Universidade de Aveiro Secção Autónoma de Ciências da Saúde
Ano 2013

NUNO DUARTE RIBEIRO LOPES

iNKT CELLS IN MUCOPOLYSACCHARIDOSIS TYPE II PATIENTS

LINFÓCITOS iNKT EM PACIENTES COM MUCOPOLISSACARIDOSE TIPO II

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

Apoio financeiro do POCTI no âmbito do III Quadro Comunitário de Apoio.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

Dedico este trabalho ao meu pai.

o júri

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agradecimentos

Agradeço à Doutora Fátima Macedo pela preciosa orientação e revisão ao longo de todo o trabalho assim como pelo apoio e total disponibilidade para esclarecer dúvidas. Agradeço também à Doutora Clara Sá Miranda pela orientação que prestou no desenvolvimento do trabalho escrito assim como por me ter permitido desenvolver este trabalho no seu laboratório.

Às minhas colegas de laboratório, Cátia, Ana e Luz, por me terem ensinado todas as técnicas necessárias ao desenvolvimento deste trabalho mas sobretudo pela dedicação e paciência que tiveram nesse processo. Agradeço especialmente à Cátia pela disponibilidade constante para me auxiliar e pela supervisão do meu trabalho.

A todos os membros da UniLiPe e OBF pela simpatia com que sempre me trataram.

À Kika, o meu norte e grande apoio nos momentos mais difíceis ao longo dos últimos tempos. Obrigado pela compreensão e persistência ao longo deste ano de distâncias enormes e ausências prolongadas.

À minha família, tanto a de Fafe como a de Viseu, por acreditarem sempre em mim e nunca me deixarem desistir. Não teria chegado tão longe sem o apoio deles, sobretudo dos meus avós, os meus segundos pais.

Aos meus amigos, especialmente ao ‘quarteto do trio maravilha’ por todos os momentos especiais destes últimos cinco anos.

À Fundação para a Ciência e Tecnologia, cujo financiamento foi essencial para o desenvolvimento deste trabalho.

palavras-chave

doenças de sobrecarga lisossomal, mucopolissacaridose tipo II, antigénios lipídicos, células apresentadoras de antigénios, linfócitos iNKT

resumo

A Mucopolissacaridose tipo II (MPS II) é uma Doença de Sobrecarga Lisossomal (LSD) pertencente às mucopolissacaridoses. É caracterizada pela acumulação de sulfato de heparan e dermatan devido à deficiência na enzima lisossomal Iduronato 2-Sulfatase. O lisossoma é um compartimento importante para a atividade dos linfócitos iNKT (iNKT). Os linfócitos iNKT são linfócitos T restritos a lípidos envolvidos na infeção, autoimunidade e vigilância tumoral. Estudos anteriores em modelos de murganhos de LSDs demonstraram uma redução do número de linfócitos iNKT assim como alterações nas subpopulações de linfócitos iNKT. Apesar destes resultados, investigação similar em doentes humanos foi ainda pouco abordada. Aqui, analisamos pela primeira vez os linfócitos iNKT de doentes com MPS II. Os dados foram recolhidos através da análise por citometria de fluxo de Células Mononucleares do Sangue Periférico de doentes com MPS II. Os doentes com MPS II não apresentavam diferenças nos linfócitos iNKT totais nem nas subpopulações de linfócitos iNKT. Fenotipicamente, não foram encontradas, nestas células, alterações na expressão de marcadores de ativação e de linfócitos NK. Uma vez que a ativação de linfócitos iNKT requer o funcionamento do lisossoma das células apresentadoras de antigénios, analisámos as suas frequências e fenótipos. Foram encontradas reduções significativas nos monócitos de doentes e não foram encontradas alterações nas restantes células. Não foram encontradas alterações fenotípicas nas células apresentadoras de antigénios. A comparação dos resultados apresentados nesta tese com os resultados previamente obtidos no nosso laboratório para outras LSD sugere que o desenvolvimento dos linfócitos iNKT é influenciado pela natureza das moléculas acumuladas. Descrevemos ainda pela primeira vez alterações na percentagem de monócitos no sangue de doentes com MPS II.

keywords

lysosomal storage disorders, mucopolysaccharidosis type II, lipid antigens, antigen presenting cells, iNKT cells

abstract

Mucopolysaccharidosis type II (MPS II) is a Lysosomal Storage Disorder (LSD) belonging to the group of mucopolysaccharidoses. It is characterised by the accumulation of heparan and dermatan sulfate due to deficiency of the lysosomal enzyme Iduronate 2-Sulfatase. The lysosome is an important compartment for the activity of invariant Natural Killer T cells (iNKT). iNKT cells are lipid-specific T cells that were shown to be important in infection, autoimmunity and tumour surveillance. Previous studies in mouse models of LSDs have shown a decrease in iNKT cell numbers and alterations in iNKT cell subsets. In spite of these findings, similar research in human patients has been poorly addressed. Herein, we analysed for the first time iNKT cells from Mucopolysaccharidosis type II patients. Data was acquired through flow cytometry analysis of Peripheral Blood Mononuclear Cells from MPS II patients. MPS II patients did not present differences in total iNKT cells neither in iNKT cell subsets. Phenotypically, no differences have been found in the expression of activation and NK cells markers. Since iNKT cell activation requires a functioning lysosome of antigen presenting cells, we analysed their frequency and phenotype. We have found a significant reduction in monocytes from patients and no differences in the other cells. Furthermore, no phenotypical alterations have been found in antigen presenting cells. The comparison of the results presented in this thesis with the results previously obtained by our laboratory in other LSD suggests that iNKT cell development is influenced by the nature of the accumulated molecules. We also described for the first time alterations in the percentage of monocytes in the peripheral blood of MPS II patients.

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ABBREVIATIONS

α-GalACer	α-Galacturonosylceramide
α-GalA-GSL	α–Galacturonosylceramide
α-GalCer	α-Galactosylceramide
αGalDAG	α-Galactosyldiacylglycerol
αGlcDAG-s2	α-Glucosyldiacylglycerol
β-GlcCer	β-Glucosylceramide
ACK	Ammonium-Chloride-Potassium
AP	Adaptor Protein
APC	Allophycocyanin
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CDR	Complementarity Determining Region
CG	Cholesteryl-6-O-Acyl-α-Glucoside
Cy	Cyanine Dye
DM1	Diabetes Mellitus Type 1
DN	Double Negative
EDTA	Ethylenediamine Tetraacetic Acid
ER	Endoplasmic Reticulum
ERT	Enzyme Replacement Therapy
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycan
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Factor
GM3	α-N-Acetylneuraminyl-2,3-β-D-Galactosyl-1,4-β-D-Glucosylceramide
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IFN	Interferon
iGb3	Isoglobotrihexosylceramide
IL	Interleukin
iNKT	Invariant Natural Killer T
I2S	Iduronate-2-Sulfatase
LSD	Lysosomal Storage Disorder
LTP	Lipid Transfer Protein
MHC	Major Histocompability Complex
MPS	Mucopolysaccharidoses
MPS II	Mucopolysaccharidosis Type II
MS	Multiple Sclerosis
NK	Natural Killer
NKT	Natural Killer T
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered Saline
PE	Phosphatidylethanolamine
PerCP	Peridinin Chlorophyll Protein Complex

PI	Phosphatidylinositol
PIM	Phosphatidylinositolmannoside
pLPE	1-Palmitoyl-2-Linoleoyl-Pe
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper
TNF	Tumour Necrosis Factor

1. INTRODUCTION

1.1. Lysosomal Storage Disorders

The lysosome is an acidic organelle existent in all nucleated cells. It is involved in macromolecule digestion but also in lipid antigen presentation [1-4]. The dysfunction of lysosomal proteins may originate Lysosomal Storage Disorders (LSDs), a group of inherited metabolic disorders [5]. Defective proteins can be directly involved in lysosomal macromolecule digestion, such as in the Mucopolysaccharidoses, or in molecule transport, such as in Niemann-Pick disease type C. In these diseases, lysosomal dysfunction causes a progressive intracellular accumulation of macromolecules. As a consequence, cell and tissue function become altered. This occurs either as a direct result of substrate accumulation - cell enlargement and meganeurites due to accumulation of hydrophilic metabolites – or by the activation of secondary pathways - altered gene expression and defective intracellular signalling [4, 5].

Individually, LSDs are a vast group of rare disorders, but combined they account for more than 1 case per 8000 births. In Portugal the incidence is even higher, with 1 case per 4000 births [6]. At birth, LSD patients can have a normal clinical presentation. Symptoms develop with age, resulting in a wide spectrum of clinical phenotypes and often in premature death [3, 5]. Distinct phenotypes and onsets are explained by different thresholds of enzyme activity, by the nature of the accumulated molecule and the anatomical location of the accumulation [3, 7].

LSDs are classified according to the nature of the stored molecule: lipidoses, mucopolysaccharidoses, glycogenoses, glycoproteinoses, mucolipidoses and neuronal ceroid lipofuscinosis [4, 7]. The focus of this study is Mucopolysaccharidosis type II, later described in this section.

1.1.1. Mucopolysaccharidoses

Mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders caused by dysfunctional lysosomal proteins involved in the degradation of glycosaminoglycans (GAGs), also known as mucopolysaccharides [8]. Most GAGs are a constitutive part of proteoglycans, extracellular matrix proteins involved in several biological functions such as tissue lubrication, chemical signalling and regulation of protein activity [9, 10].

GAG metabolisation requires their entrance in the lysosome, where they are digested by several enzymes. When the lysosome fails, these molecules are stored in the lysosome and/or excreted in the urine. Phenotypically, MPS patients present several similarities due to the similar nature of the accumulated molecules [8].

1.1.1.1. Mucopolysaccharidosis type II

Mucopolysaccharidosis type II (MPS II), also known as Hunter Syndrome, is an X-linked recessive LSD caused by mutations in the IDS gene. The IDS gene encodes for the lysosomal enzyme iduronate-2-sulfatase (I2S). This protein degrades dermatan sulfate and heparan sulfate through the removal of a sulfate group from α-L-iduronic acid [8]. The reduction or absence of I2S activity leads to incomplete or null degradation of dermatan and heparan sulfate. As a consequence, these GAGs accumulate in the lysosome [8, 11, 12].

Epidemiology

MPS II is a rare disorder despite being one of the more prevalent MPSs. In Europe, its prevalence is between 0.64-0.71:100.000 births and more than two thirds of these cases are of severe phenotypes [12]. The prevalence of MPS II varies with geographic location [6-8]. In Portugal, twenty four cases of MPS II have been reported between 1982 and 2004, most of them in the North. From 1967 to 2000, Portugal presented a prevalence of 1.09:100.000 births, a higher prevalence than the one from Netherlands (0.67:100.000 births) and Australia (0.74:100.000 births) [6].

Given the X-linked nature of MPS II, this pathology is almost exclusive of males. However, there are some reported cases of mildly affected heterozygous females due to structural abnormalities or skewed inactivation of the X chromosome [8, 11, 12].

Molecular basis

The gene that codifies for I2S (IDS gene) is located in the long arm of the X chromosome (Xq28.1). It has a length of 24 kb and comprises nine exons. To date, there are more than three hundred known mutations responsible for MPS II. Point mutations (missense, nonsense, frameshift, altered splicing sites and small deletions/insertions) are the more frequent ones. These mutations can either result in a mild or severe phenotype [8, 12]. Major mutations (major deletions and rearrangements) are less frequent, representing only one fifth of the total mutations and often lead to severe outcomes. Null mutations (major deletions and rearrangements, frameshifts, mutations in splice-site associated consensus

regions and non-sense codons) are also associated with severe outcomes [8].

Disease severity relates to the location of mutation in the enzyme and how it influences enzyme activity. Mild patients express mutations that result in residual enzyme activity – 0.2-2.4% of wild-type enzyme activity; on the other hand, severe phenotypes have null levels of enzyme activity [13].

Clinical presentation

MPS II patients appear normal at birth and disease onset occurs during childhood with a series of unspecific symptoms, leading to considerable delay in diagnosis [8, 12]. Like other LSDs, age of onset and disease severity (e.g. neurological symptoms) is highly variable [8, 11, 12]. Severely affected patients appear normal at birth, with a few cases of overweight, abdominal hernia or Mongolian blue spots [12]. First symptoms appear in early life with neurological and somatic involvement. Patients develop coarsen facial features, dysostosis multiplex, organomegaly, mental retardation, chronic diarrhoea and ear infection. Natural death generally occurs in the mid-teenage years due to neurological deterioration and cardiorespiratory failure [8, 12]. Mildly affected patients are also born clinically normal but onset occurs later, around 4-8 years of age, with somatic involvement. Patients develop many somatic features like short stature and joint stiffness, besides carpal tunnel syndrome, cervical myopathy and ivory-coloured skin lesions; neurological impairment is limited or inexistent and intelligence is preserved [8]. Survival is frequent until the 2nd or 3rd decade of life, with some cases of nearly normal life span [12]. Natural death often occurs due cardiorespiratory problems [8, 12].

Hunter patients have a multisystem involvement with more prominence in the skeletal, respiratory, cardiovascular and neurological systems alteration (neurological systems are almost exclusively affected in severe phenotypes):

- *Musculoskeletal system* (Figure 1.1) – patients express distinctive features such as coarse facies, irregularly shaped teeth, short stature and macrocephaly as well as locomotion difficulties as a result of joint stiffness and contractures; spinal deformities such as kyphosis or scoliosis are also common [8, 11, 12];
- *Cardiovascular system* – valvulopathies, ventricular hypertrophy, arrhythmia, heart failure and hypertension are common clinical findings [8, 11, 12];
- *Respiratory system* – both airway and pulmonary anomalies are common in these patients, such as airway obstruction and sleep apnoea secondary to macroglossia, supraglottic narrowing and tracheomelacia caused by deposition of GAGs in the thong and trachea [8, 11, 12];
- *Nervous system* – neurological symptoms are highly variable among patients, however, in severe phenotypes, these are common; severe patients present mental impairment, loss

of auditory and/or visual acuity (even deafness or blindness) secondary to nerve lesion or blockage; rare cases of seizures and behavioural disturbances like aggressive behaviour have also been described [8, 11, 12].

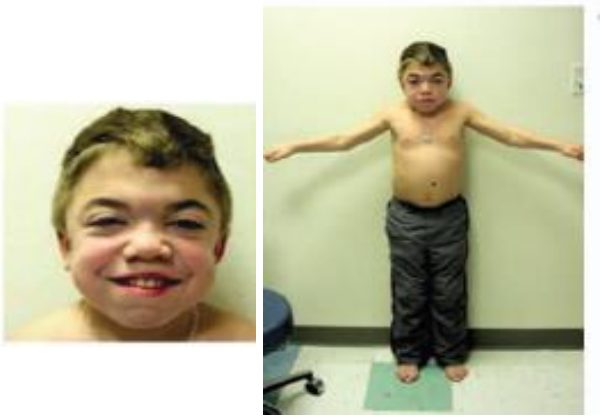

Figure 1.1 - Characteristic features of MPS II patients [11]. A typical MPS II patient presents characteristic facial features such as enlarged head, broad nose, large jowls and thickened lips (left) and musculoskeletal alterations such as short neck and stature as well as joint stiffness (right).

