Kb [11, 12]. To date, there are more than 130 described mutations in *Human Gene Mutation Database* including nonsense and missense mutations, which are the most common mutations in this pathology, in addition can happen splicing mutations, deletions and insertions [14].

Clinical Manifestations

The characteristic symptoms of MPS VI are: hearing and visual impairment, which can lead to a loss of hearing and blindness; upper airway obstructions and enlargement of the tongue, which can cause asphyxia; skeletal deformities and compromised growth; nervous system disorders including hydrocephalus, spinal cord disorders and compressive neuropathies, but normal intelligence. Frequently poor clearance of airway secretions leads to a recurrent pneumonia. The most common cause of death of these patients are cardiac diseases caused by abnormal storage of dematan sulfate in the heart and blood vessels [13, 14].

The severity of clinical manifestation of MPS VI depends of the onset age and rate of the disease progression. The rapid progress of the disease is characterized by high levels of GAGs in urine, between 100 and 200µg/mg creatinine. In most cases the onset is before 2 or 3 years of age, symptoms include impaired mobility by 10 years old, absent or delayed puberty, cervical spinal cord compression, respiratory insufficiency and surgical complications. Patients frequently die from heart complications at 20 or 30 years. The slow progress is characterized by levels of GAGs in urine lower than 100µg/mg creatinine and in this case the symptoms have a later onset and the diagnosis generally occurs after 5 years of age. These patients eventually develop serious manifestations, such as joint degeneration, cardiac valve disease, sleep apnea, a decrease in pulmonary function and reduced endurance [14].

Diagnosis

Diagnosis is necessary to distinguish between type VI and other types of MPS, because they share similar symptoms. The diagnosis can include evidence of clinical phenotype, analysis of GAGs quantity in urine and analysis of ABS enzyme activity in isolated leukocytes, cultured skin fibroblasts or in dried blood spot this activity is generally less than 10% of the lower limit of normal ABS activity [13, 14]. The biochemical diagnosis in our laboratory is done by measurement of the ABS enzyme activity in dried blood spot and genotypic alterations were identified by DNA sequencing.

Treatment

The main focus of the treatment of MPS before the use of recent therapies like enzyme replacement therapy was to prevent complications and to improve the quality of life in the patients. This first treatment was symptomatic and palliative, based on a multidisciplinary team with diverse clinical specialties, including nutrition counseling, occupational and physical therapy and management of individual symptoms. Surgical intervention may be needed, depending on the severity of the symptoms [14, 15].

Bone marrow transplantation has been used in rare cases, because the risk of death, morbidity and difficulty obtaining adequate or optimal HLA-matched donors [14].

Enzyme replacement therapy for the treatment of MPS VI is available since 2005, and is performed by intravenous administration of Naglazyme (galsulfase) a recombinant form of the enzyme ASB. Naglazyme can be administered to all symptomatic patients [15]. The patients under this therapy reveled endurance improvement and a decrease of GAGs level in urine [16].

1.2 Presentation of lipid antigens to T cells

The presentation of lipid antigen to T cells is mediated by CD1 molecules present at the surface of antigen presenting cells (APCs). The lipid antigen bound with CD1 molecules is recognized by the T cell receptor (TCR) that causes the activation of T cells, which leads to their proliferation, cytokine production and in some cases cytolytic activity. A design of this process is shown on Figure 2.

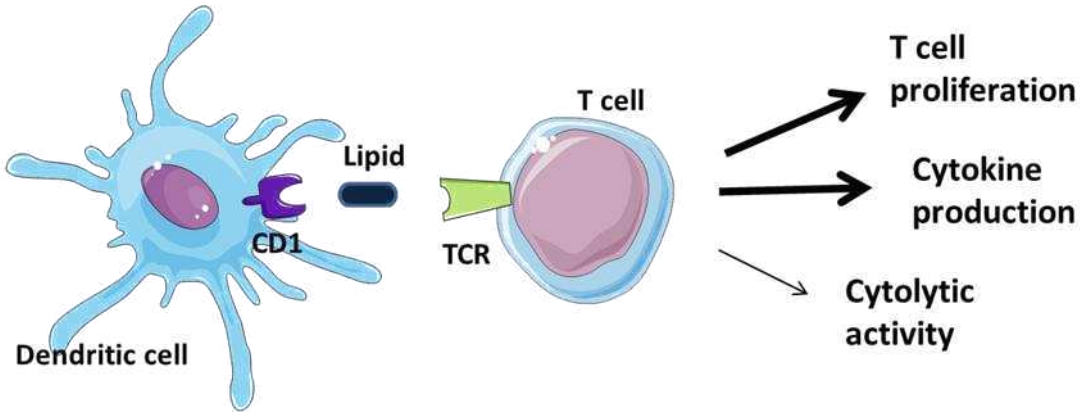

Figure 2. Activation of CD1-restricted lipid-specific T cells by dendritic cells.

1.2.1 The nature of lipid antigens

The lipid antigens can be divided in two groups, the self lipid antigens that have origin in the body and the non-self that originate from external microbial or environmental sources. Lipid antigens can also be classified depending on the location of synthesis, as endogenous if they are synthesized in the same APC that interacts with the T cells or exogenous if they are synthesized in other cells and then transferred to the APC [17]. *Mycobacteria*, *Spingomonas*, *Ehrlichia* and *Borrelia* are examples of bacteria for which were identified lipid antigens [17]. The α-galactosylceramide (α-GalCer) is the non-self lipid antigen most potent and studied, and was originally isolated from the *Agelas mauritianus* sponges [18]. The great majority of identified lipid antigens are glycosphingolipids. Several glycosphingolipids of self origin were identified as lipid antigens, such as GM1,

sulfatide, GD3 and β-D-glucopyranosylceramide $C_{24:1}$ [19, 20]. Mono-alkyl glycerophosphates were recently identified as an important lipid family that contributes to the maturation of iNKT cells [21].

1.2.2 CD1 molecules

The CD1 molecules are MHC class I-like glycoproteins composed by a heavy and a light chain. The heavy chain, which is the α chain is divided in three extracellular domains: α1, α2 and α3. The α3 domain is noncovalently associated with β2-micoglobulin which corresponds to the light chain of the CD1 molecule. The α1 and α2 domains are organized in two α-helices and compose the lipid antigen binding pocket [22, 23].

CD1 expression

CD1 molecules are expressed by a variety of cell types including dendritic cells (DCs), B cells, monocytes, Langerhans cells, stellate hepatic cells, epithelial cells, microglial cells and keratinocytes. Mice only have one isoform, the CD1d, although in humans five CD1 genes have been identified on chromosome 1. These genes encode CD1 proteins that represent distinct CD1 isoforms, which are CD1a, CD1b, CD1c, CD1d and CD1e [23, 24]. These isoforms can be divided in three groups according to homology of the α1 and α2 domains. Group I is composed by CD1a, CD1b and CD1c isoforms, group II by CD1d and group 3 by CD1e [19]. The CD1e isoform is involved in lipid antigen processing, but this isoform does not reach the cell surface and consequently is not involved in lipid antigen presentation [23].

CD1 synthesis and trafficking

The CD1 proteins are synthesized in the endoplasmatic reticulum (ER) and in this organelle the glycosylation is initiated. The following process is the association of β2-microglobulin with CD1 α chain. The correct folding of CD1 proteins is assisted by the interaction with chaperone proteins such as calnexin,

calcireticulin and ERp57 [23]. Endogenous lipids in the ER bind CD1 molecules during assembly and probably prevent their collapse [24].

After being synthesized in the ER CD1 molecules migrate to the Golgi apparatus where the glycosylation process is completed, and then move to the cell surface with exception of CD1e [23].

Some potent lipid antigens can be presented by the CD1 isoforms at the cell surface. However, for presentation of other lipid antigens, CD1 molecules need to be internalized, following the endocytic pathway [19]. CD1 isoforms pathways are described in Figure 3.

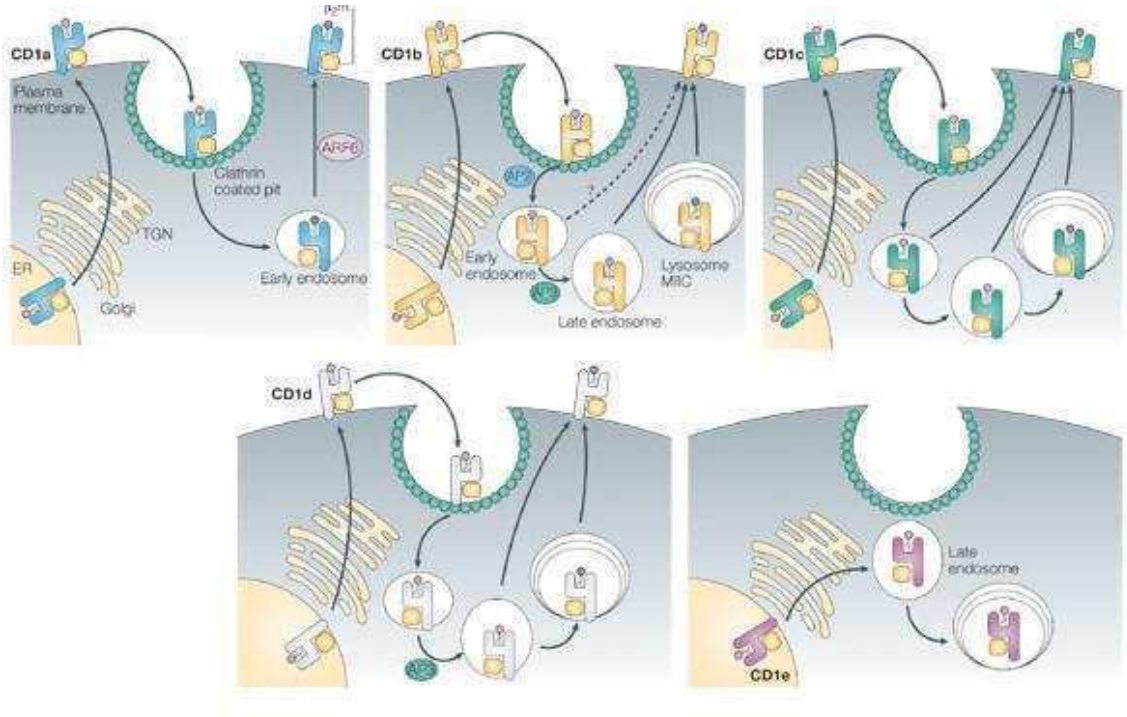

Figure 3. CD1 isoforms are transported through the Golgi and trans Golgi network (TGN) to reach the cell surface, with the exception of CD1e, which remains in the cell. Image from [25].

1.2.3 CD1 molecules loading with lipid antigens

Lipids are poorly soluble in water, so they need to form a complex with plasma lipoproteins, which facilitate their uptake by APC. The hidrolases α-galactosidade A, hexosaminidase B and acidic α-mannosidase are involved in

partial degradation of complex glycolipid antigens. This degradation is needed for the binding of lipids containing large hydrophilic moieties to CD1 molecules [24].

Lipid transfer proteins (LTPs) such as saposins, GM2-activator protein and Niemann-Pick C2 protein facilitate the extraction of lipids from lysosomal membranes and loading in CD1 molecules [24, 26]. CD1e is also an LTP required for the processing of complex mycobacterial antigens [27]. Another LTP with immunological relevance is the microssomal triglyceride transfer protein which has been shown to regulate the loading of self-lipids onto CD1 molecules in the ER [26, 28]. Besides LTPs other factor that facilitates the loading of lipid antigens onto CD1 molecules is the low pH present in the lysosome [28].

1.2.4 CD1-restricted T cells

CD1-restricted T cells have a TCR that recognize the CD1 molecules at the surface of an APC. These CD1-restricted T cells are classified in two groups according to the CD1 isoforms to which they are restricted.

Group I CD1-restricted T cells

Group I includes T cells restricted to CD1a, CD1b and CD1c, which can express a αβ or γδ TCR. Group I restricted T cells can express at the surface CD4 ($CD4^+$), CD8 ($CD8^+$) or neither of the molecules, being CD4-CD8 double negative (DN). Group I CD1-restricted T cells can release different types of cytokines, including Th1, Th2 and Th17 cytokines [17].

In the peripheral blood of healthy adults the CD1a restricted T cells are the most frequent, followed by CD1c, CD1d and CD1b restricted T cells which are the least frequent [29, 30].

