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IMPACT OF GLYCOSPHINGOLIPID ACCUMULATION ON THE IMMUNOLOGICAL SYSTEM

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Preceitos legais

De acordo com o disposto no nº 2 do artigo 8º do Decreto-lei nº 388/70, nesta dissertação foram utilizados os resultados dos trabalhos publicados abaixo indicados. No cumprimento do disposto no referido Decreto-lei, a autora desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação de resultados e na redacção dos resultados publicados sob o nome **Balreira A**:

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Summary, Sumário, Résumé

Summary

The lysosome is the main digestive compartment of the cell, and its role is highlighted by the existence of several inherited metabolic disorders caused by lysosomal enzymes deficiency. These enzymes are responsible for the catabolism of glycosphingolipids, and when impaired originate lysosomal storage disorders. How the storage of a specific glycosphingolipid is linked to the pathology of the respective lysosomal storage disorder remains unknown. Due to the absence of genotype/phenotype correlations, other mechanisms arise as having a possible determinant role on the pathophysiology of these disorders. Besides the digestive function, lysosomes are also essential for the normal functioning of the immune system. So, an understanding of the impact of glycosphingolipid accumulation on the immune system could broaden our knowledge on the mechanisms underlying the symptoms and the clinical heterogeneity of lysosomal storage disorders. In order to extend our understanding on the association between glycosphingolipid accumulation and the immunological phenotype, we studied two lysosomal storage disorders: Gaucher and Fabry disease.

MHC-family molecules are involved in the selection and shaping of the T lymphocyte pool. CD1d, along with MHC-class I and II, plays a key role in selecting populations of T cells. Trafficking pathways of these molecules involve shuttling through the endosomal/lysosomal system where glycosphingolipids accumulate in lysosomal storage disorders. We wanted to determine whether anomalies of sphingolipids metabolism in Gaucher disease patients are linked to anomalies in the expression of molecules of MHC-family and the existence of imbalances in T cell subsets. Gaucher disease is an autosomal recessive disease, in which the primary defect is a deficient activity of the lysosomal enzyme β -glucocerebrosidase that leads to the lysosomal accumulation of glucosylceramide.

Hereby we show that glucocerebrosidase-deficient monocytes from Gaucher Disease patients express higher levels of CD1d and MHC-class II molecules at the cell surface, when compared to monocytes from healthy controls. Gaucher disease patients under enzyme replacement therapy were also studied. Like untreated patients, patients under treatment showed increased levels of CD1d and MHC-class II, as well as imbalances in the percentage of CD4⁺, CD8⁺ and V α 24⁺ T cells. Follow-up studies showed that enzyme replacement therapy induces a partial correction on the CD4⁺ T cell imbalances and a reduction in MHC-class II expression in monocytes that positively correlates with chitotriosidase activity, reflecting the activation state of lipid-laden macrophages. Several approaches were used in order to induce glycosphingolipid accumulation in peripheral blood monocytes from healthy controls and the monocytic cell line THP-1 (i.e.

development of an *in vitro* model of a Gaucher cell), like retinoic acid and/or conduritol-B-epoxide treatment, and a two-fold increase of CD1d molecules at cell surface was observed. Co-cultures experiments, performed *in vitro*, showed that RA-treated THP-1 cells induced a higher proliferation in isolated CD4⁺ T cells than in CD8⁺ T cells, as determined by CFSE loss, when compared with untreated THP-1 cells. Addition of iGb3, a putative endogenous CD1d ligand, increased the percentage of dividing CD4⁺ T cells, but interestingly, no V α 24⁺ T cells were found within the expanded T cells. These studies demonstrate a link between lipid accumulation and the regulation of the expression of some MHC molecules. This may result in imbalances of regulatory T cells subsets and contribute for the wide clinical heterogeneity in Gaucher disease.

Fabry disease, is caused by the deficient activity of α -galactosidase A, leading to storage of Gb3, an isomer of the CD1d putative ligand iGb3, in lysosomes. In the light of this, we performed a similar study to the one described above, using Fabry disease patients and Fabry knockout mice. We show that Fabry mice present several alterations in hepatic and splenic T and iNKT cells. In contrast, Fabry disease patients do not present any imbalances in T and iNKT cell populations. However, circulating monocytes presented a MHC-class II upregulation. The divergence between the human and mice results suggested that the impact of sphingolipid accumulation on the immunological system may be depend on the tissue distribution of the accumulated sphingolipids, and the species where it accumulates.

Taken together, the results presented in this thesis show that sphingolipid accumulation, or disturbances resulting from it, are involved in the regulation of CD1d expression in monocytes, and contribute to a better understanding of the clinical heterogeneity of lysosomal storage diseases patients.

Sumário

Os lisossomas são organelos citoplasmáticos que têm por função a digestão intracelular. A sua importância é evidenciada pela existência de várias doenças hereditárias do metabolismo que resultam da deficiência em enzimas lisossomais. Estas enzimas encontram-se envolvidas no metabolismo dos glicoesfingolípido, e originam as doenças lisossomais de sobrecarga. O modo como a acumulação de um determinado glicoesfingolípido está associada com a patologia de uma determinada doença lisossomal de sobrecarga permanece sem explicação. Devido à inexistência de uma correlação entre o genótipo e o fenótipo nestas doenças, pensa-se que outros mecanismos possam estar envolvidos na patofisiologia destas doenças. Além da função de digestão intracelular, os lisossomas são também essenciais para o normal funcionamento do sistema imunológico. Deste modo, a compreensão do impacto da acumulação de glicoesfingolípido no sistema imunológico poderá contribuir para o conhecimento dos mecanismos responsáveis pelos sintomas e pela heterogeneidade clínica associada às doenças lisossomais de sobrecarga. No sentido de aprofundar o conhecimento da associação entre a acumulação de glicoesfingolípido e o fenótipo imunológico, foram estudadas duas doenças lisossomais de sobrecarga: a doença de Gaucher e de Fabry.

As moléculas da família MHC, como as moléculas CD1d, MHC-classe I e II, estão envolvidas na selecção e modelação da população de linfócitos T. As vias de transporte intracelular destas moléculas envolvem circulação através do sistema endossomal/lisossomal onde ocorre a acumulação de glicoesfingolípido nas doenças lisossomais de sobrecarga.

De forma a investigar se anomalias no metabolismo de esfingolípido estão associadas a anomalias na expressão de moléculas da família MHC e à existência de desequilíbrios nas subpopulações de células T, foram efectuados estudos em doentes de Gaucher. A Doença de Gaucher é uma doença hereditária do metabolismo de transmissão autossómica recessiva, que resulta da deficiência na actividade da enzima lisossomal β -glucocerebrosidase que conduz à acumulação lisossomal de glucosilceramida. Os resultados obtidos mostraram que monócitos deficientes em glucocerebrosidase de doentes de Gaucher, quando comparados com monócitos de controlos saudáveis, expressam níveis elevados de CD1d e MHC-classe II à superfície celular. Foram também estudados Doentes de Gaucher submetidos a tratamento de substituição enzimática. Tal como os doentes não tratados, pacientes em tratamento, apresentaram níveis elevados de CD1d e MHC-classe II, assim como desequilíbrios nas percentagens de células T CD4⁺, CD8⁺ e V α 24⁺. Estudos de acompanhamento dos doentes ao longo do tratamento

mostraram que a terapia de substituição enzimática induz a correcção parcial das percentagens de células T CD4⁺ bem como a redução dos níveis de expressão de MHC-classe II nos monócitos. A diminuição dos níveis de MHC-classe II correlaciona-se positivamente com a actividade enzimática da quitotriosidase, que reflecte o estado de activação dos macrófagos onde ocorre a acumulação dos glicoesfingolípido. Várias abordagens foram utilizadas de modo a induzir a acumulação de glicoesfingolípido em monócitos de sangue periférico de doadores saudáveis e na linha celular monocítica THP-1 (i.e., de modo a desenvolver um modelo *in vitro* das células de Gaucher), através do tratamento das células com ácido retinóico e/ou conduritol-B-epóxido, tendo sido observada uma duplicação dos níveis de expressão de CD1d nas células tratadas. Coculturas realizadas *in vitro* mostraram que células THP-1 tratadas com ácido retinóico, quando comparadas com células THP-1 não tratadas, induziam uma maior proliferação das células T CD4⁺ do que das CD8⁺, o que foi avaliado pela perda de fluorescência do CFSE. A adição às coculturas do ligando endógeno do CD1d, o iGb3, induziu uma maior divisão das células T CD4⁺, embora, curiosamente, não tenham sido detectadas quaisquer células Vα24⁺ entre as células T CD4⁺ em divisão. Estes estudos estabeleceram uma associação entre a acumulação de lípidos e a regulação da expressão de moléculas MHC, nomeadamente das moléculas CD1d e MHC-classe II, o que poderá resultar em desequilíbrios nas subpopulações de células T regulatórias e desta forma contribuir para a grande heterogeneidade clínica observada na Doença de Gaucher.

A Doença de Fabry resulta da deficiência na actividade da enzima α-galactosidase A, levando à acumulação lisossomal de Gb3, um isómero do iGb3, o ligando endógeno do CD1d. Assim, um estudo semelhante ao desenvolvido com os doentes de Gaucher foi realizado com doentes de Fabry e no modelo animal desta doença. Mostramos que o ratinho Fabry apresenta várias alterações nas células T e iNKT tanto do fígado como do baço. Pelo contrário, não foram encontradas quaisquer alterações nas células T e iNKT do sangue periférico dos doentes de Fabry, tendo sido apenas observado o aumento da expressão de MHC-classe II nos monócitos em circulação. A divergência nos resultados obtidos entre humanos e ratinhos sugere que o impacto da acumulação de esfingolípido no sistema imunológico poderá depender dos tecidos onde a acumulação ocorre, bem como da espécie em causa.

Os resultados descritos nesta tese mostram que a acumulação de esfingolípido ou outras alterações que possam daí advir estão envolvidos na regulação da expressão de CD1d nos monócitos e poderão contribuir para aumentar o conhecimento sobre a heterogeneidade clínica nos doentes com doenças lisossomais de sobrecarga.

Résumé

Le lysosome c'est l'organite cellulaire chargé de la digestion de plusieurs substrats, l'importance de cette fonction est démontrée par l'existence de différentes maladies métaboliques héréditaires originées par le déficit dans des enzymes lysosomales. Ces enzymes sont impliquées dans le catabolisme des glycosphingolipides et leur déficit origine les maladies lysosomales de surcharge. La liaison entre l'accumulation d'un glycosphingolipide spécifique et la pathologie de la maladie lysosomale de surcharge associée n'est pas encore connue. L'absence d'une corrélation génotype/phénotype dans les maladies lysosomales de surcharge indique l'influence d'autres mécanismes dans la pathophysiologie de ces maladies. Au delà de sa fonction dans la digestion intracellulaire les lysosomes ont aussi un rôle essentiel dans le fonctionnement du système immunologique. En conséquence, l'étude de l'effet de l'accumulation des glycosphingolipides sur le système immunologique doit contribuer pour la connaissance des mécanismes causaux des symptômes et pour l'hétérogénéité clinique associée à ces maladies. Dans le but d'approfondir la compréhension sur la liaison entre l'accumulation de glycosphingolipides et le phénotype immunologique, on a étudiée deux maladies lysosomales de surcharge, la maladie de Gaucher et la maladie de Fabry.

Les molécules de la famille MHC, les molécules CD1d, MHC-classe I et II sont tous impliquées dans la sélection et dans la modulation de la population des lymphocytes T. Le transport intracellulaire de cette molécules se fait à travers du système endosome/lysosome où l'accumulation des glycosphingolipides dans les maladies lysosomales de surcharge a lieu.

Dans ce travail on a voulu rechercher, en étudiant des malades de Gaucher, si des anomalies dans le métabolisme des sphingolipides sont associées à des anomalies dans l'expression des molécules de la famille MHC et à l'existence des déséquilibres dans les sous populations de cellules T. La maladie de Gaucher est une maladie lysosomale de surcharge due à une activité déficiente de la glucocérébrosidase qui conduit à une accumulation du glucocérébroside non hydrolysé dans le lysosome. La maladie de Gaucher est une maladie héréditaire de transmission autosomique et récessive. Les résultats obtenus dans ce travail montrent que les monocytes des malades de Gaucher, déficients en glucocérébrosidase, expriment à la surface des niveaux élevés de CD1d et de MHC-class II en comparaison avec les monocytes des individus normaux. Des malades de Gaucher traités avec de la thérapie enzymatique de substitution, ont été aussi étudiés. Les malades traités avec de la thérapie enzymatique ont montré comme les malades non traités des niveaux élevés de molécules CD1d et MHC-class II. Ils

présentaient aussi des déséquilibres dans les pourcentages de cellules T CD4⁺, CD8⁺ et Vα24⁺. Les études de surveillance des malades pendant le traitement ont montré que la thérapie enzymatique induit la correction partielle des pourcentages de cellules T CD4⁺ et aussi la réduction des niveaux de l'expression des molécules MHC-class II dans les monocytes. Il a été observé une corrélation positive entre la diminution des niveaux de MHC-class II et l'activité enzymatique de la chitotriosidase, enzyme sécrétée par les macrophages activés, qui traduit l'état d'accumulation lipidique. On a utilisée des différents méthodes (ex. l'époxyde de conduritol B) pour induire l'accumulation de glycosphingolipides dans les monocytes de sang périphérique, obtenu à partir des individus normaux. Les mêmes méthodes ont été utilisés dans un lignage cellulaire monocytique THP-1 (acide rétinoïque et l'époxyde de conduritol B), dans le but d'obtenir un modèle de cellules de Gaucher *in vitro*. Les résultats obtenus montrent une augmentation de deux fois dans le nombre de molécules CD1d présentes à la surface cellulaire. Des essais de co-culture *in vitro* ont montré que les cellules THP-1 traitées avec l'acide rétinoïque provoque une prolifération plus élevée de cellules T CD4⁺ comparativement aux cellules CD8⁺. L'addition d'iGb3, le lipide reconnu par le CD1d, fait augmenter le pourcentage de cellules T CD4⁺ en division, mais curieusement, par contre, on n'a pas trouvé des cellules Vα24⁺ parmi les cellules T en expansion. Ces études ont permis l'établissement d'une association entre l'accumulation de lipides et la régulation de l'expression des molécules MHC, notamment des molécules CD1d et MHC-class II, ce qui peut conduire à des déséquilibres dans les sous-populations des cellules T régulatrices et en conséquence contribuer pour la vaste hétérogénéité clinique de la maladie de Gaucher.

La maladie de Fabry est due au déficit de l'enzyme α-galactosidase A, ce qui origine l'accumulation du Gb3 dans le lysosome. Le Gb3 est un isomère du iGb3 le ligand endogène du CD1d. Donc, on a développé une étude similaire à celui sur la maladie de Gaucher, dans des malades avec la maladie de Fabry et aussi dans le modèle animal de la maladie. On a pu montrer que la souris Fabry présente des différentes anomalies dans les cellules T et iNKT du foie et de la rate. Par contre on n'a pas pu trouver aucune altération dans les cellules T et iNKT du sang périphérique dans des malades de Fabry, ces malades présentaient seulement une augmentation dans l'expression de MHC-class II dans les monocytes. Les différents résultats obtenus dans la souris et dans les malades Fabry suggèrent que l'effet de l'accumulation de sphingolipides dans le système immunologique peut être dépendant du type de tissu ou l'accumulation à lieu et aussi de l'espèce.

En conclusion, les résultats présentés dans cette thèse montrent que l'accumulation des sphingolipides ou d'autres altérations originés par cette accumulation, sont impliquées dans la régulation de l'expression des molécules CD1d dans les monocytes ce qui peut contribuer pour l'hétérogénéité clinique observée dans les maladies lysosomales de surcharge.

Abbreviations

α -gal A	α -galactosidase A
α -GC	α -galactosylceramide
β_2 m	β_2 -microglobulin
4-MU	4-Methylumbelliferone
AHR	Airway hyperreactivity
AP	Adaptor protein
APC	Antigen presenting cells
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CBE	Conduritol-B-Epoxyde
CLIP	Invariant chain peptide
CSFE	Carboxyfluorescein succinimidyl ester
DC	Dendritic cell
DN	Double negative
DP	Double positive
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
FD	Fabry disease
Gb3	Globotriaosylceramide
GD	Gaucher disease
GluCer	Glucosylceramide
GluCerase	β -glucocerebrosidase
GSL	Glycosphingolipids
iFBS	Heat inactivated foetal bovine serum
IFN- γ	Interferon- γ
iGb3	Isoglobotrihexosylceramide
Ii	Invariant chain
IL	Interleukin
iNKT	Invariant Natural Killer T
KO	Knock-out
LIMP-2	Lysosomal integral membrane protein type 2
LSD	Lysosomal storage disorder
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MIIC	MHC class II compartments
MPS	Mucopolysaccharidosis
MTP	Microsomal triglyceride transfer protein
n.s.	Not significant
NaN ₃	Sodium azide
NB-DNJ	<i>N</i> -butyldeoxynojirimycin
NK	Natural killer
NOD	Non-obese diabetic
NPC	Niemman-Pick type C
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline solution
PC	Phosphatidylcholine
PI	Phosphatidylinositol
RA	Retinoic acid
RBC	Red blood cells
RT-PCR	Reverse transcription polymerase chain reaction
Sap C	Saposin C
SRT	Substrate reduction therapy
TCR	T cell receptor
TNF- α	Tumor necrosis factor- α

I – General Introduction

1. Lysosomal Storage Disorders

1.1. Lysosomes

Lysosomes, described for the first time in 1955 by Christian De Duve ¹, are membrane-bound organelles that occur in all mammalian cell types with the only exception of mature erythrocytes. Lysosomes are the cell main digestive compartment, being the terminal degradative compartment of the endosomal/lysosomal system. This system is also constituted by early endosomes, which tend to be tubular and are located at the cell periphery, and by late endosomes, spherical organelles that tend to be perinuclear ². The endosomal/lysosomal system is responsible for the trafficking and digestion of macromolecules that result from autophagy, phagocytosis or endocytosis ³. The endocytosed molecules associated with the corresponding recycling receptor are internalized through clathrin-coated vesicles and delivered to early endosomes where efficient sorting occurs. In that mildly acidic luminal pH, receptor-ligand uncouples and recycling receptors are rapidly transported along the recycling pathway, while the ligand follows the degradation pathway to late endosomes and lysosomes (reviewed in ⁴). A morphological criterion such as size or internal structure does not allow for the identification of lysosomes due to the wide variability of these structures ⁵. In relation to size, lysosomes can vary between less than 1 μm in cells like hepatocytes and neurons and several microns in macrophages ⁶, thus being defined by functional rather than structural properties. The lysosomal luminal environment is kept at a pH of 4.6-5.0 by the proton-pumping vacuolar ATPases ⁷ and are distinguished from endosomes and other related vesicles by the absence of mannose-6-phosphate receptors ⁸. The functional properties of these organelles are linked to the fact that they contain more than 50 hydrolases with an optimum pH below 6 ⁹. Lysosomal degradation is crucial to a number of physiological processes like turnover of normal proteins and other macromolecules and removal of abnormal ones, downregulation of surface receptors, release of endocytosed nutrients, inactivation of pathogenic organisms and antigen processing ¹⁰.

1.2. Sphingolipidosis

The role of lysosome as the main digestive organelle in cell is highlighted by the existence of a number of inherited human metabolic disorders that result from defects in the lysosomal enzymes involved in the glycosphingolipids (GSL) catabolism. More than 60

lysosomal storage disorders (LSD) are known to date ¹¹, and defects for almost every step in the GSL catabolic pathway have been described in humans ³ (Figure 1). The majority of LSD are caused by a deficiency of single lysosomal enzyme, with the subsequent substrate accumulation within lysosomes, leading to pathology, but can also result from defects in cofactors or in the lysosomal proteins targeting ³. The accumulation of undegraded substrate gradually occurs within the lysosome, causing a progressive increase in the size and number of these organelles and leading to the malfunction of the affected organ ¹². These diseases are generally multisystemic and always progressive ², and they are inherited in an autosomal recessive manner, with the only exception of Fabry and Hunter disease that are X-linked recessive, and Danon disease that is X-linked dominant ¹³. LSD are in general monogenic but a wide range of mutations has been described for the same gene in different patients, and different mutations have different consequences in the protein, i.e., while some mutations result in a complete loss of function of the enzyme others result only in decreased activity ¹⁴. LSD can be grouped mainly according with the type of substrate that accumulates. In a review published by Futerman and van Meer in 2004, LSD are classified as: i) sphingolipidosis (which accumulate sphingolipids); ii) mucopolysaccharidosis (which accumulate glycosaminoglycans also known as mucopolysaccharides); iii) oligosaccharidosis (which accumulate oligosaccharides); iv) diseases caused by defects in integral membrane proteins and v) others ¹⁴ (Table I). Despite the fact that, individually, LSD are considered rare diseases, when grouped they represent a significant and severe set of diseases with a described prevalence of 1 in 4000 live births in Portugal ¹⁵, which is twice the Australian and the Dutch prevalence of 1 per 7700 ¹⁶ and 1 per 7143 ¹⁷ living births, respectively.

Sphingolipidosis are a group of LSD that are caused by defects in genes encoding for enzymes involved in lysosomal catabolism of sphingolipids.

GSL are essential components of eukaryotic cell membranes. These lipids are composed of a ceramide backbone linked to a sugar headgroup. In the ceramide backbone, the hydrophobic part consists of a sphingoid base and a fatty acid and is embedded in the outer leaflet of the cellular membrane, while the sugar headgroup is exposed to the cell surface. GSL display a high structural diversity, both in sphingoid base and in headgroup, being known more than 60 different sphingoid bases and more than 300 different oligosaccharide chains ¹⁸.