Diagnosis and Treatment

The clinical suspicion for MPS II usually occurs with the analysis of the patient's facial features. During clinical evaluation, anamnesis and radiological examinations help the physician to distinguish the MPSs from other LSD. However, MPS II is phenotypically similar to other MPSs – organomegaly, dysostosis multiplex and facial features are common findings [8]. After clinical evaluation, biochemical and genetic tests are made to confirm the diagnosis. Biochemical analysis of urinary GAGs is a quick and inexpensive method, very useful in the differentiation among MPSs; it is, however, prone to false results [8, 11, 12]. Enzyme assays with leukocytes or cultured fibroblasts are the gold standard for disease confirmation [12]. Nevertheless, dry blood spots are a more popular alternative because these require low quantities of blood, facilitating blood collection from paediatric patients and reducing shipping problems. Finally, genetic analysis by gene sequencing can also be performed; this is particularly important for cases of abnormal phenotype, inconclusive enzyme assays or affected females [11].

For many years, the management of MPS II was merely supportive. It was focused on palliative relief of symptoms and life quality improvement, with nutritional, occupational, and physical therapy as well as surgical interventions [11, 12]. Since 2007, disease-specific therapy is available. Enzyme replacement therapy (ERT) for Hunter Syndrome acts by replacing the missing or abnormal I2S by a recombinant form of the enzyme. After administration of the recombinant I2S (Elaprase, Shire), it enters the lysosome where it

restores the function of the organelle and degrades accumulated GAGs. ERT has vast effects in quality of life and somatic function. It improves respiratory function, decreases organomegaly and allows more mobility due to enhanced joint mobility. Nonetheless, it has limited effects in the nervous and cardiovascular systems and can cause adverse effects such as infusion-related reactions [11]. Other therapeutic options based on transplantation such as stem cell therapy and bone marrow transplantation are not commonly used. Such treatments have promising effects in neurologic functions probably thought the replacement of deficient macrophages with marrow-derived donor macrophages that constitute a source of enzyme capable of gaining access to various storage sites. However, there is a high risk in terms of mortality and morbidity and therefore need further investigation [3, 11]. Promising new therapies such as gene therapy and substrate reduction therapy are in development [3, 11].

1.2. Lipid Antigen Presentation

T cells are commonly known by their capability to recognise peptide antigens bound to major histocompability complex (MHC) molecules. However, some T cell subsets are capable of recognising lipid antigens bound to MHC-like molecules. Lipid antigens exposed at the cell-surface, bound to CD1 molecules, are presented by antigen-presenting cells to CD1-restricted T cells. After lipid-CD1 recognition, CD1-restricted T cells become activated and respond through proliferation, cytokine production and cytolytic activity [14-21].

1.2.1. CD1 molecules

CD1 molecules are MHC-I-like molecules, capable of lipid-antigen presentation (Figure 1.2). These are composed by a heavy chain (α-chain) and a light chain (β2-microglobulin). The heavy chain is a transmembrane chain with three extracellular domains. The α1 and α2 domains form a deep hydrophobic antigen-binding groove with 2-4 pockets delimited by hydrophobic amino acids. The α3 domain supports the α1 and α2 domains and is non-covalently associated with the light chain - β2-microglobin. The cytoplasmic tail is involved in the recycling of the CD1 molecules [18, 19, 22, 23].

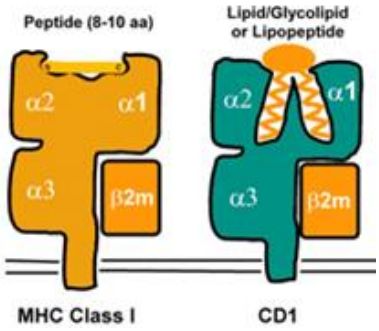

Figure 1.2 –Structure of the MHC-I and the CD1 molecules [22]. MHC-I and CD1 have three extracellular domains of similar size that bind β2-microglobulin. The CD1 groove has a narrow opening with a contiguous large and deep hydrophobic surface. It binds and shields the hydrophobic tails of lipids, anchoring the ligand so that its hydrophilic portions are exposed at the groove entrance.

Expression

CD1 molecules are expressed by a myriad of different cell types including professional antigen presenting cells such as dendritic cells (DCs), B cells and monocytes as well as tissue-specific antigen presenting cells such as thymocytes, hepatic cells (hepatic stellate cell, Kupffer cells), epithelial/mucosal (Langerhans cells) and neuronal (microglial) antigen presenting cells. CD1 molecules are also expressed by cells with non-professional antigen presenting capability such as epithelial cells, hepatocytes and keratinocytes [15, 18, 21, 23, 24].

In humans there are five isoforms of the CD1 α-chain (CD1a-e) whereas in mice only one isoform is present (CD1d) [18]. Human CD1 isoforms can be divided into three groups according to their homology, expression pattern and function:

- *Group 1* (CD1a-c) – expressed in cortical thymocytes and some antigen presenting cells (DCs and Langerhans cells); capable of presenting lipid antigens to several T cell subpopulations; upregulated by GM-CSF during *in vitro* monocyte differentiation to dendritic cells (DCs) [18];
- *Group 2* (CD1d) – expressed in cells of hematopoietic origin, mostly antigen presenting cells (DCs, B cells, monocytes and thymocytes) but also non antigen presenting cells such as hepatocytes; presents lipids exclusively to natural killer T cells (NKT cells); their expression is upregulated by inflammatory cytokines (IFN-β, IFN-ϒ) and down-regulated by immunoregulatory cytokines (IL-10, TGF-β), infectious agents (e.g. HIV, HSV) and during *in vitro* monocyte differentiation to DCs [18];
- *Group 3* (CD1e) – mainly expressed in DCs; intracellular molecule involved in lipid antigen processing but not in lipid antigen presentation [18].

Trafficking and lipid loading

The CD1 molecule is synthesized in the endoplasmic reticulum (ER). Upon synthesis, it translocate through the ER's membrane due to the presence of N-terminal signal sequences. In the ER glycosylation occurs. This allows the association with the light chain, with help of chaperones (calreticulin, calnexin and oxidoreductase ERp57) that stabilize the heavy chain during the folding process. When the CD1 molecule is assembled, its binding pockets are usually loaded with endogenous lipids ("spacers") such as phosphatidylinositol that prevent the collapse of the molecule. CD1 molecules then move to the Golgi complex for further glycosylation. Finally, CD1 molecules follow the secretory pathway to the plasma membrane, with the exception of CD1e. This isoform does not reach the plasma membrane and therefore is not capable of presenting antigens [4, 18, 25-27].

After reaching the plasma membrane CD1 molecules are recycled by the endosome-lysosome pathway. CD1 moves to the early/late endosomes and then to the lysosome [4]. In these compartments, the low pH allows the relaxation of CD1 structure, which facilitates lipid loading (Figure 1.3). CD1 isoforms vary in number, length and total volume of their binding pockets; this allows the selective binding of different lipids [22].

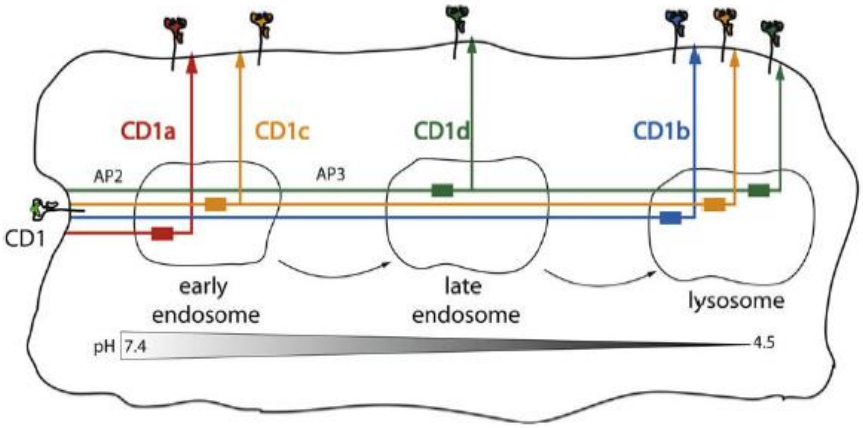

Figure 1.3 - Trafficking of the CD1 isoforms in the recycling compartments [17]. Different isoforms traffic through various endosomal compartments, each with different pH, and then move to the plasma membrane. Coloured boxes indicate where antigen loading occurs.

Different CD1 molecules recycling pathways are particularly important because different lipids also have distinct intracellular pathways. The entrance of lipids to the intracellular medium is required for their loading into CD1; this process is helped by lipid-transfer proteins (LTP). Inside the cell, their trafficking varies according to their features: neutral/acid lipids undergo fast recycling pathways whereas charged head groups (such as the common immunogenic lipids) undergo slow recycling pathways (from early endosomes

to the plasma membrane) [27]. In these organelles, some immunogenic lipids need to be reduced to smaller sizes to enable CD1 binding and TCR recognition. Lipid degradation is accomplished by physiological processes of degradation of oligosaccharides and lipids, fundamentally in the lysosome [19, 27]. After digestion, lipids are ready for CD1 loading. This process is achieved through the action of several LTPs (e.g. saposins, CD1e). Saposins are responsible for the internalization of membrane lipids and their binding to CD1 molecules, after the removal of "spacer" lipids. This process is facilitated by the low pH of recycling organelles. Finally, the CD1-lipid complex externalizes to the plasma membrane where it can be recognised [19, 27, 28].

The capability of these complexes to activate T cells depends on the stability of the complexes. CD1b-d molecules bind to lipids in the late endosome and lysosome, where the pH is lower, and therefore the formed complexes are less stable (until 24 hours). On the other hand, CD1a binds to lipids in the early endosome with a higher pH and the complexes are more stable (over three days) [19, 27].

1.2.2. Invariant Natural Killer T cells

Some T cell subsets are capable of recognising lipid antigens. Among these, invariant natural killer T cells (iNKT cells) are the most frequently used to study the biology of lipid antigen presentation due to the existence of a specific marker for their identification [29]. iNKT cells are a T cell population expressing a CD1d-restricted, lipid-specific T cell receptor combining a canonical Vα14-Jα18 α chain with a variable Vβ8, Vβ-7, or Vβ-2 β chain in mouse or Vα24-Jα18/Vβ11 in human [15]. Active iNKT cells induce immune responses, fundamentally through rapid and strong cytokine production [14, 15]. This action allows the protection and regulation of a broad spectrum of diseases such as tumour surveillance, infections and autoimmunity [15]. Phenotypically, iNKT cells are characterised by the expression of T and NK cell markers. Additionally, iNKT cells also express markers of a memory phenotype $CD62L^{Low}$ (memory), $CD44^{High}$ (effector-memory) [29].

Besides iNKT cells, there is another NKT cell subset – diverse NKT cells. These cells are also CD1d-restricted T cells capable of lipid antigen recognition. However, due to the variance in their TCR there is a lack of a specific marker for their identification [26, 27]. Hence, these cells remain poorly understood and will not be considered for this work.

Discovery

The term 'iNKT cell' was used for the first time in 1995 to define a mouse T cell lineage with invariant TCR α-chain, biased set of TCR β-chains, NK cell marker expression

and cytokine production. Later, this definition was extended to cells with the same features in other species such as humans. Currently, iNKT cells are a well-characterised population. However, to achieve the current state of the art, a long and tortuous process had to be traversed for several years (Figure 1.4) [16].

In the mid-1980s, an unusual and recurrent TCR was identified. This TCR's, expressed by murine suppressor T cells, was composed by an invariant α-chain - Vα14-Jα18. Later (1994), a similar TCR, composed by Vα24-Jα18 was found in human peripheral blood $CD4^-$ $CD8^-$ (DN) T cells. However, the major outbreaks in iNKT cells research occurred in the thymus. Several groups identified a small DN murine T cell subset with only intermediate expression of TCR and a two/three-fold increase in Vβ8 expression [15, 16, 30]. Slightly afterwards, a hepatic population of murine T cells with the same features was discovered. This allowed the establishment of a lineage relationship between the two populations [30]. Relatively to human iNKT cells, Vβ11 expression was only detected late (1993) [15].

In 1990, a subset of mouse T cells from spleen and bone marrow expressing intermediate levels of TCR, with a bias towards Vβ8 expression was identified. These cells expressed NK1.1, a NK cell marker until then only observed in NK cells; NK1.1 expression was later discovered in thymocytes. Another key feature of iNKT cells is cytokine production, observed for the first time in thymic $CD4^+$ mice T cells – IL-4 and IFN-Υ [16, 30]. Despite the previously shown features that nowadays are associated with iNKT cells, at the time, the relationship between these studies was still missing. Finally, in 1995 the connections were established with research in DN and $CD4^+$ T cells from mice and later in humans. Immediately afterwards, the search for iNKT cell ligands began with the discovery of the CD1d-restriction (1995) and activation by α-galactosylceramide (1997) [16, 30]. The last outbreak in iNKT cell research was the discovery of CD1d-αGalCer tetramers (2000) because it enables the specific detection of these cells [30].

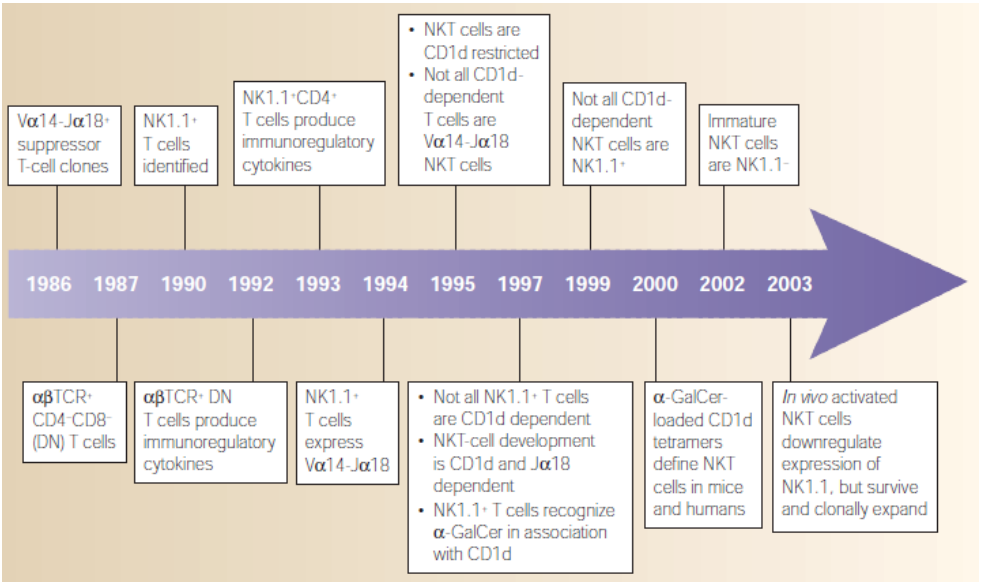

Figure 1.4 - Key events in iNKT cell research [16].