Group II CD1-restricted T cells

The group II is composed by CD1d-restricted T cells, which include two populations of natural killer T (NKT) cells. NKT cells express markers of the natural killer (NK) cells and the TCR. Two populations are divided according to the TCR properties. The first population Type I NKT cells, includes T cells that express

a semi-invariant TCR and are called as invariant NKT (iNKT) cells. The second population Type II NKT cells, have variable α and β chains [17]. The iNKT cells have a TCR with specificity for glicolipid antigens, like the glycosphingolipid α-GalCer. Because of the invariant chain and this specificity, the iNKT cells can be detected with α-GalCer-loaded CD1d tetramers. Type II NKT cells recognize hydrophobic antigens including sulfatide and lysophosphatidylcholine [31].

Little information is available about Type II NKT cells because they are difficult to identify. Due to the variable TCR it is hard to found surface markers to identify these cells. Therefore the study of CD1d-restricted T cells is centered in iNKT cells, because they can be identified by the use of CD1d tetramers.

iNKT cells

iNKT cells are cytotoxic T lymphocytes with a TCR that in humans is composed by an Vα24Jα18 chain combined with a limited but not invariant β chain repertoire, most usually is Vβ11[32]. iNKT cells are capable of modulate immunity responses in a broad spectrum of diseases, by the production of a broad range of cytokines. These cells are associated with protective and regulatory functions in tumor surveillance, chronic infectious diseases, transplants and autoimmunity [31]. And a role in the pathogenesis of inflammatory bowel diseases and in asthma was also described [33]. Other study shows that in cases of HIV infection and *Mycobacterium tuberculosis* infection iNKT cells had phenotypes associated with immune activation, and the absolute number of these cells was reduced in peripheral blood [34].

iNKT cell numbers are highly variable in humans. The number can range from 0.001% to over 3% of blood lymphocytes [31]. The iNKT cells in humans can be divided in three functional subsets, the $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ DN. In the human thymus the iNKT cells are mainly $CD4^+$. The $CD4^-CD8^-$ DN and $CD8^+$ are highly proliferating cells in the periphery [35].

iNKT cell development begins in the thymus with the expression of the invariant TCR. In the differentiation pathway cells are positively selected by the interaction of the TCR with CD1d molecules expressed by thymocytes. In mice, the iNKT cells maturation has at least four stages. Stage 0, which is characterized

by a population CD24-positive, $CD44^{lo}NK1.1^{lo}$, in this stage the cells are extremely rare and are not proliferating. After CD24 downregulation the cells start to proliferate, this is stage 1 where the cells are $CD24^{lo}CD44^{lo}NK1.1^{lo}$. The cells continue to proliferate and start to maturate. As they maturate they upregulate CD44 entering in stage 2 where the cells are $CD24^{lo}CD44^{hi}NK1.1^{lo}$. The last maturation phase, stage 3 is defined by upregulation of NK cell markers such as NK1.1 and is accompanied by much less proliferation as they migrate to the periphery. In humans, iNKT cell maturation is also completed at the periphery. The earliest detectable iNKT cell precursors are $CD4^{+}$ and $CD161^{lo}$ (human equivalent to NK1.1) these cells leave the thymus in this stage and continue their maturation in the periphery, were they become $CD4^{-}CD161^{+}$. Mature iNKT cells are extremely rare in human thymus, in contrast to their frequency in human blood [31].

iNKT cells are activated in the presence of endogenous and exogenous antigens loaded in CD1d molecules. This activation process may occur by three different pathways. The first one is foreign antigen-driven pathway were the antigen can be internalized and loaded in CD1d molecules or directly loaded in CD1d molecules at the cell surface. This complex CD1d-lipid antigen present at the surface of the APC interacts to the TCR of the iNKT cell, causing its activation. The second one is cytokine and self-antigen-driven pathway. In this case the antigens bind to toll-like receptors (TLRs) present at the surface of the APC promoting the loading of endogenous antigens in CD1d molecules and IL-2 secretion, causing double activation of the iNKT cells. In the third case, dominantly cytokine-driven pathway the antigens that bind to the TLRs promotes IL-2 secretion by the APCs, without the need for additional TCR-mediated stimulation [36].

1.3 CD1-restricted T cells in Lysosomal storage diseases

The lysosome is an important organelle in lipid antigen presentation by CD1 molecules, and therefore important to the CD1-restricted T cells. In patients with LSDs the function of the lysosome is impaired and so the lipid antigen presentation could be affected. This impairment of the lipid antigen presentation can lead to an alteration of the CD1-restricted T cell population. The majority of the studies regarding lipid specific CD1 restricted T cells in LSDs were performed in mice analyzing the iNKT cells. It was described for several mouse models of LSDs a decrease of the percentage of iNKT cells in the thymus and at the periphery. These studies showed a decrease of the iNKT cell percentage in mouse models of Sandhoff disease [37-39], GM1 gangliosidosis [37, 40], Niemann Pick C1 [37, 41], Tay-Sachs disease [37], Multiple sulfatase deficiency [39], and Fabry disease[37, 42, 43]. On the other hand, metachromatic leukodystrophy, Krable disease and MPS I presented no alterations in the frequency of iNKT cells [39]. Those results, that show a reduction in iNKT cells raise a question, of this happened due to a specific defect that leads to an accumulation of a specific lipid or simply arise from a non-specific effect of macromolecules accumulation in the lysosome.

In humans, both patients with Fabry and Gaucher diseases were analyzed regarding iNKT cell percentage. In Fabry disease patients no alterations on the iNKT cell percentage were found [42, 44] however, a difference in the subset $CD4^+$ was observed in a previous work (Cátia Pereira et al unpublished results). In Gaucher disease patients no alterations on the percentage of iNKT cells were found too [45].

The capacity of APCs from LSDs mouse models to present lipid antigens to iNKT cells was tested, to identify the cause of this reduced percentage of iNKT cell population in LSDs. In the mouse models tested of Sandhoff, Fabry, GM1 gangliosidosis, NPC1 deficiency and NPC2 deficiency the decrease in the number of iNKT cells was confirmed to be associated with deficient lipid antigen presentation [37, 38, 40, 41, 43, 46].

2. Aims

Considering the previous studies that found alterations in the iNKT cells percentage and in lipid antigen presentation in mouse models of LSDs, the aim of this work was to study the effects of metabolic alterations in Mucopolysaccharidosis type VI lymphocytes including iNKT cells and in lipid antigen presentation. The more specific aims were:

- To quantify the lymphocytes in Mucopolysaccharidosis type VI patients
- To quantify the iNKT cells and subsets in Mucopolysaccharidosis type VI patients
- To assess the phenotype of Mucopolysaccharidosis type VI patients dendritic cells
- To test the capacity of dendritic cells from Mucopolysaccharidosis type VI patients to present lipid antigens by the CD1b isoform.

3. Methods

All the experimental analyses were performed using peripheral blood samples from MPS VI patients and control subjects. PBMCs were isolated and used for:

- Flow cytometry analyses, to study the lymphocyte population;
- Dendritic cell generation.

For five of the patients, the first of the two determinations were performed by Dr. Fátima Macedo and Cátia Pereira prior to the start of this thesis. Dendritic cells were cultured for 7 days and then analyzed by flow cytometry to confirm the success of the differentiation and to assess CD1, MHC-class I and MHC-class II 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 CD1b molecule. The capacity of antigen presentation was evaluated by the amount of GM-CSF produced by the activated T cell and measured by enzyme linked immunosorbent assay (ELISA). Experimental design of the study is described in Figure 4.

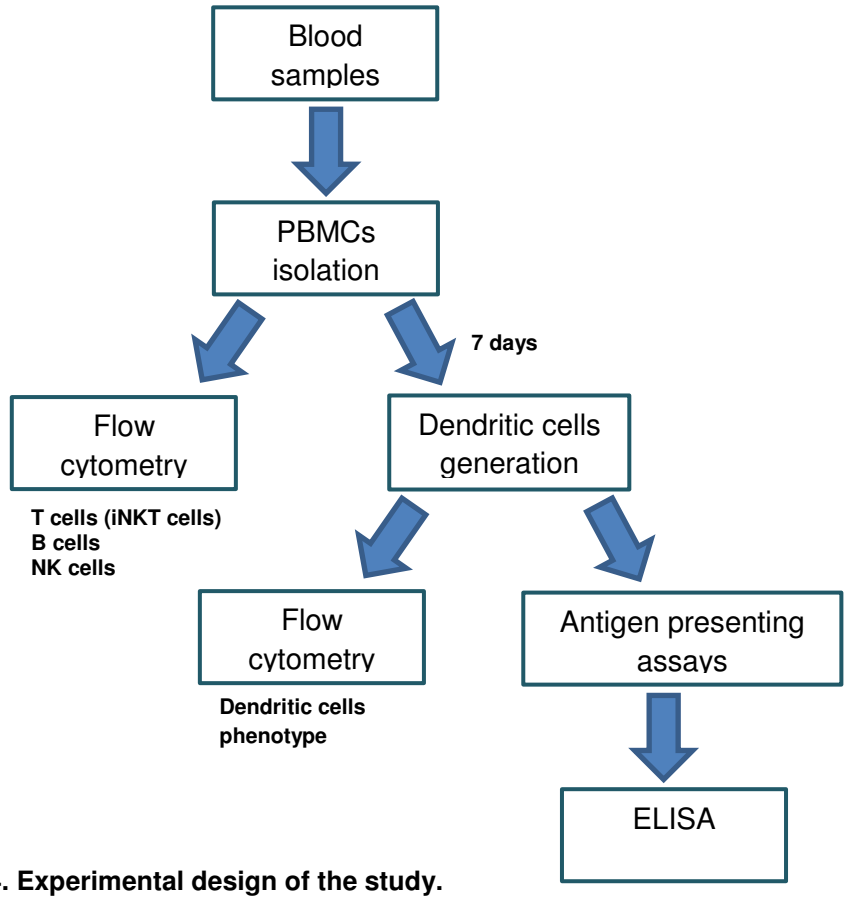

Figure 4. Experimental design of the study.

Subjects and sample collection

Seven MPS VI patients and seven control subjects were analyzed in this study. The MPS VI patients group was composed by five females and two males. In the control group, there were five females and two males. Control subjects were blood donors at the *Centro Regional de Sangue do Porto – Instituto Português do Sangue* (IPS), at the *Hospital São João do Porto or* at the *Hospital de Santo António do Porto*. Samples were obtained under a protocol between IBMC and these institutions. MPS VI patients were recruited by their physicians, Dr. Elisa Leão Teles from *Hospital São João* and Dr. Esmeralda Martins from *Hospital de Santo António*. In the beginning of this study, all the patients were already under ERT. This treatment consists in the administration of a recombinant form of the ASB enzyme (Naglazyme) via regular intravenous infusions. The characteristics of the patients included in the study are described in the results section 4, on table 4.

Peripheral blood mononuclear cells (PBMCs) isolation

After the samples arrive, PBMCs were isolated using density gradient centrifugation with Histopaque-1077 from 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 plasma and Histopaque-1077. PBMCs were then collected and washed once by adding 10mL of phosphate buffered saline 1x (PBS 1x, see appendix) and centrifuged at 250xg for 10 minutes. Then the remaining erythrocytes were lysed by incubating cells for 10 min with 15mL of ACK lysis solution (see appendix). Cells were washed once more with 10mL of PBS 1x and counted using a Neubauer chamber. After cell count, PBMCs were used for flow cytometry or for dendritic cell differentiation.

Flow Cytometry

Up to 1 x 10^6 PBMCs or about 0.05 x 10^6 dendritic cells were stained per well in a round-bottomed 96-well plate. To use few dendritic cells 0.5 x 10^6 of Jurkat cells were added per well and we used an anti-human CD3 antibody to exclude the Jurkat cells in our analyses. The cells were added to the plate, and

then centrifuged at 1200 rpm for 2 min. For PBMCs, supernatant was rejected and the 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). The antibodies listed in Table 2 were used.

Table 2. Antibodies used in PBMCs flow cytometry to identify the T cells, B cells and NK cells population.

Antibody	Clone	Flourochrome	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
Anti-human CD19	HIB19	PE-Cy7	eBioscience
Anti-human CD56	MEM188	FITC	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; FITC – Fluorescein.

To identify iNKT cells, the CD1d tetramer loaded with PBS57 and labeled with the flourochrome phycoerythrin (PE), from National Institute of Health tetramer core facility was used. PBS57 is an 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 [47]. An unloaded CD1d tetramer was used as a control to identify unspecific staining.

For dendritic cells flow cytometry is needed one more step because they need to be blocked to avoid unspecific staining, so they were resuspended in 50µL of PBS 25% human serum (see appendix) and incubated for 20 min at 4ºC. Then dendritic cells were washed twice by adding 100µL of flow cytometry solution and centrifuged at 1200 rpm for 2 min. After that, 25µL of the antibody mix were added. The antibodies used for dendritic cell staining are described in Table 3.