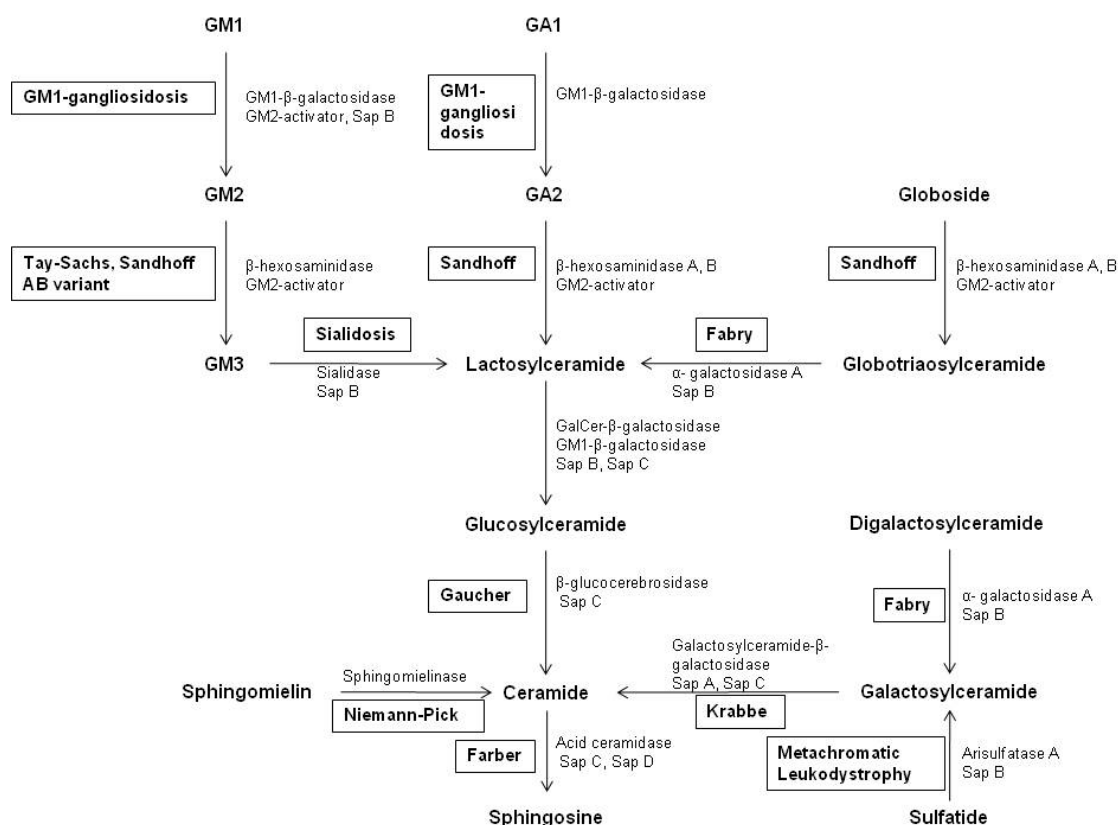


Figure 1. Lysosomal sphingolipids catabolic pathway. Inside boxes are indicated the eponyms of individual LSD. Activator proteins required for the respective degradation step *in vivo* are indicated next to the lysosomal enzymes. Sap, saposin. (Adapted from ³).

GSL are synthesized in the endoplasmic reticulum (ER) and Golgi apparatus, reside in the plasma membrane and are internalized by endocytosis ¹⁹. Besides being components of cell membranes, GSL are essential for development and survival of multicellular organisms ²⁰, play a role in cell adhesion ²¹, in the regulation of membrane proteins and a number of studies have shown that they are also involved in cell signaling (reviewed in ¹⁸).

Table I- Lysosomal storage disorders. ^{a)}

Disease	Deficient enzyme or cofactor	Main storage materials
Sphingolipidosis		
Fabry	α -galactosidase A	Gb3 and blood-group-B compounds
Farber lipogranulomatosis	Ceramidase	Ceramide
Gaucher	β -glucocerebrosidase Saposin-C activator	Glucosylceramide
GM1 gangliosidosis	β -galactosidase	GM1 ganglioside
GM2 gangliosidosis (GM2-activator protein)	GM2-activator protein	GM2 ganglioside and related glycolipids
GM2 gangliosidosis (Sandhoff)	β -hexosaminidase A and B	GM2 ganglioside and related glycolipids
GM2 gangliosidosis (Tay-Sachs)	β -hexosaminidase A	GM2 ganglioside and related glycolipids
Niemann-Pick A and B	Sphingomyelinase	Sphingomyelin
Sphingolipid activator protein	Sphingolipid activator	Glycolipids
Mucopolysaccharidosis (MPS)		
Morquio A disease	<i>N</i> -acetylglucosamine-6-sulphate-sulphatase	Keratan sulphate, chondroitin-6-sulphate
Morquio B disease	β -galactosidase	Keratan sulphate
MPS I (Hurler, Scheie, Hurler/Scheie)	α -iduronidase	DS and HS
MPS II (Hunter)	Iduronate-2-sulphatase	DS and HS
MPS IIIA (Sanfilippo)	Heparan <i>N</i> -sulphatase (sulphamidase)	HS
MPS IIIB (Sanfilippo)	<i>N</i> -acetyl- α -glucosaminidase	HS
MPS IIIC (Sanfilippo)	Acetyl-CoA: α -glucosamide <i>N</i> -acetyl transferase	HS
MPS IIID (Sanfilippo)	<i>N</i> -acetylglucosamine-6-sulphatase	HS
MPS VI (Maroteaux-Lamy)	<i>N</i> -acetylgalactosamine-4-sulphatase (arylsulphatase B)	DS
MPS VII (Sly)	β -glucuronidase	HS, DS, chondroitin-4-sulphate, chondroitin-6-sulphate
Oligosaccharidosis		
Pompe (glycogen storage disease type II)	α -glucosidase	Glycogen
Defects in integral membrane proteins		
Cystinosis	Cystinosis	Cystine
Danon disease	LAMP-2	Cytoplasmic debris and glycogen
Infantile sialic-acid-storage disease and Salla disease	Sialin	Sialic acid
Mucopolipidosis IV	Mucolipin-1	Lipids and acid mucopolysaccharides
Niemann-Pick C	NPC1 and 2	Cholesterol and sphingolipids
Others		
Galactosialidosis	Cathepsin A	Sialyloligosaccharides
I cell and pseudo-Hurler polydystrophy	<i>N</i> -acetylglucosaminyl-1-phosphotransferase	Oligosaccharides, mucopolysaccharides and lipids
Multiple sulphatase deficiency	Co-formylglycine-generating enzyme	Sulphatides
NCL1 (Batten disease)	CLN1 (protein palmitoylthioesterase-1)	Lipidated thioesterases
NCL2 (Batten disease)	CLN2 (tripeptidyl amino peptidase-1)	Subunit c of the mitochondrial ATP synthase
NCL3 (Batten disease)	Arginine transporter	Subunit c of the mitochondrial ATP synthase

^{a)} adapted from ¹⁴. Gb3, globotriaosylceramide; MPS, mucopolysaccharidoses; DS, dermatan sulphate; HS, heparin sulphate; NPC, Niemann-Pick; NCL, neuronal ceroid lipofuscinosis; ATP, adenosine triphosphate

Sphingolipidosis present a high degree of phenotypic variability, where several factors can be determinant for the pathogenesis of disease, like the cell-type specific partner of glycosphingolipid expression. Sphingolipid storage occurs mainly in cells and organs where the unmetabolized lipid substrate is preferentially synthesized or taken up by phagocytosis³. Another factor with relevance for pathogenesis is the residual activity of the deficient enzyme, which is responsible for the onset and severity of the disease. While residual activity allows a later onset of the symptoms and a slower progression of the disease, complete absence of activity is responsible for the early onset and the severe course of the disorder³.

The major consequence of deficiency in sphingolipid catabolic pathway is the accumulation of the respective enzyme substrate. However, other factors as the nature of the stored material can be responsible for the pathogenesis. In addition, and secondary to the lipid storage, downstream effects of lysosomal storage have been described as contributing to pathogenesis, such as inflammatory responses, e.g., macrophage activation and cytokine release in Gaucher disease patients and in a mouse model of Sandhoff disease^{22,23}. Other secondary effects were reported as a result of lipid accumulation in lipid trafficking, calcium ion homeostasis and mitochondrial function³.

1.2.1. Gaucher Disease

Gaucher disease (GD) is the most frequent LSD in Portugal, with a prevalence of 1.4 per 100000 live births¹⁵ and was first described by Philippe Gaucher, a French dermatologist, in 1882. GD results from a deficiency in the lysosomal hydrolase β -glucocerebrosidase (GluCerase, EC.3.2.1.45), which is responsible for the intralysosomal hydrolysis of glucosylceramide (GluCer) into ceramide and glucose²⁴. GluCerase is encoded by the *GBA* gene that has been mapped to chromosome 1q21²⁵⁻²⁷, a housekeeping gene with widespread low-level expression in most cells and tissues²⁸. The *GBA* gene is 7.5 kb long and contains 11 exons²⁹. A highly homologous pseudogene sequence located 16 kb downstream from *GBA* gene has also been described²⁹. The *GBA* gene and pseudogene share 96% of homology and are in the same orientation. More than 200 different mutations have been described in *GBA* gene³⁰.

GD is a disorder with a wide phenotypic variability, with manifestations ranging from death in uterus to asymptomatic octogenarians³¹. Based upon the presence or absence and grading of neurological manifestations, GD can be divided into 3 types. The most common is GD type 1 (MIM 230800), characterized by the absence of neuronopathic involvement,

and with onset occurring either in childhood or adulthood. GD type 2 (MIM 230900), is characterized by the infantile onset and a clinical presentation of severe neurological involvement with a survival prognosis of less than two years. In GD type 3 (MIM 231000), neuronopathic disturbance usually begins at the end of the first decade of life ²⁴.

In GD, the unmetabolized GluCer accumulates primarily throughout the reticulo-endothelial system. The lysosomes within macrophages become engorged with the stored sphingolipid (can be up to 100 μm), originating the so called “Gaucher cells”, the hallmark of GD. Gaucher cells present a distinctive appearance by light microscopy, with a displaced nuclei and a cytoplasm with a striated, fibrillary or tubular pattern described as similar to “wrinkled tissue paper” ²⁴. In electron microscopy images, Gaucher cells present twisted, elongated, tubular structures that completely deform the lysosome and result from the polymerization of GluCer in a polar head-to-polar tail manner ³². Alterations in the cellular structure of monocytes are also observed, namely the presence of smaller membrane-bound tubule-like structures similar to the ones observed in macrophages, indicating that monocytic cells are precursors of tissue-bound Gaucher cells ³³. Pathophysiologically, the involvement of macrophages in GD can be explained by the role of these cells in scavenging waste material such as old and damaged erythrocytes and leukocytes by phagocytosis, which are the main source of stored glycolipids ^{24,34}.

Clinically, GD is a multi-systemic disorder affecting most organs, resulting in cirrhosis and liver disease, pulmonary, cardiac and renal disease. Parkinson’s disease, lymphoproliferative disease, complications in pregnancy and increased foetal loss have been associated to GD (reviewed in ³⁴). GD is characterized by peripheral inflammation resulting from circulation or tissue-resident macrophage dysfunction causing cytopenia, hepatosplenomegaly and skeletal abnormalities. Bone involvement is the most disabling feature of adult GD patients and originates pain, immobility, impairment of bone marrow function and fracture, frequently followed by infection and osteomyelitis ³⁴. Clinical diagnosis is confirmed by measuring the activity of GluCerase in peripheral blood lymphocytes (PBL) ³⁵.

Despite the difficulty in the establishment of a genotype-phenotype correlation, Zhao & Grabowsky in their 2002 review, point out some links between the type of mutation and the clinical phenotype ³³. The presence of a N370S allele, either in homozygosity or heterozygosity, is associated with the nonneuronopathic form of GD (type 1), while the genotypes L444P/L444P and D409H/D409H correlate with the neuronopathic forms of the disease. However, differences between the two phenotypes can be pointed: patients with L444P/L444P present varying degrees of visceral involvement but do not have cardiac

valvular calcifications, cataracts, hydrocephalus and other milder visceral involvement, like patients with D409H/D409H³³. GD can also result from mutations in the saposin C domain of the prosaposin gene, which encodes for saposin C (Sap C) protein³⁶, resulting in a clinical presentation similar to GD type 3³⁷. Sap C was first isolated from the spleen of a GD patient³⁸ and is required for the full catalytic activity of GluCerase, apparently because it binds to GluCerase instead of GluCer. At acidic pH values, Sap C presents a high affinity for cell membranes³⁹, a fact that could facilitate the association of GluCerase to these structures⁴⁰. This association is vital for maintaining sufficient steady-state amounts of enzyme activity, facilitating GluCer hydrolysis⁴⁰.

GluCerase is a 497 amino acid glycoprotein with 4-5 occupied *N*-glycosylation sequences. Upon trans-ER membrane passage, the GluCerase leader peptide is cleaved, and the enzyme is cotranslationally glycosylated in the ER with the subsequent sequential oligosaccharide modifications and movement through the cis, mid and trans Golgi apparatus⁴¹⁻⁴³. GluCerase is not phosphorylated and does not contain mannose-6-phosphate target residues for lysosomal sorting^{44,45}. Instead, GluCerase requires the lysosomal integral membrane protein type 2 (LIMP-2) to reach the lysosome⁴⁶. Once delivered to the lysosome, GluCerase becomes bound to its inner membrane³³. Patients with LIMP-2 deficiency have normal GluCerase activity levels in leukocytes, but a severe enzymatic deficiency in cultured skin fibroblasts, resulting in progressive myoclonic epilepsy without intellectual impairment, and in a nephrotic syndrome with a strong accumulation of C1q in the capillary loops and mesangium of kidneys⁴⁷. These differences in GluCerase activity among different cell types, suggest the existence of more than one GluCerase sorting mechanism.

Tybulewicz *et al* in 1992, described a mouse model of GD, generated with a null allele that resulted in a neonatal lethal phenotype. These mice were cyanotic, akinetic, did not feed, had dry and rough skin and died within a few hours after birth⁴⁸. The inviability of these most probably results from the important role of GluCer in maintaining the skin permeability barrier⁴⁹. Another frustrating attempt to create a viable mouse model of GD was described in 1998. At that time, Liu *et al* created a mouse homozygous for the L444P mutation that were viable and had few symptoms, however died within 24-48 hours after birth⁵⁰. Homozygous mouse for the characteristic type I mutation N370S, die immediately after birth, which was totally unexpected since humans with this genotype have a less severe GD phenotype⁵¹.

1.2.2. Fabry Disease

Fabry disease (FD) (MIM 300644), also known as Anderson-Fabry Disease and *angiokeratoma corporis diffusum universal*, is one of the most common LSD, with a prevalence of 1:40000 to 1:60000 males⁵². FD results from a deficiency in the lysosomal hydrolase α -galactosidase A (α -gal A) (EC 3.2.1.22). This enzyme is responsible for the hydrolysis of the terminal, non-reducing α -D-galactose residues in α -D-galactosides, namely the breakdown of globotriaosylceramide (Gb3) into lactosylceramide and of digalactosylceramide into galactosylceramide. α -gal A deficiency is caused by mutations in the *GLA* gene that are transmitted in a X-linked recessive manner⁵³. FD patients present with accumulation of neutral GSL containing a terminal α -galactose, mainly Gb3 and digalactosylceramide, into plasma and tissues throughout the body, causing a multisystemic disorder⁵⁴. Lipid deposits occur mainly in the endothelium and the media of small vessels, causing blood vessel occlusions and pre-thrombotic state. In kidneys, storage occurs in renal tubules and glomeruli, resulting in proteinuria and renal failure, while in the heart, the cardiac muscle and conducting fibers are affected, manifesting as cardiomyopathy and striated muscle dysfunction^{54,55}. Gb3 accumulation was observed also in autonomic ganglia and specific cortical and brain stem structures (reviewed in⁵⁶). Clinical manifestations of FD are very heterogeneous, ranging from pain to ocular abnormalities. Acute episodic pain is the most striking feature of FD⁵³, and these pain episodes are accompanied by fever and increased erythrocyte sedimentation rate⁵². Normally, pain episodes are triggered by stress, heat, fatigue or exercise and are related to the hypohidrosis and to the inability of affected individuals to relieve their body heat through sweating, resulting in increased body temperature⁵². Acroparesthesia, a second type of pain, can be presented by affected males⁵². FD may present gastrointestinal disturbances, which include diarrhea, anorexia, weight loss, abdominal discomfort, nausea, vomiting and acute abdominal pain^{57,58}. One of the first manifestations of FD, and usually the one responsible for the diagnosis during childhood, is angiokeratomas⁵³. These skin manifestations are small, slightly raised, purplish-red, nonblanching with pressure lesions, that became progressively larger and more numerous with age⁵³. FD patients also present ocular involvement with a characteristic corneal opacity and despite the absence of skeletal abnormalities, FD patients can present musculoskeletal involvement and retarded growth⁵³. In the adult age, dysmorphic facial features are present in about half of the affected individuals^{59,60}.

A mouse model of FD was developed by Ohshima *et al* in 1997, through the disruption of the *GLA* gene. Knock-out (KO) mice display totally absent α -gal A activity, with marked

accumulation of Gb3 in the liver and kidneys ⁶¹. α -gal A⁻⁰ mice, described as being phenotypically normal at 10 weeks of age, show no evidence of organ failure at 80 weeks of age ⁶². This relatively mild phenotype observed in KO mice, when compared to humans, could result from the significant lower basal concentration of Gb3 in mouse erythrocytes ⁶². The major precursor of Gb3, globoside, is the main neutral GSL of senescent erythrocytes, and is the major source of circulating Gb3 ⁶³. Ohshima *et al* in 1999, showed that globoside and Gb3 were undetectable in mouse erythrocyte glycolipid fraction, while in humans, Gb3, despite lower than globoside, can be detected ⁶².

1.3. Treatment of LSD

For many years, splenectomy and bone marrow transplantation (BMT) were the available therapies for GD. Despite the achieved correction of haematological and visceral symptoms, the risks involved in the case of splenectomy and the significant morbidity and mortality in the case of BMT led to the abandonment of these procedures (reviewed in ⁶⁴). The replacement of defective enzymes by functional ones was first suggested by Christian De Duve in 1964 ⁶⁵. However, only almost thirty years later, the R.O. Brady group reported the improvement of clinical symptoms from GD patients when treated intravenously with GluCerase purified from human placenta ^{66,67}. This procedure was designated as enzyme replacement therapy (ERT). Currently, GD patients in ERT are no longer treated with GluCerase purified from human placenta, but instead with recombinant human enzyme produced in Chinese hamster ovary cells ⁶⁸. The success of ERT in GD disease led to the development of ERT to other LSD, namely for FD ^{69,70}, mucopolysaccharidosis (MPS) I ^{71,72}, MPS II ⁷³ and MPS VI ⁷⁴, as well as for Pompe's disease ⁷⁵⁻⁷⁷. In the case of GD, and despite the efficiency of ERT, which is capable of reversing years of substrate accumulation, recombinant GluCerase is unable to cross the blood-brain barrier. Consequently neurological deterioration continues in neuronopathic forms of GD even with ERT ⁶⁴. An alternative approach to ERT is substrate reduction therapy (SRT) that aims to reduce the rate of GSL biosynthesis in order to reduce the amount of GSL stored (reviewed in ⁷⁸). The described inhibitors of GSL synthesis described inhibit the GluCer synthase (E.C. 2.4.1.80), which catalyzes the first step in GSL biosynthesis. The capacity of *N*-butyldeoxynojirimycin (NB-DNJ) to inhibit GluCer synthase was first described by Platt *et al.* in 1994 ⁷⁹. This therapy is available in Europe since 2002, is administrated orally, and proved to be efficient in reversing the clinical symptoms of GD type I (reviewed in ⁷⁸) and in Niemann-Pick type C ⁸⁰. Enzyme enhancement therapy is another possible approach towards LSD treatment, and it

consists in the use of low molecular weight pharmacological chaperones to rescue misfolded or unstable proteins. However, this is only effective when disease results from missense mutations or some small *in frame* deletions that do not impair the functional domains of mutant proteins. In the ER, where mutated proteins are retained, chaperones associate and restore the native conformation of these proteins (reviewed in ⁸¹). Promising results were obtained in five LSD, namely GD ⁸², FD ⁸³, GM1 gangliosidosis ⁸⁴ and Tay-Sach and Sandhoff disease ⁸⁵. Clinical trials of gene therapy for GD were carried out with transplantation of genetically corrected autologous CD34⁺ cells with promising results ⁸⁶. However, the effect of this therapy decreases with time ⁸⁶. Human CD34⁺ hematopoietic progenitor cells from a MPS VII patient were targeted directly with a therapeutic lentiviral vector and then xenotransplanted into MPS VII mouse model with therapeutic benefits ⁸⁷.

2. The immune system and lipid metabolism

Lysosomes are the main degradative compartment of eukaryotic cells. They are involved in degradation of proteins, lipids, nucleic acids and saccharides among other cellular components ¹². In general, endocytosed material is initially sorted into early endosomes where lipids and proteins are either recycled back to the plasma membrane or follow to late endosomes and lysosomes to be degraded. The pathways of lysosomal degradation are of physiological relevance in several cellular processes, including the normal function of the immune system. The catabolism of macromolecules in lysosomes is essential for the correct functioning of several immune system processes including antigen processing and presentation (reviewed in ⁸⁸), cytokine secretion ⁸⁹, phagocytosis (reviewed in ⁹⁰) and secretion of macromolecules (reviewed in ^{91,92}).

The importance of lysosomes for the correct function of the immune system is supported by a number of studies describing immune function alterations in LSD, where lysosomal function is compromised, as described in table II. A possible role for the immune system in the pathology of LSD, namely the inflammatory response directed against tissues with pathological storage, was hypothesized by Cox TM in 2003 ⁹³.

2.1. CD1 molecules and invariant Natural Killer T cells

Besides recognizing peptidic antigens presented by MHC-class I and II, lymphocytes are also capable of recognizing glycolipids which are presented by a group of molecules belonging to the CD1 family ⁹⁴⁻⁹⁷, thus being considered the third way of antigen presentation ^{98,99}.