Frequency and tissue distribution

Mice and human iNKT cell populations are different. In mice, the hepatic population is the more substantial, constituting 15–35% of hepatic lymphocytes and 30–50% of hepatic T cells. In other locations such as the thymus, spleen, peripheral blood and bone marrow, this population is smaller – represents 0.2–0.5% of total lymphocytes and 0.5–2% of total T cells. In humans, the total frequency is inferior, possibly because of the higher frequency of other innate and innate-like T cells that might compensate iNKT cell function [14]. The frequency of this population is highly variable between individuals but it is relatively stable in each individual [14, 31] , with the exception of some slight increases that can occur with adulthood [32]. Human iNKT cells are more numerous in the adipose tissue, omentum and liver, constituting 15-35%, 10% and 1% of the lymphocytes, respectively. In peripheral blood, bone marrow and spleen the frequency is lower, accounting for 0.001%-3% of total lymphocytes [14].

Ligands

Many iNKT cell ligands remain unidentified and the nature and origin of those already identified remains poorly elucidated. iNKT cell ligands have a distinctive structure – a lipid tail, buried in the CD1d binding pocket, and a sugar head group, protruding out of CD1d for recognition by iNKT cell TCR [33].

Exogenous agonists are fundamentally derived from microorganisms - *Sphingomonas* spp. (α-GalACer and α-GalA-GSL), *M. bovis* (PIMs), *S. pneumoniae* (α-GlcDAG-s2), *H. pylori* (CG) and *B. burgdorferi* (α-GalDAG). Some of these lipids are α-Galactosylceramide (α-GalCer) analogues, a glycosphingolipid extracted from a marine sponge. Despite its lack of physiological function, this is the strongest activator of iNKT cells and due to its wide and specific recognition by a myriad of species it is used for iNKT cell identification [15, 21].

Endogenous lipids are also recognised by iNKT cells. Many of these were extracted from tumours such as the cases of phosphatidylinositol (PI) and phosphatidylethanolamine (PE); these two phospholipids are capable of weak stimulation. Non-tumoural ligands also exist, such as iGb3, produced in response to bacterial infection, and the ganglioside GM3 [15, 34]. Recently two important endogenous lipids have been identified – β-GlcCer and 1-Palmitoyl-2-Linoleoyl-Pe (pLPE): β-GlcCer is a danger-induced self-lipid and accumulates in antigen presenting cells that were stimulated with Toll-like receptor (TLR) agonists]23]; pLPE is a peroxisomal lipid capable of strong activati9on of iNKT cells [29].

Activation

iNKT cells are activated by three pathways: direct (TCR activation via CD1d lipid interaction), indirect (cytokine receptor activation) and combined (TCR and cytokine receptor activation) (Figure 1.5) [35].

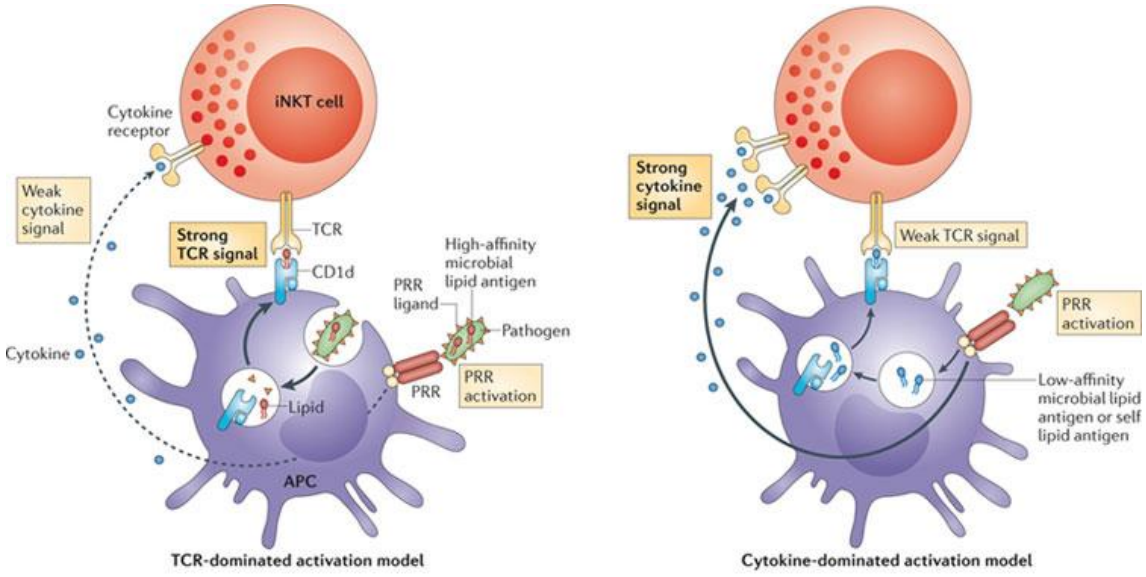

Figure 1.5 - TCR- and cytokine-driven activation of iNKT cells [23]. Two signals are involved in iNKT cell activation. A TCR signal provided by a foreign antigen–CD1Dd complex (left) and a cytokine signal dependent on the expression of their receptor by iNKT cells (right).

For direct or combined iNKT cell activation, CD1d lipid complex formation and cell surface exposure is required. After synthesis, CD1d reaches the plasma membrane and undergoes extensive internalization and recycling between the plasma membrane and endosomal/lysosomal compartments through the action of adaptor proteins. In mice, internalization occurs fundamentally through AP-2 and AP-3 to the lysosome; in humans, AP-2 transfers CD1d to the late endosomes. Then, CD1d is loaded with endogenous lipids [15]. iNKT cell TCR recognition is possible by their complementary-determining regions (CDRs) that form contacts with both ligand and CD1d. These regions determine the affinity of TCR to the lipid-CD1d complex [36-38]. Despite the invariance of iNKT cell TCR, these recognise distinct lipids by the same way: they reduce the energy expenditure required for forming a lipid-TCR complex and therefore induce the fit of the lipid. This explains the different strength of lipid-TCR interaction and the lower affinity of endogenous ligands [20, 23, 36, 38, 39].

Another way to activate iNKT cells is through cytokine production (indirect pathway). Antigen presenting cells secrete cytokines are secreted and recognised by the iNKT cells leading to their activation. This process is facilitated by the high basal levels of cytokine receptors in resting iNKT cells and by the high levels of cytokine transcripts in antigen

presenting cells. This allows a rapid immune response after iNKT cell activation, without the need of foreign antigen recognition by their TCR [23].

Many times the presence of cytokines alone is not sufficient to activate iNKT cells and there is a need for a weak costimulatory TCR signal for robust activation [40]. IL-12 is the best example. High levels of IL-12 receptor in resting iNKT cells allow a rapid response after cytokine production by antigen presenting cells; however, a weak TCR signal is still required despite the dominance of cytokine for the activation process [23, 40].

Immune responses

iNKT cells have a broad effect in the immune system, being involved in several processes such as infection, tumour development and autoimmunity. This is achieved fundamentally by cytokine production. After iNKT cell activation, these are capable of rapid and large secretion of several cytokines – IFN-ϒ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, TNF-α, TGF-β, GM-CSF [17-19, 21, 31, 36]. This capability is explained by the high levels of cytokine mRNAs in resting iNKT cells and the several transcription factors involved in the transcription of cytokine genes [36, 38].

Cytokines are capable of immunomodulation through crosstalk between several immune cells or by changing cytokine production from T helper (Th) type 1, type 2 and type 17 cytokines (Table 1). Cytokine production varies with: CD4 and CD8 expression, strength of activation, agonist type, antigen presenting cell involved [14-19, 21, 31].

Table 1.1 - Mouse iNKT cell classification according to the cytokine expression profile; adapted from [23, 40].

iNKT cell subsets	Location	Phenotype	Transcription factor	Cytokine
Th1-like	Liver, spleen	NK1.1$^+$, CD4$^+$/ CD4$^-$, IL-2R	T-bet	IFN-ϒ
Th2-like	Lungs	CD4$^+$, IL-17R$^+$	GATA3	IL-4, IL-9, IL-10, IL-13
Th17-like	Lymph nodes, lungs, skin	NK1.1$^-$, CD4$^-$, CCR6$^+$, IL-23R$^+$	RORYt	IL-17, IL-21, IL-22

Considering CD4 and CD8 expression, human iNKT cells can be classified into three subsets: CD4+CD8-, CD4-CD8+ and CD4-CD8-. CD4- iNKT cells are mainly producers of Th1-type cytokines (e.g. IFN-ϒ and TNF-α) whereas CD4+ produce both Th1- and Th2-type cytokines (IFN-ϒ, TNF-α, IL-4, IL-10, IL-13). CD8 expression also influences cytokine

production – e.g. Th1-like CD4-CD8+ iNKT cells produce more IFN-Ɣ than DN iNKT cells [14, 16, 23, 29, 37]. In mice iNKT cells, CD8 is not expressed and CD4 does not appear to relate with cytokine production [23, 40]. Strength of iNKT cell activation also relates with cytokine production profile. Strong activation induces both Th1 and Th2-like cytokine production but weak activation leads to a Th1-like profile [38]. The agonist also influences the response. Many agonists induce both Th1 and Th1-like responses (e.g. iGb3), however, others induce biased responses such as Cholesteryl-6-O-Acyl-α-Glucoside (Th1-biased response) and truncated αGalCer (Th2-biased response) [38, 41]. Th2-biasing ligands can bind to CD1d at cell surface whereas Th1-biasing ligands require intracellular trafficking [38].

Similarly to NK cells, iNKT cells are also capable of cytolitic activity and high levels of granzyme B, perforin and FasL are consistent with this. iNKT cells can kill antigen-pulsed antigen presenting cells in a CD1d-dependent manner in processes such as tumour surveillance and bacterial infection [14, 15, 36].

iNKT cells have a vast effect in other immune cells – macrophage, NK, B and conventional CD8+ T cell activation and DC recruitment and maturation. This occurs through cytokine production, as previously mentioned, and through upregulation of MHC-I and MHC-II antigen presentation (Figure 1.6) [14, 15, 36]. In the conventional iNKT cell activation, DCs and iNKT cells become activated mutually. Lipid-CD1d complexes activate iNKT cells and induce their upregulation of CD-40L, chemokines, Th1 and Th2 cytokines. This set of molecules triggers several signalling pathways that further induce iNKT cell activation. As a result of the increased activation of iNKT cells, produced cytokines (e.g. IL-12) activate NK and other iNKT cells. These produce IFN-Ɣ, with vast effects on immune cells, of both innate (NK cells) and adaptive immunity (conventional CD8+ T cells) [14, 15, 31].

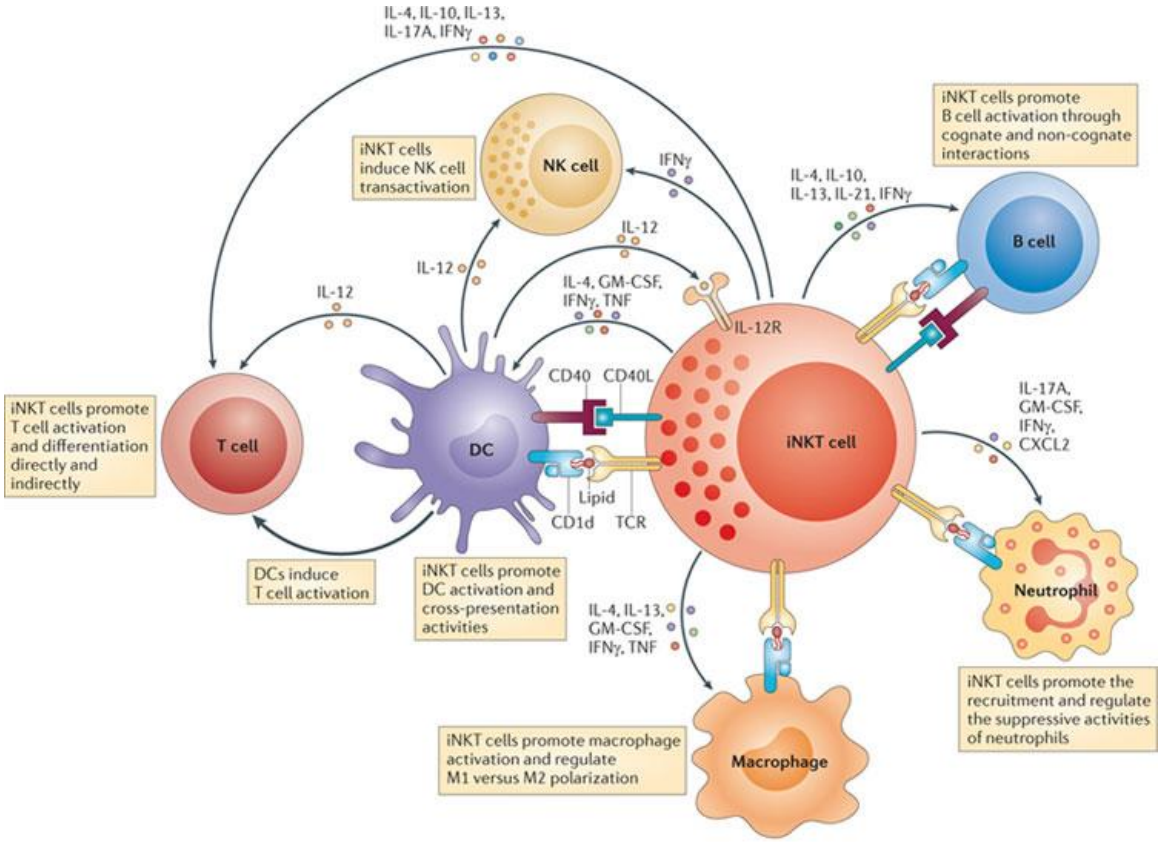

Figure 1.6 - Interactions between iNKT cells and other immune cells [23]. Lipid antigen presentation facilitates cognate interactions between iNKT cells and antigen presenting cells, leading to a reciprocal activation. iNKT and antigen presenting cell-derived factors can activate other cells.

iNKT cells in pathologic conditions

In several pathological conditions there are alterations in iNKT cell frequency and function suggesting their involvement in the development of the disease [31].