Table 3. Antibodies used in dendritic cells flow cytometry to confirm differentiation and to assess CD1, HLA-ABC (MHC-class I) and HLA-DR (MHC-class II) 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 CD1d	CD1d42	PE	BDBioscience
Anti-human CD11c	3.9	PE-Cy7	eBioscience
Anti-human HLA-ABC	W6/32	FITC	eBioscience
Anti-human HLA-DR	LN3	APC	eBioscience
Anti-Human CD80	2D10	APC	Biolegend

PE – Phycoerythrin; FITC – Fluorescein; PerCP – Peridinin chlorphyll protein; PE-Cy 7 – Phycoerytrin conjugated with Cy7; APC – Allophycocyanin.

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, centrifuged at 1200 rpm for 2 min, rejected supernatant and resuspended cells. After the last wash, PBMCs were resuspended in 350µL of PBS 1% formaldehyde (see appendix) and dendritic cells were washed twice and resuspended too in 350µL of PBS 1% formaldehyde. All samples were transferred to FACS tubes (BD bioscience).

For PBMCs (iNKT cells), up to 2000 events were acquired inside the gate of iNKT cells, and for B cells, up to 10000 events were aquired in a FACSCanto (BD bioscience), using the FACSDiva software (BD bioscience). In the case of dendritic cells, 10000 events were acquired using the same equipment and software used for PBMCs. 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 these two cell types. Immunomagnetic labeling using MACS anti-human CD14 MicroBeads from Miltenyi Biotec were used to achieve this separation. The

technique uses magnetic beads bound to an anti-human CD14 antibody that attaches to the monocytes, which are $CD14^+$ cells (Figure 5).

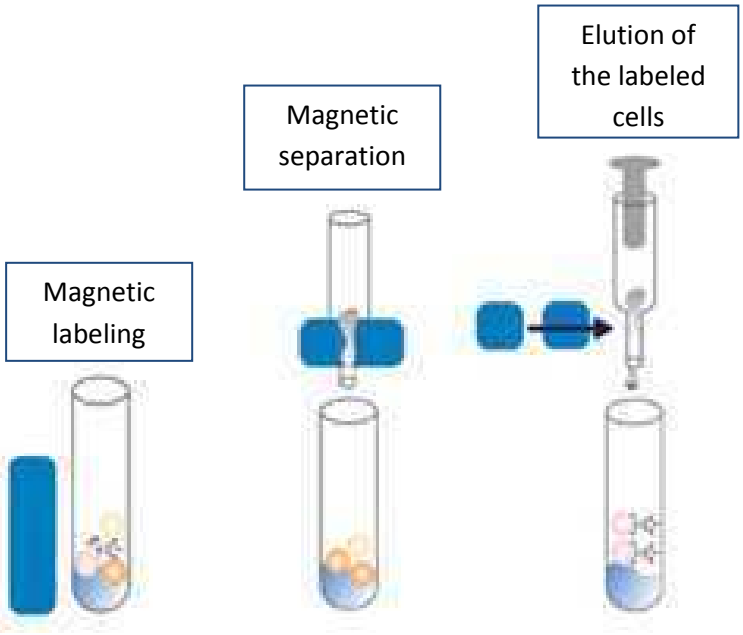

Figure 5. Separation of CD14+ cells by positive selection using MACS beads. Figure adapted from http://www.proimmune.com

PBMCs were washed once with 5mL of 1x PBS 2mM EDTA 0.5% BSA (MACS buffer, see appendix). 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. In this step the anti-human CD14 antibodies present in the magnetic beads 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 100 x 10^6 of PBMCs, use 475µL of MACS buffer and 62.5µL of anti-human CD14 magnetic beads. For few cells (less than 20 x 10^6) the proportion changes to: for 10 x 10^6 of PBMCs, use 80µL of MACS buffer and 20µL of anti-human CD14 magnetic beads. After incubation, cells were washed twice with 5mL of MACS buffer. The columns (can be MS or LS) are chosen depending on the number of PBMCs available. According to the manufacturer's instructions, MS columns were used if there were less than 20 x 10^6 PBMCs available and LS columns if there were more. The columns were placed in the magnetic support and pre-wetted with MACS buffer. The magnetic

force existent between the beads and the support keeps the labeled cells from eluting. For LS columns, the cells were resuspended in 4mL and for MS columns the cells were resuspended in 0.5mL of MACS buffer and applied. The monocytes that bond to the magnetic beads remained in the column, while the non-labeled cells are eluted. The columns were 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. The columns were removed from the magnetic support and placed in 15mL falcon tubes. Depending on the columns used, 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. Finally the $CD14^+$ cells are eluted, since the column is no longer in the magnetic support. After the isolation process, cells were counted and plated at 1 x 10^6/mL in six-well plates, 3mL per well in dendritic cell culture medium, RPMI 10% heat inactivated fetal bovine serum (iFBS), containing 25ng/mL of recombinant human interleukin 4 (rhIL-4 from ImmunoTools) and recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF from ImmunoTools). Cells were incubated at 37ºC, 5% CO_2. At day 4 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 of rhIL-4 and 75ng/mL of rhGM-CSF). Cells were used for activation assays and flow cytometry at day 7.

T cell clone

T cell clone used for the lipid antigen presentation assays were kindly provided by Prof. Gennaro De Libero from Basel University, Switzerland. T cell clone was established and maintained as described [48, 49]. 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 [48]. After 3 weeks, growing T cells were restimulated with heterologous dendritic cells plus the mixture of glycolipids. T cell clone were obtained by limiting dilution and scored for antigen specificity and CD1 restriction using CD1-transfected APCs [48, 49]. In this study

the T cell clone used was GG33A that is restricted to CD1b molecule and specific for lipid antigen GM1.

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 rhIL-2. PBMCs are isolated following the protocol presented above. Then, PBMCs are ressuspended in culture medium (see appendix) at a concentration of 5 x 10^6 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 ressuspended in T cell culture medium with 2µg/mL of PHA (see appendix). The T cell clones to restimulate 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 approximately in 15 days.

Lipid antigen presentation assays

In these *in vitro* assays, dendritic cells are incubated with the lipid antigen and with T cell clones. T cell clones recognize the lipid antigen bound to CD1 at the cell surface of the dendritic cell, and when T cell clones bound to this complex are activated and start producing cytokines. The cytokines can be then detected, indicating the efficiency of the lipid antigen presentation.

These *in vitro* assays were performed as previously described [48, 49]. Dendritic cells were collected, counted and resuspended in culture medium (see appendix) without 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 (50 µL of the solution of 0.4x10^6 cells) were added to each well of 96-well flat-bottomed plate and incubated at 37ºC, 5% CO_2.

Then, antigen dilutions were performed. GM1 was initially diluted in a solution of chlorophorm:methanol (1:1) in a concentration of 10mg/mL. The lipid was diluted 4x in the activation volume, so a solution of 200µg/mL was necessary. Then, was dried under N_2 and dissolved only in methanol to obtain the needed

concentrations, because chlorophorm is toxic for the cells. After this, the lipid antigen was sonicated for 3 min in a water bath sonicator. Eight serial dilutions of 1:3 in duplicates were prepared in 50 µL of the culture medium with iFBS and added to the wells containing the dendritic cells. The lipid antigen concentrations tested were: 16.7; 5.6; 0.62; 0.07 µg/mL and a blank without antigen. Dendritic cells were incubated with the lipid antigen at 37ºC, 5% CO_2 for 4 hours. In the meantime, the T cell clone was collected washed once with PBS and counted. 100µL of the culture medium with 20% iFBS containing 100000 T cells were added per well. This medium 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 clone, dendritic cells and lipid antigen were incubated at 37ºC, 5% CO_2 for 36 hours, 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 used for determination of cytokine concentration by ELISA.

ELISA

ELISA plates (MaxiSorp, Nunc) were coated with 50µL per well of purified anti-human GM-CSF (BVD2-23B6, Biolegend) at 4ºC, overnight. The coating solution was then removed and wells were washed once by adding 300µL of washing buffer, PBS, 0.05%Tween 20 (see appendix) to each well. Then the cells were blocked by adding 100µL of blocking buffer, PBS, 0.05% Tween 20, 1%BSA (see appendix) and incubated at room temperature for 1 hour. After this process, wells were washed twice and incubated with 50µL of the sample. In some wells, 50µL of the standard rhGM-CSF were added in eight different concentrations, starting in 20 ng/mL (1:3 serial dilutions) in duplicates. After an incubation of 1 hour and 30 min, wells were washed three times and 50µL of the biotinilated anti-human GM-CSF antibody (BVD2-21C11, Biolegend) were added to each well. After 1 hour and 30 min, wells were washed again with washing buffer three times. Then for detection of the biotinilated antibody, 70µL of streptavidin conjugated with horseradish peroxidase from Invitrogen were added to each well and incubated for 1 hour at room temperature, in the dark. Wells were washed four times, and after 90µL of SigmaFast™ o-phenylenediamine dihydrochloride (OPD) from Sigma, a

substrate for the horseradish peroxidase, were added. The OPD reagent was prepared according to the manufacturer's instructions. The reaction occurred for about 30 min, at room temperature, in the dark. Then the reaction was stooped adding 50µL of H_2SO_4. Absorbance was read at 490 nm 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 Student's T-Test. P-values bellow 0.05 were considered significant.

4. Results

Mucopolysaccharidosis type VI patients and control subjects characteristics

In this study seven MPS VI patients and seven control subjects were analyzed. The general characteristics of MPS VI patients, such as, sex, age, genetic mutations in *ARSB* gene and the date they started the enzyme replacement therapy, are described in Table 4. All the patients are currently under treatment. The control group of this study is composed by two males and five females, with ages between 15 and 40 years old.

Table 4. Mucopolysaccharidosis type VI patients' characteristics: sex, age, mutations and starting date of enzyme replacement therapy.

Patient	Sex	Age	Mutation	ERT
1	F	18	R315Q	2003
2	M	20	R315Q	2003
3	M	18	NA	2004
4	F	4	NA	2010
5	F	23	L72R	2003
6	F	14	NA	NA
7	F	26	NA	NA

M – male; F – female; ERT – enzyme replacement therapy; NA – Not available

The percentage of iNKT cells and their subsets are not altered in peripheral blood of Mucopolysaccharidosis type VI patients in comparison with control subjects

In this study, peripheral blood iNKT cells were identified by flow cytometry, using an antibody anti-human CD3 and the human CD1d tetramer loaded with PBS57. The gating strategy used to identify the iNKT cells is exemplified in Figure 6. First in this analysis the lymphocyte gate is defined using a side scatter and forward scatter cytogram, after this the T lymphocytes (CD3+) were selected using

a histogram, and the percentage of iNKT cells (CD1d-PBS57 tetramer positive cells) was determined inside the $CD3^+$ gate.

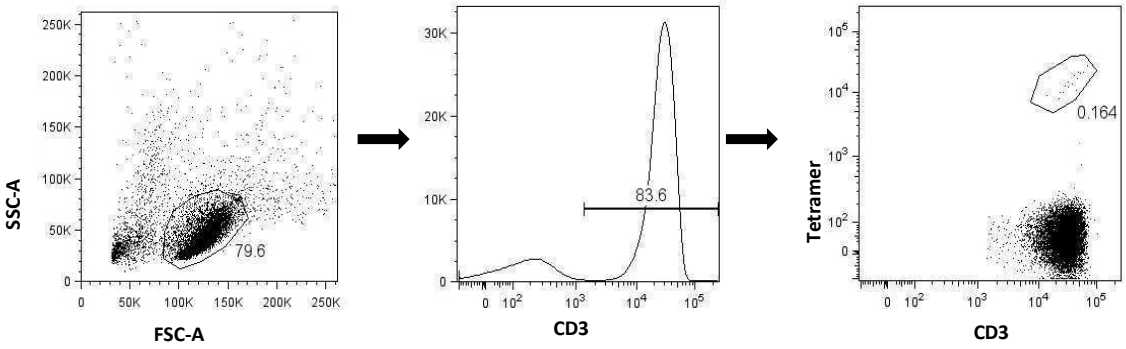

Figure 6. Flow cytometry analysis for iNKT cell frequency quantification.

The percentage of iNKT cells among MPS VI patients and among control subjects is highly variable, as shown in Figure 7. Six MPS VI patients and seven control subjects were analyzed. The percentage of iNKT cells were slightly decreased in MPS VI patients, but this difference was not statistically significant.