CD1 molecules, discovered by César Milstein group, are classified as non-classical MHC molecules. Despite having structural similarities with MHC class I molecules, CD1 molecules allelic variation is extremely limited, contrary to the characteristic polymorphism of MHC-class I and II ¹⁰⁰. CD1 molecules, like MHC-class I, are constituted by a heavy chain not covalently associated with the β_2 -microglobulin (β_2m) ^{101,102}. The heavy chain folds into five domains, three extracellular domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$) membrane anchored by a transmembrane domain, ending in a short cytoplasmic tail (Figure 2). The $\alpha 1$ and $\alpha 2$ domains fold to form the antigen-binding groove, which is laterally confined by the two α -helices and is supported at the bottom by the two β -sheets, each of them made up of four antiparallel β -strands. The $\alpha 3$ domain and β_2m light chain, supports the superdomain created to form the antigen-binding groove (reviewed in ¹⁰³). In contrast to MHC-class I molecules, $\alpha 1$ and $\alpha 2$ domains of CD1 molecules are hydrophobic and the shape of the binding groove is narrowed and deeper ¹⁰⁴. The CD1 family comprises three groups of molecules: group I constituted by CD1a, CD1b and CD1c, group II formed by CD1d and group III by CD1e. From the five CD1 molecules, only group II molecules are expressed both in mice and humans, being the group I and III molecules only expressed in humans ^{105,106}. Group I CD1 molecules, are primarily expressed by cortical thymocytes. CD1d molecules are expressed weakly in thymus, but instead are expressed extrathymically by professional antigen-presenting cells (APC), such as activated monocytes and B cells ¹⁰⁷, dendritic cells (DC) ^{108,109} and monocytes-derived macrophages ¹¹⁰ as well as by activated T cells ¹⁰⁷. In terms of tissue distribution, CD1d staining was detected in intestinal epithelial cells ¹¹¹, hepatocytes, bile ducts epithelium, pancreas, epididymis, kidney, endometrium, testis, conjunctiva, breast, skin and weakly in tonsils ¹¹².

CD1d molecules were described as existing in two distinct isoforms in epithelial cells. The main isoform, expressed in apical and lateral membranes of intestinal epithelium cells, is a fully glycosylated 48 kDa form, associated with β_2m ¹¹³, while the second form has 37 kDa and is expressed on the cell surface independently of β_2m ¹¹⁴. Besides the differences in tissue distribution of CD1 molecules, they also differ in the type of lipids presented, while group I CD1 molecules can present both self and foreign lipids to conventional T cells ^{106,115}, CD1d molecules predominantly present self lipids to a specialized T cell subset, the invariant Natural Killer T cells (iNKT). iNKT cells are lymphoid cells that are distinct from conventional T cells and natural killer (NK), being characterized by the simultaneous expression of T cell antigen receptor (TCR) and C-lectin-type receptor, NK1.1 ^{116,117}. iNKT cells will be more extensively described below.

Table II. Immune system alterations in lysosomal storage disorders (adapted from ¹¹⁸)

LSD	Main storage cells	Immune system alterations	References
Gaucher disease	Macrophages	B cell lymphocytosis; increased serum IgG levels	119,120
		Chronic stimulation of humoral immune system	121
		AL amyloid deposition in the liver, spleen and kidney	122
		Impaired function of monocytes	123
		Impaired host-defense mechanisms	124
		Abnormal neutrophil chemotaxis	125
		Upregulation of HLA-DR in Gaucher cells	126
		Upregulation of IL-1 β , IL-1Ra, IL-6, IL-10, TNF- α , sIL-2R	127-129
		Upregulation of CCL18/PARC	130
		Upregulation of CD163 and correlation with disease severity	131
		Decreased numbers of peripheral blood T lymphocytes and relation with bone involvement	132
		Multisystemic inflammation and B cell hyperproliferation in Gaucher mutant mice	23
		Increased levels of MIP-1 α and MIP-1 β in plasma and spleen	133
		Enhanced cathepsin S expression in tissues, plasma and serum	134
		Anomalies in cellular immunity and NK cells	135
GM2 gangliosidosis	Neurons	Lysosomal inclusions in T, B and NK cells	136,137
		Infiltration of inflammatory cells of CNS	22
		Macrophages and microglia activation	22,138
		Increase of brain cytokine production in mouse model of Sandhoff	22
		Increased expression of inflammatory genes	139
Globoid cell leukodystrophy	Macrophages (mesodermal origin)	Upregulation of MIP-1 α in astrocytes and recruitment of macrophages to CNS from the periphery in Sandhoff mice	140
		Upregulation of IL-6, TNF- α , MCP-1 and IL-10 in CNS of twitcher mice and infiltration of hematogenous lineage cells into CNS	141,142
Neuronal ceroid lipofuscinosis 3	Neurons (predominantly)	Increased production of TNF- α by PBMC of patients	143
		Storage of undegraded material in lymphocytes	144
		Presence of anti-GAD65 antibodies in CLN3 knockout mice	145
		Serum highly reactive to CNS antigens	146
α -Mannosidosis	Various tissues	IgG entry and deposition within CNS	147
		Low chemotactic responsiveness and impaired phagocytosis of bacteria	148
		Upregulation of TNF- α induced by psychosine	149
Niemman Pick type B	Macrophages	Upregulation of CCL18	150
Niemman Pick type C (NPC)	Macrophages	Respiratory infections	151-153
		Lack of V α 14 J α 18 NKT cells in NPC1-deficient mice and impairment of <i>Sphingomonas</i> clearance	154
Mucopolysaccharidosis VII	Various tissues	Upper respiratory and middle ears infections	155,156

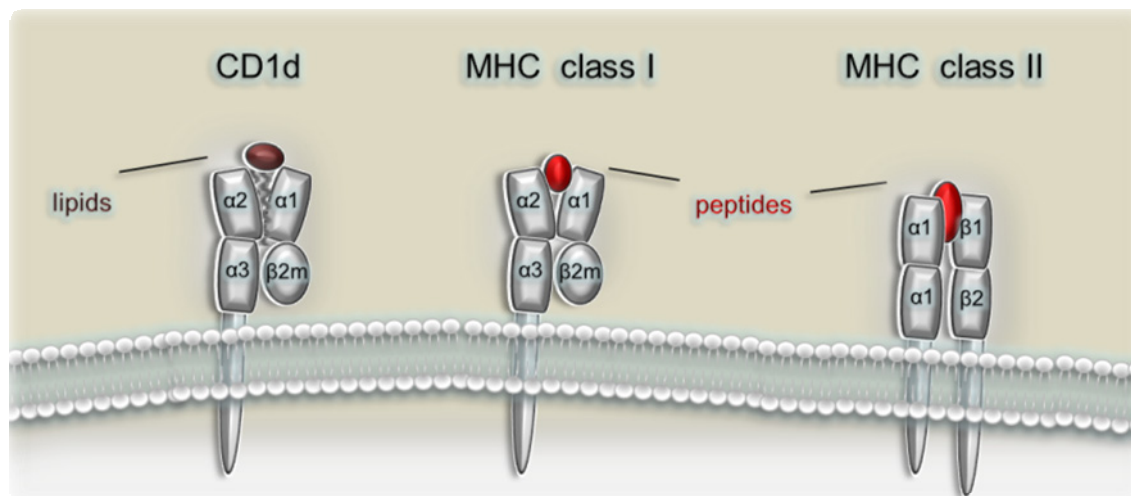


Figure 2. MHC family molecules. Three classes of antigen-presenting molecules are represented. CD1d and MHC-class I molecules are heterodimers formed by a heavy chain that folds in α_1 , α_2 and α_3 domains non-covalently associated with β_2m and a transmembrane domain. MHC-class II are heterodimers formed by an α and a β chains. (Adapted from ¹⁵⁷)

The first identified ligand of iNKT cells, α -galactosylceramide (α -GC) ⁹⁵, emerged in a screen of products derived from the marine sponge *Agelas mauritianus* ¹⁵⁸ with described antimetastatic activity in mice ⁹⁵. α -GC can be distinguished from endogenous lipids by the stereochemistry of the bond that joints the asymmetric 1' carbon of the sugar to the lipid (reviewed in ¹⁵⁹). However, as α -anomeric GSL are absent in mammals, since mammalian cells cannot synthesize α -linked sphingolipids, α -GC is not a physiological antigen for iNKT cells ¹⁶⁰. In 2004, Zhou *et al*, proposed the lysosomal isoglobotrihexosylceramide (iGb3) as the natural ligand, since it is recognized by both mouse and human iNKT cells, with capacity of selectively expand iNKT *in vitro* ¹⁶¹. This finding was supported by the fact that β -hexosaminidase b deficient mice, which lack the capacity to degrade isoglobotetrahexosylceramide into iGb3 and have normal CD1d expression, have a severe decrease in the number of iNKT cells ¹⁶¹. β -hexosaminidase b deficient mice, developed by Phaneuf and coworkers, are a LSD mice model of the Sandhoff disease that accumulates GM2-ganglioside ¹⁶². However, having iGb3 as a ligand for iNKT cells has generated controversy. First, because iGb3 was recently shown to be absent in mouse and human thymus ¹⁶³. A second reason is the absence of α 1,3-galactosyltransferase, the enzyme thought to be responsible for the synthesis of iGb3, in humans ¹⁶⁴, as well as the normal development of iNKT cells in mice lacking this enzyme ¹⁶⁵. Also, a study by Gadola

SD, 2006, described anomalies in iNKT of several mouse models of LSD, suggesting that the GSL storage, and not the lack of a specific enzyme of the GSL pathway, is responsible for these alterations ¹⁶⁶. Amidst this controversy, in a study published very recently, the authors were capable to detect isoglobos series of GSL are expressed in human thymus by electrospray ionization-ion trap mass spectrometry ¹⁶⁷. Other self lipid described as a ligand for iNKT was the ganglioside GD3 that is present in high levels in melanomas ¹⁶⁸ and is capable to induce an iNKT cell response among splenocytes of mice immunized with a melanoma cell line or with APCs loaded with GD3 ¹⁶⁹. Apparently, also β -galactosylceramide is capable of stimulating iNKT cells, however in a substantially reduced intensity than with α -GC ^{170,171}.

2.1.1. CD1d synthesis, intracellular loading and transport

Despite the structural similarity between MHC class I and CD1d molecules, intracellularly, CD1d molecules behave like MHC class II molecules ¹¹⁵. The trafficking routes and requirements appear to be different for mouse and humans (reviewed in ¹⁷²). After translocation into the ER, CD1d forms a ternary complex with calnexin and calreticulin, and the complex recruits the thiol oxidoreductase Erp57, that catalyzes intradisulfide bond formation in the CD1d heavy chain ¹⁷³. Then, CD1d dissociates from this complex and associates with β_2m . The complex formed by the heavy chain and β_2m is a structure receptive to ER resident lipids such as phosphatidylcholine (PC) and phosphatidylinositol (PI). The association with lipids in the ER is thought to have two functions: protect the antigen binding groove and occupy the groove with a ligand that could be easily exchanged for other antigen in late secretory vesicles ¹⁷⁴. In order to bind CD1d molecules, lipids need to be extracted from membranes, in a process mediated by lipid transfer proteins such as microsomal triglyceride transfer protein (MTP) and saposins. MTP was initially described as acting like a chaperone in the ER, loading CD1d upon assembly with PC and PI (reviewed in ¹⁷⁵). Saposins are lysosomal sphingolipids activator proteins that make GSL available for degradation (reviewed in ¹⁷⁶). Even though the exact role of saposins in CD1d ligand loading is not clear, a study by Zhou *et al* in 2004, suggested that they are essential for presentation of self and non-self lipids in mice ¹⁷⁷. Mice deficient in prosaposin, besides exhibiting a LSD phenotype ¹⁷⁸, lack iNKT cells ¹⁷⁷. In the case of self-derived ligands, saposins would be involved either in release of the endogenous ligands from the membrane bilayer or in assisting the catabolism of endogenous lipids in order to originate iNKT cell ligands. In humans, saposins are not essential for presentation of endogenous or exogenous ligand ^{179,180}, probably due to the

differential trafficking requirements of human CD1d to mouse CD1d ¹⁷². However, in humans, saposins can enhance the rate of exogenous ligands presentation, mainly through saposin B ¹⁸⁰, by increasing the load of CD1d in the endocytic system ¹⁷². After PC/PI loading, CD1d molecules follow the secretory pathway to cell surface and recycle between the plasma membrane and the endocytic system ¹⁸¹. A part of CD1d molecules that are synthesized in the ER associate with the chaperone invariant chain (Ii) rather than with self lipids. Due to the presence of a modified dileucine motif on the cytoplasmic tail of Ii the CD1d molecule is redirected from the ER to endolysosomal compartments ¹⁸¹ rich in MHC-class II molecules, also known as MHC-class II compartments (MIIC) ¹⁷³, where exogenous peptides are loaded into MHC-class II molecules (reviewed in ¹⁸²) (Figure 3).

2.1.2. CD1d cell surface expression and function

After synthesis and assembly in the ER, most of the CD1d molecules are directed to the plasma membrane, via secretory pathway, from where they are reinternalized via adaptor protein (AP)-2 in humans, and AP-3 in mouse. The cytoplasmic tail of CD1d molecules, both in humans and mice, contains a tyrosine based motif of the YXXZ type, where Y is tyrosine, X is any amino acid and Z is a bulky hydrophobic motif ¹⁸³. The tyrosine based motif mediates binding of the μ -subunit of AP heterotetramers, which play a crucial role in many intracellular protein sorting events (reviewed by ¹⁸⁴). Mice lacking AP-3 have increased cell surface expression of CD1d that results from the redistribution of these molecules from endosomal vesicles and cell surface, and iNKT cell numbers are reduced, suggesting an iNKT cell-selecting ligand presentation ^{185,186}. CD1d undergoes intensive recycling between the plasma membrane and intracellular compartments ¹⁸¹ (Figure 3). Comparatively, MHC class II molecules, by virtue of the modified dileucine motif present in Ii, are directed directly from the *trans* Golgi network (TGN) to MIIC (Figure 4). In both cases, cathepsin S cleaves the Ii, remaining the invariant chain peptide (CLIP) on the peptide/lipid groove until being exchanged for peptides/lipids with higher affinity (reviewed in ¹⁸⁷) (Figures 3 and 4).

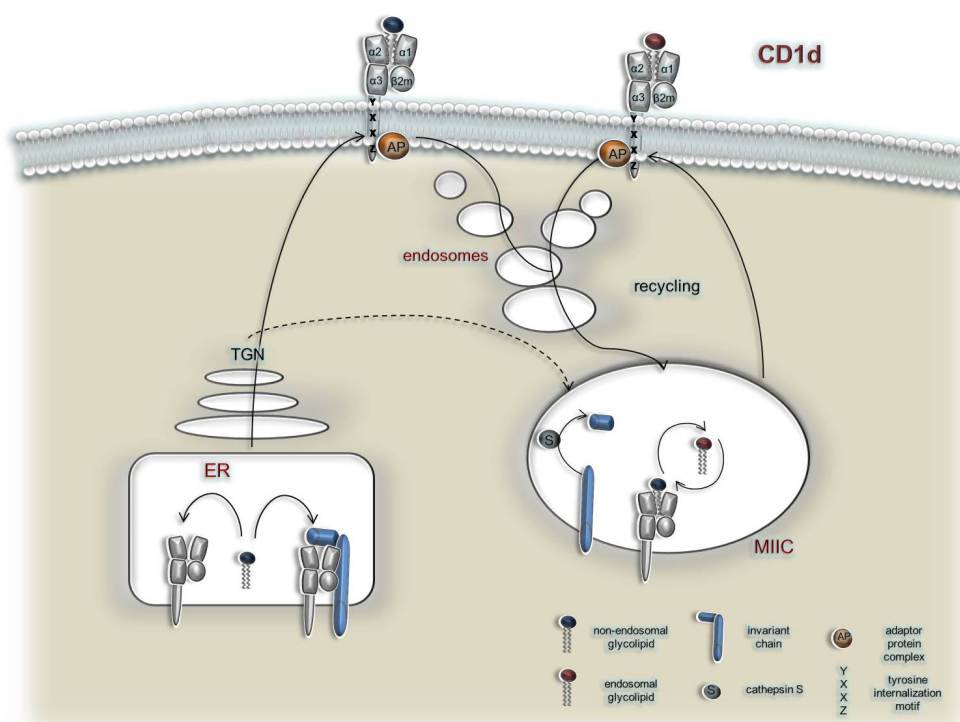


Figure 3. Intracellular pathway for antigen presentation by CD1d. Trafficking pathways of CD1d are represented. The majority of CD1d proteins move rapidly to the cell surface and are subsequently delivered to MIICs by the internalization and recycling pathway that is controlled by the cytoplasmic tail tyrosine-based motif and its interaction with clathrin AP complexes (solid arrows). (Adapted from ¹⁸⁷)

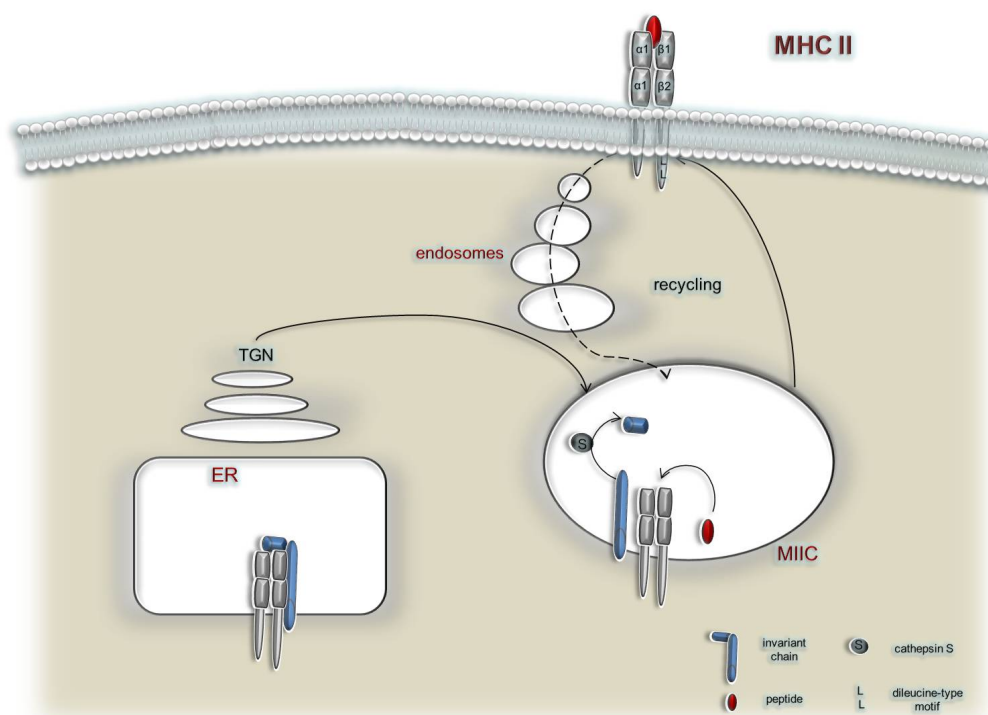


Figure 4. Intracellular pathways for antigen presentation by MHC-class II. Trafficking pathways of MHC-class II are represented. MHC class II, associated Ii blocks the antigen binding groove and directs MHC-class

II from the TGN to MHC, where cathepsin S (S) cleaves Ii, releasing the MHC-class II associated CLIP and exposing the groove for peptide exchange prior to release of MHC-class II to the cell surface (solid arrows). A second, less dominant pathway (dashed arrow) into the endosomes for MHC class II involves recycling from the cell surface as a result of the dileucine motif in the cytoplasmic tail of the chain. (Adapted from ¹⁸⁷)

2.1.3. CD1d and disease

Several studies have shown variations in CD1d expression during gastrointestinal, hepatic and dermatological diseases and cancer. Increased expression of CD1d was reported in colonic epithelium of inflammatory bowel disease ¹⁸⁸, Chron disease, ulcerative colitis ¹⁸⁹ and in duodenal lamina propria of patients with cow milk hypersensitivity ¹⁹⁰. In liver disorders, CD1d was upregulated in primary biliary cirrhosis and sarcoidosis ¹⁹¹. Infiltration of CD1d⁺ B cells was observed in periodontal disease ¹⁹², as well as its overexpression in psoriatic lesions and other inflammatory dermatoses ¹⁹³. CD1d is also expressed in a number of tumors, namely in brain tumors where a very strong expression of CD1d on angiogenic vessels was reported ¹⁹⁴, in B-cell chronic lymphocytic leukemia ¹⁹⁵, T-acute lymphoblastic leukemia ¹⁹⁶, acute myeloid leukemias and juvenile myelomonocytic leukemias ¹⁹⁷. Upregulation of CD1d was also reported in lipid-laden foam cells of atherosclerotic lesions ¹⁹⁸.

Despite the role of GSL as endogenous ligands for CD1d ¹⁹⁹, studies addressing the involvement of CD1d molecules in LSD have been absent. There are few published studies reporting iNKT cells alterations in LSD mouse models where some attention was provided to CD1d expression as referred below (see 2.2.3. iNKT cells and disease).

2.2. Invariant Natural Killer T cells

The existence of a distinct subset of $\alpha\beta$ -T cell receptor⁺ cells in mice, which expresses an intermediate level of T cell receptor (TCR) and lack the expression of CD4 and CD8 molecules ²⁰⁰, with a higher frequency of V β 8 expression than conventional T cells ²⁰¹, was described in 1987. Still, the term NKT was only proposed in 1995 ²⁰².

In contrast to conventional T cells that recognize peptide antigens bound to MHC molecules, iNKT cells recognize glycolipid antigens presented by CD1d. Association between NKT cells and CD1 molecules in mice started when several groups showed that cells expressing the NK mouse marker NK1.1⁺ T in thymus ²⁰³⁻²⁰⁵ or liver ²⁰⁶, as well as the related V α 14⁺ peripheral T cell subset ²⁰⁷, failed to develop in β_2m -deficient mice,

suggesting that a β_2m -associated molecule was a component of the ligand of these cell's TCR.

NKT cells are actually divided into three subgroups²⁰⁸. CD1-dependent NKT cells can be divided into two classes, the type I, also called iNKT cells, and type II. Both are CD1d dependent, but while iNKT cells express an invariant T-cell receptor formed by the canonical rearrangement of the V α 24 gene segment in humans (V α 14 in mice) to the J α 18 segment in combination with V β 11 in humans²⁰⁹⁻²¹¹ (V β 8.2, V β 7 or V β 2 in mice²¹²), type II NKT cells express more diverse TCR V α chains. A third population of NKT cells that does not depend on CD1d recognition is designated non-CD1d-restricted NKT cells²⁰⁸.