Diabetic patients have lower peripheral blood iNKT cells and cytokine production. However these have a dual role, according to the stage of the disease. Before disease development, if predisposed mice receive iNKT cells or αGalCer, their risk of developing Diabetes mellitus type 1 decrease. An explanation lies in the impaired differentiation of auto-reactive T cells into Th1 effector cells, the suppression of diabetogenic T cells and the increase of anti-inflammatory DCs. On the other hand, iNKT cells from diabetic mice appear to accelerate disease progression through the activation of diabetogenic T cells [31]. Multiple sclerosis patients have reduced iNKT cells and cytokine production. In the mouse model of multiple sclerosis, there is an overexpression of IL-14, a cytokine that has an inhibitory effect on the production of IFN-ϒ by splenocytes; also, TNF-treated DCs induce Th2 cytokine production in iNKT cells [14, 31].

In the case of LSDs, similar reductions in mouse iNKT cell frequency and cytokine production have been observed in a number of diseases – Sandhoff disease, Tay-Sachs disease, Fabry disease, GM1 gangliosidosis, Niemann-Pick disease type C and multiple sulfatase deficiency [44-51]. Despite the promising results in animals, similar studies in humans are scarce. In Fabry and Niemann-Pick type C mice, iNKT cell frequency is reduced in the liver, spleen and thymus of mice but normal in human peripheral blood [21, 42, 43]. Despite the several alterations of iNKT cells in many LSDs mouse models, it is not clear yet how these affect disease progression. These lysosomal inclusions can entrap endogenous ligands, impair antigen presentation, reduce CD1d loading or compete with storage lipids for CD1d binding [21].

2. Aims

The lysosome is an important organelle for lipid antigen presentation, a process that has been shown to be altered in many mouse models of LSDs and that is fundamental for the development and activation of iNKT cells by the CD1d molecule. Given this, the aim of this thesis is to study the iNKT cells in Mucopolysaccharidosis type II. To achieve it, we propose:

- To quantify the iNKT cells and their subsets in MPS II patients;
- To characterise phenotypically the iNKT cells from MPS II patients;
- To quantify the frequency of the antigen presenting cells in MPS II patients and to assess their phenotype, namely MHC-II and CD1d expression.

3. Materials and Methods

All the experimental analyses were performed using peripheral blood samples from MPS II patients and control subjects. Peripheral blood mononuclear cells (PBMCs) were isolated and used for flow cytometry analysis.

As these are rare patients, this thesis include all the analyses of the MPS II patients that were available in the lab, this means that the earlier analyses of the patients were done by Cátia Pereira. During the period of my thesis, I also contributed to the study of other LSD patients like MPS VI patients.

3.1.Subject selection and sample collection

Seven MPS II patients and thirteen control subjects were analysed in this study. The MPS II patient group was composed entirely by males, recruited by their physicians at the Hospital de Faro (Portugal), Centro Hospitalar São João (Portugal), Centro Hospitalar do Porto (Portugal) and Hospital de Clínicas de Porto Alegre (Brazil). In the beginning of the study, six of the patients were already under ERT - intravenous administration of a recombinant form of I2S (Elaprase, Shire). The only patient that was not already under ERT in the beginning of the study started ERT during the course of this study. The age, sex and ERT information of the patients included in this study, provided by their physicians, are described in the Results section, on Table 4.1. The control group was also composed exclusively by male blood donors. Blood samples from control subjects were obtained under a protocol between the Institute for Molecular and Cell Biology (Portugal) and the Centro Hospitalar São João.

Blood samples from patients were collected to ethylenediamine tetraacetic acid (EDTA)-containing tubes. In the case of patients under ERT, blood collection was performed before the infusion. Blood samples from blood donors came in buffy coats.

3.2.PBMC isolation

Upon arrival, which occurred until twenty four hours after blood collection, peripheral blood mononuclear cells (PBMC) from patients and control subjects were isolated using gradient centrifugation with Histopaque-1077® (Sigma-Aldrich), under sterile conditions (Figure 3.1). The blood was carefully laid over an equal amount of Histopaque-1077® and centrifuged at 400g during 30 minutes, without break. After centrifugation, the plasma was almost completely discarded and the PBMC ring (in the PBMC-interphase) collected.

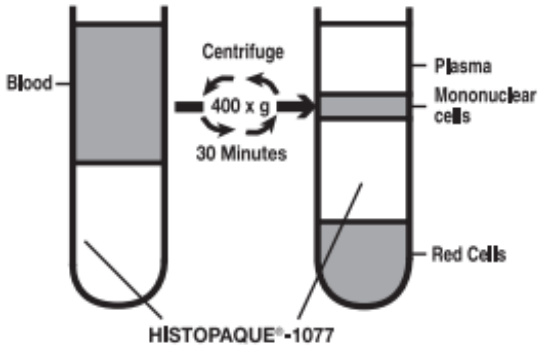

Figure 3.1 - PBMC isolation using Histopaque®-1077 (Sigma-Aldrich) [extracted from www.sigmaaldrich.com].

PBMCs were then washed with 10 mL of phosphate-buffered saline 1x (PBS 1x; see appendix) and centrifuged at 250g for 10 minutes. PBMCs were then incubated with 10 mL of Ammonium-Chloride-Potassium lysing buffer (ACK; see appendix) for 10 minutes. After incubation, 10 mL of PBS 1x was added and cells were centrifuged at 250g for 10 minutes. Cells were washed again with 10 mL of PBS 1x and centrifuged at 250g for 10 minutes. Finally, cells were counted using a microscope counting chamber (Neubauer chamber). After cell count, PBMCs were used for flow cytometry if from Portuguese patients or cryopreserved if from Brazilian patients.

3.3.PBMC cryopreservation

The analysis of cells from Brazilian patients required PBMC isolation and freezing before their transportation to Portugal. After PBMC isolation by the same protocol as described in section 3.3., cells were resuspended in 1 mL of freezing medium (see appendix) and transferred to a cryovial. The cryovial was transferred to a pre-cooled freezing box at -80ºC and then to liquid nitrogen, at -198ºC, until their transportation in dry ice. The analysis of these samples was performed using frozen PBMCs from Portuguese blood donors, cryopreserved by the previously mentioned technique.

3.4.PBMC thawing

Frozen PBMCs were thawed before flow cytometry analysis. Cryovials were placed in a 37ºC water bath until cell suspension was almost completely melted. To each vial, 1 mL of warm PBS 1x (see appendix) was added and cells were transferred to a centrifuge tube containing 9 mL of warm PBS 1x (see appendix). Cells were centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded and cells resuspended in 5 mL of PBS 1x (see appendix).

Finally, cells were counted using a microscope counting chamber (Neubauer chamber). After cell count, PBMCs were used for flow cytometry.

3.5.Flow cytometry

Up to $1x10^6$ PBMCs were stained per well in a round-bottomed 96-well plate. The cells were added to the wells and centrifuged at 1200 rpm for 2 minutes. The supernatant was then rejected and PBMCs were resuspended in 25 μL of antibody mixes diluted in PBS 0,2% BSA 0,1% NaN_3 (see appendix) for 20 minutes, in the dark at 4º C. The used antibodies and tetramers are listed in Tables 3.1-3.3.

Table 3.1 – Tetramers and antibodies used in flow cytometry to identify T and iNKT cells and their respective subsets.

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-eFluor780	eBioscience
Anti-human PBS57-loaded CD1d tetramers	-	PE	NIH

Table 3.2 – Antibodies used in flow cytometry to characterise phenotypically T, iNKT and NK cells.

Antibody	Clone	Fluorochrome	Brand
Anti-human CCR7	150503	FITC	R&D Systems
Anti-human CD45RA	MEM-56	APC	ImmunoTools
Anti-human CD56	MEM-188	FITC	eBioscience
Anti-human CD69	FN50	APC	eBioscience
Anti-human CD161	HP-3G10	eFluor450	ImmunoTools

Table 3.3 – Antibodies used in flow cytometry to identify and characterise antigen presenting cells.

Antibody	Clone	Fluorochrome	Brand
Anti-human HLA-ABC	W6/32	FITC	eBioscience
Anti-human HLA-DR	LN3	APC	eBioscience
Anti-human CD1c	L161	PerCP	BioLegend
Anti-human CD1d	51.1	PE	BioLegend
Anti-human CD14	M5E2	Pacific Blue	BioLegend
Anti-human CD19	HIB19	PE-Cy7	eBioscience

After incubation, PBMCs were washed three times with 150 µL of PBS 0.2% BSA 0.1% NaN3 (see appendix), centrifuged at 1200 rpm for 2 minutes, supernatant was rejected and cells resuspended. After the last wash, PBMCs were resuspended in 150 μL of PBS 1% formaldehyde (see appendix) and transferred to round-bottom Falcon tubes (BD Biosciences) containing 250 μL of PBS 1% formaldehyde (see appendix). PBMCs were kept in the dark at 4ºC until sample acquisition. Sample acquisition occurred in the same day of antibody incubation or in the following day.

PBMCs were acquired in a FACS Canto II (BD Biosciences) flow cytometer, using FACS Diva (BD Biosciences) software. Before data acquisition, it was required to analyse single stained PBMCs to adjust the voltage of sample acquisition. Data acquisition was performed as explained in Table 3.4. Data analysis was performed using FlowJo (Tree Star) software.

Table 3.4 – Sample acquisition in FACS Canto II (BD Biosciences).

Cell analysis	Events	Gate
Unstained PBMCs	10.000	Monocytes
iNKT cells	2.000	NKT cells
B cells and DCs	20.000	B cells

3.6.Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad) software. Mean and standard deviation values were calculated and p-values below 0.05 were considered statistically significant. Normally distributed parameters were tested using

Student's T-test and parameters without a normal distribution were tested with Mann-Whitney U test.

4. Results

4.1.Patient and control subject characteristics

Seven MPS II patients and thirteen control subjects were included in this study. The patients samples were thawed after flow cytometry analysis. The general features of each patient, that were available for this study, are described in Table 4.1.

Table 4.1 – Main features of the MPS II patients.

Patient	Sex	Age (study)	ERT	Country
1	Male	16, 15, 14	Yes	Portugal
2	Male		Yes	Portugal
3	Male		Yes	Portugal
4	Male	33	No	Portugal
5	Male		Yes	Brazil
6	Male		Yes	Brazil
7	Male		Yes	Brazil

The control group was composed by adult males with a mean age of 33 years old (standard deviation of ± 5.5 years).

4.2.T cell and T cell subset frequency

T cells ($CD3^+$) are key players in cell-mediated immunity that recognise peptide and also lipid antigens. These cells can be divided into two subsets: T helper ($CD4^+$) and T cytotoxic ($CD8^+$). The T cells and T cell subsets were identified by flow cytometry. To identify T cells, a lymphocyte gate is defined in a side scatter and forward scatter cytogram and, from this gate, the $CD3^+$ population is selected to define T cells. The analysis of the T helper and T cytotoxic subsets is performed by the definition of a gate to select $CD3^+CD4^+$ and $CD3^+CD8^+$ cells, respectively (Figure 4.1). All patients and controls were analysed for these parameters.

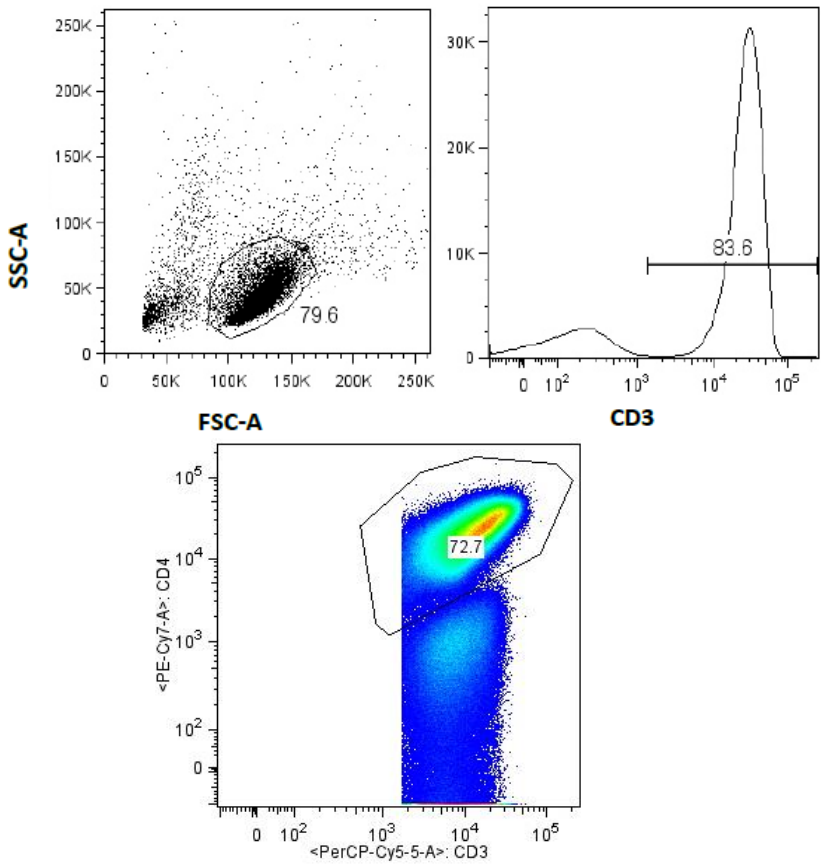

Figure 4.1 - Flow cytometry analysis of T cells and T cell subsets. A lymphocyte gate is established (left), T cells are selected by CD3 expression (right) and T cell subsets are identified through the expression of CD3 and CD4 (down) or CD3 and CD8.

Results concerning T cell frequency are presented in Figure 4.2. No significant differences have been found between the patient and the control groups in terms of T cell, $CD4^+$ T cell or $CD8^+$ T cell frequency (Figure 4.2). The untreated patient showed one of the highest frequencies of T helper cells. The patients from Brazil presented some of the highest frequencies of total and $CD8^+$ T cells.