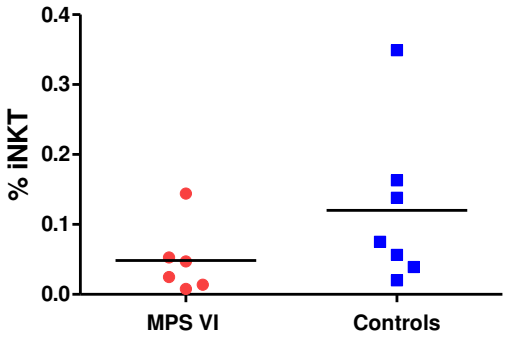

Figure 7. iNKT cells frequency among T lymphocytes (CD3+) in MPS VI patients. iNKT cells were identified in the gate of CD3+ cells as positive for CD1d-PBS57 tetramer. Horizontal bars represent means.

The percentage of iNKT cells activated (that express CD69) were also analyzed, and the results are shown in Figure 8. We found that some patients had

a more activated phenotype in comparison with controls, but in general the differences were not significant. Interestingly the two MPS VI patients with higher expression of CD69 in the iNKT cells are the two male patients analyzed.

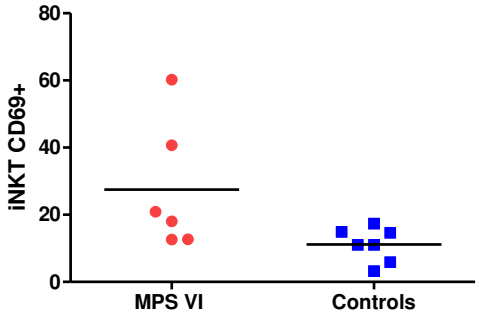

Figure 8. The percentage of activated iNKT cells in MPS VI patients and in control subjects. Horizontal bars represent means.

In humans, the iNKT cell population can be divided in three different subsets according to the expression of CD4 and CD8 molecules. These subsets are $CD4^+$ (that only express CD4); $CD8^+$ (that only express CD8) and $CD4^-CD8^-$ (do not express neither of these molecules). The subsets were analyzed after selected the gate of iNKT cell population (Figure 9).

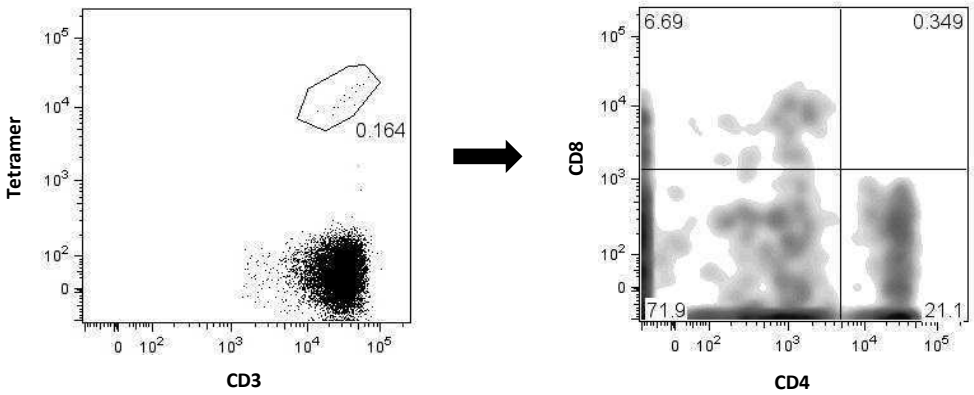

Figure 9. Representative example of iNKT cell subsets frequency determined by flow cytometry.

In the analyses of the three subsets of iNKT cell population, no alteration was found in their frequency between MPS VI patients and the control subjects (Figure 10). For the iNKT cells $CD4^+$ subset we had a patient with a higher percentage of this subset, which is the younger patient analyzed with only 4 years

old. This patient had a 92.1% of $CD4^+$ iNKT cells, and is also one of the patients with lower percentage of total iNKT cells. In the case of $CD8^+$ iNKT cells percentage the patient that showed higher percentage was patient number 2.

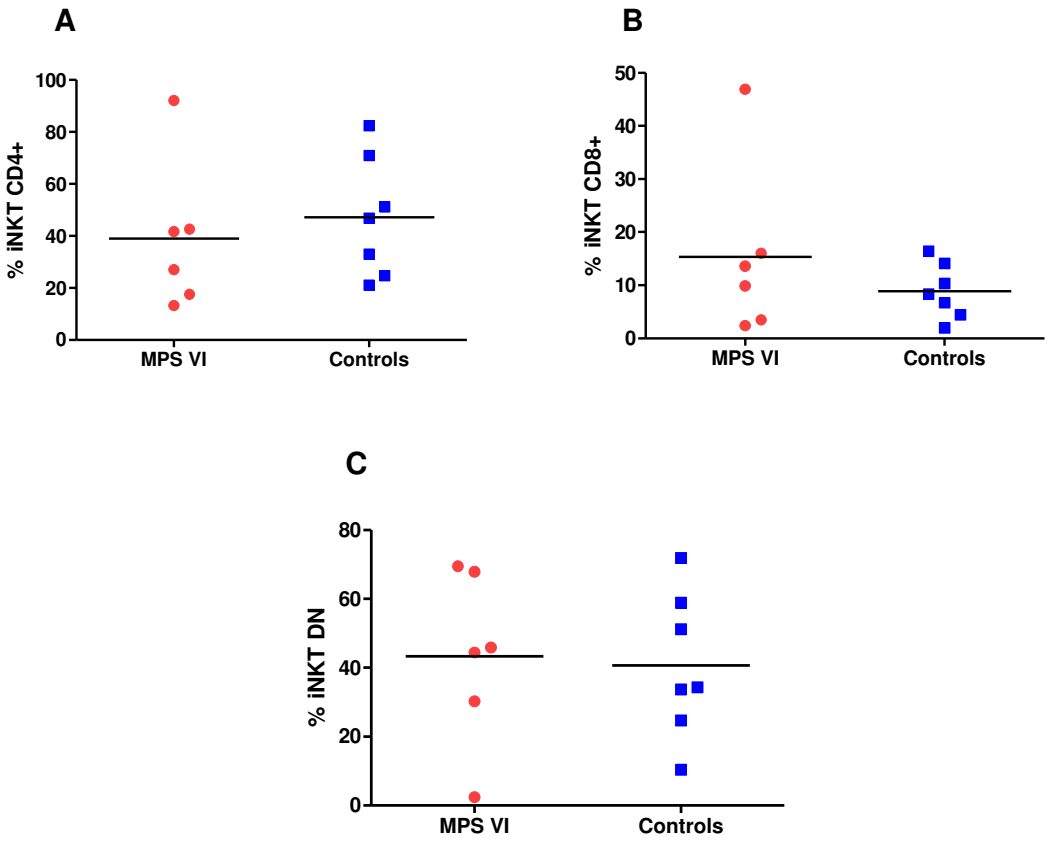

Figure 10. iNKT cell subsets frequency in MPS VI patients and control subsets. Horizontal bars represent means. A – iNKT CD4+ cell frequency; B – iNKT CD8+ cell frequency; C – iNKT DN cell frequency.

<u>The percentage of iNKT cells in peripheral blood is not altered overtime (one year) in patients with Mucopolysaccharidosis type VI</u>

For five of the patients it was possible to analyze the percentage of iNKT cells and the $CD4^+$ iNKT cell subset twice with one year of interval. The results are described in Figure 11 and show that there is a slight decrease of the percentage of iNKT cells between the analyses made in 2011 and 2012 in three of the patients (Figure 11A) and for the other two patients the values keep stable. However even for the patients for which there is a small decrease in the percentage of iNKT cells,

patients that had higher iNKT cells levels maintained high levels and patients with lower levels maintained lower levels. The percentage of $CD4^+$ iNKT cells in the MPS VI patients remained stable in the two analysis made, with the exception of the patient number three that had a slight increase (but maintained the same range level) in the percentage of this subset, results shown in Figure 11 (B). These small variations did not appear to be correlated with age or sex of the patients.

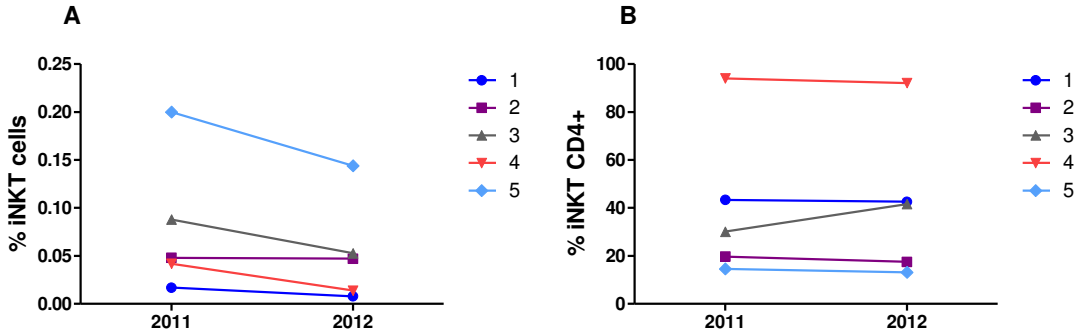

Figure 11. Variation of iNKT cells over one year period (A) and their CD4+ subset (B) frequency in five MPS VI patients.

T cells in Mucopolysaccharidosis type VI patients

Total T lymphocyte population was analyzed too. This population was identified by the expression of $CD3^+$ molecule and can be divided into two major subsets, the cytotoxic T cells ($CD8^+$) and the helper T cells ($CD4^+$). The naïve and memory subsets within T cells were also analyzed, these data are not shown here, because currently the age of the control subjects and the MPS VI patients are not matching and age clearly influences the naïve and memory subsets [50].

Table 5. The mean and standard deviation values of the percentage of T lymphocytes (CD3+), and their subsets (CD4+ and CD8+) in MPS VI patients and control subjects.

Cells	Patients	Controls
T lymphocytes	78 ± 3	80 ± 6
CD4	51 ± 8	51 ± 5
CD8	23 ± 7	23 ± 4

The results described (Table 5) for T cells and their subsets showed no differences statistically significant. The values were very similar between MPS VI patients and control subjects.

Alteration of the B cell population in Mucopolysaccharidosis type VI patients

The B lymphocytes that are identified by the expression of CD19 and the NK cells, which express CD56 at the cell surface, were also analyzed. The results of B cells are shown in Figure 12, and for the NK cells in Figure 13.

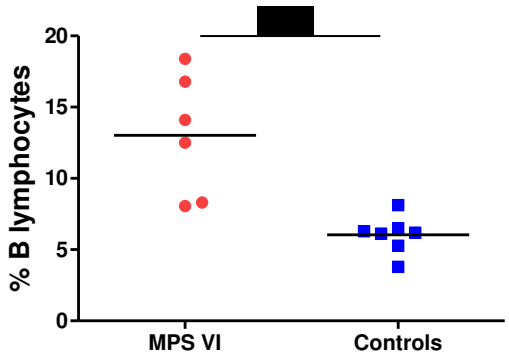

Figure 12. Percentage of B cell population is higher in MPS VI patients. B cells were identified in the lymphocytes gate as cells positive for CD19. Horizontal bars represent means. Statistical significance was calculated by Student's T test, $p<0.05$.

For the B cell population were found differences between control subjects and MPS VI patients that were statistically significant ($p<0.05$). The MPS VI patients have an increase of the percentage of B cells when compared with control subjects.

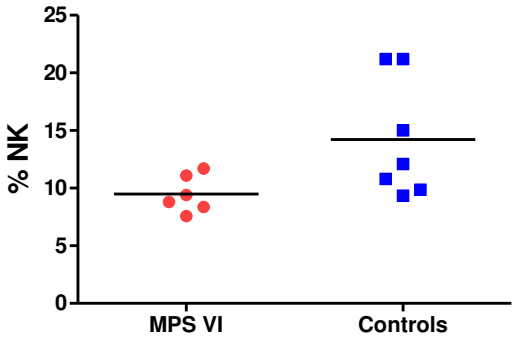

Figure 13. Percentage of NK cells in MPS VI patients. NK cells were identified in the lymphocytes gate as cells positive for CD56 and negative for CD3. Horizontal bars represent means.

In the case of NK cells no significant differences were found between MPS VI patients and control subjects (Figure 13).

MPS VI dendritic cells phenotype

Dendritic cells were generated *in vitro* from MPS VI patients and from control subject's monocytes isolated from peripheral blood. Dendritic cells were differentiated from monocytes over 7 days in culture with GM-CSF and IL-4. After this, dendritic cells were analyzed by flow cytometry to confirm differentiation. For this study only 3 patients were analyzed, because of the low volume of blood received from the patients. Two control subjects were analyzed at the same time. To confirm differentiation the expression of CD80 and CD11c molecules were analyzed. In all the control subjects and MPS VI patients, dendritic cells had a CD80 and a CD11c expression compatible with dendritic cell differentiation (Figure 14).