2.2.1. iNKT cells development and distribution

Studies in nude and thymectomized mice have shown that iNKT cells are a thymus dependent population²¹³⁻²¹⁷ that appear in thymus slightly after other T cell subsets^{216,218-220}. iNKT cells differentiate from double positive (DP) thymocytes²²¹⁻²²³ that have undergone random TCR gene rearrangement and expression, and are positively selected by CD1d-expressing cortical CD4⁺CD8⁺ DP thymocytes^{213,224}, in contrast with mainstream thymocytes that recognize their positively selecting MHC ligand on epithelial cells. Once selected, iNKT cell precursors go through a series of differentiation steps that give rise to the iNKT-cell pool. NK1.1 expression is a downstream event in maturation of iNKT cells, in most cases acquired outside the thymus although some NK1.1⁺ cells can move abroad to periphery^{216,218,220}. Mature NK1.1⁺ iNKT cells undergo clonal expansion in the periphery after activation, which is associated with downregulation of this receptor^{225,226}. Interleukin (IL)-15 and/or IL-2 play an important role in iNKT cell maturation in the thymus, since iNKT cells upregulate the expression of the IL-2/IL-15 receptor β -chain (CD122) as they progress from NK1.1⁻ to NK1.1⁺ stage²²⁷, which is supported by previous reports where mice deficient in IL-15 or CD122 lack NK1.1⁺ T cells^{228,229}. Previous studies have shown that human iNKT cells development occurs in thymus and follows a similar maturation pathway to that of mouse iNKT cells²³⁰⁻²³². However, some differences should be pointed out. It is important to note that blood mature human iNKT cells include CD4⁺, CD8⁺ and double negative (DN, CD4⁻CD8⁻) subsets. The CD8⁺ iNKT cells subsets is absent in mice. Lantz *et al* in 1994, suggested that thymic CD8⁺ iNKT cells after upregulation of their TCR level, reached the negative selection threshold and are eliminated²¹².

The frequency of iNKT varies among tissues and differs from mice to humans. In mice, iNKT cells represent about 30% of liver T cells ^{233,234}, 30% of bone marrow T cells ²³⁵, 0.5% in whole thymus ²³³, about 1.2% in splenic T cells ²³³, around 0.4% of T cells in peripheral nodes ²³⁶ and less than 1% in peripheral blood ²³³. In human liver, about 30% of hepatic CD3⁺ lymphocytes are NKT cells ²³⁷. However, contrarily to mice, human liver NKT cell TCR repertoire is not skewed to V α 24 ²³⁸. The frequency of iNKT cells is less than 1% in human peripheral blood ²³⁹.

2.2.2. iNKT cells: phenotype and function

In humans, three subsets of iNKT cells can be defined upon expression of CD4 and CD8: i) the DN subset that represents about 49% of iNKT cells; ii) the CD4⁺ subset, representing on average 27% and iii) the CD8⁺ subset, representing about 24% of iNKT cells ²⁴⁰.

A hallmark of iNKT cells is their unusual ability to rapidly secrete both Th1 and Th2 cytokines upon activation. Different functional phenotypes can be observed in iNKT cells if we consider the presence or absence of CD4 expression in human peripheral blood ^{239,241}. While both CD4⁺ cells and DN iNKT cells can produce Th1 cytokines, the release of Th2 cytokines such as IL-4 and IL-13 is exclusive of CD4⁺ iNKT cells ²³⁹. In contrast, Th1 cytokines interferon γ (IFN- γ) and tumor necrosis factor α (TNF- α) are selectively produced by CD4⁺ iNKT cells ²⁴¹. Human CD4⁺ and CD4⁻ iNKT cells subsets also present different patterns of chemokine receptors ^{239,240}. iNKT cells express inflammatory lymphocyte chemokine receptors in at higher levels than other memory or effector T cells subsets and also exhibit distinct and subset specific expression profiles of other chemokine receptors, namely CCR4 by CD4⁺ iNKT cells and CXCR6, CCR1 and CCR6 by CD8⁺ and DN iNKT cells ²⁴⁰.

Regulation of other immune cells has also been attributed to iNKT cells. A prompt communication network between innate and adaptative immune systems *in vivo*, is initiated upon iNKT cells activation ²⁴². Reciprocal activation of iNKT cells and DC is instigated by antigen activated iNKT cells that express CD40 ligand, which in turn engages CD40 in DC and stimulate them to produce IL-12 ²⁴³. IL-12 production by DC, precedes and is necessary for iNKT cells IFN- γ production ^{243,244}. The specific activation of iNKT cells, in liver and spleen, leads to a rapid induction of extensive NK cell proliferation and cytotoxicity, which is dependent, at least in part, on IFN- γ production by iNKT cells and IL-12 by APC ²⁴⁵.

2.2.3. iNKT cells and disease

iNKT cells have been implicated in a wide variety of immune responses ranging from transplants to tumors, autoimmunity, atherosclerosis, allergy and microbial immunity.

iNKT cells play a role in the regulation of several autoimmune diseases in mice and humans, where alterations in number and/or function have been reported (reviewed in ²⁴⁶). Decreased numbers of iNKT cells were described in patients with Sjogren's syndrome ^{247,248}, rheumatoid arthritis ²⁴⁷⁻²⁵⁰, systemic sclerosis ^{247,248,251}, systemic lupus erythematosus ^{247,248,252} and type 1 diabetes ^{253,254} as well as in non-obese diabetic (NOD) ^{214,255-261} and multiple sclerosis ²⁶²⁻²⁶⁴ mice models. The role of iNKT cells in diabetes type 1 is strongly supported by exacerbated manifestation of the disease in CD1d^{-/-} NOD mice ^{265,266} and by studies where stimulation of iNKT cells by α -GC treatment protects mice models from diabetes ^{261,266-268} and autoimmune encephalomyelitis ^{263,269}. Functional abnormalities in iNKT cells were reported in diabetes where both humans ²⁵⁴ and mice present impaired capacity to produce IL-4 ^{214,258-261,266,268}.

The involvement of iNKT cells in the immune response after transplantation is not completely understood. iNKT cells may promote rejection or tolerance, and Jukes *et al* suggested that they can either be activated by cytokines generated by nonspecific inflammation during transplantation or in a CD1d-dependent manner by GSL resulting from tissue damage ²⁷⁰. Survival of islet allografts in liver of streptozotocin-induced diabetic CD1d^{-/-} or iNKT^{-/-} mice is prolonged ²⁷¹. In contrast, the presence of iNKT cells seems to induce tolerance in CD1^{-/-} mice with sex-mismatched skin grafts ²⁷² and in models of cardiac transplant mouse ²⁷³, rat xenograft islets ²⁷⁴ and corneal transplantation ²⁷⁵. In these models, tolerance has been attributed to the production of various cytokines such as IL-10, IFN- γ and the decrease of IL-4 (reviewed in ²⁷⁰).

In tumors, iNKT cells, like conventional T cells, are decreased or functionally impaired both in mice and humans. A study in advanced prostate cancer patients, reported a significant decrease in peripheral blood iNKT cells with diminished *ex vivo* expansion with α -GC and impaired function with decreased production of IFN- γ ²⁷⁶. IFN- γ deficient production was also observed in patients with progressive myeloma, despite the fact that iNKT cells could be detected ²⁷⁷. In mice, iNKT cells were implicated in the immunosurveillance of methylcholanthrene-induced sarcomas ²⁷⁸ and in the inhibition of antitumor activity *in vivo*, since CD1d^{-/-} and J α 18^{-/-} mice inoculated with cells transfected with CD1d survived significantly longer than mice inoculated with vector-transfected cells ²⁷⁹. Despite the major consensus on the role of iNKT cells in the prevention of cancer,

some reports contradict this, namely a study where CD1d-deficient mice subcutaneously transplanted with a tumor cell line presented to be resistant to recurrence²⁸⁰.

iNKT cells appear to play a critical role in allergic inflammatory responses, particularly in the development of asthma and airway hyperreactivity (AHR). In mice, a number of studies suggested that iNKT cells are absolutely required for the development of AHR. CD1d^{-/-} and J α 18^{-/-} mice showed reduced eosinophilic inflammation and failed to develop allergen-induced AHR^{281,282}. However, iNKT cells may not be required in all forms of AHR, since $\beta_2m^{-/-}$ mice, when sensitized and challenged with allergen, develop AHR^{283,284}. Co-administration of α -GC along with exogenous antigen results in enhanced sensitization and subsequent development of AHR²⁸⁵. In humans, 60% of pulmonary CD4⁺CD3⁺ T cells from patients with moderate to severe persistent asthma are iNKT cells²⁸⁶, suggesting that iNKT cells play a major pathogenic role in human asthma. Most of these pulmonary iNKT cells are a Th2 subset, capable of producing IL-4 and IL-13 but not IFN- γ . Apparently, iNKT cells could drive a particular form of asthma, since in some patients studied, the numbers of iNKT cells in the lungs are not affected by treatment with corticoids²⁸⁷, probably accounting for the 10-25 % of patients with corticosteroid-resistant asthma^{288,289}.

iNKT cells play an important role on the immune response to mycobacteria infections (reviewed in²⁹⁰). However, despite being capable of recognizing glycolipids from mycobacteria, iNKT cells are not crucial for *Mycobacterium tuberculosis* resistance since mycobacterial cell-wall antigens are presented by CD1b and CD1c (reviewed by¹¹⁵). iNKT cells play a primary role in the granulomatous response to injection of glycolipids derived from mycobacteria, which was proven by the observation that the same does not occur in the iNKT cells deficient mice J α 281^{-/-}²⁹¹. Recruitment of iNKT cells to granulomas does not require antigen recognition by iNKT cells TCR since it can occur even when iNKT cells are adoptively transferred to CD1d^{-/-} mice²⁹². So, it was proposed that neither a functional CD1d-TCR axis nor the expression of CD1d is required for iNKT cell migration. Moreover, the iNKT cell accumulation at infection sites was antigen independent, and their recruitment to inflammatory sites is most likely driven by cytokines²⁹². iNKT cells were also reported to be involved in immune responses to *Listeria*, *Toxoplasma*, *Salmonella* and *Plasmodium* (reviewed in¹¹⁷).

In LSD, studies of iNKT cells are scarce and limited to mouse models. Frequency and percentages of iNKT cells were reported to be decreased or even absent in several LSD mice models, namely in Nieman-Pick Type C1 (NPC1)^{154,166}, Fabry, Tay-Sachs, GM1-gangliosidosis, Sandhoff and late-onset Tay-Sachs disease models¹⁶⁶. In most of the

cases, decreased numbers of iNKT cells resulted in impairment of cytokine production in response to α -GC, and reduced iNKT cell-mediated lysis of wild-type targets loaded with α -GC¹⁶⁶. NPC1 mice exhibited marked defects in presentation of *Sphingomonas* antigens to iNKT cells as well as in bacteria clearance¹⁵⁴.

II – Aims of the thesis

How accumulation of a specific GSL leads to alterations in cell and tissue function, and ultimately to disease, remains unexplained. Furthermore, no single unifying theory has been proposed to explain the link between GSL accumulation and pathology²⁹³. In the case of GD, it was suggested that pathology does not result exclusively from the burden of GluCer storage but, at least in part, results from macrophage activation. It is known that the concentration of many macrophage-associated molecules like IL-1 β , IL-6, TNF- α and M-CSF, for example, are increased in serum of GD patients (reviewed in²⁹⁴). Many of these molecules are associated with inflammation, osteopenia and haematological alterations (reviewed in²⁹⁴). In LSD, the main unsolved question is related to how GSL accumulation results in cellular dysfunction. Furthermore, it became clear that the genotype/phenotype correlation is difficult to establish for these monogenic disorders¹⁴, suggesting that other mechanisms may have a determinant role in the pathophysiology of these disorders.

An understanding of the impact of GSL accumulation on the immunological system could provide new insights on the mechanisms underlying the symptoms and the clinical heterogeneity of LSD. In this thesis we propose to study the effect that glycosphingolipid accumulation has on the expression of CD1d and MHC-class II molecules and on the functional phenotype of NKT cells in two LSD, Gaucher and Fabry disease. In this context, the specific aims were:

- Ascertain whether abnormalities in sphingolipid metabolism are linked to anomalies in the expression of molecules of MHC family and to imbalances in T cell subsets, in GD and FD patients, as well as in a Fabry mouse model.

- Understand the effect of glycosphingolipid accumulation on the expression of MHC family molecules, and consequently on T and iNKT cell populations, through the development of an *in vitro* model of GD.

III – Research work

1. Gaucher disease: impact of glycosphingolipid accumulation on the immunological phenotype

Adapted from Balreira A, Lacerda L, Sá Miranda MC and Arosa FA.
Brit J Haematol. 2005; 129: 667-676

1.1. Abstract

GD is an autosomal recessive inherited defect of the lysosomal enzyme GluCerase that leads to GluCer accumulation. We previously demonstrated the existence of imbalances in certain lymphocyte populations in GD patients. We now show that GluCerase-deficient monocytes from GD patients or monocytes from healthy subjects treated with conduritol-B-epoxide (CBE), an irreversible inhibitor of GluCerase activity, display high levels of surface expression of the lipid-binding molecule CD1d. GluCerase-deficient monocytes from GD patients also showed increased surface expression of major histocompatibility complex (MHC)-class II, but not of other lysosomal trafficking molecules, such as CD63 and MHC-class I. However, CD1d and MHC-class II mRNA levels were not increased. GluCerase-deficient monocytes from GD patients undergoing ERT also exhibited increased levels of CD1d and MHC-class II and imbalances in the percentage of CD4⁺, CD8⁺, and V α 24⁺ T cells. Interestingly, follow-up studies revealed that ERT induced a decrease in MHC-class II expression and partial correction of the CD4⁺ T cell imbalances. These results reveal a new link between sphingolipid accumulation in monocytes and the expression of certain MHC molecules that may result in imbalances of regulatory T cell subsets. These immunological anomalies may contribute to the clinical heterogeneity in GD patients.

1.2. Introduction

GD is the most common LSD in the Portuguese population ¹⁵. The primary defect is a deficient activity of the lysosomal enzyme GluCerase, which cleaves the β -glucosidic linkage of GluCer to produce ceramide and glucose. In the absence of GluCerase activity, the uncleaved lipid substrate is stored in lysosomes within cells of the macrophage lineage, mostly affecting the spleen, liver, bones and bone marrow ²⁴. Depending on the involvement of the central nervous system, GD can be divided into three types ²⁹⁵. Type 1 GD is non-neuropathic and the most prevalent form seen in adults. It is characterized by hepatosplenomegaly, haematological abnormalities and bone involvement. Type 2 is an acute neuropathic form resulting in death before 2 years of age, while type 3 is a sub-acute neuropathic form that usually results in death between the second and fourth decade of life. Even though a number of different mutations have been described in the gene encoding GluCerase, this genetic variability has not provided major advantages in predicting clinical phenotype. Members of the MHC family are involved in the selection and shaping of the T lymphocyte pool. Along with MHC-class I and II molecules, CD1d plays a key role in selecting subsets of NKT cells, namely TCRV α 24-expressing cells ^{296,297}. NKT cells are a specialized subset of T cells that secrete large amounts of

regulatory cytokines, some of which are elevated in GD, upon triggering of the T cell receptor¹¹⁷. While structurally CD1d resembles classical MHC-class I molecules, in terms of intracellular trafficking it behaves like classical MHC-class II molecules¹¹⁵. Thus, CD1d molecules are abundantly expressed in endolysosomal compartments, where lipid accumulation in GD takes place^{24,298}. Despite a body of evidence in the literature linking lipids and the immunological system both at the systemic and cellular level²⁹⁹⁻³⁰², studies addressing the role of immunological cells and molecules in lysosomal storage disorders, such as GD, are scarce. Earlier studies consistently reported anomalies of the B cell compartment, namely B cell lymphocytosis and benign monoclonal gammopathies, as well as some cases of lymphocytic leukaemia^{119,120,303}. Concurrently, a study described the existence of abnormalities of cellular immunity and NK cells in GD¹³⁵. More recently, electron microscopy studies detected lysosomal inclusions in B and T cells and in CD4⁺, CD8⁺ and CD56⁺ subpopulations^{136,137}. Although the relevance of the reported lymphocyte anomalies to the clinical course of the disease is unknown, another study showed a relationship between anomalies in the numbers of PBL and some clinical manifestations of the disease, namely bone involvement¹³². This study was undertaken to ascertain whether anomalies of sphingolipid metabolism in GD patients are linked to anomalies in the expression of molecules of the MHC family and to the existence of imbalances in T cell subsets. The results support the notion that expression of CD1d and MHC-class II molecules by monocytes is influenced by the intracellular sphingolipid content. Because certain T cell subsets are altered in GD patients, we suggest that the alterations in CD1d and MHC-class II as a result of sphingolipid accumulation may underlie imbalances in regulatory NKT and T cells, which may contribute to the clinical heterogeneity of the clinical manifestations.

1.3. Material and methods

1.3.1. Patients population

The biochemical diagnosis of the GD patients was done by determining the residual activity of GluCerase in PBL³⁰⁴. Patients were genotyped using previously described methods. Most of their genotypes have already been reported³⁰⁵⁻³⁰⁸. Patients were divided into two groups. Group 1 included untreated patients and group 2 patients were undergoing ERT. In group 2, 13 of the 49 patients were splenectomized. Untreated patients had been recently diagnosed or had milder clinical forms that did not require ERT. Clinical relevant data of both groups are summarized in Table I. Patients submitted to intravenously administered ERT started treatment with alglucerase, human GluCerase

purified from human placenta and modified in order to be correctly targeted to the macrophages⁶⁶. Currently, all treated patients are receiving the recently available imiglucerase (human glucocerebrosidase obtained by recombinant methods)⁶⁸. All the patients started the treatment with 60 U/kg/every 2 weeks; treatment regimens were modified according to the patient's clinical response. All studies were performed with the patients' consent and with the approval of the Ethical Committee of the Institute for Molecular and Cell Biology.

1.3.2. Expression of cell surface molecules

PBL were obtained from whole blood unless indicated. Red blood cells (RBC) were first lysed in lysis solution (10 mM Tris, 150mM NH₄Cl, pH 7.4) for 10 min at 37°C. Cells were washed twice in phosphate-buffered saline solution (PBS) supplemented with 0.1% sodium azide (NaN₃) and 0.2% bovine serum albumin (BSA) (PBS-BSA-NaN₃, staining solution). Staining was performed on ice for 30 min in the dark, in staining solution in round-bottomed microtiter plates (Greiner-Nurtingen, Germany) with $\sim 0.5 \times 10^6$ cells/well. After staining, cells were washed twice with staining solution and immediately acquired in a FACScalibur (Becton Dickinson, Mountain View, CA, USA). Mononuclear cells (lymphocytes and monocytes) were identified and gated according to forward scatter/side scatter (FSC/SSC) characteristics. For each sample, 50000 events within the mononuclear gate were acquired and analyzed using the CellQuest (Becton Dickinson, Mountain View, CA, USA) or FlowJo softwares (Treestar Inc., Ashland, OR, USA). The following fluorochrome-conjugated mouse anti-human monoclonal antibodies were used: anti-CD4 (clone MT310), anti-CD8 (clone DK25), anti-CD19 (clone HD37), anti-CD14 (clone TÜK4), anti-CD3 (clone UCHT1), anti HLA-DR,DP,DQ (clone CR3/43) and anti HLA-A,B,C (clone W6/32), from DakoCytomation (Glostrup, Denmark); anti-V α 24 (clone C15), from Beckman Coulter (Fullerton, CA, USA); anti-CD1d (clone 42), from BD Biosciences (Erembodegem, Belgium), anti-CD1d (clone Nor3.2), from AbD Serotec (Oxford, UK) and anti-CD63 (clone MEM-259), from Immunotools (Friesoythe, Germany). Mouse immunoglobulins (Igs) were used as negative control. Expression of the different molecules at the cell surface was determined by mean fluorescence intensity (MFI) values. The percentage of CD8⁺ T cells and CD8⁺V α 24⁺ T cells were determined after setting quadrants on CD8^{bright} cells, and they are referred to as CD8⁺ T cells. Staining with CD3 confirmed that CD8^{bright} cells were T cells.

1.3.3. Inhibition of glucocerebrosidase activity

Fresh peripheral blood mononuclear cells (PBMC) were obtained from leukocyte preparations of healthy subjects by centrifugation over the density gradient Lymphoprep (Axis-Shield, Oslo, Norway). PBMC were then cultured for 24 to 96 hours in normal media or media supplemented with two different concentrations (0.66 mM and 1 mM) of CBE (Biomol, Plymouth Meeting, PA, USA), prepared as stock solution in 20 mM Hepes buffer, pH 7.4 as described³⁰⁹. After the incubation period, PBMC were harvested, washed and processed for flow cytometry studies as indicated above. Cells were acquired without fixation in a FACScalibur (Becton Dickinson, Mountain View, CA, USA) and analyzed using the CellQuest software (Becton Dickinson, Mountain View, CA, USA). Inhibition of GluCerase activity was confirmed by enzymatic activity measurement following previous described protocols³⁰⁴. Briefly, PBMC pellets were extracted in 0.1% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) by brief sonication and centrifuged. Supernatant was collected and incubated for 30 min at 37 °C with 5 mM 4-methylumbelliferyl- β -glucoside (Sigma-Aldrich, St. Louis, MO, USA) as substrate in 100 mM citrate-200 mM phosphate (pH 5.2), 5mM sodium taurocholate (Calbiochem, La Jolla, USA), and 0.02% (v/v) Triton X-100. After incubation, the reaction was stopped with 1 mL of 1 M glycine/NaOH buffer, pH 10.0. Fluorescent 4-methylumbelliferone (4-MU) was measured at 445 nm with a fluorescence spectrophotometer (Hitachi HTA, Illinois, USA).