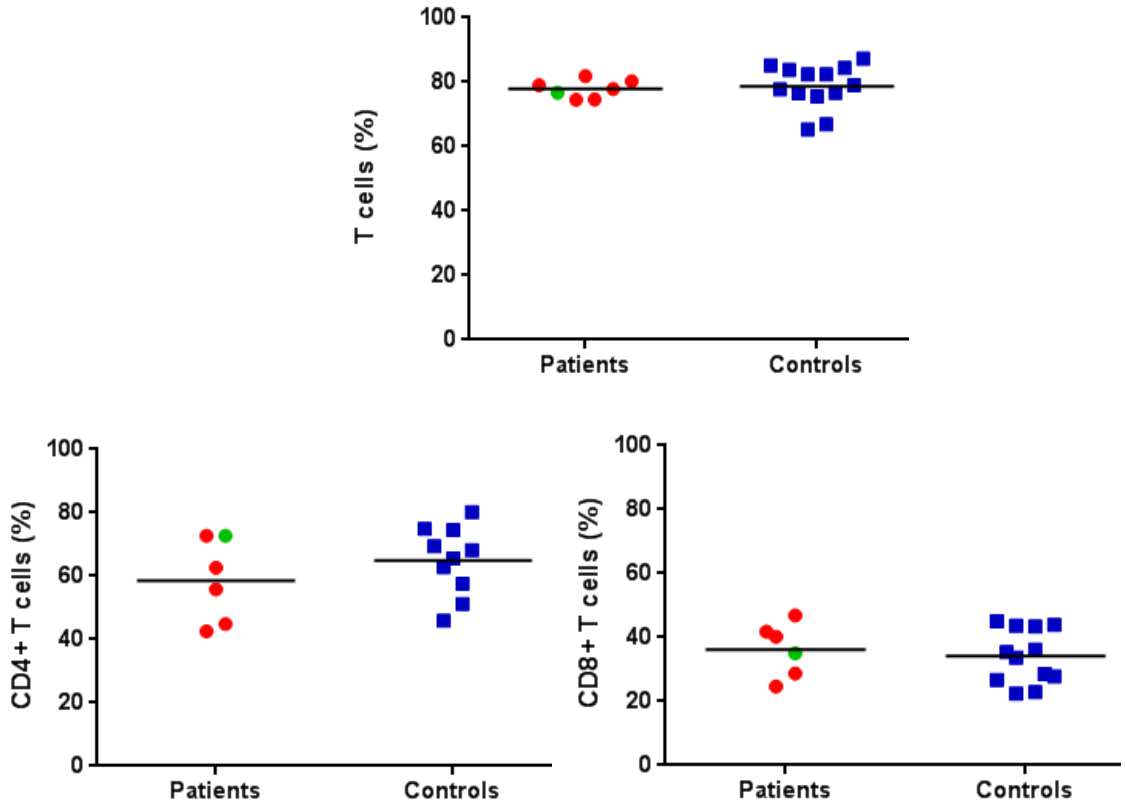

Figure 4.2 – Frequency of T cells and T cell subsets in MPS II patients. Red circles represent the T cell frequency in patients under ERT; green circles represent the T cell frequency in untreated patients; blue squares represent the T cell frequency in control subjects. Horizontal bars represent mean values.

4.3.NK cell frequency and activation

NK cells are cytotoxic cells involved in innate immunity. Their identification is performed through the definition of a lymphocyte gate in a forward scatter and side scatter cytogram and in this gate, $CD3^{-}CD56^{+}$ cells are selected (Figure 4.3). Activated NK cells were also studied. For this purpose, CD69 expression in NK cells was studied: geometric mean of CD69 fluorescence was analysed and auto fluorescence removed. These parameters were analysed in six patients and all control subjects.

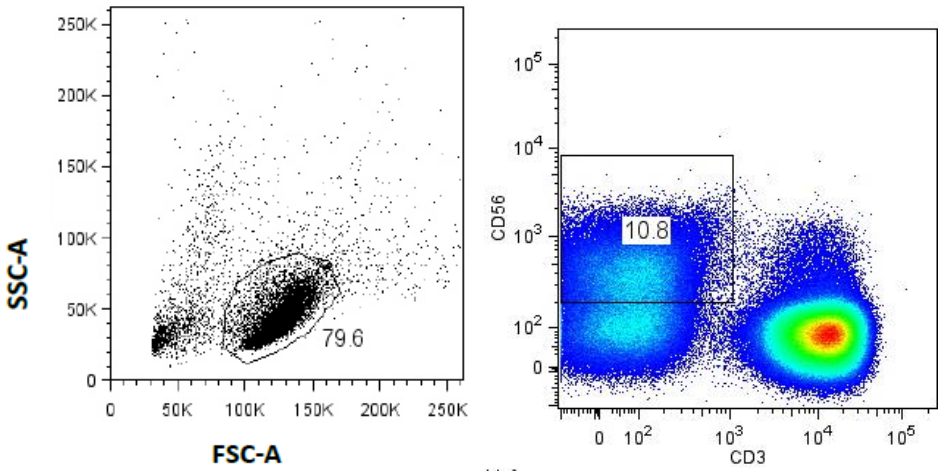

Figure 4.3 - Flow cytometry analysis of NK cells. A lymphocyte gate is established (left) and $CD3^-$ $CD56^+$ cells are selected (right).

No significant differences have been found in NK cell frequency and NK cell activation despite of the two cases of higher CD69 expression (Figure 4.4). No differences have been found in the analysis of frozen and fresh cells. In the patients, there is a great variability in terms of NK cell activation.

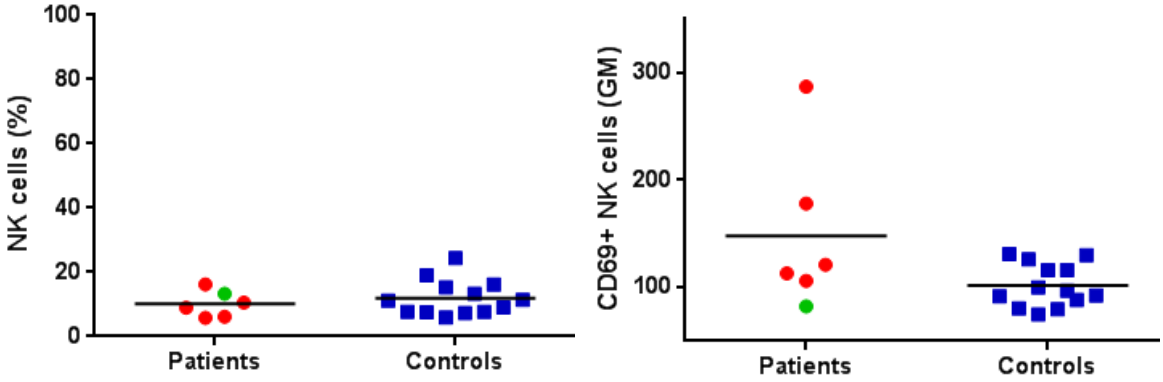

Figure 4.4 – Frequency and activation of NK cells in MPS II patients. Red circles represent the patients under ERT; green circles represent the untreated patients; blue squares represent the control subjects. Horizontal bars represent mean values.

4.4.iNKT cell and iNKT cell subset frequency

iNKT cells are involved in lipid antigen presentation and share many properties with NK and T cells. iNKT cell identification requires the definition of a lymphocyte gate and from

this gate, the CD3⁺ cells are selected to define T cells. Among T cells, the CD3⁺CD1d-PBS57 Tetramer⁺ population defines iNKT cells (Figure 4.5). All patients and control subjects were analysed for this parameter.

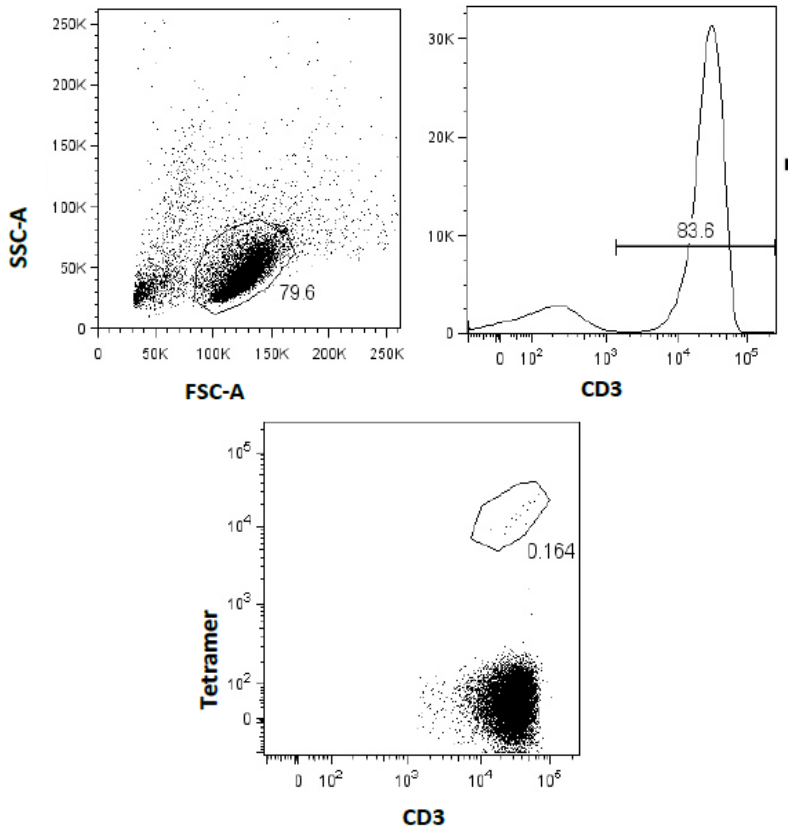

Figure 4.5 - Flow cytometry analysis of iNKT cells. A lymphocyte gate is established (left), T cells are selected by CD3 expression (right) and CD3 and tetramer positive cells are gated (down).

Results concerning iNKT cell frequency are presented in Figure 4.6. MPS II patients presented a higher variability in results than control subjects. The untreated patient presents a very low percentage of iNKT cells. The mean percentage of iNKT cells in MPS II is increased, however, not in a significant way and partly due to an outlier. Such outlier comes from a frozen sample, and can be partially explained by the presence of dead cells. To study the variability of results over time, patient #1 was studied for three years. He presented initial low levels of iNKT cells that increased during the first year of analyses and remained stable in the second year. Overall their frequency was kept very low.

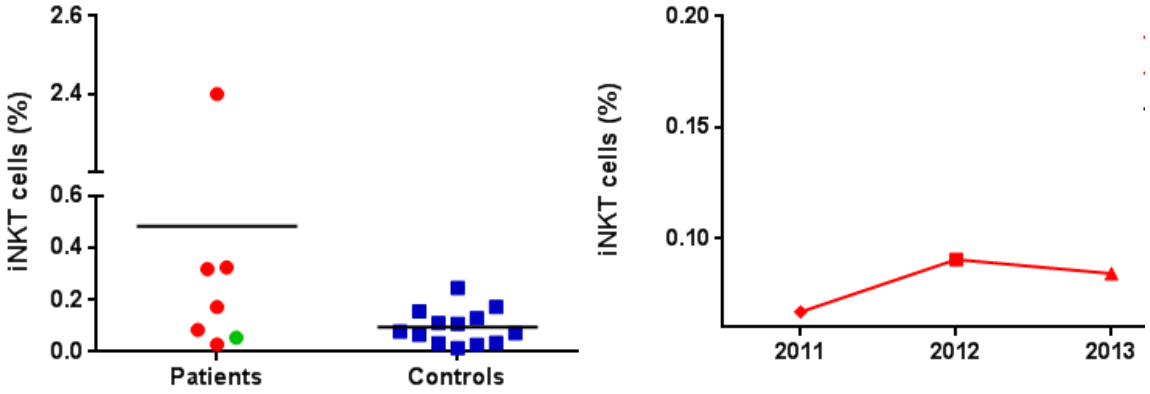

Figure 4.6 - iNKT cell frequency in MPS II patients (left) and a follow-up of iNKT frequency (right). Red circles represent the iNKT cell frequency in patients under ERT; green circle represent the iNKT cell frequency in untreated patients; blue squares represent iNKT cell frequency in the control subjects. Horizontal bars represent mean values.

In humans the CD4 and CD8 expression by iNKT cells is associated with different cytokine production profiles. To better characterise the iNKT function, these cells can be divided into three subsets according to CD4 and CD8 expression: $CD4^+$, $CD8^+$ and DN iNKT cells. After iNKT cell identification as described in Figure 4.5, four quadrants are draw to characterise CD4 and CD8 expression (Figure 4.7). All patients and controls were analysed for CD8 and CD4 expression.

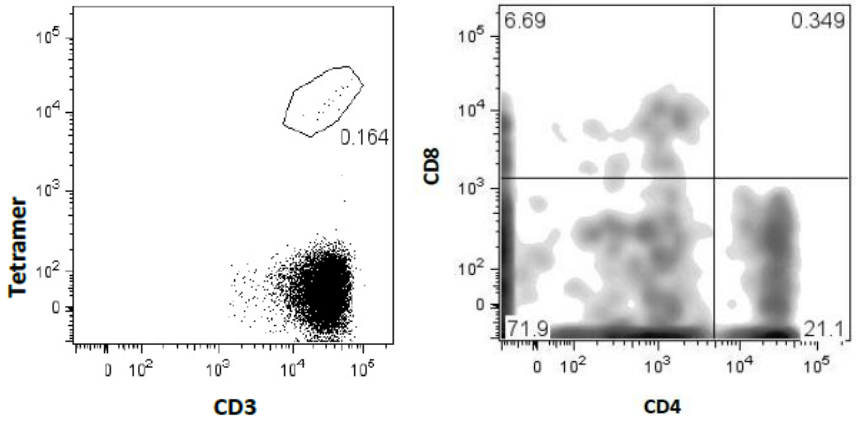

Figure 4.7 - Flow cytometry analysis of iNKT cell subsets. Inside of the iNKT cell gate (left), four quadrants are formed (right) according to CD8 and CD4 expression.

The frequencies of iNKT cell subsets are shown in Figure 4.8. Two patients presented almost exclusively $CD4^+$ iNKT cells and almost no DN iNKT cells. In both $CD4^+$ and DN iNKT cells the variability of results is very large in both groups. In patients, there is a lower presence of $CD8^+$ iNKT cells and the distributions of results falls within a narrow range. However, no significant differences were found among iNKT cell subsets between the patient and the control groups. In Brazilian patients, the percentage of $CD8^+$ iNKT cells is very small.