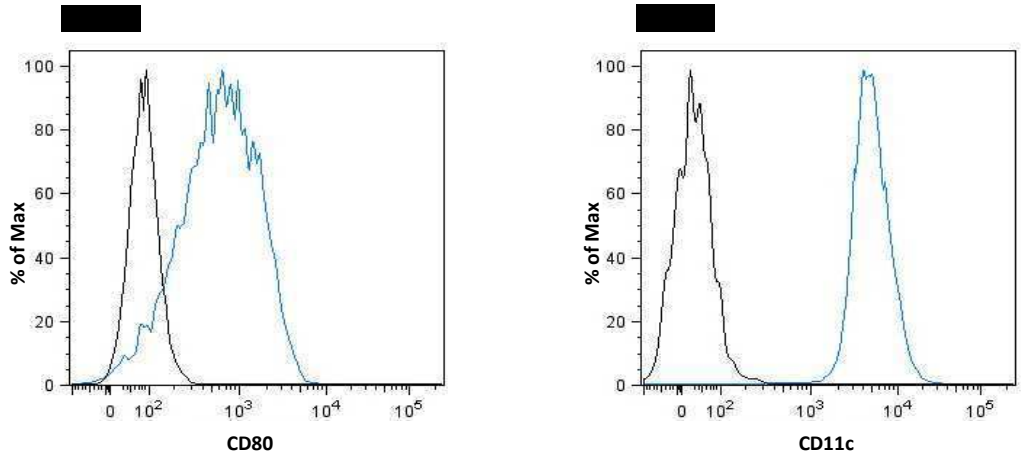

Figure 14. Representative example of monocyte derived dendritic cells phenotype. A - CD80 cell surface expression; B – CD11c cell surface expression. Cells were stained with anti-human CD80 and CD11c and analyzed by flow cytometry. The black line corresponds to unstained and blue line to stained sample.

The expression of CD80 was similar between MPS VI patients and control subjects (Figure 15). The three MPS VI patients showed similar values and when compared with the control subjects no differences were found.

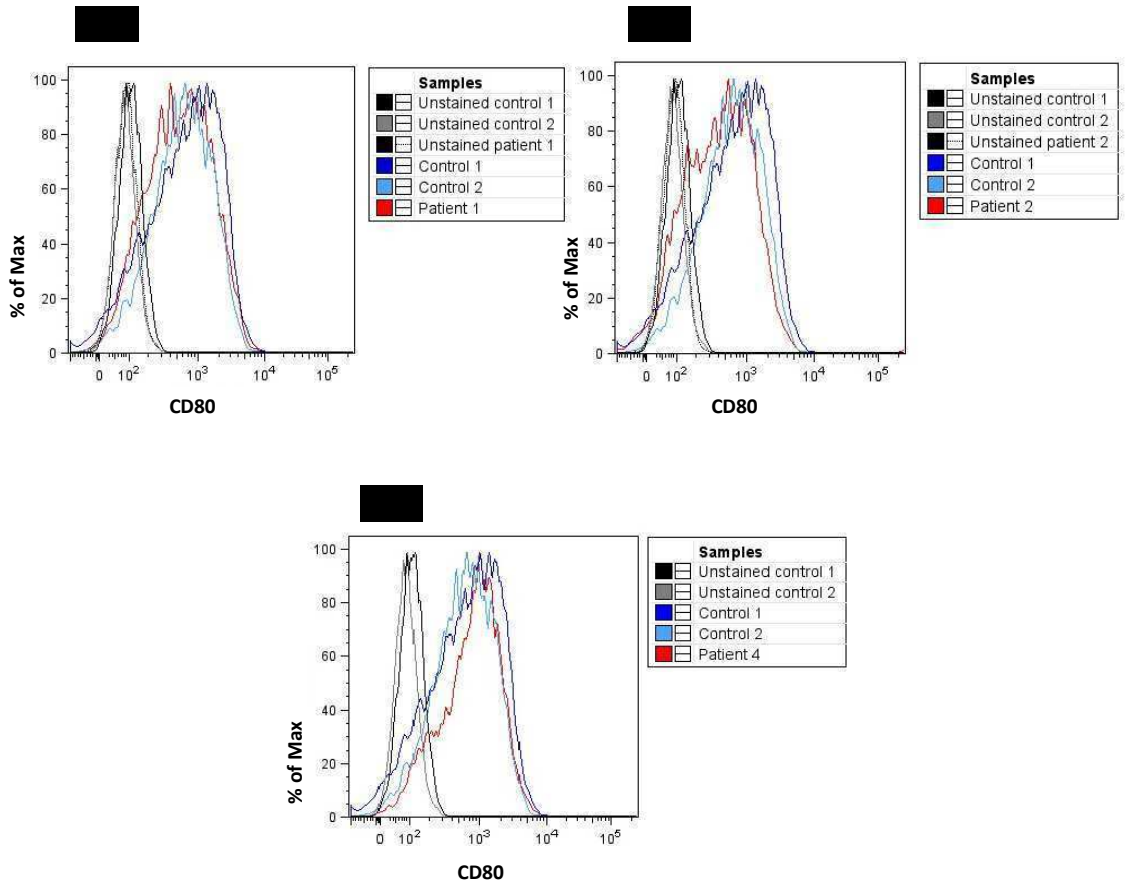

Figure 15. Histogram plots of CD80 expression at the cell surface of dendritic cells from control subjects and MPS VI patients. Flow cytometry analyses of dendritic cells differentiated from monocytes was performed at day 7. A – Patient number 1; B – Patient number 2; C – Patient number 4.

However, for the expression of CD11c molecule, two of the MPS VI patients analyzed showed a decrease in comparison with control subjects (Figure 16). We can see for patient number 1 (Figure 16 A) and for patient number 2 (Figure 16 B) a decrease on the expression of CD11c molecule when compared with control subjects.

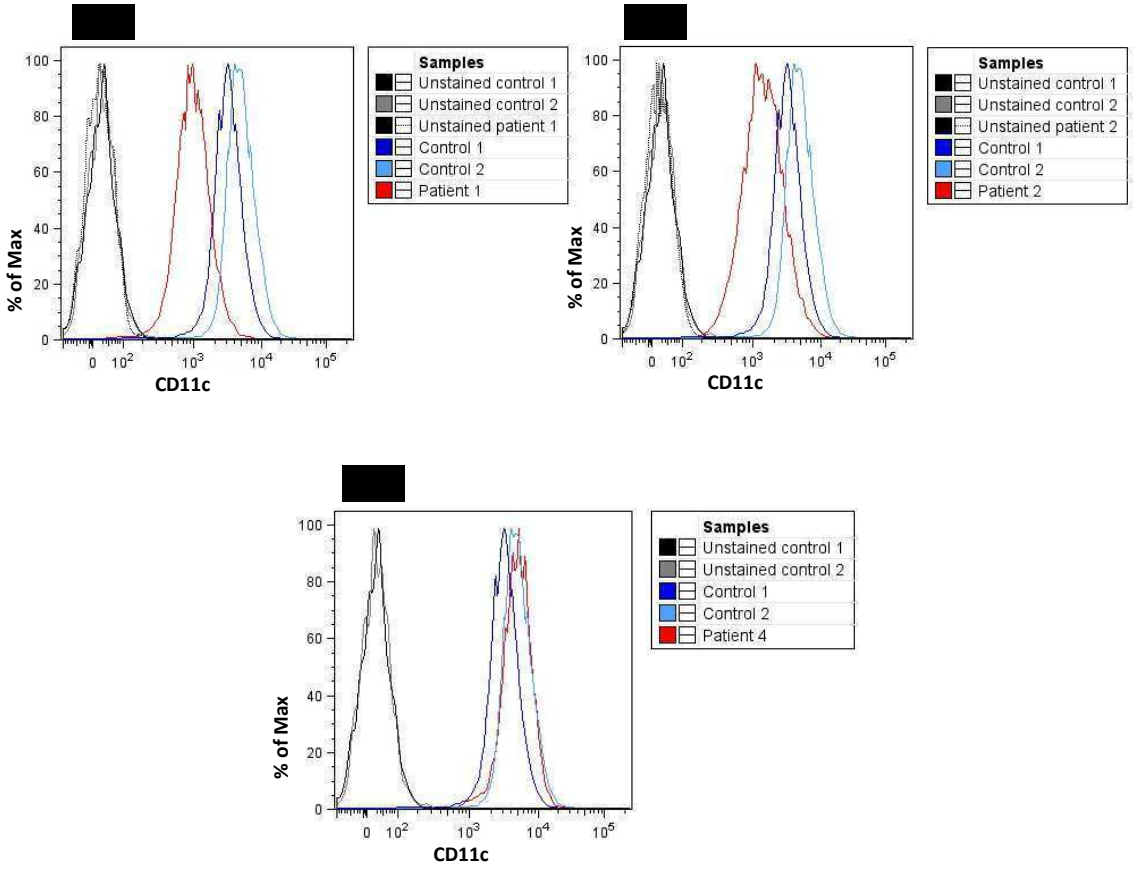

Figure 16. Histogram plots of CD11c expression at the surface of dendritic cells from control subjects and MPS VI patients. Flow cytometry analyses of dendritic cells differentiated from monocytes was performed at day 7. A – Patient number 1; B – Patient number 2; C – Patient number 4.

Concerning CD1a expression, one of the patients, patient number 1, showed similar values compared with control subjects (Figure 17 A). For the other two MPS VI patients, patient number 2 (Figure 17 B) and patient number 4 (Figure 17 C) when compared with control subjects showed dendritic cells with a bimodal expression, with a population that had lower expression of CD1a.

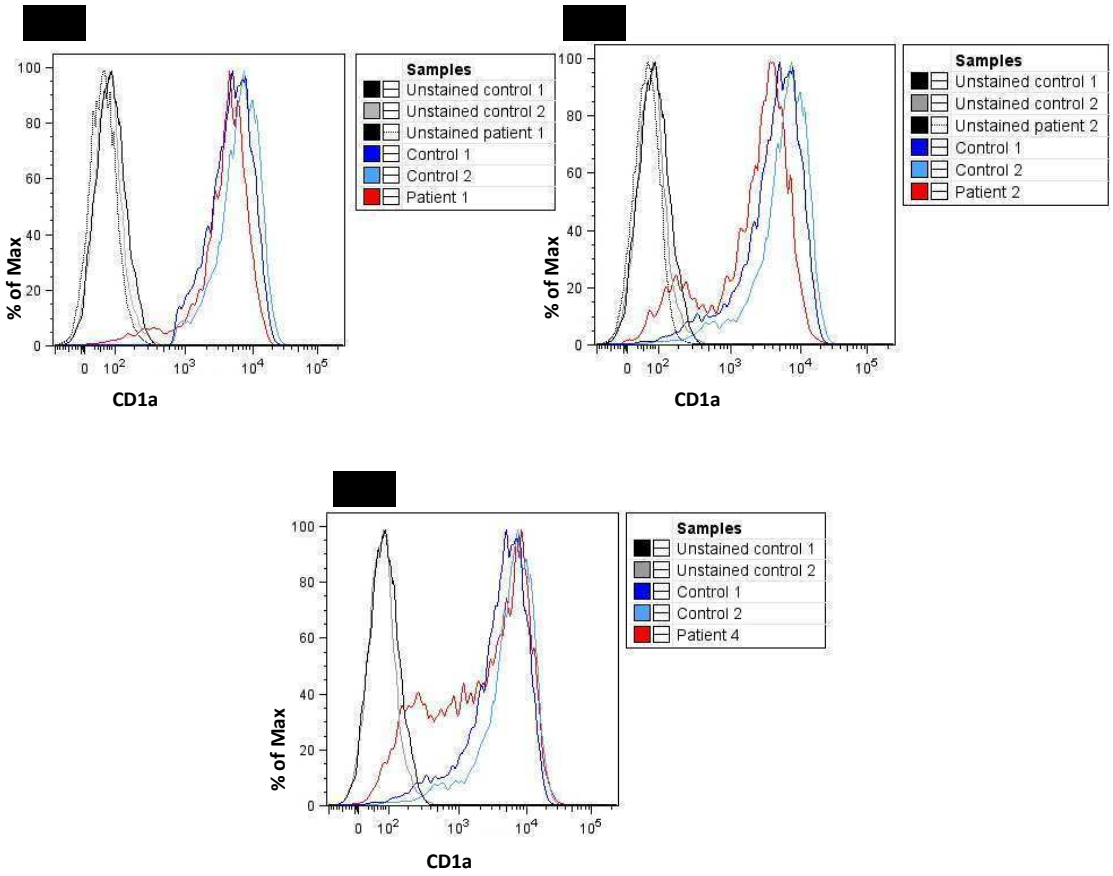

Figure 17. Histogram plots of CD1a expression at the cell surface of dendritic cells from MPS VI patients and control subjects. Flow cytometry analyses of dendritic cells differentiated from monocytes was performed at day 7. A – Patient number 1; B – Patient number 2; C – Patient number 4.