1.3.4. Reverse transcription polymerase chain reaction (RT-PCR)

PBMC were isolated as described above. From each sample, total RNA was isolated using the High Pure RNA Isolation Kit (Roche, Carlsbad, USA). Total RNA concentration was measured by spectrophotometry and its quality assessed by agarose gel electrophoresis. Subsequently, 1 μ g of total RNA was converted into cDNA with the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. The CD1d gene was amplified by PCR using the following primers: 5'-GGGCACTCAGCCAGGGGACATCCTGCCCAA-3' as forward and 5'-GATACAAGTTTGCACACCTTTGCACTTCTG-3' as reverse. The HLA-DR gene was amplified by using the following primers: 5'-CAGAGGTAAGTGTGCTCACGAACAGCCC-3' as forward and 5'-CCCCAGTGCTTGAGAAGAGGCTCATCC-3' as reverse. To ensure that the cDNA samples were of similar concentration and quality, the β -actin gene was also amplified with the following primers: 5'-CCATGGAGAAGGCTGGGG-3' as forward and 5'-CAAAGTTGTCATGGATGACC-3' as reverse. The PCR amplification was

performed in a total volume of 50 µl reaction mix containing 1 µl of cDNA, 10 pmol of each primer, 10× reaction buffer, 0.5 mM dNTPs and 1.25 units of Taq polymerase (Invitrogen, Prat de Llobregat, Spain). For the amplification of CD1d, we used the following PCR program: an initial denaturing step of 3 min at 92 °C, followed by 35 cycles (92 °C for 30 s, 55 °C for 1 min and 72 °C for 3 min) and a final extension step for 10 min at 72 °C. For the amplification of HLA-DR and β-actin we used the same program but with 30 cycles of amplifications instead of 35. Subsequently, 10 µl of the PCR products were loaded into a 2% agarose gel with 0.05% ethidium bromide, the electrophoresis performed and the PCR fragments visualized under ultraviolet light.

1.3.5. Statistical analysis

Statistical evaluation was performed using paired Student's *t*-test at the level of significance of 0.05.

1.4. Results

1.4.1. Patients' characteristics

GD patients were divided into two major groups: untreated patients and patients undergoing ERT. As summarized in Table I, the two groups did not differ significantly in age or gender. Patients *GBA* gene was previously characterized and N370S/N370S and N370S/L444P were the most frequent genotypes³⁰⁵⁻³⁰⁸ (Figure 5). For ethical reasons it was not possible to include young healthy controls less than 18 years old in the study. However, age had no significant impact on the data from GD patients.

Table I. Characterization of the groups of controls and patients studied.

Group	Controls	Gaucher	Gaucher ERT
Number	25	13	49
Age (years)	38±14	35±26	39±17
(range)	(22-73)	(6-83)	(5-76)
Gender (M/F)	12/13	7/6	23/26
Splenectomy	0/25	1/13	13/49

Gaucher, untreated patients with GD; Gaucher ERT, GD patients receiving ERT

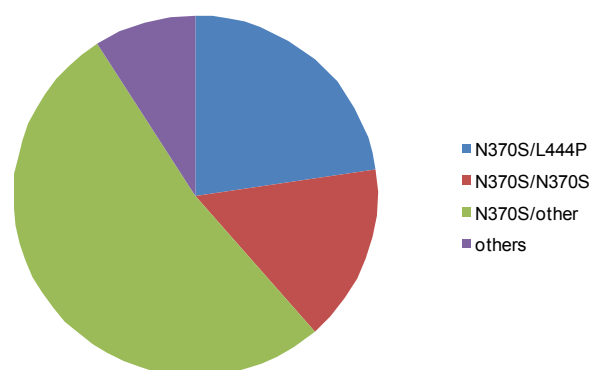


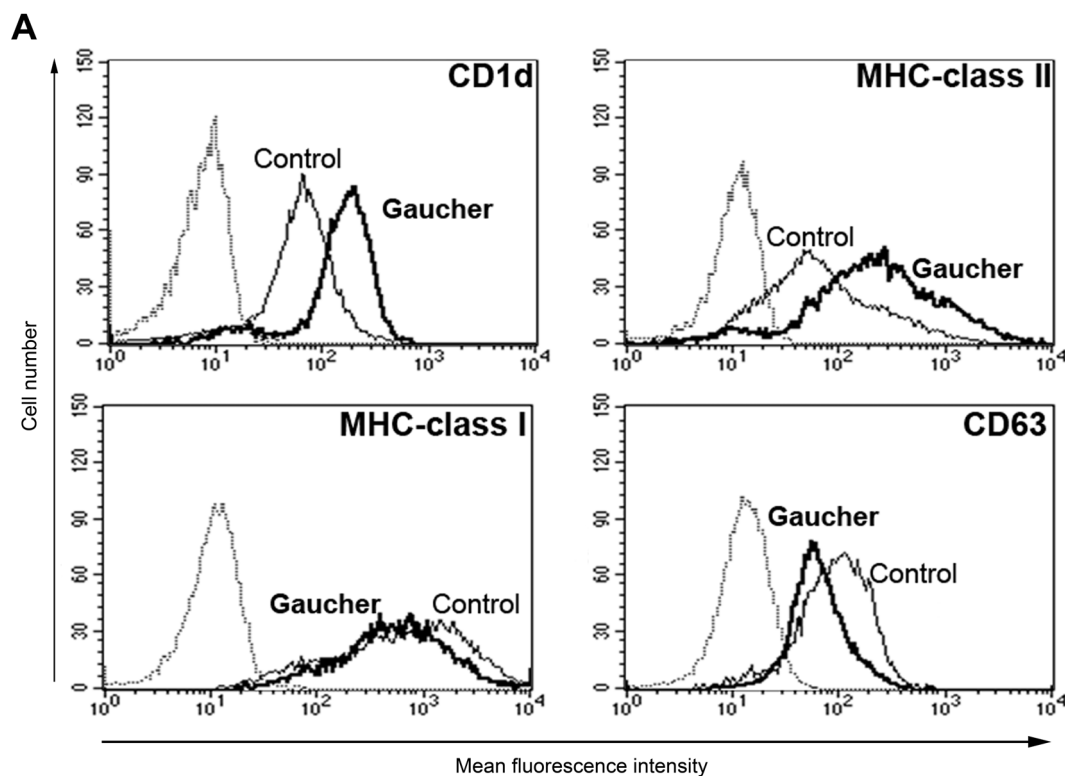
Figure 5. Genotypes distribution among the GD patients studied. Frequency of main genotypes of the GD patients studied are represented, namely N370S/N370S, N370S/L444P, N370/any mutation other than N370S or L444P (N370S/other) or any genotype without N370S and L444P mutations (others).

1.4.2. Increased expression of CD1d and MHC-class II in Gaucher disease monocytes

Flow cytometry determination of cell surface expression of CD1d and MHC-class II on freshly isolated peripheral blood CD14⁺ monocytes showed higher levels of expression of CD1d and MHC-class II molecules in GD patients, both untreated and undergoing ERT, when compared to the control group (Figure 6). Indeed, the average MFI values of CD1d and MHC-class II expression were significantly increased both in untreated GD patients and in GD patients undergoing ERT (Figure 6B). Although some GD patients receiving ERT were splenectomized, spleen removal did not influence expression of either molecule (Figure 7). Analysis of cell surface expression of other endolysosomal trafficking molecules on GluCerase-deficient monocytes, such as MHC-class I and CD63, showed no significant differences (Figure 6A, bottom panel). Furthermore, analysis of CD1d and MHC-class II mRNA levels showed no significant differences between GD patients and controls (Figure 6C).

1.4.3. Upregulation of CD1d in monocytes from healthy subjects after treatment with CBE

The results obtained with GluCerase-deficient monocytes suggested a link between GluCer accumulation and the upregulated expression of CD1d and MHC-class II. In order to investigate this relationship further, GluCerase activity in monocytes from healthy subjects was inhibited by treatment of PBMC samples with 1mmol/L of CBE, an irreversible inhibitor of GluCerase activity that leads to a several-fold increase in GluCer levels³⁰⁹. Short-term treatment (24-48 hours) of PBMC samples with CBE induced only slight increases or no change in CD1d expression. However, longer treatment periods (up to 96-hour) induced a specific upregulation of CD1d in four out of five samples studied, as determined by an increase in the MFI values (Figure 8A). Importantly, CD1d upregulation was paralleled by an almost complete inhibition of GluCerase activity (97% inhibition, 84.71 nmol/h/mg in untreated PBMC vs 2.47 nmol/h/mg in PBMC treated with CBE). In contrast, CBE treatment did not augment the expression of other molecules studied, including MHC-class II (Figure 8B). Thus, inhibition of GluCerase activity with the subsequent lipid accumulation resulted in CD1d upregulation *in vitro*.



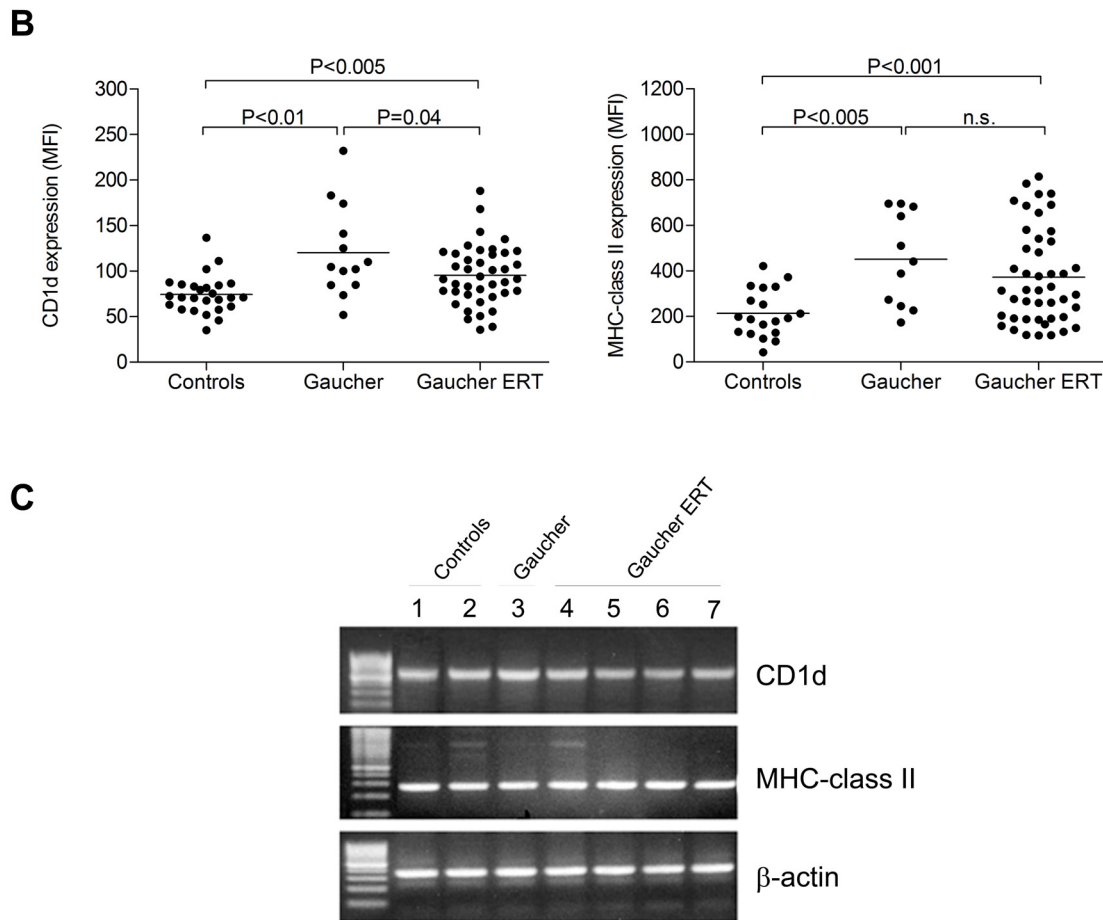


Figure 6. GluCerase-deficient monocytes show upregulation of CD1d and MHC-class II. (A) Cell surface expression of CD1d, MHC-class II, MHC-class I and CD63 was determined by flow cytometry on total PBL. Histograms show results of CD1d, MHC-class II, MHC-class I and CD63 expression on gated CD14⁺ monocytes from healthy control (thin line) and an untreated GD patient (thick line). Background staining with mouse Igs is included (dotted line). (B) Figures show individual MFI for each group under study with each point representing a different individual (Gaucher, untreated patients with GD; Gaucher ERT, GD patients receiving ERT). Statistical significant differences (*t*-test) are indicated. Straight lines represent mean values. n.s., not significant (C) RT-PCR of total RNA isolated from PBMC preparations of healthy controls (lanes 1 and 2), untreated GD patients (lane 3) and GD patients under ERT (lanes 4-7). Primers specific for CD1d, HLA-DR and β -actin were used.

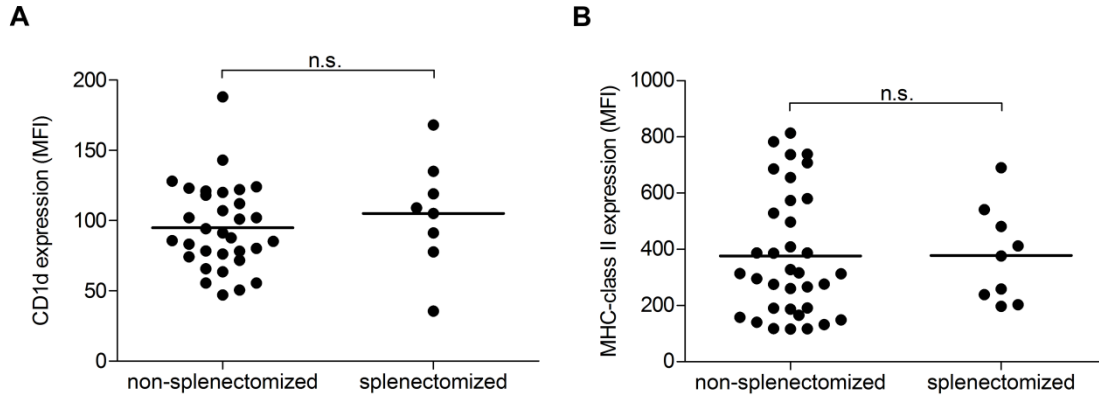


Figure 7. CD1d and MHC-class II cell surface expression is not altered in splenectomized patients. Expression of CD1d (A) and MHC-class II (B) in CD14⁺ monocytes was determined by flow cytometry. Gaucher ERT patients were divided in two groups: non-splenectomized and splenectomized. Figures show individual MFI for each group under study with each point representing a different individual. Statistical significant differences (*t*-test) are indicated. Straight lines represent mean values. n.s., not significant.

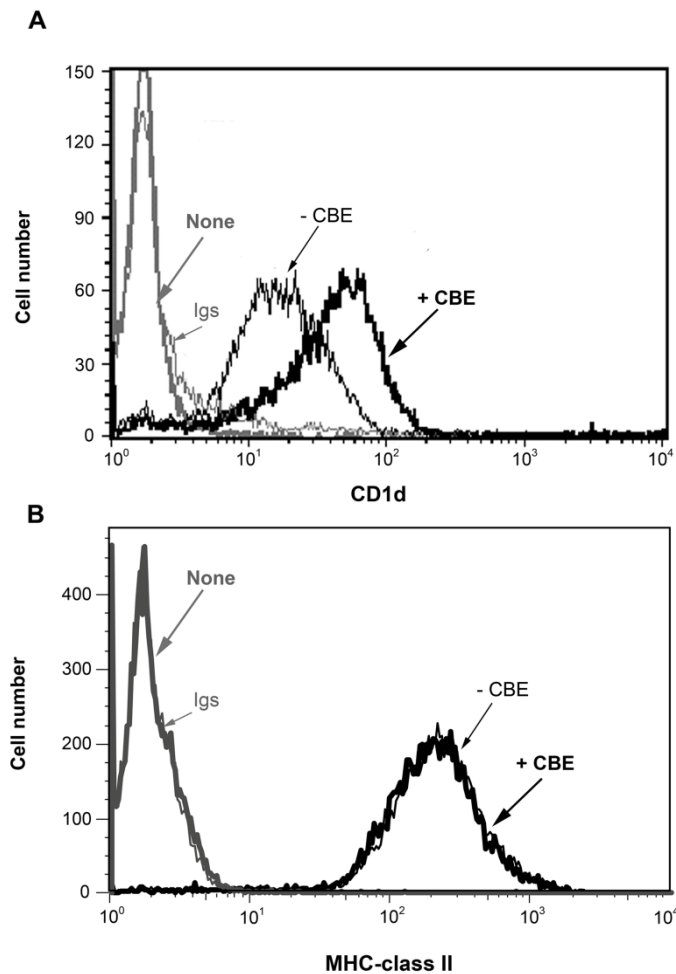


Figure 8. CBE treatment of healthy monocytes induces CD1d upregulation. Mononuclear cells from a healthy control were treated with 1 mM of CBE for 96 hours. Histogram shows (A) CD1d and (B) MHC-class II expression in control untreated (-CBE, thin line) and treated (+CBE, thick line) CD14⁺ monocytes. Background staining without primary antibodies (None) or mouse IgG are also shown.

1.4.4. Expression of V α 24 by peripheral blood CD4⁺ and CD8⁺ T cells

CD1d and MHC-class II play key roles in the homeostasis of V α 24-carrying NKT cells and CD4⁺ T cells, respectively, and anomalies in the expression of these molecules are known to have an impact on the numbers of these T cell subsets. Thus, we analyzed the percentage of CD4⁺ and CD8⁺ T cells in peripheral blood as well as the expression of V α 24. As shown in Figure 9, a large fraction of GD patients under ERT showed low percentages of CD4⁺ T cells when compared to the control group (Figure 9A). The mean percentage of CD4⁺ (40.8 $\pm$ 9.1 vs 50.1 $\pm$ 7.5 in controls, mean $\pm$ SD, P <0.0001) and CD8⁺ (33.2 $\pm$ 9.3 vs 27.5 $\pm$ 8.4 in controls, mean $\pm$ SD, P =0.02) T cells were significantly altered. Interestingly, statistically significant differences in the percentage of CD4⁺ and CD8⁺ T cells were observed between the two groups of patients (Figure 9A and B). Moreover, analysis of V α 24 percentage showed that this conserved TCR variable chain was over represented within the CD4⁺ T cell pool of GD patients under ERT when compared to controls (0.83 $\pm$ 0.41 vs 0.62 $\pm$ 0.23 in controls, mean $\pm$ SD, P <0.05, Figure 9C). Of note, some expansions of V α 24⁺ cells were observed in CD8⁺ T cell subset (Figure 9D).

So GD patients presented imbalances in conventional T cells, namely low numbers of CD4⁺ T cells and higher numbers of CD8⁺ T cells, and also increased numbers of iNKT cells within CD4 subset.

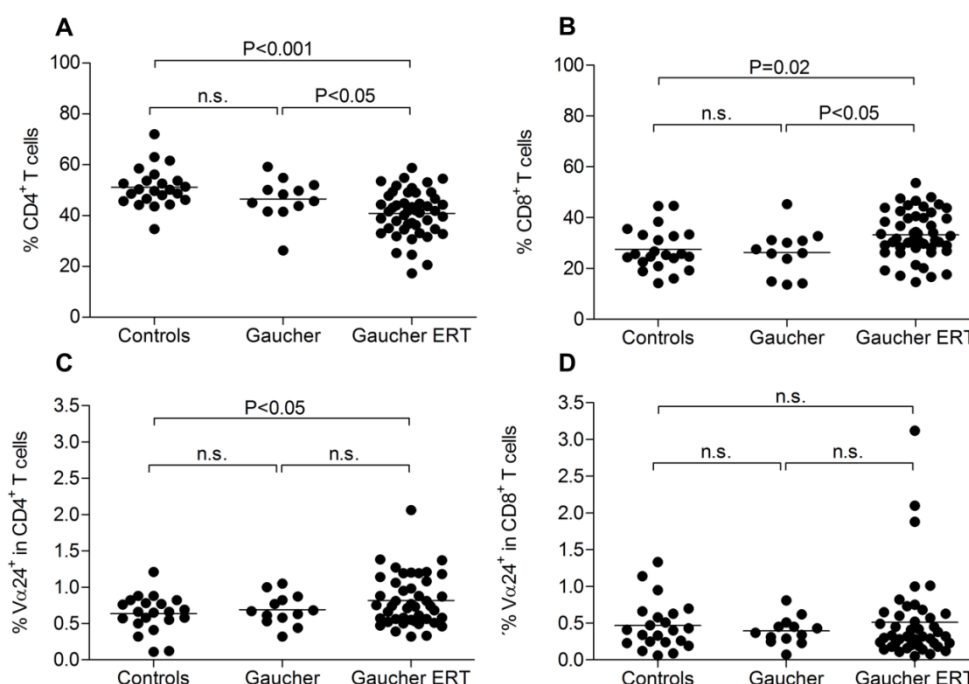


Figure 9. Anomalies in peripheral blood T cell subsets in GD patients. Percentages of CD4⁺ T cells, CD8⁺ T cells and V α 24⁺ cells were determined by flow cytometry. The percentage of (A) CD4⁺ T cells, (B) CD8⁺ T

cells, (C) V α 24⁺ cells in CD4⁺ T cells and (D) V α 24⁺ cells in CD8⁺ T cells in each group under study is indicated. Each point represents a different individual. Statistical significant differences between groups (*t*-test) are indicated. Straight lines represent mean values. n.s., not significant.

1.4.5. ERT induces a decrease in MHC-class II expression

Since ERT is known to restore normal values of GluCer, we wanted to ascertain whether the therapy influenced the level of expression of CD1d and/or MHC-class II molecules on monocytes. For that purpose, several GD patients were followed during their treatment. Characterization of surface expression of CD1d and MHC-class II on monocytes showed that ERT induced a time-dependent down-modulation in MHC-class II, but not CD1d, in the majority of the patients studied (Figure 10), despite the fact that a decrease in CD1d expression was observed in Gaucher patients under ERT when compared with untreated patients (Figure 10B). Importantly, down-modulation of MHC-class II was accompanied by an increase in the percentage of CD4⁺ T cells in most patients (Figure 10). Notably, the increase in the percentage of CD4⁺ T cells was more evident in GD patients with low numbers of CD4⁺ T cells at the start of the therapy (see Figure 10A). Comparison of the average MFI values of MHC-class II between monocytes from GD patients undergoing ERT for less than 50 months and for more than 50 months showed statistically significant differences between the two groups (423.7 ± 232.9 in < than 50 months vs 289.2 ± 131.0 in > than 50 months, mean $\pm$ SD, $P < 0.05$) (Figure 11).