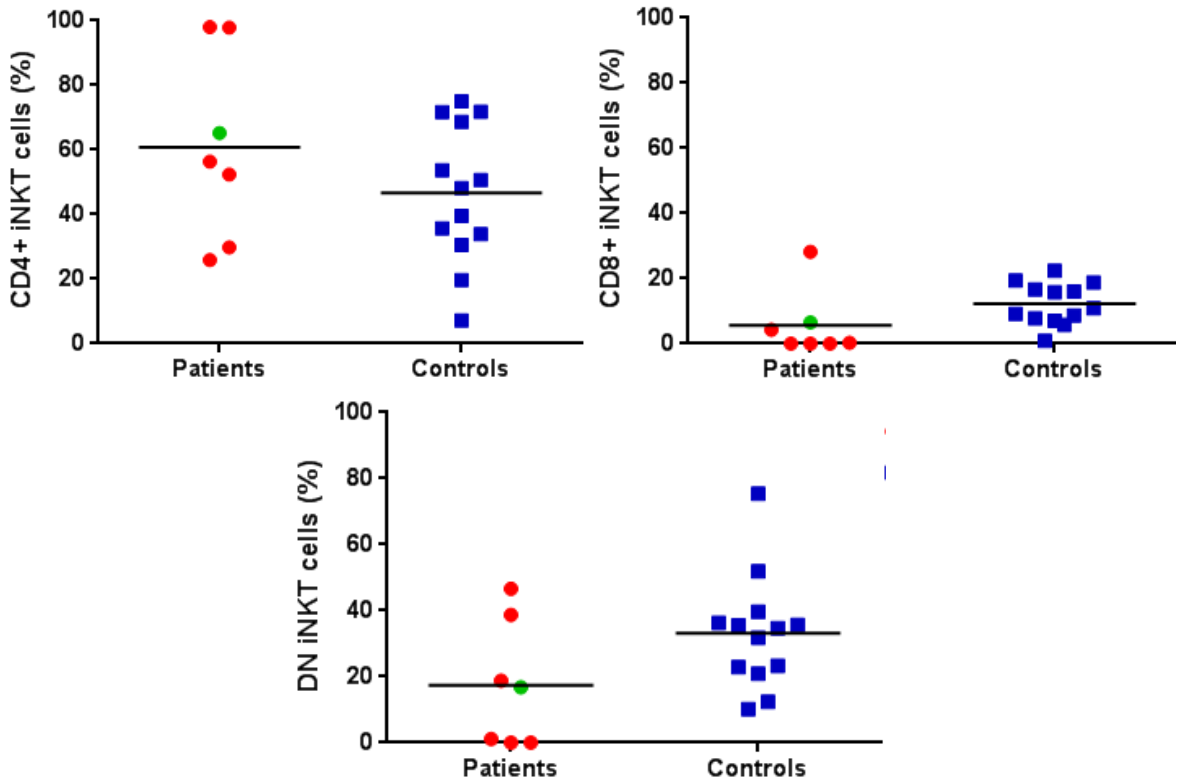

Figure 4.8 - iNKT cell subset frequency in MPS II patients. Red circles represent the iNKT cell frequency in patients under ERT; green circles represent the iNKT cell frequency in untreated patients; blue squares represent the iNKT cell frequency in the control subjects. Horizontal bars represent mean values.

The evolution of patient #1's iNKT cell subsets was also analysed during three years (Figure 4.9). During this period, there was an increase in the $CD4^+$ iNKT cell frequency and a variation in DN and $CD8^+$ iNKT cells.

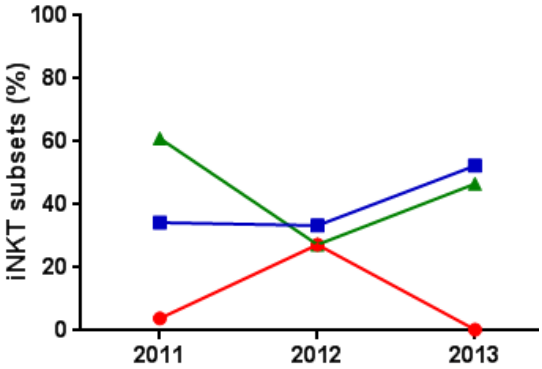

Figure 4.9 – Follow up of a patient's iNKT cell subset variation over time. Green line represents the DN iNKT cell frequency; blue line represents the CD4+ iNKT cell frequency; red line represents the CD8+ iNKT cell frequency.

4.5.iNKT cell activation

The activation of iNKT cells is fundamental for their action. To determine the degree of activation of these cells, CD69 expression is assessed by flow cytometry. To determine the expression of CD69, the geometric mean of CD69 fluorescence was determined in the iNKT cell gate (Figure 4.5). All patients and controls were analysed for this parameter.

Results relative to the activation of iNKT cells are show in Figure 4.10. The expression of CD69 by iNKT cells was similar between the two groups. No significant alterations were found. The cells with the lowest CD69 expression were the frozen ones.

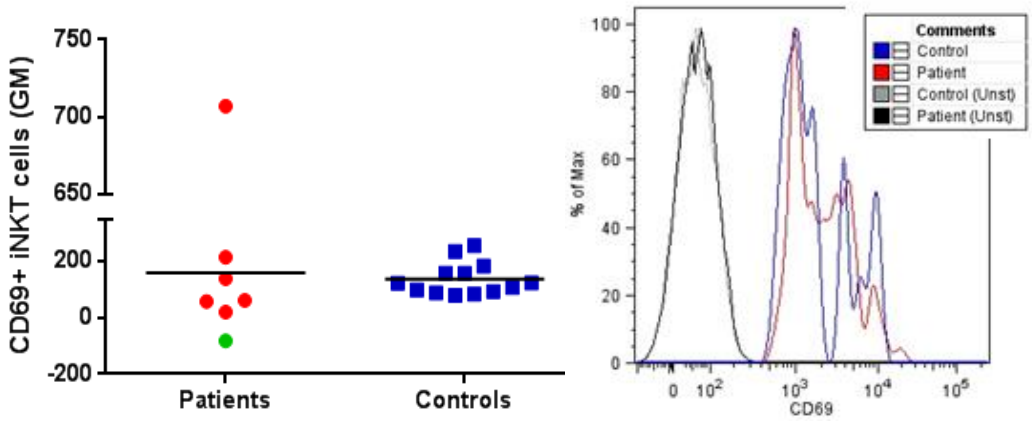

Figure 4.10 – Activation of iNKT cells in MPS II patients (right) and a representative example. Red balls represent the expression of CD69 in iNKT cells from patients under ERT; green balls represent the expression of CD69 in iNKT cells from untreated patients; blue squares represent the expression of CD69 in iNKT cells from control subjects. Horizontal bars represent mean values.

4.6. NK cell markers expression in iNKT cells

The characterisation of iNKT cells also involves the analysis of the NK cell markers CD56 and CD161. To determine the percentage of cells that expresses these markers, the $CD56^+$ and $CD161^+$ populations are selected from the iNKT cell gate. Both parameters were analysed in six patients and in all control subjects.

Results relative to the expression of NK cell markers are shown in Figure 4.11. In terms of CD56 expression, patients have a small reduction in the percentage iNKT $CD56^+$, with almost null in the untreated patient. Relatively to the expression of CD161, there is a larger variability in the percentage of $CD161^+$ cells, either from patients and controls and no alterations were found. Overall, there are no significant differences between patients and

controls in terms of the NK cell markers analysed.

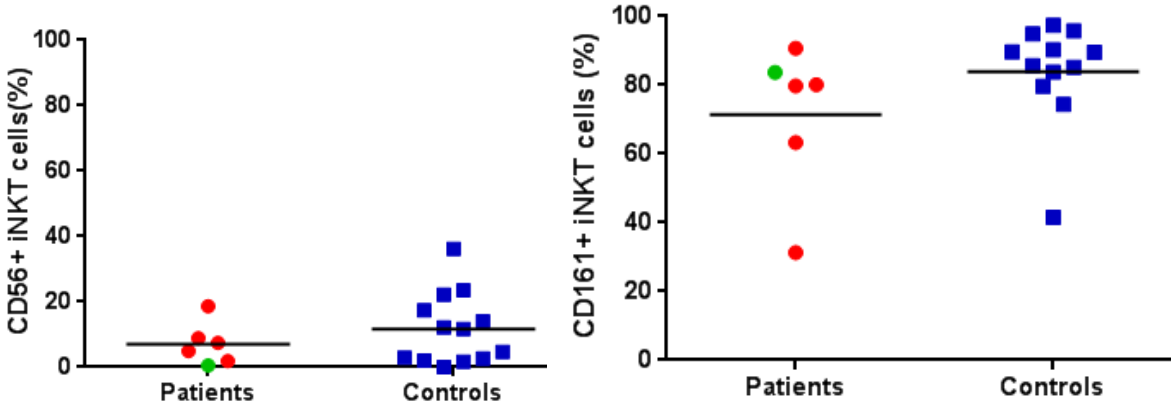

Figure 4.11 - Expression of natural killer markers in iNKT cells from MPS II patients. Red balls represent the iNKT cell frequency in patients under ERT; green balls represent the iNKT cell frequency in untreated patients; blue squares represent the iNKT cell frequency in the control subjects. Horizontal bars represent mean values.

4.7.Memory T and iNKT cells

The naïve and memory subsets of T and iNKT cells were also analysed (data not shown). However, the interpretation of these results requires an age-matched comparison and patient's age is unknown in most cases, therefore these results will not be presented herein.

4.8.Antigen Presenting Cell frequency

Lipid antigen recognition by iNKT cells is highly dependable on antigen presentation by antigen presenting cells such as DCs, B cells and monocytes. The frequency of these cells was accessed by flow cytometry. To identify B cells the CD19 marker was used on the lymphocyte gate. In the case of monocytes, a gate containing monocytes and lymphocytes was created in the FSC SSC gate and from this gate, monocytes were selected (Figure 4.12), therefore we are calculating the frequency of monocytes among PBMCs. DCs were identified as $CD14^-$ cells of the monocyte gate (Figure 4.12). It is important to refer that the used method for DC identification was not the ideal. The ideal one would be CD11c and CD123. Due to the frequent lack of cells from patients to perform flow cytometry analysis, the use this simpler method was the ideal method to use without the need for another antibody mix (that would require more patient blood). Monocyte and B cell frequency was studied in six patients and all control subjects and DCs frequency was studied in four patients and four control subjects.

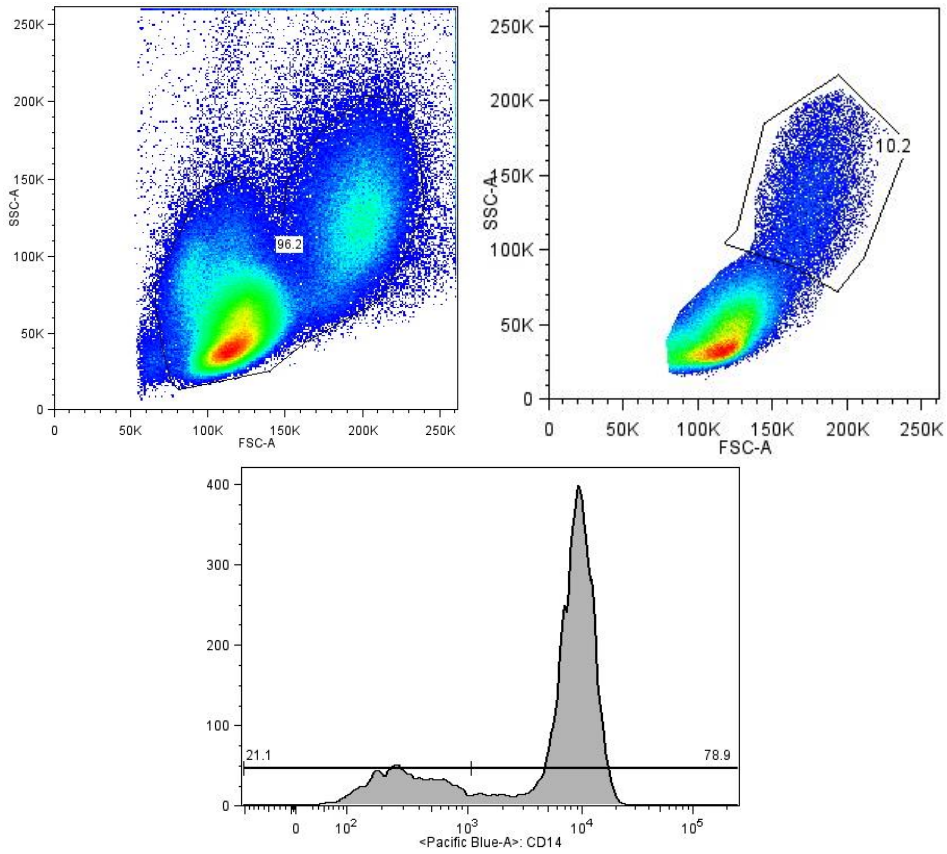

Figure 4.12 - Flow cytometry analysis of monocytes and dendritic cells. A gate including monocytes and lymphocytes is established and monocytes are selected (top) and, for the study of dendritic cells, the $CD14^-$ population is selected from the monocyte gate (down).

Results for antigen presenting cell frequency are shown in Figure 4.15. No significant differences have been observed between patients and control subjects in terms of DC and B cell frequency. Patients presented a significant reduction in monocyte frequency, comparatively to the control group (Figure 4.15 and 4.16). Curiously, the patient not under ERT presented one of the highest frequencies of monocytes.

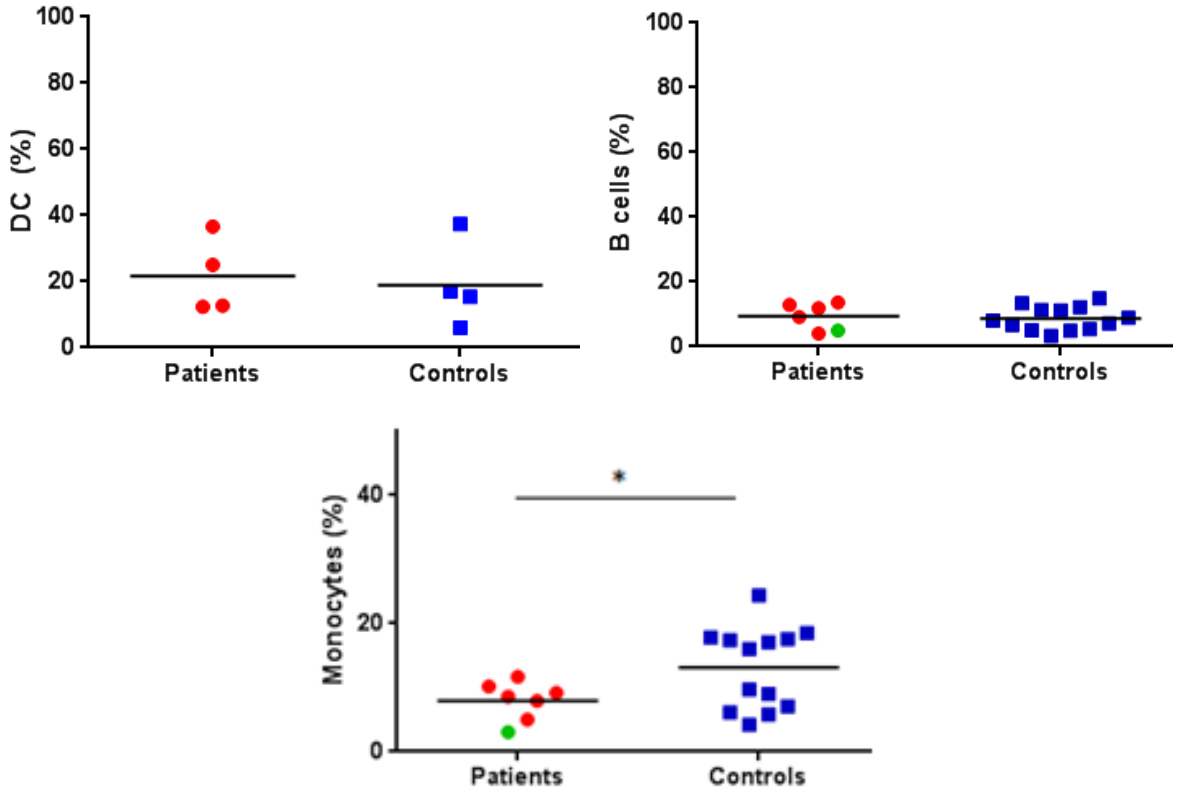

Figure 4.15 - Antigen presenting cell frequency in MPS II patients. Red balls represent the cell frequency in patients under ERT; green balls represent the cell frequency in the untreated patients; blue squares represent cell frequency in the control subjects. Horizontal bars represent mean values. Statistical significance was determined by Student's T-test, $p<0.05$.