In the case of CD1b molecule, the results showed no alterations in dendritic cell surface expression of this molecule in two patients (Figure 18). Patient number 1 (Figure 18 A) showed a slight decrease in dendritic cell surface expression of CD1b.

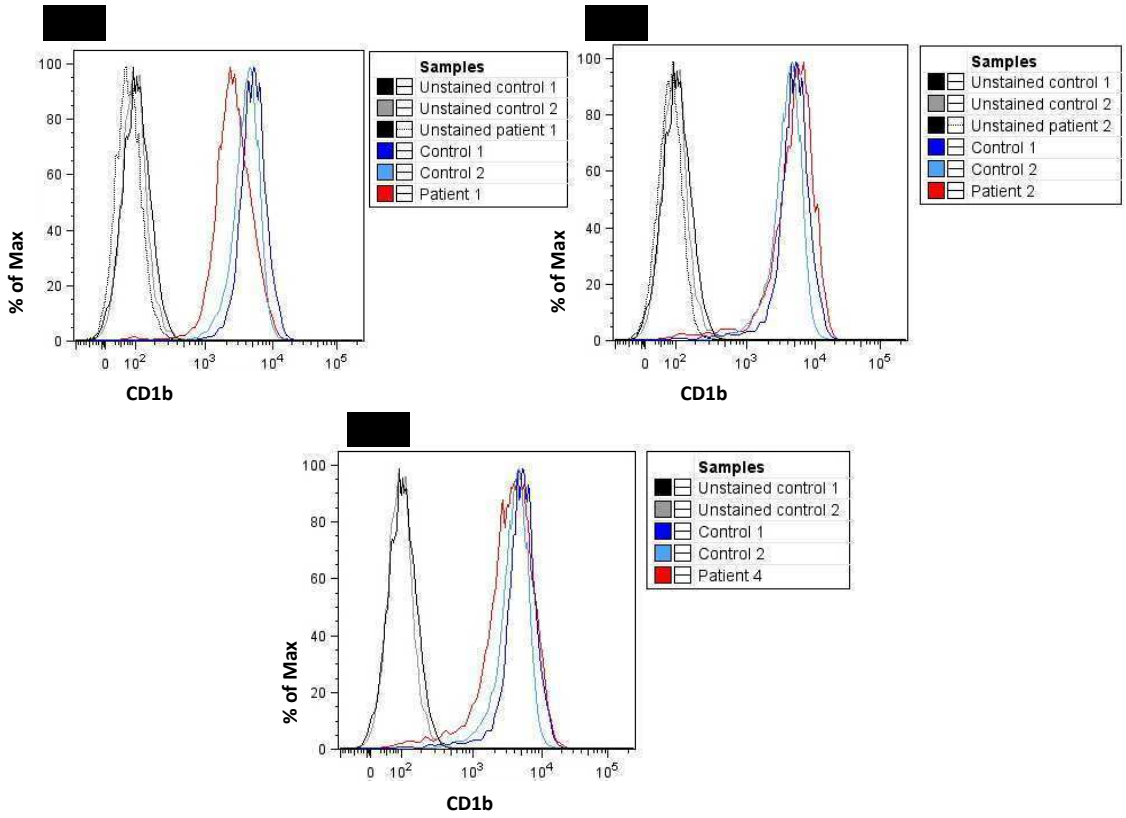

Figure 18. Histogram plots of CD1b expression at the cell surface of dendritic cells from MPS VI patients and control subjects. Flow cytometry analyses of dendritic cells differentiated from monocytes was performed at day 7. A – Patient number 1; B – Patient number 2; C – Patient number 4.

No differences were found in dendritic cell surface expression of CD1c molecule. The results obtained for the three MPS VI patients were very similar among them and when compared with the control subjects (Figure 19).

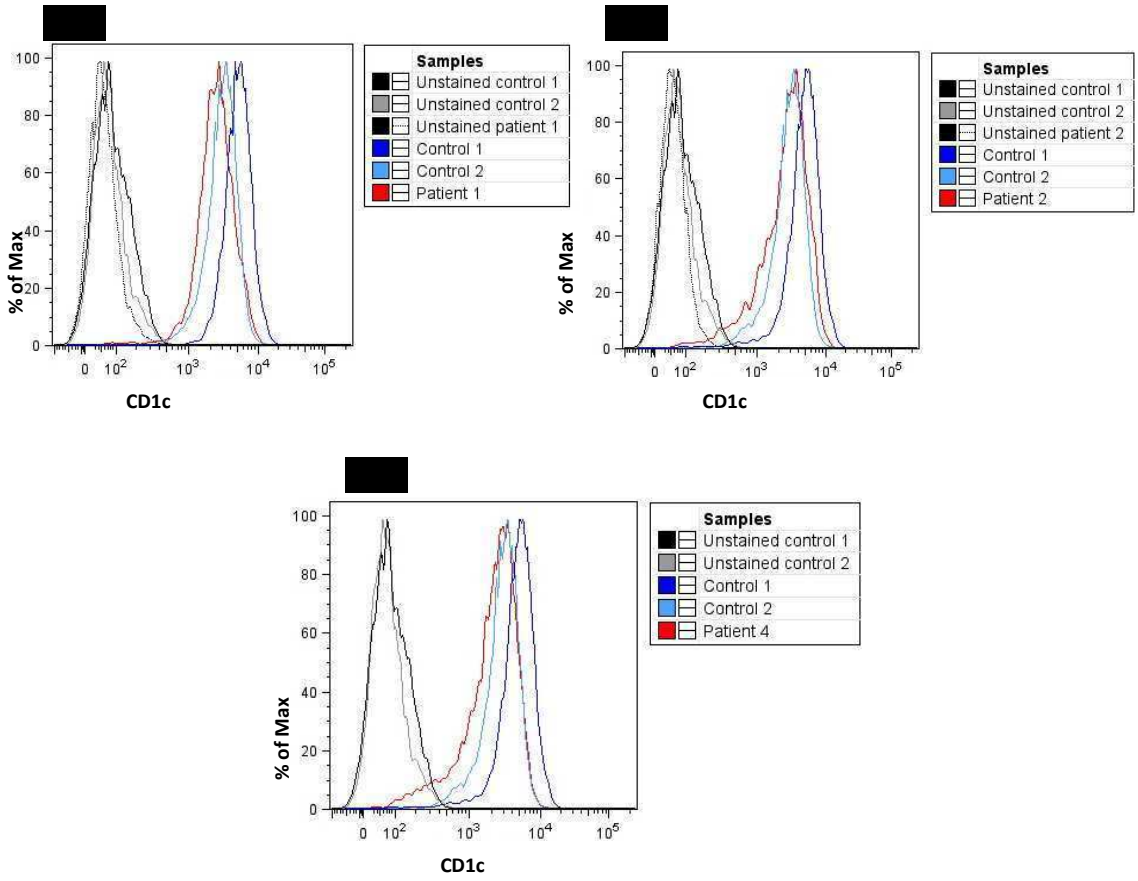

Figure 19. Histogram plots of CD1c expression at the cell surface of dendritic cells from MPS VI patients and control subjects. Flow cytometry analyses of dendritic cells differentiated from monocytes was performed at day 7. A – Patient number 1; B – Patient number 2; C – Patient number 4.

In this study, Group II CD1 molecules were not tested. Justified by the fact that monocyte derived dendritic cells cultured in media containing FBS do not express CD1d at the surface in amounts detectable by flow cytometry [51].

The same MPS VI patients and control subjects were analyzed for cell surface expression of MHC class I (HLA-ABC) and MHC class II (HLA-DR). Figure 20 shows the results for HLA-ABC, no alterations were found in the dendritic cell surface expression of this molecule in two MPS VI patients tested, patient number 1 (Figure 20 A) and patient number 2 (Figure 20 B) when compared with control subjects. In patient number 4 (Figure 20 C) two populations of dendritic cells can be identified by the expression of HLA-ABC, a population similar to the other subjects and a population with higher expression of HLA-ABC.

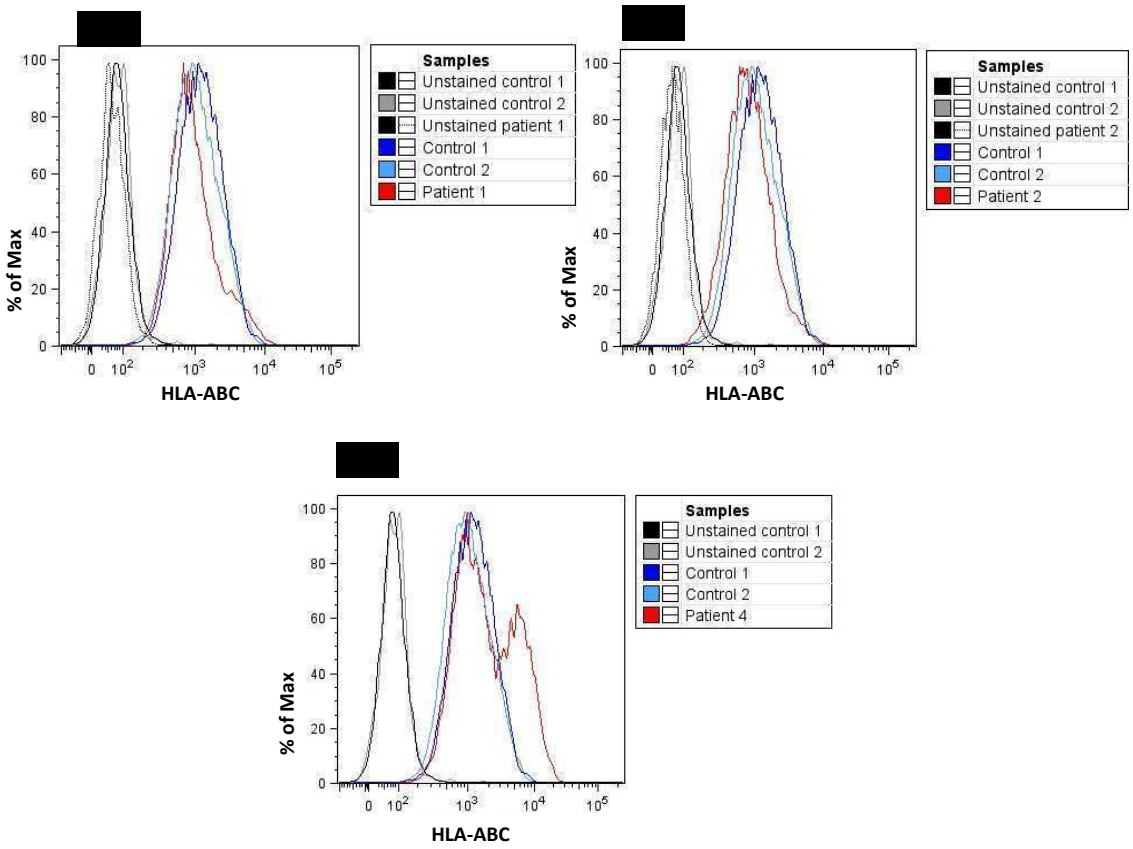

Figure 20. Histogram plots of HLA-ABC expression at the cell surface of dendritic cells from MPS VI patients and control subjects. Flow cytometry analyses of dendritic cells differentiated from monocytes was performed at day 7. A – Patient number 1; B – Patient number 2; C – Patient number 4.

In the case of HLA-DR two of the MPS VI patients, patient number 1 (Figure 21 A) and patient number 2 (Figure 21 B) had a slight decrease in the cell surface expression of this molecule compared with the control subjects. Patient number 4 (Figure 21 C) showed a bimodal expression of HLA-DR.