1.5. Discussion

Characterization of the molecular mechanisms that regulate CD1d and MHC-class II expression on the surface of APC is important in order to provide insights on their functional role. Expression of MHC-class II and CD1d are regulated by external factors, such as cytokines, and share intracellular trafficking pathways and molecular chaperones, such as the invariant chain^{173,181,310,311}. Still, little is known about other pathways of regulation. The present study demonstrated that sphingolipid accumulation represents a novel regulatory component for CD1d and MHC-class II expression in monocytes.

A number of previous studies in GD have identified imbalances in peripheral blood lymphocytes, including anomalies of B cells, T cells and NK cells^{119,120,132,135-137,303}.

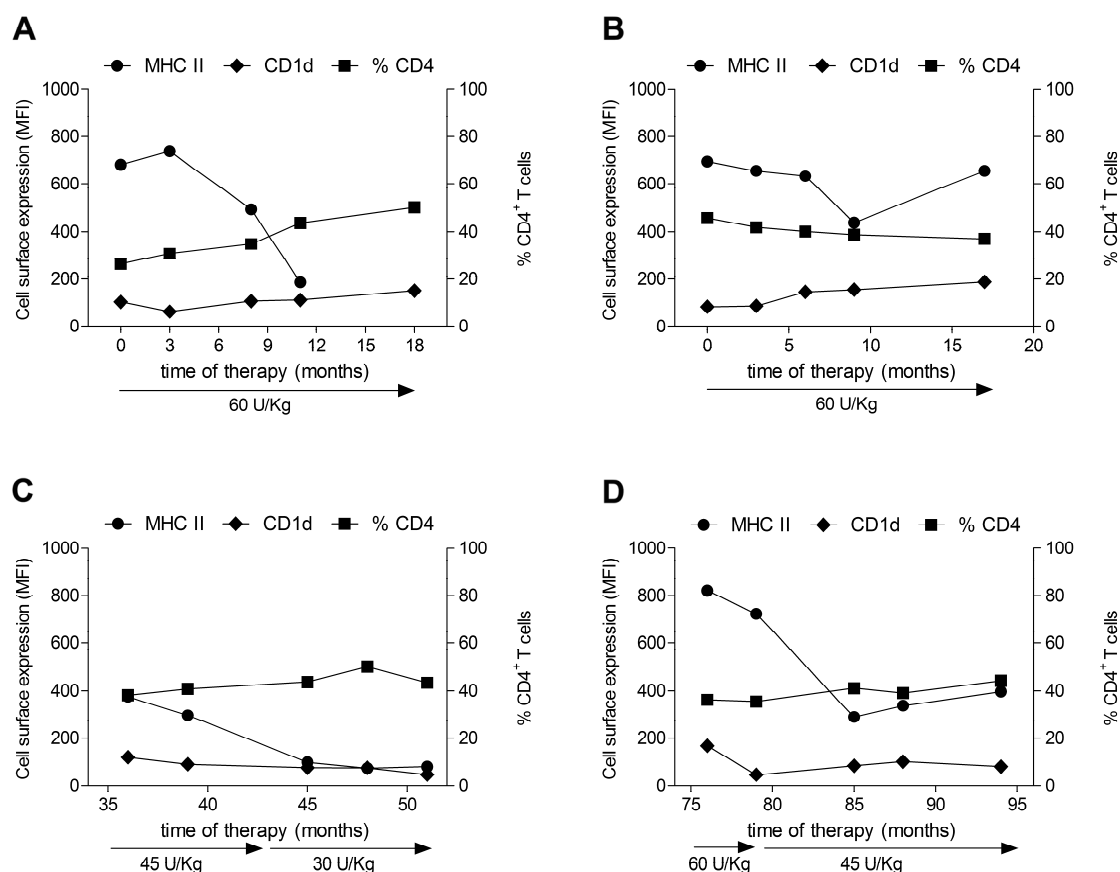


Figure 10. ERT alters MHC-class II expression and the percentage of CD4⁺ T cells. Expression of CD1d and MHC-class II in CD14⁺ monocytes and the percentage of CD4⁺ T cells were determined by flow cytometry. Each graph represents a different GD patient and the results show the levels of CD1d and MHC-class II (left vertical axis) in combination with the percentage of CD4⁺ T cells (right vertical axis) along the time of therapy (horizontal axis). The dose of imiglucerase administrated every two weeks and the duration are also indicated. (A, B) GD patients studied since the start of the therapy. (C, D) GD patients with 36 and 76 months of therapy at the first time of study, respectively. Each point represents a single determination.

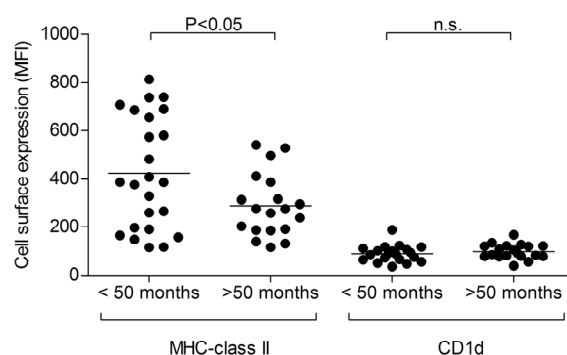


Figure 11. MHC-class II cell surface expression decreases with ERT. Expression of MHC-class II and CD1d in CD14⁺ monocytes were determined by flow cytometry. Patients were divided in two groups: patients with less than 50 months of ERT (< 50 months) and with more than 50 months of ERT (> 50 months). Figure

show individual MFI values for each group under study with each point representing a different individual. Statistical significant differences (*t*-test) are indicated. Straight lines represent mean values. n.s., not significant.

Given the critical role played by molecules of the MHC family in maintaining homeostasis of T cell populations, the level of expression of CD1d and MHC-class II molecules in peripheral blood monocytes were examined in controls and in two groups of GD patients, one untreated and one under ERT. These studies showed that cell surface expression of CD1d and MHC-class II by CD14⁺ monocytes was increased in both groups of patients when compared with the controls (Figures 2, 3 and 4). The anomalies appear to be specific since no significant alterations were found in the expression of other endolysosomal trafficking molecules, such as CD63 and MHC class I (Figure 6). Moreover, the demonstration that monocytes from healthy controls rendered deficient in GluCerase activity by treatment with CBE, express increased levels of CD1d at the cell surface (Figure 8) strongly suggest a connection between expression of these endolysosomal trafficking molecules and GluCer accumulation.

To our knowledge, this is the first study showing anomalies in the expression of CD1d and MHC-class II in a storage disease characterized by accumulation of sphingolipids. The exact molecular mechanism(s) of CD1d and MHC-class II upregulation in GD monocytes is not known but two possible scenarios can be envisaged. In the first, upregulation of CD1d and MHC-class II may result from an increased transcription of the encoding genes induced by factors present in the patients. Such factors may include the cytokines IL-1, IL-6, IL-10 and transforming growth factor- β ^{127,129,312}. However, these cytokines have not been reported to influence levels of CD1d expression and are known negative regulators of MHC-class II expression³¹¹. To our knowledge, IFN- γ is the only cytokine described to increase expression of CD1d and MHC-class II molecules but it is not altered in GD patients^{127,193,311}. The possibility that other inducing factors, such as the chemokine CCL18/PARC¹³⁰ may be associated with the upregulation of CD1d and MHC-class II remains to be ascertained. Nevertheless, the absence of anomalies in the levels of mRNA for CD1d and MHC-class II (Figure 6C) rules out increased transcription as a likely mechanism. In the second scenario, upregulation of CD1d and MHC-class II molecules could result either from an accelerated transport from endolysosomal compartments to the plasma membrane, from downregulation of endocytosis, or a combination of both. If we consider that CD1d and MHC-class II molecules have internalization motifs involved in the trafficking between the plasma membrane and endolysosomal compartments^{173,181,187}, and that egress of CD1d and MHC-class II molecules from endolysosomal compartments to the cell surface is influenced by a number of shared chaperones and also by lipid

transfers proteins such as saposins, GM2 activator protein and triglyceride transfer protein^{177,187,313}, any biochemical alteration in the endolysosomal compartment may influence intracellular trafficking and, ultimately, expression at cell surface. In this context, it is worth mentioning that GluCer accumulation is known to modulate traffic between endolysosomal compartments³¹⁴ and that increased levels of Sap C and cathepsin D have been observed in lysosomes from GD patients³¹⁵. Thus, it could be hypothesized that GluCer accumulation in lysosomes in GD patients could interfere in a specific manner with the normal trafficking pathways used by CD1d and MHC-class II molecules and, as a result, increase the number of molecules present at the cell surface. The lack of anomalies on cell surface expression of CD63 and MHC-class I, which follow similar intracellular trafficking pathways^{183,316}, supports this assumption.

Importantly, follow-up studies demonstrated that ERT induced a time-dependent decrease in the levels of MHC-class II molecules, but not in CD1d, at the cell surface of CD14⁺ monocytes in most of the patients studied (Figure 6). Moreover, some of these patients showed a simultaneous recovery in CD4⁺ T cells. The differences in down-modulation of MHC-class II and CD1d observed in the follow-up studies could result from the slightly different circuits followed by these two molecules. Although both molecules traffic through endolysosomal compartments designated as MIIC, nascent CD1d molecules move first to the cell surface and then recycle between the plasma membrane and MIIC while newly synthesized MHC-class II molecules are routed directly to the MIIC¹⁸⁷. Moreover, the fact that the half-life of CD1d molecules is about fivefold higher than the half-life of MHC-class II¹⁸¹ may have also contributed for these differences. Alternatively, it could be argued that reversal of the inflammatory status of the GD patients by ERT may impact primarily at the level of MHC-class II recycling.

In vitro studies aimed at reproducing the metabolic defect of GD monocytes by using CBE showed upregulation of CD1d (Figure 8). Thus, GluCerase-deficient monocytes from healthy controls generated *in vitro* by treatment with CBE reproduced the CD1d anomalies observed in GluCerase-deficient monocytes from GD patients. Interestingly, a recent report has shown that CBE-treatment altered PC metabolism in monocyte-derived macrophages but not in lymphocytes, most likely due to the preferential targeting of monocytes by GluCer accumulation induced by CBE treatment³¹⁷⁻³¹⁹. Thus, upregulation of CD1d, but not MHC-class II, molecules in this *in vitro* model may result from kinetic differences or to the fact that GluCer accumulation without inflammation, as it occurs *in vivo*, impacts preferentially CD1d expression. The possibility that GluCer accumulated in the lysosomal compartment binds to CD1d or to third-party molecules remains to be elucidated.

The combined *in vivo* and *in vitro* data suggest that GluCerase deficiency may preferentially influence the expression of CD1d at the cell surface of GD monocytes, most probably because of sphingolipid accumulation, while upregulation of MHC-class II could be more related to the inflammatory status of the GD patients. Preliminary studies indicate that the initial value of MHC-class II expression by monocytes from GD patients appears to be positively correlated with the levels of chitotriosidase activity, a marker of activated macrophages and ongoing inflammation (see Research Work 3). Upregulation of CD1d and MHC-class II molecules by monocytes may have clinical implications, such as impacting on the repertoire of regulatory subsets of CD4⁺ and NKT cells in the periphery. CD1d-expressing monocytes present self-sphingolipids and are potent stimulators of NKT cells^{110,320}. Accordingly, differences in the response to therapy seen in areas of resistance, such as the lung and bone, could be because of differential upregulation of CD1d (and/or MHC-class II) by monocytes or macrophages in the target organs, which could activate regulatory T cells (i.e. CD4⁺CD25⁺ or NKT). Activation of regulatory T cells may, in turn, contribute for the modulation of the clinical symptoms, namely inflammatory phenomena. In this scenario, it is surprising that most of the GD patients under ERT presented unusually low percentages of CD4⁺ T cells, akin with an expansion of the CD4⁺Vα24⁺ NKT cell pool. On the other hand, it could be argued that upregulated MHC-class II expression by monocytes may deliver strong activation signals to CD4⁺ T cells and trigger apoptotic signals, thus interfering in the balance between CD4⁺ and CD8⁺ T cells. The partial correction of CD4⁺ T cells during ERT, similar to the MHC-class II down-modulation provides indirect support to this hypothesis. Although the percentage of CD8⁺ T cells was increased, the CD8⁺Vα24⁺ NKT cell pool was within control values. Importantly, the CD4⁺ and CD8⁺ T cell imbalances were not observed in the untreated GD patient group. The small number of subjects, as well as the fact that this group included patients with milder clinical forms of GD (the more severely affected had already started therapy), may have contributed to the lack of differences.

At present, it is not known whether CD1d and MHC-class II upregulation in GD monocytes may represent a pathophysiologic process induced by sphingolipid overloading and/or inflammation. Early studies demonstrated that lymphocytes and monocytes in GD showed evidence of lipid accumulation¹³⁵⁻¹³⁷. Given the constitutive presence of sphingolipids in the plasma membrane and in the membranes of endolysosomal compartments³²¹, it might be hypothesized that sphingolipid-mediated regulation of CD1d is a physiologic process. In the context of lipid-laden monocytes, and considering that monocytes are the precursors of tissue macrophages, it is likely that upregulated expression of CD1d represents the "physiologic response" associated with a number of tissue sites populated

by lipid-laden Gaucher cells or macrophages ³³. Some evidence exists that similar pathways are associated with pathological processes. Thus, it was recently shown that accumulation of lipids by macrophages in atherosclerotic lesions coincides with upregulated expression of CD1d and abundant surrounding T cells ¹⁹⁸. In addition, we have recently reported that accumulation of lipids by an epithelial cell line results in upregulated expression of Nor3.2-reactive CD1d ³²². In agreement with this, GluCerase-deficient monocytes from GD patients also present upregulation of Nor3.2-reactive CD1d molecules.

In summary, these studies suggest that anomalies in sphingolipid metabolism may interfere with the trafficking pathways of CD1d and MHC-class II, and therefore with their immunoregulatory properties.

2. Anomalies in T and iNKT populations in Fabry mice but not in Fabry patients

Adapted from Balreira A, Macedo MF, Girão C, Rodrigues LG, Oliveira JP, Sá Miranda MC and Arosa FA. *Br J Haematol.* 2008; 143(4): 601-604

2.1. Abstract

GD patients, who accumulate GluCer, displayed anomalies in T and iNKT populations and in monocytes. Hereby, we show that Fabry knockout mice, which accumulates Gb3, also displayed anomalies in T and iNKT populations, namely in CD4⁺ T cells, CD8⁺ T cells and V α 14⁺CD4⁺ T cells akin to a marked downregulation of CD3 cell surface expression. In marked contrast, FD patients only showed anomalies in the expression of MHC class II molecules by circulating monocytes. These results suggest that the impact of sphingolipid accumulation on the immunological system may depend on the nature and tissue distribution of the accumulated sphingolipid, and the organism where it accumulates.

2.2. Introduction

FD, an X-linked sphingolipidosis, results from the defective activity of α -Gal A, leading to lysosomal accumulation of Gb3 and digalactosylceramide primarily in vascular endothelium, smooth-muscle cells, and epithelial/perithelial cells of most organs ⁵³. Whether Gb3 and digalactosylceramide accumulation impacts the immunological system remains uncertain. A mouse model of FD was originally developed in 1997 through disruption of the mouse α -Gal A gene. A mouse model of FD showed accumulation of Gb3 in several organs, namely in kidneys and liver ⁶¹ and has been used recently to reveal a reduction in CD1d-restricted iNKT cells in liver, spleen and thymus ¹⁶⁶. Elucidation of whether sphingolipid accumulation in lysosomes impact onto the immunological system may help to understand how the immunological system deals with lipids, which may give clues to develop novel immunotherapies against a number of lysosomal storage disorders. Thus, endogenous glycosphingolipids are thought to bind to CD1 members, namely CD1d, and be presented to a subset of natural killer T cells carrying a conserved TCRV α chain, V α 24⁺ in humans and V α 14⁺ in mice ³²³. These iNKT cells are unusual in the sense that they rapidly secrete a large amount of regulatory cytokines upon activation ³²⁴. Indeed, we have previously show that accumulation of glucosylceramide into monocytes/macrophages of GD patients results in overt anomalies of the immunological system, namely in T and iNKT lymphocyte populations akin to upregulated expression of CD1d and MHC class II molecules on monocytes (see Figure 6, 9 and ¹³²). In this study, we performed a comprehensive analysis of T and iNKT cell populations in Fabry mice and FD patients, and we report that contrary to FD mice, FD patients do not present significant lymphocyte anomalies.

2.3. Material and methods

2.3.1. Patients and patient's biological material

FD patients were previously diagnosed biochemically and genotyped using described methods³²⁵. All but one FD patients were undergoing ERT with recombinant α -gal A (Fabrazyme®). Fourteen FD patients and 13 healthy controls were studied. Blood samples were obtained with consent. PBL were isolated from whole blood. RBC were lysed in lysis solution (10 mM Tris, 150mM NH₄Cl, pH 7.4) for 10 min at 37°C.

2.3.2. Mice and mice biological material

FD mouse model, α -Gal A⁻⁰, and C57BL/6J background mice were maintained and genotyped according to published methods⁶¹. Male mice were sacrificed and studied at 12 and 24 weeks. Liver mononuclear cells were isolated according to a previously described protocol³²⁶. Briefly, after liver perfusion with DMEM medium supplemented with 10% heat inactivated foetal bovine serum (iFBS) (Gibco, Invitrogen, Prat de Llobregat, Spain), livers were cut in small pieces, passed first through a 50 mesh stainless steel sieve and then by a 200 mesh stainless steel sieve, washed with DMEM 10% iFBS medium, layered over Percoll 35% gradient and centrifuged at 1200 rpm, 10 minutes at 4 °C. Remaining RBC were lysed using Gey's solution (10 mM KHCO₃, 155 mM NH₄Cl, pH 7.2) for 10 minutes at 4 °C. Cells were washed twice in DMEM 10% iFBS medium. To isolate splenic mononuclear cells, mice spleens were removed, placed on a Petri dish with RPMI medium and cut in several pieces. Isolated cells were placed in solution by drowning-up and expelling the suspension through a 6 ml syringe equipped with a 20.5G needle, several times. Cells were washed twice and contaminating RBC lysed with Gey's solution as above. Cells were washed twice with complete medium.

2.3.3. Cell staining, cell surface expression and lymphocyte determination

Human and murine cells were washed twice in PBS supplemented with 0.1% NaN₃ and 0.2% BSA (PBS-BSA-NaN₃, staining solution). Cell surface staining was performed on ice for 30 min in the dark in staining solution in 96 well round-bottom microtiter plates (Greiner-Nurtingen, Germany) with $\sim 0.5 \times 10^6$ cells/well. After the staining, cells were washed twice with staining solution and immediately acquired in a FACScalibur (Becton

Dickinson, Mountain View, CA). Mononuclear cells (lymphocytes and monocytes) were identified and gated according to FSC/SSC characteristics. For each sample, 50000 events within the mononuclear gate were acquired and analyzed using the FlowJo software (TreeStar, Inc, Ashland, USA). For humans studies the following fluorochrome-conjugated monoclonal antibodies were used: anti-CD4 (clone MEM-241), anti-CD8 (clone 3B5), anti-CD3 (clone MEM-57), anti HLA-A,B,C (clone W6/32) all from Immunotools (Friesoythe, Germany); anti HLA-DR,DP,DQ (clone CR3/43) from DakoCytomation (Glostrup, Denmark); anti-V α 24 (clone C15) from Beckman Coulter (Fullerton, CA, USA); anti-CD1d (clone 42) from BD Biosciences (Erembodegem, Belgium). Mouse immunoglobulins were used as negative control. Expression of the different molecules at the cell surface was determined by MFI values. The percentage of CD8⁺ T cells and CD8⁺V α 24⁺ T cells were determined after setting quadrants on CD8^{bright} cells, and they are referred to as CD8⁺ T cells. Staining with CD3 confirmed that CD8^{bright} cells were T cells.

For mice studies, the following fluorochrome-conjugated antibodies were used: anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD3 (clone 145-2C11) all from BD Biosciences (Erembodegem, Belgium). V α 14⁺ T cells were identified using CD1d tetramers complexed with PBS57, an α -GC analog kindly provided by the NIH tetramer core facility. Expression of the different molecules at the cell surface was determined by MFI values.

2.3.4. Statistical analysis

Statistical evaluation was performed using paired Student's *t*-test at the level of significance of 0.05.

2.4. Results

2.4.1. Imbalances in Fabry mice conventional T cell populations

Hepatic and splenic T and iNKT cells were analyzed in Fabry mouse and C57BL/6J mouse males by flow cytometry. In Fabry mice, the percentage of liver CD3⁺CD4⁺ T cells was significantly decreased (55.8 $\pm$ 1.9 vs 64.6 $\pm$ 5.0 in controls, mean $\pm$ SD, P=0.02 in 12 weeks mice and 58.8 $\pm$ 1.4 vs 65.2 $\pm$ 2.9 in controls, mean $\pm$ SD, P=0.01 in 24 weeks mice) while the percentage of CD3⁺CD8⁺ T cells was increased (24.0 $\pm$ 1.3 vs 16.6 $\pm$ 5.3 in controls, mean $\pm$ SD, P=0.05 in 12 weeks mice and 19.5 $\pm$ 1.4 vs 14.4 $\pm$ 1.1 in controls,

mean $\pm$ SD, $P=0.02$ in 24 weeks mice). These differences were observed both at 12 and 24 weeks of age (Figure 12, upper panel), leading to a two-fold decrease in the CD4⁺/CD8⁺ T cell ratios between Fabry and control mice in the liver. Less obvious but still significant, imbalances in the percentages of CD3⁺CD4⁺ (46.7 $\pm$ 2.3 vs 53.5 $\pm$ 4.0 in controls, mean $\pm$ SD, $P=0.01$ in 12 weeks mice and 43.9 $\pm$ 2.8 vs 52.8 $\pm$ 0.6 in controls, mean $\pm$ SD, $P=0.01$ in 24 weeks mice) and CD3⁺CD8⁺ T cells (44.9 $\pm$ 1.1 vs 38.8 $\pm$ 0.5 in controls, mean $\pm$ SD, $P<0.0001$ in 12 weeks mice and 47.3 $\pm$ 1.5 vs 37.9 $\pm$ 2.8 in controls, mean $\pm$ SD, $P=0.0002$ in 24 weeks mice) were observed in spleen of Fabry mice (Figure 12, lower panel).