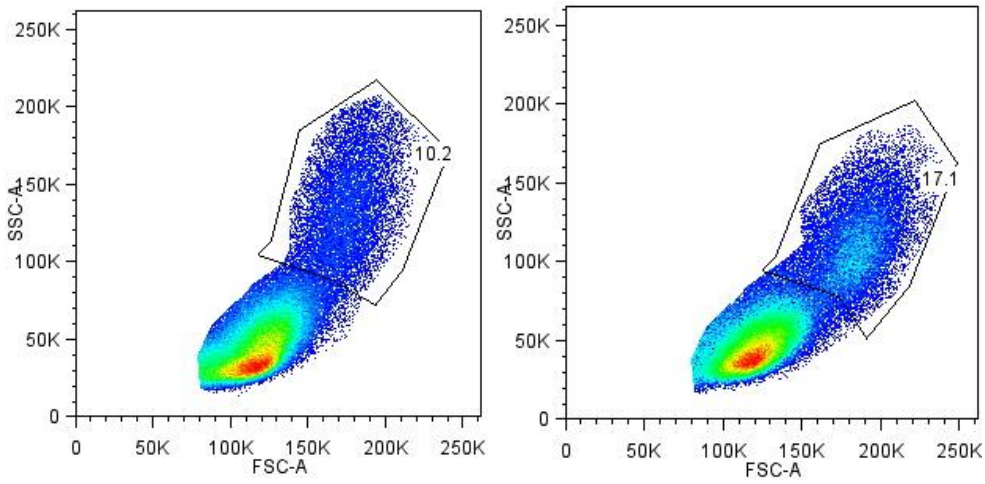

Figure 4.16 – Representative example of monocyte frequency in a patient (left) versus a control subject (right).

4.9.MHC and MHC-like molecule expression in Antigen Presenting Cells

Antigen presentation, either of lipids or peptides, to T cells requires their binding to MHC and MHC-like molecules. To assess the expression of these molecules within antigen presenting cells, the geometric mean of fluorescence was assessed for MHC-II, MHC-I, CD1d and CD1c in DCs, B cells and monocytes (CD1c was not analysed in monocytes as it is not expressed in these cells). Expression in monocytes and B cells was studied in six patients and all control subjects and in DCs, it was studied in four patients and four control subjects.

DC expression of MHC-II, MHC-I and CD1c is not significantly different between patients and controls (Figure 4.17). CD1d expression was not detected in the patients; CD1c was also undetected in one patient. In some patients, MHC-II is lower than in control subjects. However, the size of the sample is very small and the dispersion of the data is large. Three of the four samples analysed for these parameters were cryopreserved. The single fresh sample presented a smaller expression of MHC-II, MHC-I and CD1c. In the example shown in Figure 4.18, it is visible a smaller expression of MHC-II in a patient.

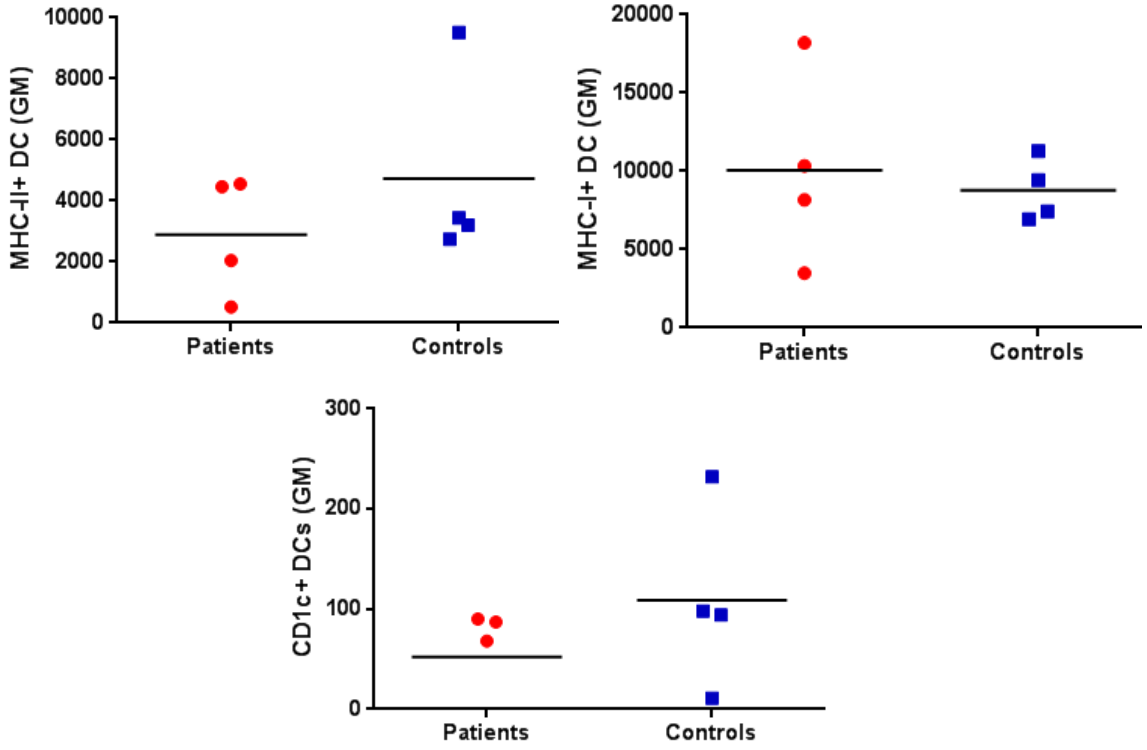

Figure 4.17 – Expression of MHC-II, MHC-I and CD1c by DCs in MPS II patients. Red balls represent the expression of the previously mentioned molecules in DCs from patients under ERT; blue squares represent the expression of the previously mentioned molecules in DCs from control subjects. Horizontal bars represent mean values.

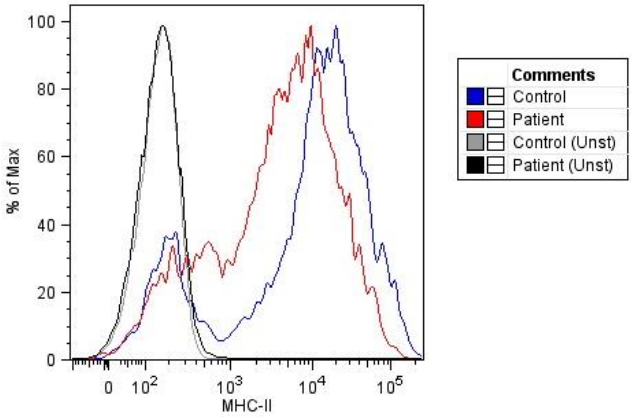

Figure 4.18 - Representative example of MHC-II expression by DCs of patients.

B cells from MPS II patients are not significantly different from the control group in terms of MHC-II, MHC-I, CD1d and CD1c expression (Figure 4.19). CD1d expression was not detected in one patient and in seven control subjects. No differences were found between fresh and frozen samples.

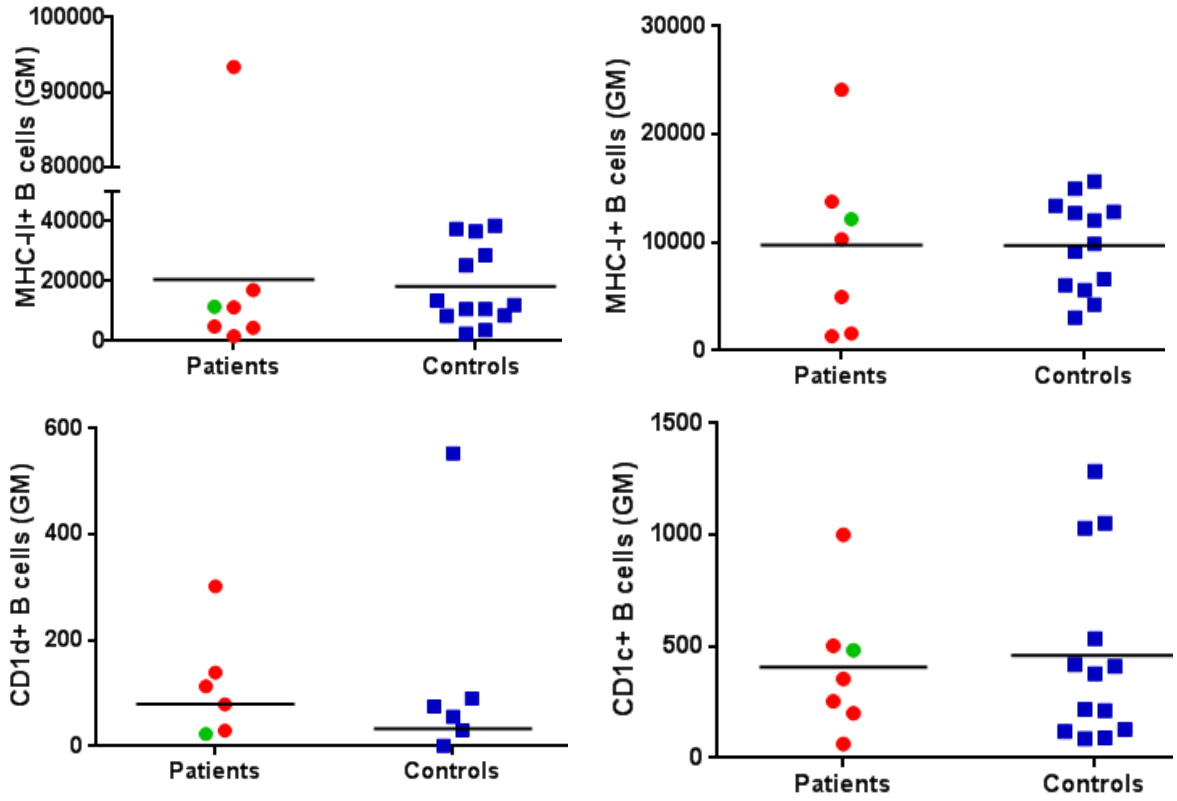

Figure 4.19 - Expression of MHC-II, MHC-I, CD1d and CD1c by B cells in MPS II patients. Red balls represent the expression of the previously mentioned molecules in B cells from patients under ERT; green balls represent the expression of the previously mentioned molecules in B cells from untreated patients not under ERT; blue squares represent the expression of the previously mentioned molecules in B cells from control subjects. Horizontal bars represent mean values.

Monocytes from MPS II patients are not significantly different from the control group in terms of MHC-II, MHC-I and CD1d expression (Figure 4.20). Monocytes expression of CD1d was not detected in the untreated patient and in one control subject. CD1d expression is reduced like in the example from Figure 4.21.

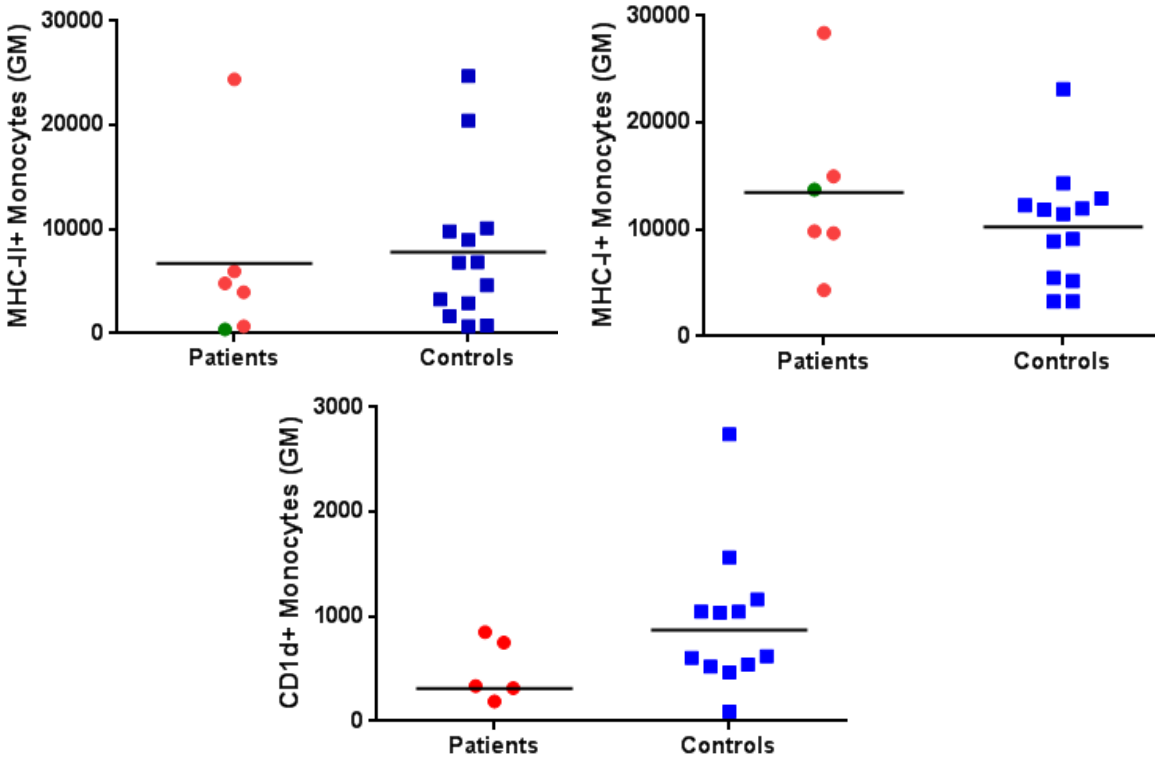

Figure 4.20 - Expression of MHC-II, MHC-I and CD1d by monocytes in MPS II patients. Red balls represent the expression of the previously mentioned molecules in monocytes from patients under ERT; green balls represent the expression of the previously mentioned molecules in monocytes from untreated patients; blue squares represent the expression of the previously mentioned molecules in monocytes from control subjects. Horizontal bars represent mean values.