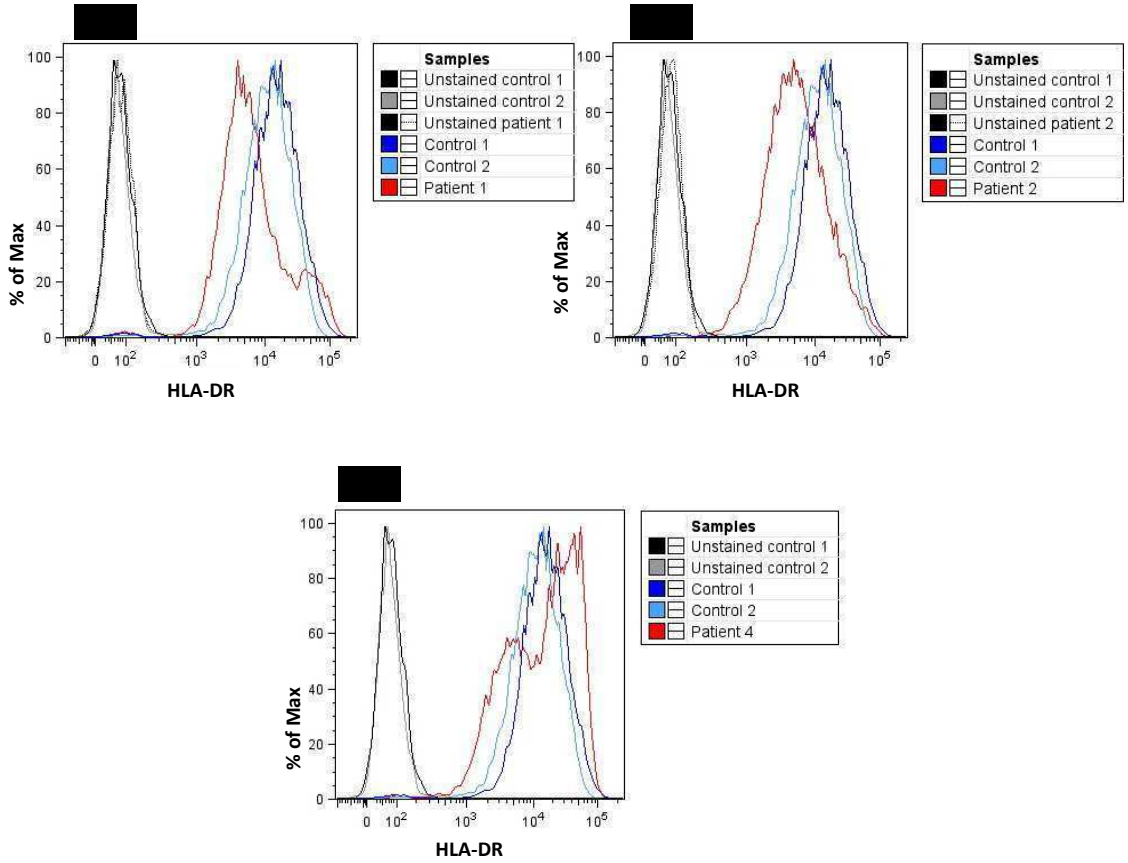

Figure 21. Histogram plots of HLA-DR expression at the cell surface of dendritic cells from MPS VI patients and control subjects. Flow cytometry analyses of dendritic cells differentiated from monocytes was performed at day 7. A – Patient number 1; B – Patient number 2; C – Patient number 4.

Lipid antigen presentation to T cells in Mucopolysaccharidosis type VI patients

The capacity of dendritic cells from MPS type VI patients to present lipid antigens and consequently activate T cells in comparison to control subjects was assessed using an *in vitro* system of lipid antigen presentation. For this part of the study only three patients were analyzed for one of the CD1 isoforms (CD1b), due to a limitation in this study that is the amount of peripheral blood sample collected from the patients that is generally small and so a few cells were collected for culture. Dendritic cells were derived from monocytes, and when differentiated were incubated with different concentrations of lipid antigen (GM1) for 4 hours. Then, a T cell clone restricted to CD1b molecule (GG33A) was added and incubated for 36 hours, to allow activation of the T cell clone and consequent cytokine production. ELISA was performed to determine cytokine concentration on the supernatant collected. The efficiency of the lipid antigen presentation by CD1b molecule is related to the amount of cytokine produced.

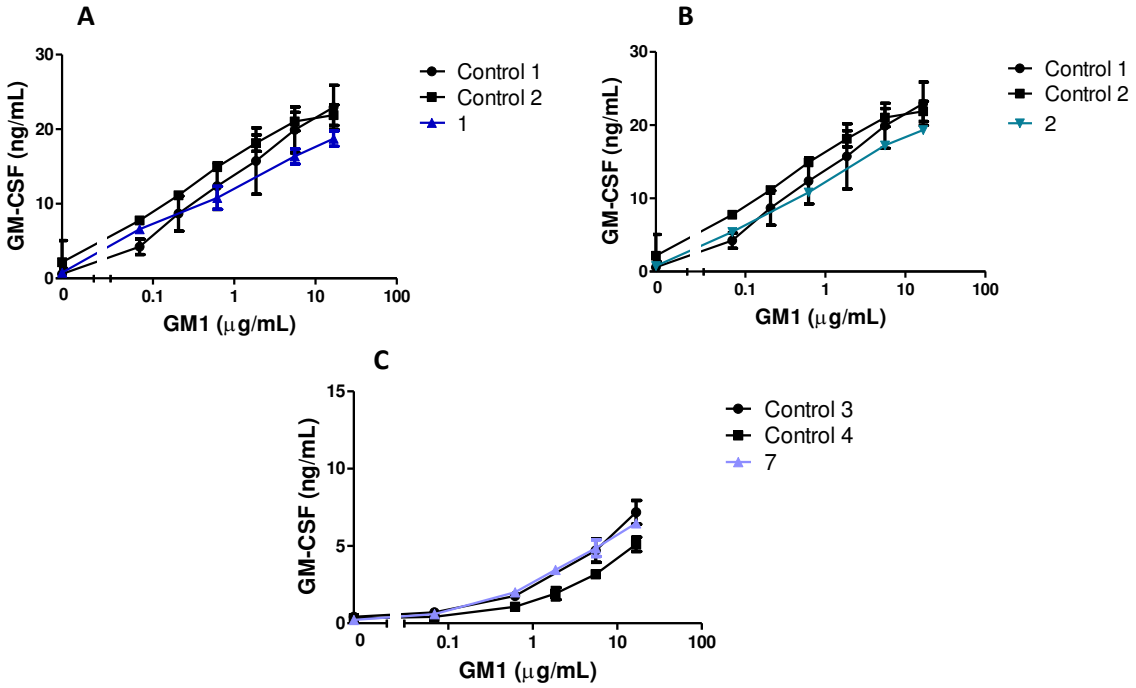

Figure 22. GM-CSF production by T cell clone GG33A activated with dendritic cells from four control subjects and three MPS VI patients. A – patient number 1; B – patient number 2; C – patient number 7. Dendritic cells were challenged with GM1 for 4h and then the T cell clone GG33A was added to the culture. 36h later supernatant was collected and

cytokine concentration determined by ELISA. The values represent means and standard deviation.

In the presentation of GM1 by CD1b, the three MPS VI patients showed a similar capacity for antigen presentation (this was assessed measuring the concentration of cytokine produced by the T cell clone when incubated with the dendritic cells plus the lipid antigen) these results are in Figure 22. Patient number 7 and the controls 3 and 4, as shown in Figure 22 C had lower cytokine production in comparison with the other subjects analyzed. This could be explained by the fact that patient number 7 and controls 3 and 4 were analyzed in a different day, and so the T cell clone used could be less responsive.

5. Discussion

Lysosomal storage diseases are metabolic disorders, characterized by the accumulation of biological materials in lysosome. This accumulation causes the impairment of lysosome, which is an intracellular compartment important for lipid antigen presentation and consequently important for the CD1 restricted lipid specific T cells. So emerged the hypothesis that patients with LSDs could have lipid antigen presentation and CD1 restricted lipid specific T cells affected. Several of the studies regarding CD1 restricted T cells were made in iNKT cells, because they are more easily identified. The percentage of the iNKT cells or their subsets was described to be altered in a variety of conditions, such as HIV infections [34], during *Mycobacterium* infections [52], during rejection of kidney transplants [53], and in cases of advanced cancer [54]. Other studies found a decrease in the iNKT cells percentage in the thymus and at the periphery in mouse models of several LSDs [37-43]. For some of these LSDs the reduced number of iNKT cells in mouse models was associated with deficient lipid antigen presentation [37, 38, 40, 41, 43, 46]. In humans Fabry, Gaucher and Niemann-Pick type C diseases were tested and no alterations in the percentage of total iNKT cells were found [42, 44, 45, 55].

The aim of our work was to study the lymphocyte family including B cells and T cells. And inside the T cells the CD1 restricted lipid specific T cells, analyzing the percentage of iNKT cells in MPS VI patients and testing the capacity of dendritic cells from the MPS VI patients to present lipid antigens to lipid specific T cells.

The iNKT cells percentage was highly variable among the MPS VI patients analyzed, similar to the variation that we and others have found on healthy subjects [31]. As previously described for other LSDs, such as Fabry, Gaucher and Niemann-Pick type C [42, 44, 45, 55] no differences statistically significant were found in the percentage of iNKT cells between the MPS VI patients and control subjects analyzed.

iNKT cells in humans can be divided in three different subsets, the $CD4^+$ subset (only express CD4), the $CD8^+$ subset (only express CD8) and DN (do not express neither of them). No significant differences in the percentage of the three

subsets between MPS VI patients and control subjects were found. Although recently our laboratory found in two sphingolipidoses alterations in the iNKT subsets. In Fabry disease patients a decrease in the percentage of $CD4^+$ iNKT cells and in Gaucher diseases patients an increase of this subset was found (Pereira et al unpublished results). Therefore the results obtained with MPS VI patients suggest that the alterations in the iNKT cells present in Fabry and Gaucher diseases are related to the specificity of the material stored rather than being associated with a general lysosomal dysfunction.

One of the MPS VI patients analyzed, the youngest showed a percentage of iNKT cells $CD4^+$ very high (92.1%) compared with others MPS VI patients and this patient was also one of the MPS VI patients with lower percentage of iNKT cells (0.013%). This is in agreement with results previously described by Montoya et al, where they saw that iNKT cells percentage have an inverse correlation with the percentage of the $CD4^+$ subset cells [56].

Five of the MPS VI patients were analyzed twice (2011 and 2012). The results showed no significant differences in the percentage of the iNKT cells and their $CD4^+$ subset between the two analyses. Longitudinal studies were already assessed in Fabry disease patients and the results showed some alterations on the percentage of iNKT cells in patients under ERT, however the percentage don't follow an increase or decrease pattern. So no significantly differences were found in the percentage of iNKT cells overtime in Fabry disease patients [57], according to the results presented here for MPS VI patients. We show that iNKT cells were stable over a period of one year in MPS VI patients under ERT.

For lipid antigen presentation assays we used DCs as APCs, a T cell clone restricted to CD1b molecule (GG33A) and a lipid antigen (GM1). For all the MPS VI patients analyzed were not found differences between them and the control subjects using CD1b molecule. These results are in agreement with previous results obtained in Fabry disease patients [57] and in Niemann-Pick disease type C [55]. In MPS patients this studies were accessed for the first time.

During this thesis I tried to develop a method to assess GAGs accumulation in few cells, to investigate the accumulation of GAGs in MPS VI patients DCs. To develop the method I used a patient fibroblasts cell line that was previously

described to accumulate GAGs and used Alcian blue cell staining followed by observation under optical microscopy. Alcian blue stains mixtures of sulfated GAGs, but does not allow distinguish between different types of GAGs [58]. This method turned out to be not sensible enough. Currently we are establishing a technique using Lysotracker, to evaluate the intralysosomal accumulation of undegraded macromolecules in DCs of LSDs patients by flow cytometry. Lysotracker was used before to measure the accumulation of cholesterol and gangliosides in cells of Niemann Pick type C2 mouse models [46] and in Niemann Pick type C1 patients [55].

For the study of DCs phenotype MPS VI patients' dendritic cells were generated from peripheral blood monocytes and used as APCs. We found small alterations in the DCs phenotype, but more subjects need to be included in the study before definitive conclusions can be drawn. We observed that expression of CD80 was similar between controls and MPS VI patients, however for CD11c expression two of the MPS VI patients shown a slightly decrease expression of this molecule when compared with control subjects. Group I CD1 molecules, was also analyzed and in this group CD1b and CD1c molecules expression were similar between MPS VI patients and control subjects, for CD1a molecule we found for the MPS VI patients two populations of DCs, one that express more CD1a and one that express less, this DCs that express low levels of CD1a can be in a different step of differentiation, supported by the notion that differentiation of $CD1a^{-}$ to $CD1a^{low}$ to $CD1a^{high}$ cells represent consecutive steps of DCs differentiation from monocytes [59]. CD1d was not analyzed due to the dendritic cells cultured in media containing FBS do not express CD1d at the surface in amounts detectable by flow cytometry [51]. In this work we analyze also the MHC-class I and MHC-class II molecules. For the HLA-ABC (MHC-class I) expression only one of the MPS VI patients (the youngest) showed alterations that was once again the observation of two populations of DCs (one that express more and one that express less HLA-ABC). The HLA-DR (MHC-class II) expression was altered in all three MPS VI patients when compared with the control subjects, the patients shown a slight decrease in the expression of this molecule, with the exception of the patient that shown a bimodal expression. Some of this alterations could be

related with a fact documented, that gangliosides can inhibit the differentiation of DCs from monocytes and influence the DCs phenotype [60]. It was reported previously that MPS VI feline models exhibit accumulation of both GM2 and GM3 gangliosides [61]. So this could be affecting the DCs phenotype of MPS VI patients and highlight the relevance of continuing the study of the DCs phenotype in these patients.