2.4.2. Imbalances in Fabry mice hepatic iNKT cells

Analysis of V α 14⁺ T cells in spleen and liver by using PBS57-loaded CD1d tetramers revealed low numbers of CD3⁺V α 14⁺ T cells in the liver of Fabry mice when compared to control mice due mostly to low numbers of CD4⁺V α 14⁺ T cells (54.24 $\pm$ 5.1 vs 68.7 $\pm$ 4.5 in controls, mean $\pm$ SD, $P=0.01$ in 12 weeks mice and 53.5 $\pm$ 9.2 vs 65.5 $\pm$ 6.8 in controls, mean $\pm$ SD, $P=0.04$ in 24 weeks mice) (Figure 13A). The anomalies in liver V α 14⁺ T cells were maintained after correction to the percentage of CD4⁺ T cells and were not observed in the spleen (Figure 13B).

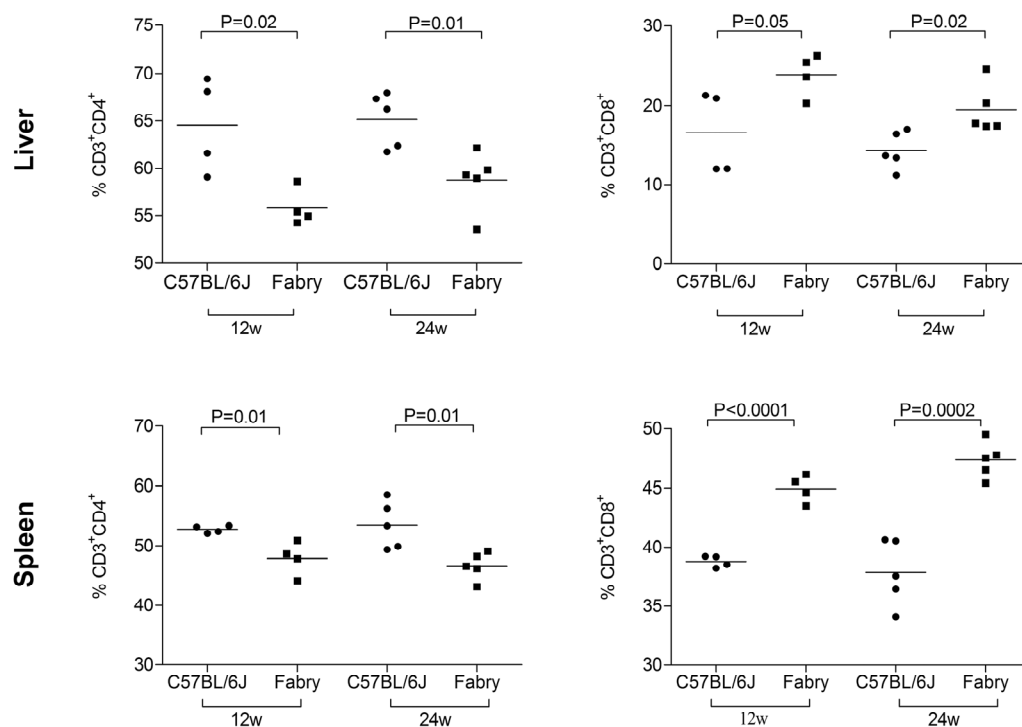


Figure 12. Decreased percentages of CD4⁺ T cells and increased percentages of CD8⁺ T cells in hepatic and splenic T cells in Fabry mice. Percentages of CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells were

determined by flow cytometry within lymphocytes gate. Figures show the percentages of total $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in liver (upper panel) and spleen (lower panel) in each group of mice studied. Each point represents a different individual. Statistical significant differences between groups (t-test) are indicated. Straight lines represent mean values. n.s., not significant.

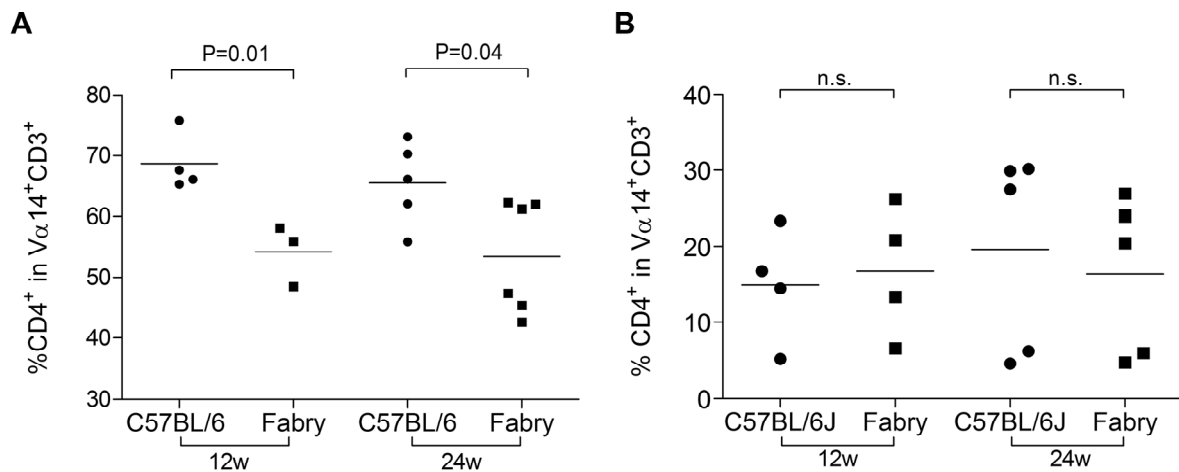


Figure 13. iNKT cells in liver and spleen of Fabry mice. Percentages of $V\alpha 14^+$ cells were determined by flow cytometry. Figure show the percentages of liver $CD4^+$ T cells within $V\alpha 14^+CD3^+$ cells for each group of mice studied in liver (A) and spleen (B). Each point represents a different individual. Statistical significant differences between groups (t-test) are indicated. Straight lines represent mean values. w, weeks, n.s., not significant.

2.4.3. Expression of CD3 molecule by liver T cells

Analysis of the different $CD3^+$ T cell subsets ($CD3^{int}$ and $CD3^{high}$ T cells) in the liver revealed a marked decrease in $CD4^+CD3^{int}$ T cells in 12 weeks old Fabry mice when compared to control mice (Figure 14), that resulted in a two-fold decrease in the $CD3^{int}/CD3^{high}$ T cell ratio (Figure 15). The differences in the $CD3^{int}/CD3^{high}$ T cell ratio between 24 weeks old Fabry mice were not statistically significant.

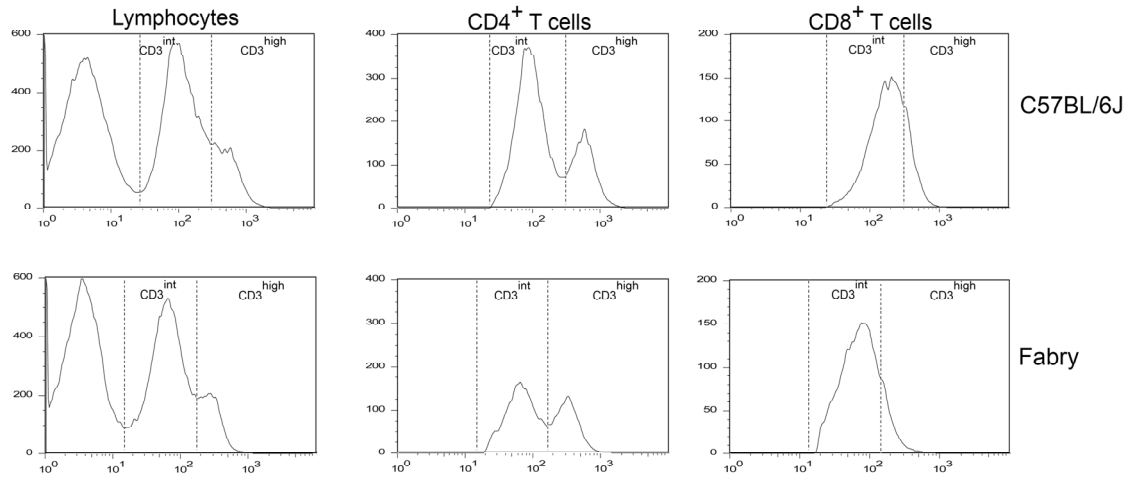


Figure 14. Anomalies in hepatic CD3^{int} T cells from Fabry mice. Histograms show MFI values of CD3 molecules in liver T cells from a representative 12 weeks old C57BL/6J and Fabry mice. CD3 cell surface expression was analyzed in total liver lymphocytes, and then in CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells. Dotted lines define CD3^{int} and CD3^{high} populations. A decrease in the percentage of CD3^{int} can be clearly observed within CD4⁺ T cells in Fabry mice.

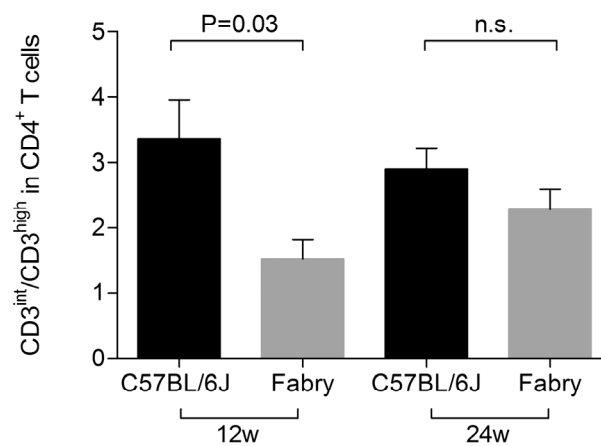


Figure 15. Reduction of liver CD3^{int}/CD3^{high} T cells ratio in Fabry mice. Results show the ratio (mean±SD) of liver CD3^{int}/CD3^{high} T cells for each group of mice studied. Statistical significant differences between groups (*t*-test) are indicated. n.s., not significant; w, weeks.

2.4.4. Peripheral blood T cells in Fabry Disease patients

A similar analysis to the one done in Fabry mice model was performed in peripheral blood samples of FD patients. Expression of $V\alpha 24$ TCR was analysed in T cells population, namely in $CD3^+$, $CD4^+$ and $CD8^+$ T cells. FD patients showed normal levels of all $V\alpha 24^+$ T populations analysed (Figure 16). No differences were observed between FD patients and healthy controls in any of T cell subset analysed (Figure 16).

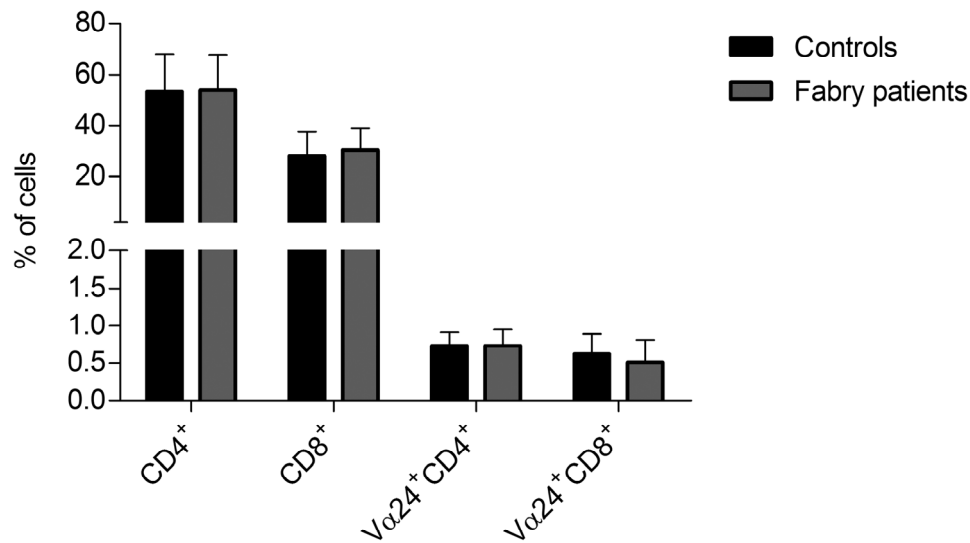


Figure 16. No anomalies in peripheral blood T and iNKT cell in FD patients. Percentages of $CD4^+$ T cells, $CD8^+$ T cells and $V\alpha 24^+$ cells were determined by flow cytometry. Results show the percentage of peripheral blood T cell subsets in healthy controls and FD patients. Values represent mean $\pm$ SD for each group studied.

2.4.5. Upregulation of MHC-class II in Fabry Disease monocytes

Analysis of surface expression of MHC family molecules in monocytes from FD patients showed that MHC class II expression was increased when compared with monocytes from healthy controls (259.9 ± 75.1 vs. 147.8 ± 60.6 , MFI $\pm$ SD, $P=0.007$) (Figure 17), as we had described for GD patients in chapter 1 (Figure 6, chapter 1). However, in contrast to GD patients, CD1d MFI in monocytes from FD patients was within normal values (Figure 17).

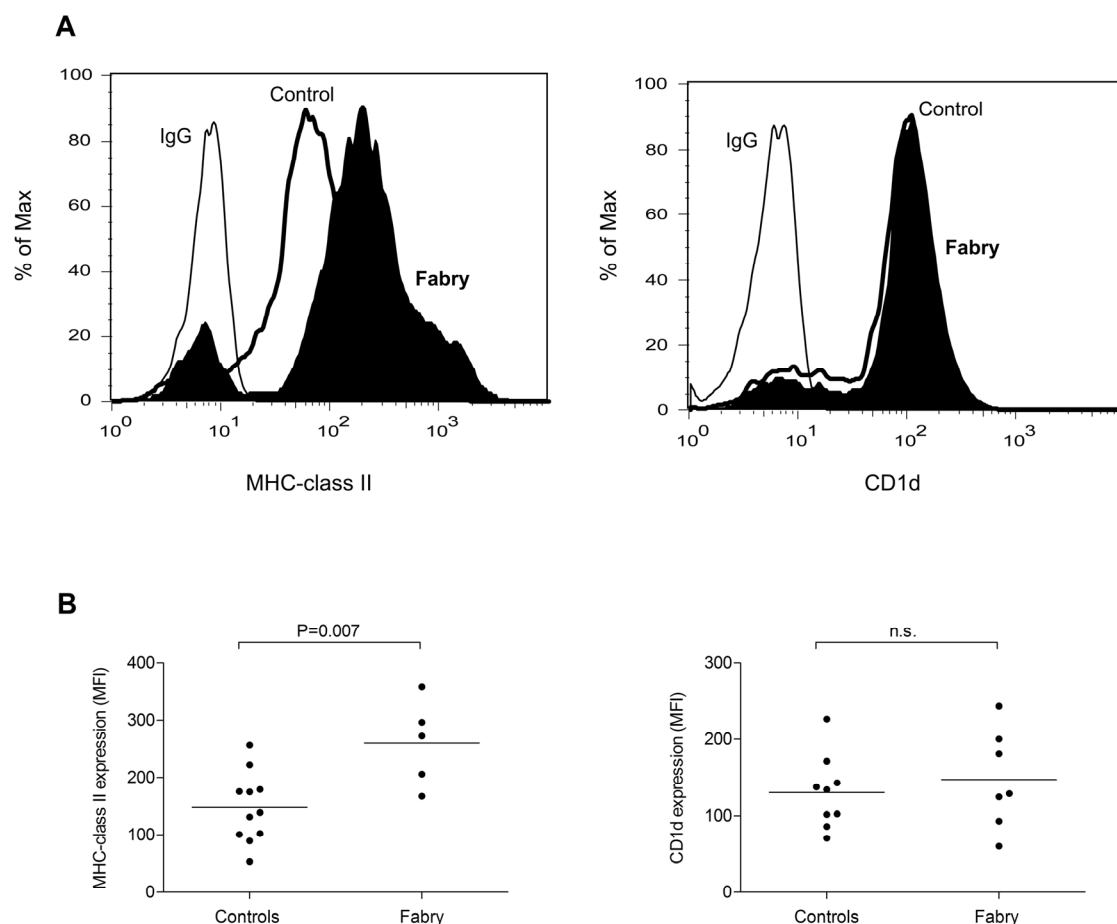


Figure 17. Upregulation of MHC-class II expression in α -Gal A deficient monocytes. Freshly collected PBMC were labeled for MHC-class II and CD1d, and cell surface expression was analyzed by flow cytometry on the monocytes gate. (A) Histograms show MFI values for each individual molecule from a representative control (thick line) and an FD patient (shaded area). Background staining with mouse immunoglobulins is included (thin line). (B) Figures show individual MFI for Fabry patients and healthy controls, with each point representing a different individual. Statistically significant differences (t -test) are indicated. Straight lines represent mean values. n.s., not significant.

2.5. Discussion

We performed a comprehensive analysis of T and iNKT cell populations in Fabry mice and FD patients.

Fabry mice presented a significant decrease in liver and spleen $CD3^+CD4^+$ T cells, while $CD3^+CD8^+$ T cells were increased, at the two time points analyzed (12 and 24 weeks) (Figure 12). Analysis of iNKT cells in liver and spleen of Fabry mice, showed that $V\alpha 14^+$ T cells were decreased in liver (Figure 13) and analysis of the percentages of $CD3^{int}$ and $CD3^{high}$ T cells in this organ revealed a marked decrease in $CD4^+CD3^{int}$ T cells in Fabry mice when compared to control mice (Figure 3), that resulted in a two-fold decrease in the $CD3^{int}/CD3^{high}$ T cell ratio (Figure 15). Overall, we have shown that Fabry mice display

overt anomalies in liver T and iNKT populations, which is in agreement with previous studies¹⁶⁶. However, in our study we did not find significant differences in splenic iNKT cells and the reduction in liver iNKT cells was not as dramatic as the described by Gadola et al¹⁶⁶. These differences may probably arise from the use of different CD1d tetramers (PBS57 vs α -GalCer) and/or the age of the mice under study. Thus, as shown here, for the case of CD3^{int}/CD3^{high} ratio (Figure 15), some differences tend to disappear with age. Importantly, we have shown that the low numbers of hepatic T lymphocytes in Fabry mice are mostly due to overt anomalies in the percentage of CD3^{int} T cells. These T cells are abundant in the liver of mice and encompass both CD1d-restricted and CD1d-unrestricted T cells with regulatory properties³²⁷. These results suggest that Gb3 accumulation in mice impact on both conventional T cells and CD1d-restricted iNKT cells, affecting negatively CD4⁺ T cells in liver and spleen. The observed increase in CD8⁺ T cells may result from homeostatic changes or from Gb3-induced CD8⁺ T cell increase or both. In any case, these imbalances may compromise CD4⁺ T cell-mediated responses under pathological situations. Noteworthy, no anomalies in T and iNKT cells were observed in Fabry patients (Figure 16), being MHC class II molecules upregulation in monocytes the only anomaly detected (Figure 17). MHC-class II upregulation has been associated with disease-associated inflammatory status (Research Work 1; ²²), which can be related to the accumulation of sphingolipids in class II-enriched endolysosomal compartments. Inflammation is common to a number of LSD³²⁸.

Lysosomes and late endosomes are simultaneously the main locals for lipid antigen load onto CD1d molecules as well as for enzymatic degradation of GSL and consequent accumulation of GSL in LSD. Different authors defend that GSL accumulation in this compartment could result of the entrapment of endogenous ligands or as a result of these ligands being out-competed by storage lipids for binding to CD1d (¹⁶⁶; Research Work 1). However, when we studied the expression of CD1d molecules on monocytes from FD patients and despite the fact that the lipid accumulated in FD (Gb3) is the isomer of the lipid reported to be the putative endogenous ligand of iNKT cells (iGb3), we did not observe any alteration in the MFI of this molecule (Figure 17). The lack of overt T cell anomalies in Fabry patients is not related with the fact that they are undergoing ERT since no significant changes in the percentage of T and iNKT cells were observed between patients at different times after ERT (data not shown).

The direct confrontation between the results observed in Fabry mice and the results in FD patients is not possible, due to the different cells analyzed. While in humans we use peripheral blood, in mice total PBL were extracted from liver and spleen. The absence in humans of equivalent anomalies seen in mice may result from the marked differences

between the innate and adaptive arms of the immunological system of these two species³²⁹ and should be taken into consideration.

3. CD1d upregulation and T cell responsiveness

Adapted from Balreira A, Cavallari M, Sá Miranda MC and Arosa FA.
Immunobiology. 2009. doi: 10.1016/j.imbio.2009.07.002

3.1. Abstract

GD is associated with upregulation of CD1d and MHC-class II expression by monocytes. While the physiological impact of CD1d upregulation remains uncertain, it has been proposed that MHC-class II upregulation is associated with inflammation. Hereby, we show that the decrease in MHC-class II expression seen in GD patients under therapy correlates positively with chitotriosidase activity, a marker of inflamed macrophages. We also show that retinoic acid (RA) and the GluCerase inhibitor CBE lead to upregulation of CD1d expression by THP-1 cells, which correlated with an increase in mRNA expression. *In vitro* co-culture experiments showed that RA treated THP-1 cells were more stimulatory for CD4⁺ than for CD8⁺ T cells, as determined by CFSE loss, in comparison to untreated THP-1 cells. Interestingly, even though addition of exogenous iGb3, a physiological CD1d ligand, augmented the percentage of dividing CD4⁺ T cells, we could not detect a significant expansion of CD4⁺Vα24⁺ iNKT cells. In contrast, addition of α-GC induced expansion of Vα24⁺ iNKT cells as determined by using α-GC-loaded human CD1d dimers. These results strengthen the existence of a cross talk between monocyte lipid accumulation, inflammation and changes in cell surface CD1d and MHC-class II in monocytes, which may result in inappropriate recognition events by immune cells and perpetuate chronic inflammation.

3.2. Introduction

GD, the most frequent LSD in Portugal ¹⁵, results from the deficiency in the lysosomal hydrolase GluCerase responsible for the hydrolysis of GluCer into glucose and ceramide. Unmetabolized GluCer, accumulates in lysosomes of macrophages, that became engorged originating the so called “Gaucher cells”, the hallmark of the disease ²⁴. GD is a multisystemic disorder with several clinical manifestations, including hepatic, splenic and bone involvement. GD is characterized by peripheral inflammation that results from dysfunction of circulating or tissue-resident macrophages ³⁴.