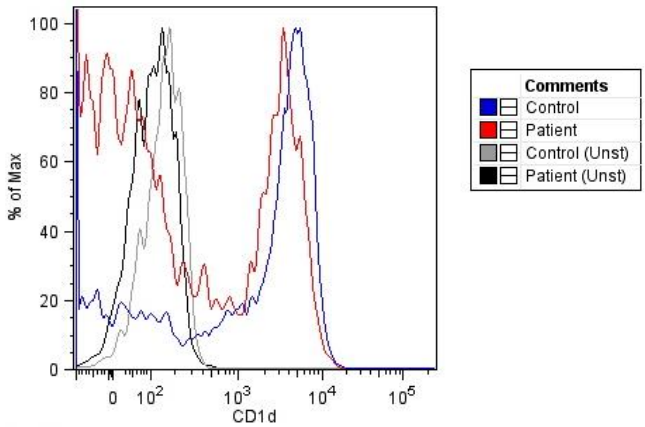

Figure 4.21 - Representative example of CD1d molecule expression by monocytes from patients.

5. Discussion

The lysosome is a fundamental organelle in the process of lipid antigen presentation due to its role in antigen loading into CD1 molecules. Consequently, alterations in the lysosome may impair the presentation and/or recognition of lipid antigens by iNKT cells. The lysosomal accumulation of undegraded molecules in LSDs led to the hypothesis that lipid antigen presentation and recognition may be altered in this type of diseases. Of particular importance is the study of iNKT cells in LSDs due to the fact that the number of these cells is reduced in several mouse models of LSDs [44-51]. This may be a direct consequence of the mutation that leads to the disease or it can be a consequence of macromolecule accumulation. In the mouse models of some other LSDs such as MPS I, no alterations have been found [46]. In patients, such studies are still rare.

In this study, we aimed to investigate the iNKT cells and the antigen presenting cells of MPS II patients. For this we analysed the frequency of iNKT cells and its subsets as well as their phenotype. Moreover, the frequency and MHC/CD1 expression by dendritic cells, B cells and monocytes was also analysed.

5.1.iNKT cell frequency

The percentage of iNKT cells from MPS II patients was not altered. It was observed a great variability of values in both groups. In other LSDs, a reduction in iNKT cells was also not observed [42, 52, 54]. iNKT cells present distinct frequencies between different individuals. Therefore, the detection of alterations in the frequency of these cells is difficult.

Patient #1 was analysed three times over a period of three years. Between the first and second analysis, it was observed a slight increase in iNKT cell frequency. In the literature, it is described an increase in the frequency of iNKT cells during puberty and a decrease in the late adult life of healthy individuals [32, 55, 56]. It is likely that the increase observed in patient #1 was due to the beginning of puberty. This patient was already on ERT for several years before the first analysis so the iNKT percentage increase was probably not ERT related. Due to the alterations of iNKT cell frequency with age, the use of age-matched controls could have improved the comparison between patients and control subjects. This would allow a better comprehension of their evolution with time.

The only untreated patient enrolled in this study presented a reduced percentage of iNKT cells. However, it was impossible to study the evolution of the iNKT cell population because of his death shortly after beginning ERT. In Fabry mice, ERT reduced macromolecule accumulation in the liver and spleen and caused an increase in splenic iNKT cells. ERT

stabilizes iNKT cell percentage in the spleen due to a specific effect on the iNKT CD4+ subset, preventing the decrease on the number of these cells that occurs with age in Fabry knockout mice [44]. In MPS II patients, it was impossible to assess the effect of ERT in iNKT cell frequency.

5.2. T and iNKT cell subset frequency

The human iNKT cell population can be functionally divided into three subsets, according to CD4 and CD8 expression: CD4+, CD8+ and DN. The CD4+ iNKT cell subset produces both Th1 and Th2 cytokines (mostly Th2 cytokines) whereas the CD8+ and DN subsets are biased towards Th1 cytokine production [45-47]. MPS II patients did not present significant alterations in the composition of iNKT cell subsets. These patients presented, however, a non-significant increase in their CD4+/CD4- iNKT cell ratio. A similar trend was not observed in the conventional T cell subsets from MPS II patients. Similar results were observed in MPS VI, where GAG accumulation also occurs. A not significant increase in the CD4+ subset was also described [48]. Due to the presence of such a trend in iNKT cell subsets, it would have been useful to include more patients in this study to confirm or dismiss if the trend was significant. To the best of our knowledge and according to the Portuguese Coordinating Committee for the Treatment of Lysosomal Storage Diseases, there are currently eight Portuguese MPS II patients. The study of the remaining patients would have been useful.

In sphingolipidoses, other type of LSDs, significant differences in the iNKT subsets were found. Gaucher patients have an increased percentage of iNKT $CD4^+$ cells whereas Fabry disease patients present a decreased $CD4^+$ subset and an increased DN subset. In both diseases, the alterations in CD4 and CD8 expression were not observed in conventional T cells, suggesting the specificity for iNKT cells [49, 50].

It was previously mentioned in the results section of this thesis that frozen $CD8^+$ iNKT cells presented a low frequency. In other LSDs studied in our lab, no significant differences were observed [Pereira, C. *et al*, unpublished results]. Hence, the low frequency of $CD8^+$ iNKT cells is hardly caused by cell cryopreservation.

In MPS II patients, the patients with the second and third highest iNKT cell frequencies (0.325 % and 0.319 %, respectively) also presented the lowest frequencies in CD4+ iNKT cells (25.9% and 29.8 %, respectively). This goes in agreement with a previously mentioned relationship between high frequency of iNKT cells and low frequency of $CD4^+$ iNKT cells [51]. The patient with the highest iNKT cell frequency was an outlier and therefore was not considered.

5.3.NK marker expression

The expression of NK cell markers was not significantly altered with disease. However, some of the patients presented a lower expression of such markers. Our results concerning CD161 expression are in agreement with the results of Niemann-Pick disease type C patients, where no alterations were detected [42]. Of our knowledge, there are no published results concerning CD56 expression in LSDs. The reduced expression in some patients may be partially explained by the non-significant increase in the $CD4^+$ subset. CD161 and CD56 are expressed by a significant number of $CD8^+$ and DN iNKT cells but only by a reduced number of $CD4^+$ iNKT cells [52]. An increase in the $CD4^+$ subset is therefore associated with a decrease in CD56 and CD161 expression and a consequent loss of cytolitic activity by iNKT cells.

5.4.NK and conventional T cells

MPS II presented no alterations in the frequency of total conventional T cells and NK cells. A similar observation was seen in Fabry disease patients [53]. On the other hand, Gaucher patients presented a normal number of T cells but a reduced number of NK cells in untreated patients [54].

5.5.Antigen presenting cell frequency and phenotype

In many LSD mouse models lipid antigen presentation was found to be impaired [55-60]. In humans, no significant alterations were observed in MPS VI and Fabry disease patients [43, 48, 49]. To assess the expression of molecules involved in antigen presentation by antigen presenting cells in MPS II patients, the frequency of antigen presenting cells (dendritic cells, monocytes and B cells) and their expression of MHC-II, MHC-I, CD1c and CD1d was studied.

5.5.1. Monocytes

MPS II patients presented a significant reduction in monocyte frequency. Similar observation was made in Fabry disease patients [43, 53, 61]. Antigen presentation by monocytes can be achieved through their differentiation into DCs or macrophages, but antigen presentation can also occur without this process. The observed reduction of monocytes can be explained by a reduction in the production of these cells by the bone marrow, a higher apoptotic rate and/or a higher migration to the peripheral tissues [53].

Despite their antigen presenting capacity, the major function of monocytes is made through their differentiation into macrophages, cells involved in primary immune responses. A reduction in monocyte frequency may therefore be behind the frequent ear infections experienced by the MPS II patients.

In monocytes from patients, no differences were found relatively to the expression of MHC-II, MHC-I and CD1d. However, some patients presented a reduction in CD1d expression. Some Fabry disease patients presented a reduction in MHC-II and CD1d expression whereas some Gaucher disease patients presented an increase in the expression of these molecules [43, 53, 61]. Further studies need to be undertaken, such as activation assays, to determine if the reduction we observed in some patients leads to an altered production of cytokines by T cells. It is possible that the reduction of CD1d expression in some of our patients without a similar reduction in MHC-II expression is due to the alteration of CD1d's trafficking. Despite of the vast similarities in terms of intracellular trafficking of MHC-II and CD1d, nascent CD1d molecules move first to the cell surface and then recycle by the endosomal pathway whereas newly synthesized MHC-class II molecules are routed directly to the recycling compartments. Also, the half-life of CD1d molecules is about fivefold higher than the half-life of MHC-class II [43]. A reduced transcription induced by low levels of factors present in the patients, a slower transport of the CD1d to the plasma membrane or an increase in endocytosis may also provide some answers [43]. Nonetheless, a reduction in CD1d expression may not influence antigen presentation [61].

5.5.2. DCs

DC frequency is normal in MPS II patients. In other LSDs, alterations were detected. Fabry and Gaucher disease patients presented reductions in circulating DCs [53, 61]. The absence of alterations in MPS II patients could be simply attributed to the few patients analysed or from the use of specific markers for DC identification (CD11c and CD123 would be the ideal ones) [62]. Curiously, despite the reduction in monocyte frequency, DC frequency was not affected. Some DCs are derived from monocytes, however, the majority derives from a common precursor [64].

In terms of MHC-II, MHC-I and CD1c expression by DCs, no significant alterations were found. It was, nonetheless, possible to observe a smaller expression of MHC-II in some patients. A similar slight decrease in MHC-II expression was observed in DCs (*in vitro* monocyte-derived DCs) from MPS VI patients, where CD1d expression also could not be studied [48]. Curiously, the accumulation of heparan sulphate, an endogenous agonist capable of inducing DC activation through decrease of antigen uptake and an increase in allostimulatory capacity [63], induced no changes in the phenotype DCs from MPS II patients.

5.5.3. B cells

In MPS II patients, no alterations were found in B cell frequency and in the expression of MHC-II, MHC-I, CD1d and CD1c. Likewise, B cells from Gaucher disease patients are also normal in number [54]. In other LSDs like Fabry disease and MPS VI, it was found also an increase in the frequency of B cells [48, 53].

The expression of heparan sulfate in B cells is smaller than in monocytes from healthy individuals [66]. Hence, it is reasonable that the accumulation of accumulation heparan sulfate in B cells from MPS II patients is larger than in monocytes from MPS II patients. The different degree of GAG accumulation in the cells may explain the absence of alterations in B cells.

6. Conclusion and Future Perspectives

This study allowed the characterisation of many lymphocytes and antigen presenting cells from MPS II patients. No alterations have been found between patients and control subjects in total iNKT cells and conventional T cells neither in their subsets. The activation and expression of NK markers in iNKT cells was also found to be unaltered in MPS II patients. No significant differences between patients and controls in the expression of MHC and CD1 molecules by antigen presenting cells. The frequency of monocytes from patients have been found to be significantly decreased and B cell and DC frequency was normal.

In some human sphingolipidoses – Fabry disease, Gaucher disease [Pereira, C. *et al,* unpublished results] and Niemann-Pick disease type C - the frequency of peripheral blood iNKT cells was also unaltered [42, 54] but its subsets were altered. Such alterations were not observed in the subsets of conventional T cells [49, 50]. In MPS VI, no alterations were observed [48]. In terms of activation and NK cell marker expression, it is only described that Niemann-Pick disease type II patients present normal expression of CD161 [42]. The frequency of monocytes of patients with Fabry disease was also found to be decrease [53]. The expression of MHC II and CD1d molecules in these cells was found to be increased in Gaucher disease patients and the expression of MHC II decreased in Fabry disease patients [43, 53]. In sphingolipidoses, DCs were reduced [48, 49, 53]. Finally, B cell frequency was found to be normal in Gaucher disease [54], but increased in Fabry disease and MPS VI patients [48, 53].

In the different Lysosomal Storage Diseases there is an accumulation of distinct macromolecules. In shingolipidoses, where there is an accumulation of sphingolipids, there is a profound influence in iNKT cells and in antigen presenting cells. In MPS II, where GAG accumulation occurs, such differences are not observed. Hence, we suggest that the chemical nature of the accumulated macromolecules influences the development of iNKT cells and the process of lipid antigen presentation. Such hypothesis is supported by the lack of alterations already observed in patients with MPS VI.

In the development of our work, we have faced some obstacles. Our work was essentially made with blood samples from paediatric patients. Therefore, the volume of blood that was collected was reduced. This lead to the collection of few cells that sometimes did not allow the study of all the desired parameters. Also, the study of a rare disease such as MPS II had some difficulties. The most obvious one was the small number of patients enrolled in the study, particularly of untreated patients. Due to the rarity of this disease, the centres where the patients are treated are much dispersed geographically. For example, in the course of this work we were able to collect and analyse clinical, biochemical and genetic information about many of the LSD patients from Centro Hospital São João due to a small internship I made. However, our patients are dispersed from the entire globe and the collection of rest of the data is impossible to be made by a single person. The collection of information from these

patients requires the commitment of their physicians and hard work due to the complexity of these diseases. Age matching was another problem experienced. Many of our patients are of paediatric age. However, all control subjects are adults. In some parameters, the lack of age match does not allow the interpretation of the results like memory phenotype that was done but not presented in this thesis. This problem is currently being addressed near the ethics committee of Centro Hospitalar São João. However, in the ending of this study, blood from paediatric donors was not yet available for analysis.

In the future, we expect to keep analysing MPS II patients to obtain data from a more representative sample. We hope that during the rest of the on-going research project we can analyse more untreated patients and follow the evolution of their iNKT cell population and antigen presenting cells. Finally, for a better comprehension of our results it would be useful to have the clinical, biochemical and genetic data from all the analysed patients.

With our work, we hope to have contributed to a better comprehension of this rare disease, namely the influence of the metabolic alterations in the immune system.

7. Literature

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Appendix – Composition of the solutions used in the experimental work

PBS 10x (for a final volume of 1000 mL)

- Add 80g of NaCl, 2g of KCl, 6.09g of Na_2HPO_4, 2g KH_2PO_4 to 1000 mL of H2O
- Adjust pH – pH=7.3

PBS 1x (for a final volume of 1000 mL)

- Add 100 mL of PBS 10x to 900 mL of H2O

ACK Lysis Solution (for a final volume of 500 mL)

- Add 4.15g of NH_4Cl and 0.5g of KCO_3 to 500 mL of H2O
- Adjust pH – pH=7.2

Freezing medium (for a final volume of 11 mL)

- Add 1 mL of dimethyl sulfoxide to 10 mL of fetal bovine serum

Flow Cytometry Solution (for a final volume of 100 mL)

- Add 0.2g of bovine serum albumin and 0.1g of NaN_3 to 100 mL of PBS 1x

PBS 1% Formaldehyde (for a final volume of 1000 mL)

- Add 1mL of 16% formaldehyde to 3mL of PBS 1x