During this study T cell and B cell populations were also analyzed; T cell population can be divided in two subsets, the cytotoxic T cells ($CD8^+$) and the Helper T cells ($CD4^+$). We don't found significantly differences between MPS VI patients and control subjects for T lymphocytes and their subsets. However for B cell population we found an increase of the percentage of these cells in MPS VI patients when compared with control subjects. Our results for B cells are in agreement with previous work of Rozenfeld et al, where they described a significantly higher percentage of B lymphocytes in Fabry disease patients, both under ERT or without enzymatic treatment, compared with control subjects [44]. All the MPS VI patients that were studied in this work were under ERT. It is known that some patients develop antibodies against the infused enzyme [62]. Therefore the increase percentage of B cells may be related with chronic activation of the B cells caused by ERT. However, other yet not-identified mechanism could be responsible for the increase in the percentage of B cells.

6. Final considerations

This study allowed the characterization of the T and B lymphocytes in MPS VI patients. For the percentage of total iNKT cells, as the same as for the iNKT cells subsets no alterations were found in MPS VI patients, contrary to that was found for sphingolipidoses. The longitudinal study shows that iNKT cells from MPS VI patients under ERT continuo stable over a period of one year. Regarding lipid antigen presentation no alterations on the capacity of DCs from MPS VI patients to present lipid antigens by CD1b molecule were found. For the DCs phenotype, small alterations were found, but more subjects need to be included in the study before conclusions can be drawn. B lymphocyte population were found to be increased in patients with MPS VI , as previously described for Fabry disease by Rozenfeld et al [44]. With our studies regarding T cells, B cells and antigen presenting assays, we hope to contribute for the better understanding of this disease and the effect of their metabolic defects in immune system.

Since MPS VI is rare disease and we work with patients, some limitations appear in the course of the work. Such as, few patients that have this disease, small amount of blood samples and consequently few cells available, that sometimes, does not allow make all the assays in the same experiment. Other limitation of this work and of this thesis in particular was that we receive the blood samples from the MPS VI patients only in May. Age matching is another problem in our work, because some of the MPS VI patients are pediatric, and the controls that we obtain from the blood bank are all adults. This is still an ongoing project and we are trying at this time make a protocol to obtain youngest controls, to make possible an age match in our work. And we want to get access to more MPS VI patients in order to draw more conclusions about the DCs phenotype.

7. References

1. Duve, C.d., et al., *Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue.* biochemical journal, 1955. **60**(4): p. 604-617.
2. Vellodi, A., *Lysosomal storage disorders.* British Journal of Haematology, 2005. **128**(4): p. 413-431.
3. Schultz, M.L., et al., *Clarifying lysosomal storage diseases.* Trends in Neurosciences, 2011. **34**(8): p. 401-410.
4. Parkinson-Lawrence, E.J., et al., *Lysosomal Storage Disease: Revealing Lysosomal Function and Physiology.* Physiology, 2010. **25**(2): p. 102-115.
5. Pinto, R., et al., *Prevalence of lysosomal storage diseases in Portugal.* European Journal of Human Genetics, 2004. **12**(2): p. 87-92.
6. Meikle, P.J., et al., *Prevalence of Lysosomal Storage Disorders.* JAMA: The Journal of the American Medical Association, 1999. **281**(3): p. 249-254.
7. 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.
8. Dionisi-Vici, C., et al., *Inborn errors of metabolism in the Italian pedriatic population: A national retrospective survey.* The journal of pediatrics, 2002. **140**(3): p. 321-329.
9. Poupětová, H., et al., *The birth prevalence of lysosomal storage disorders in the Czech Republic: comparison with data in different populations.* Journal of Inherited Metabolic Disease, 2010. **33**(4): p. 387-396.
10. Hers, H.G., *α-Glucosidase Deficiency in Generalized Glycogen-Storage Disease (Pompe´s Disease).* The Biochemical Journal, 1963. **86**: p. 11-6.
11. Neufeld, E.F. and J. Muenzer, *The Mucopolysaccharidoses*, in *In the metabolic and molecular bases of inherited disease*, C.R. Scriver, et al., Editors. 2001, McGraw-Hill medical publishing division: New York.
12. Coutinho, M.F., L. Lacerda, and S. Alves, *Glycosaminoglycan Storage Disorders: A Review.* Biochemistry Research International, 2012. **2012**: p. 1-16.
13. Giugliani, R., P. Harmatz, and J.E. Wraith, *Management Guidelines for Mucopolysaccharidosis VI.* Pediatrics, 2007. **120**(2): p. 405-418.
14. Valayannopoulos, V., et al., *Mucopolysaccharidosis VI.* Orphanet Journal of Rare Diseases, 2010. **5**(1): p. 5.
15. Giugliani, R., et al., *Mucopolysaccharidosis I, II, and VI: brief review and guidelines for treatment.* Genetics and Molecular Biology, 2010. **33**: p. 589-604.
16. Harmatz, P., et al., *Long-term follow-up of endurance and safety outcomes during enzyme replacement therapy for mucopolysaccharidosis VI: Final results of three clinical studies of recombinant human N-acetylgalactosamine 4-sulfatase.* Molecular Genetics and Metabolism, 2008. **94**(4): p. 469-475.
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. Kobayashi, E., et al., *KRN7000, a novel immunomodulator, and its antitumor activities.* Oncology research, 1995. **7**(10-11): p. 529-34.
19. Pereira, C.S. and M.F. Macedo, *Lipid antigen presentation to T cells.* Current research in Immunology, 2011. **5**: p. 65-78.
20. Brennan, P.J., et al., *Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals.* Nature Immunology, 2011. **12**(12): p. 1202-1211.
21. Facciotti, F., et al., *Peroxisome-derived lipids are self antigens that stimulate invariant natural killer T cells in the thymus.* Nature Immunology, 2012. **13**(5): p. 474-480.
22. Porcelli, S.A. and R.L. Modlin, *The CD1 system: Antigen-presenting molecules for T cell recognition of lipids and glycolipids.* Annual review of immunology, 1999. **17**: p. 297-329.

23. Bricard, G. and S.A. 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.
24. 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.
25. De Libero, G. and L. Mori, *Recognition of lipid antigens by T cells.* Nat Rev Immunol, 2005. **5**(6): p. 485-496.
26. Mori, L. and G. De Libero, *Presentation of lipid antigens to T cells.* Immunology Letters, 2008. **117**(1): p. 1-8.
27. de la Salle, H., *Assistance of Microbial Glycolipid Antigen Processing by CD1e.* Science, 2005. **310**(5752): p. 1321-1324.
28. 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**(1): p. 81-88.
29. de Lalla, C., et al., *High-frequency and adaptive-like dynamics of human CD1 self-reactive T cells.* European Journal of Immunology, 2011. **41**(3): p. 602-610.
30. 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.
31. Godfrey, D.I., S. Stankovic, and A.G. Baxter, *Raising the NKT cell family.* Nat Immunol, 2010. **11**(3): p. 197-206.
32. O'Reilly, V., et al., *Distinct and Overlapping Effector Functions of Expanded Human CD4+, CD8α+ and CD4-,CD8α- Invariant Natural Killer T Cells.* PLoS ONE, 2011. **6**(12): p. e28648.
33. Olszak, T., et al., *Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function.* Science, 2012. **336**(6080): p. 489-493.
34. 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.
35. 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.
36. 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.
37. Gadola, S.D., et al., *Impaired selection of invariant natural killer T cells in diverse mouse models of glycosphingolipid lysosomal storage diseases.* Journal of Experimental Medicine, 2006. **203**(10): p. 2293-2303.
38. Zhou, D., et al., *Lysosomal Glycosphingolipid Recognition by NKT Cells.* Science, 2004. **306**(5702): p. 1786-1789.
39. 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.
40. 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.
41. Sagiv, Y., et al., *Cutting Edge: Impaired Glycosphingolipid Trafficking and NKT Cell Development in Mice Lacking Niemann-Pick Type C1 Protein.* The Journal of Immunology, 2006. **177**(1): p. 26-30.
42. 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.
43. Prigozy, T.I., et al., *Glycolipid Antigen Processing for Presentation by CD1d Molecules.* Science, 2001. **291**(5504): p. 664-667.

44. Rozenfeld, P., et al., *Leukocyte perturbation associated with Fabry disease.* Journal of Inherited Metabolic Disease, 2009.
45. 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.
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. 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.
48. Shamshiev, A., et al., *Self glycolipids as T-cell autoantigens.* European Journal of Immunology, 1999. **29**(5): p. 1667-1675.
49. Shamshiev, A., et al., *Presentation of the Same Glycolipid by Different CD1 Molecules.* The Journal of experimental medicine, 2002. **195**(8): p. 1013-1021.
50. Yan, J., et al., *The effect of aging on human lymphocyte subsets: comparison of males and females.* Immunity & Ageing, 2010. **7**(1): p. 4.
51. Leslie, D.S., et al., *Serum lipids regulate dendritic cell CD1 expression and function.* Immunology, 2008. **125**(3): p. 289-301.
52. 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.
53. Galante, N.Z., et al., *Frequency of Vα24+Vβ11+ NKT cells in peripheral blood of human kidney transplantation recipients.* International Immunopharmacology, 2005. **5**(1): p. 53-58.
54. Muhammad Ali Tahir, S., et al., *Loss of IFN-γ Production by Invariant NK T Cells in Advanced Cancer.* The Journal of Immunology, 2001. **167**(7): p. 4046-4050.
55. Speak, A.O., et al., *Invariant natural killer T cells are not affected by lysosomal storage in patients with Niemann-Pick disease type C.* European Journal of Immunology, 2012: p. n/a-n/a.
56. 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.
57. Pereira, C., *Lipid antigen presentation in Fabry disease patients (Master Thesis)*, in *Department of Biology*. 2011, University of Aveiro.
58. Frazier, S.B., et al., *The Quantification of Glycosaminoglycans: A Comparison of HPLC, Carbazole, and Alcian Blue Methods.* Open Glycoscience, 2008. **1**(1): p. 31-39.
59. Gogolak, P., et al., *Differentiation of CD1a− and CD1a+ monocyte-derived dendritic cells is biased by lipid environment and PPARγ.* Blood, 2007. **109**(2): p. 643-652.
60. WöLfl, M., et al., *Gangliosides inhibit the development from monocytes to dendritic cells.* Clinical & Experimental Immunology, 2002. **130**(3): p. 441-448.
61. Walkley, S.U., et al., *Abnormal neuronal metabolism and storage in mucopolysaccharidosis type VI (Maroteaux-Lamy) disease.* Neuropathology and Applied Neurobiology, 2005. **31**(5): p. 536-544.
62. Brooks, D.A., *Immune Response to Enzyme Replacement Therapy in Lysosomal Storage Disorder Patients and Animal Models.* Molecular Genetics and Metabolism, 1999. **68**(2): p. 268-275.

Appendix

Composition of the solutions used in the experimental work:

PBS 10x

For a total volume of 1000mL, add:

80g of NaCl
2g of KCl
6.09g of Na_2HPO4
2g KH_2PO4
to H_2O

Adjust pH to 7.3

PBS 1x

For 1000mL, add 100mL of PBS 10x to 900mL of H_2O.

ACK lysis solution

For a final volume of 500mL, add:

4.15g of NH_4Cl
0.5g of KCO_3
to H_2O

Adjust pH to 7.2

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

For 100mL of solution, add:

0.2g of bovine serum albumin (BSA)
0.1g of NaN_3
to PBS 1x

MACS buffer

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

PBS 25% HS

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

PBS 1% formaldehyde

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

Culture medium

500mL RPMI 1640 (Invitrogen)
5mL of non essential aminoacids (Invitrogen)
5mL of kanamycin (Invitrogen)
5mL of sodium pyruvate (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

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

Washing buffer (ELISA)
PBS 0.05% Tween20

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

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

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