The catabolism of macromolecules in lysosomes is crucial for the correct functioning of immune system as shown, what is supported by several studies describing immune alterations in LSD (reviewed in ¹¹⁸). Lipids are presented to a particular T cell subset, among them the iNKT cells, by CD1 molecules ⁹⁴⁻⁹⁷. iNKT cells express an invariant TCR formed by the canonical rearrangement of the Vα24 segment to the Jα18 segment with Vβ11 ²⁰⁹⁻²¹¹, and are characterized by the singular capacity of rapidly secreting cytokines presenting molecule ¹¹⁷. CD1d molecules are structurally similar to MHC-class I molecules, but intracellularly behave like MHC-class II molecules ¹¹⁵, in that most CD1d

molecules are directed from the ER to the plasma membrane and from where they are reinternalized to the endocytic compartments. Some CD1d molecules are found associated with the li and are directed to MIIC¹⁷³.

We have shown in a previous study that monocytes from GD patients presented upregulation of CD1d and MHC-class II, and we hypothesized that CD1d upregulation resulted from alterations in intracellular trafficking as a consequence of lipid accumulation while MHC-class II upregulation appeared to be related with the inflammatory status of the patients (see Research Work 1). We also reported on upregulation of MHC-class II, but not CD1d, in monocytes from other LSD, Fabry disease (Figure 17). The association of CD1d upregulation with sphingolipid accumulation was further supported by results obtained with PBMC from healthy controls treated with an irreversible inhibitor of GluCerase, CBE (Figure 8). Nevertheless, whether alterations in CD1d and MHC-class II due to sphingolipid accumulation underlie imbalances in regulatory iNKT and T cells and contribute to the heterogeneity of the clinical manifestations remains to be elucidated. In order to address these questions, the development of *in vitro* models of the Gaucher cell (a lipid-laden macrophage) could be an important tool to dissect the molecular mechanism responsible for the upregulation of CD1d/MHC-class II and ascertain whether this upregulation has any impact on conventional and invariant T cell populations. In this study we report on the impact that an *in vitro* Gaucher cell model, a “lipid-laden” macrophage-like cell line, has on T cell responsiveness

3.3. Material and methods

3.3.1. Patients population

GD patients were diagnosed through the determination of residual activity of GluCerase in PBL³⁰⁴. Patients were genotyped using previously described methods and most of their genotypes have already been reported³⁰⁵⁻³⁰⁸. The study included healthy controls, untreated GD patients (GD patients) and GD patients submitted to ERT with imiglucerase. Treatment were initiated with 60 U/kg/every 2 weeks and adjusted according to the patient's clinical response. All studies were performed with the patients' consent and with the approval of the Ethical Committee of the Institute for Molecular and Cell Biology.

3.3.2. Expression of cell surface molecules

Cell surface staining of peripheral mononuclear and THP-1 cells was performed on ice for 30 min in the dark in PBS supplemented with 0.1% NaN₃ and 0.2% BSA (PBS-BSA-NaN₃, staining solution) in round-bottom microtiter plates (Greiner-Nurtingen, Germany) with $\sim 0.5 \times 10^6$ cells/well. After the staining, cells were washed twice with staining solution and immediately analyzed in a FACScalibur (Becton Dickinson, Mountain View, CA, USA). Monocytes were identified and gated according to FSC/SSC characteristics. For peripheral blood samples, 50000 events within the mononuclear gate were acquired, and for THP-1 cells, 20000 events were acquired and analyzed using the FlowJo software (TreeStar, Inc, Ashland, USA). The following fluorochrome-conjugated monoclonal antibodies were used: anti HLA-A,B,C (clone W6/32) from Immunotools (Friesoythe, Germany); anti HLA-DR,DP,DQ (clone CR3/43) from DakoCytomation (Glostrup, Denmark); anti-CD1d (clone 42) from BD Biosciences (Erembodegem, Belgium) and anti-CD1d (clone Nor 3.2), from AbD Serotec (Oxford, UK). Mouse immunoglobulins were used as isotype controls. Expression of the different molecules at the cell surface was determined by MFI values.

3.3.3. Determination of Chitotriosidase activity

Chitotriosidase activity was measured as previously described³³⁰. Briefly, 5 μ L of plasma were incubated with 100 μ L of 0.026 mM 4-MU- β -D-N-N'-N''-triacetylchitotriosidase (Sigma-Aldrich, St. Louis, MO, USA) as substrate in 50 mM citrate-100 mM phosphate, pH 5.2, at 37 °C, for 15 minutes. After incubation, the reaction was stopped with 1 mL of 1 M glycine/NaOH buffer, pH 10.0. Fluorescent 4-MU was measured at 445 nm with a fluorescence spectrophotometer (Hitachi HTA, Illinois, USA). Whenever necessary, samples were previously diluted 50x in BSA 1%.

3.3.4. Cells

PBMC were obtained from leukocyte preparations of healthy subjects by centrifugation over the density gradient Lymphoprep (Axis-Shield, Oslo, Norway). Contaminating RBC were lysed in lysis solution (10 mM Tris, 150mM NH₄Cl, pH 7.4) for 10 min at 37 °C. PBMC were kept in culture overnight in Petri dishes (Orange Scientific, Braine-l'Alleud, Belgium) in RPMI-1640 with Glutamax I (Gibco, Invitrogen, Barcelona, Spain) supplemented with 1% penicillin/streptomycin/amphotericin B (P/S/A), 5% heat-inactivated iFBS (Sigma-

Aldrich, St. Louis, MO, USA) at 37 °C, 5% CO₂, 99% humidity. After overnight culture, non-adherent cell suspensions (PBL) were recovered for co-culture assays. Pure CD4⁺ and CD8⁺ T cells were obtained from PBL, by positive selection using MACS microbeads and columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytic leukemia cell line THP-1 (ECACC, Salisbury, UK) was kept in culture and grown in RPMI-1640 with Glutamax I complete medium with 1% P/S/A and 10% iFBS, at 37 °C, 5% CO₂, 99% humidity.

3.3.5. Inhibition of GluCerase activity in THP-1 cells

GluCerase activity was inhibited as described for PBMC in Research Work 1. Briefly, THP-1 cells were cultured for 96 hours in normal media or media supplemented with 1 mM of CBE (Biomol, Plymouth Meeting, PA, USA), prepared as stock solution in 20mM Hepes buffer, pH 7.4³⁰⁹. Cells were then harvested, washed and processed for flow cytometry or thin layer chromatography. Cells processed for flow cytometry were acquired in a FACScalibur (Becton Dickinson, Mountain View, CA, USA) and analyzed using the FlowJo software (Tree Star Inc., Ashland, USA).

3.3.6. Thin layer chromatography (TLC)

After sonication of cell samples, total lipids were extracted by two cycles of 1 min vortexing with 4.5 mL chloroform/methanol (1:2, v/v). The extract was evaporated under a stream of nitrogen, at 40 °C. Total lipids were fractionated into acidic and neutral lipids by polarity differences. Thus, dried total lipids were dissolved in chloroform/methanol/water (2:8:4, v/v) and passed through prepacked reverse-phase C18 columns (Chromabond, Macherey-Nagel, Düren, Germany), in order to remove non-lipid contaminants³³¹. Column-bounded acidic lipids were eluted with methanol/water (12:1, v/v) and neutral lipids were eluted with chloroform/methanol (1:2, v/v). Both fractions were evaporated with a stream of nitrogen at 40 °C. In order to remove phosphoglycerides, neutral fraction was subjected to alkaline hydrolysis with 0.1M NaOH in methanol, at 37 °C, for 30 min³³². After hydrolysis, dried fractions were dissolved in chloroform/methanol (1:2, v/v) and the equivalent to 10 mg of extract of each sample was applied to HPTLC plates (Merck, Darmstadt, Germany) previously activated at 100 °C for 30 min. A mixture of lipid standards (ST), composed by GluCer; phosphatidylethanolamine (PE); lactosylceramide (LacCer) and sphingomyelin (Sph) from Sigma-Aldrich (St. Louis, MO, USA); globotriaosylceramide (Gb3) from Nacalai Tesque Inc. (Kyoto, Japan); ganglioside GM1 (Cer 4) from Calbiochem (La Jolla, CA, USA) was also applied. Plates were developed in

chloroform/methanol/water (70:30:5, v/v) and then dried under a flow of air. Lipids were visualized with anisaldehyde in 80% (v/v) sulfuric acid, followed by heating at 120 °C until the appearance of the bands. Plates were digitalized and bands quantified by Un-Scan-It software (Silk Scientific Inc, Utah, USA).

3.3.7. Treatment of THP-1 cells with retinoic acid

In order to induce differentiation of monocytic THP-1 cells into macrophages, retinoic acid (RA) (Sigma-Aldrich, St. Louis, MO, USA) was prepared as 10 mM stock solution in ethanol and kept at -80 °C, before being diluted to 0.1 µM in 10% iFBS culture medium RPMI 1640 with Glutamax I (Gibco, Invitrogen, Prat de Llobregat, Spain). Cells were incubated for different time periods. Differentiation of monocytes into macrophages was assessed by ICAM-1 expression increase. For that, we used a monoclonal mouse anti-human CD54 primary antibody, followed by a polyclonal rabbit anti-mouse secondary antibody (DakoCytomation, Glostrup, Denmark). In order to analyze the cell surface expression of MHC family molecules, cells were harvested, washed and processed for flow cytometry after RA treatment. Cells were acquired in a FACScalibur (Becton Dickinson, Mountain View, CA, USA) and analyzed using the FlowJo software (Tree Star Inc., Ashland, USA).

3.3.8. Reverse transcription polymerase chain reaction (RT-PCR)

From each sample of THP-1 cells, untreated or treated with RA, total RNA was isolated using the High Pure RNA Isolation Kit (Roche, Carlsbad, USA). Total RNA concentration was measured by spectrophotometry and its quality assessed by agarose gel electrophoresis. Subsequently, 1 µg of total RNA was converted into cDNA with the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. The CD1d and β-actin genes were amplified as described before (Research work 1). Then, 10 µl of the PCR products were loaded into a 2% agarose gel with 0.05% ethidium bromide, electrophoresis was performed and the PCR fragments visualized under ultraviolet light.

3.3.9. Co-culture assays

For monitoring T cell proliferation, bulk PBL, purified CD4⁺ and CD8⁺ T cells or an iNKT cell clone were incubated for 6 min at 37 °C with 5 µM carboxyfluorescein succinimidyl ester (CFSE) from Cell Trace™ CFSE Cell Proliferation Kit (Molecular probes, Invitrogen,

Barcelona, Spain) with occasional mixing. CFSE labeled T cells were washed twice with PBS 1x/20% iFBS and cultured in a 1:2 ratio with irradiated THP-1 cells (either untreated or RA treated), using Gammacell 1000 Elite irradiator (MDS Nordion, Ottawa, Canada) in the absence or presence of α -GC (Kirin Brewery Co. Ltd, Tokyo, Japan) or iGb3 (Alexis Biochemicals, New York, USA). After five-seven days in culture, cells were harvested, washed and stained with anti-CD4, anti-CD8, anti-CD3 (Immunotools, Friesoythe, Germany) and anti-TCRV α 24 antibodies (Beckman Coulter, Fullerton, CA, USA) and/or α -GC-loaded human dimmers (Cavallari, manuscript in preparation), and acquired in a FACScalibur (Becton Dickinson, Mountain View, CA, USA) or a CYAN ADP (Beckman Coulter, Fullerton, CA, USA). Dead cells were excluded by propidium iodide or 7AAD staining. Proliferation was determined by loss of CFSE fluorescence intensity using the FlowJo software (Tree Star Inc., Ashland, USA).

3.3.10. Statistical analysis

Statistical evaluation was performed using paired Student's *t*-test at the level of significance of 0.05.

3.4. Results

3.4.1. Correlation between MHC-class II expression and Chitotriosidase activity

We have previously showed that CD1d and MHC-class II expression are increased in *ex vivo* peripheral blood monocytes from GD patients (Figure 6 and see also Figure 18). Interestingly, CD1d upregulation in Gaucher monocytes was also observed when Nor 3.2 antibodies recognizing β_2 m-free CD1d molecules³²², were used (76.82 ± 18.23 vs 30.20 ± 3.44 in controls, mean $\pm$ SEM, $P=0.02$, see Figure 18). We have also shown that ERT, which restores normal levels of GluCer, was paralleled by a decrease in MHC-class II expression, which led us to propose that these molecules could be a marker of inflammation (Research Work 1). In order to corroborate this assumption, we performed a comparative analysis between the level of MHC-class II expression by Gaucher monocytes and the efficiency of ERT, as determined by the reduction in chitotriosidase activity. Chitotriosidase is an enzyme that is expressed by lipid-laden macrophages and has been reported to decrease with ERT^{330,333}. As shown in Figure 19, the decrease in MHC-class II expression observed in Gaucher patients undergoing ERT correlated with a decrease in chitotriosidase activity, supporting the view that MHC-class II expression by monocytes could be a marker of inflammation in LSD. Interestingly, the correlation

between the two markers was stronger in patients undergoing longer ERT (compare Figure 19A and B to C and D).

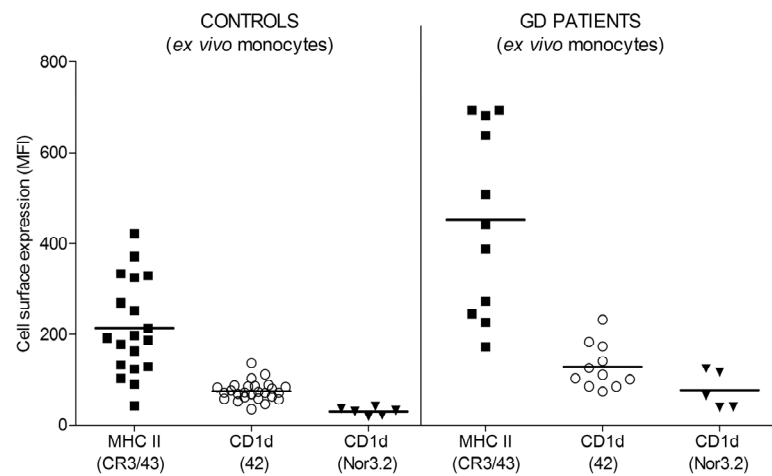


Figure 18. Analysis of MHC molecules on GluCerase-deficient monocytes from GD patients. Cell surface expression of MHC-class II (CR3/43 antibody), β_2 m-associated CD1d (42 antibody) and β_2 m-free CD1d (Nor 3.2 antibody) molecules was determined by flow cytometry. Figures show MFI for each molecule in controls and GD patients with each point representing a different individual. Straight lines represent mean values.

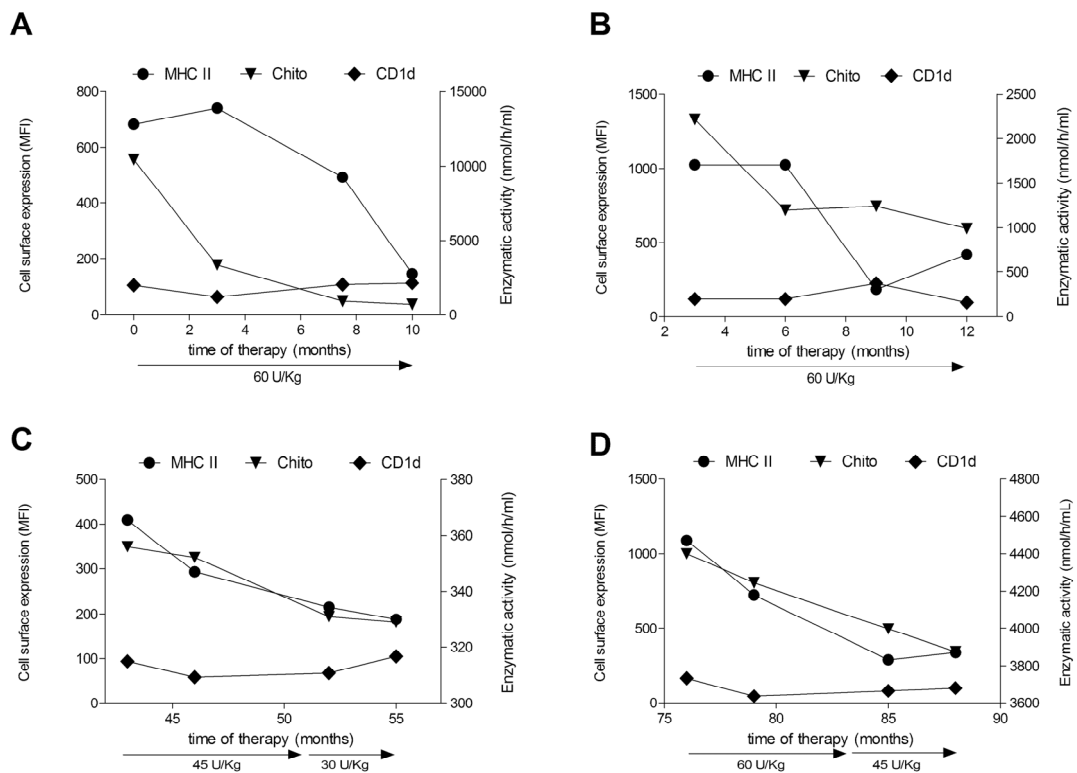


Figure 19. Correlation between MHC-class II expression and chitotriosidase activity. Expression of MHC-class II and CD1d molecules in $CD14^+$ monocytes was determined by flow cytometry. Chitotriosidase

activity was measured in GD patients' plasma. Each graph represents a different GD patient and shows the levels of MHC-class II and CD1d (left vertical axis) in combination with chitotriosidase activity (right vertical axis) along the therapy (horizontal axis). The dose of imiglucerase administered every 2 weeks and the duration of treatment are also indicated in the graphs. (A) GD patient studied since the beginning of therapy; (B, C, D) GD patients with 3, 43 and 76 months of therapy at the first time of study, respectively. Each point represents a single determination.

3.4.2. Glucocerebrosidase inhibition in THP-1 cells leads to GluCer accumulation and CD1d upregulation in THP-1 cells

In order to ascertain the impact of CD1d upregulation by monocytes may have on T cell responsiveness, we developed an *in vitro* model of lipid accumulation feasible of study. To overcome the limitations posed by the use of monocytes obtained from *ex vivo* blood samples, we decided to use THP-1 cells, a monocytic cell line that can be cultured *in vitro* and manipulated to generate lipid-laden cells through the use of CBE. CBE is an irreversible inhibitor of GluCerase activity that leads to an increase in intracellular GluCer³⁰⁹. We have shown that treatment of *ex vivo* blood monocytes with CBE resulted in a two-fold upregulation of CD1d expression at the cell surface (Figure 8). As shown in Figure 20A, THP-1 cells treated with CBE showed a time-dependent increase in CD1d surface expression that reached statistically significant at day 3. In contrast, cell surface expression of MHC-class I and MHC-class II molecules was not affected by CBE treatment (data not shown). Semi-quantitative determination of GluCer accumulation in THP-1 cells by TLC analysis demonstrated that CBE treatment induced an actual increase in the amount of GluCer (Figure 20B).

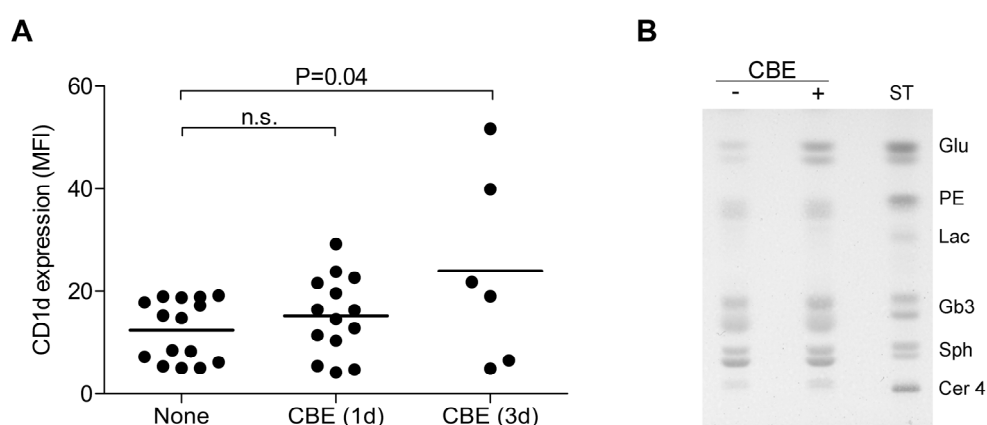


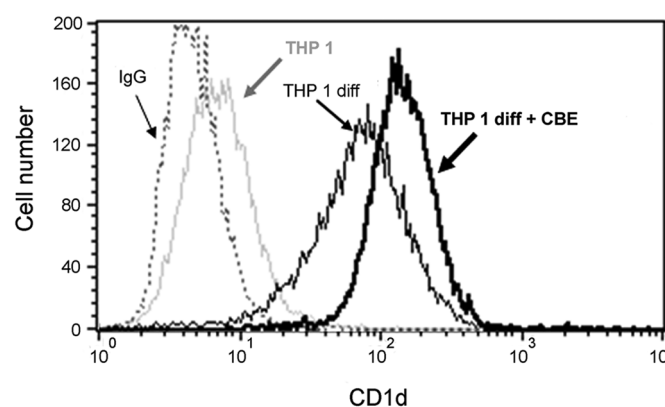
Figure 20. CBE treatment induces CD1d upregulation on THP-1 cells: correlation with accumulation of glycosphingolipids. (A) THP-1 cells were left untreated (none) or treated for 1 (CBE (1d)) or 3 (CBE (3d)) days with CBE. After treatment, CD1d molecules expression was analyzed by flow cytometry. Figures show

CD1d MFI with each point representing a single experiment. Statistical significant differences between untreated and treated cells (*t*-test) are indicated. Straight lines represent mean values. n.s., not significant. (B) THP-1 cells were treated for 3 days with CBE. Total lipids were extracted, fractionated and hydrolyzed. Neutral lipids fraction was analyzed by TLC. Image show a TLC plate from a representative experiment, where equivalent amounts of untreated cells (- CBE) and treated cells (+ CBE) were loaded, as well as 2 µg of each lipid standard (ST): GluCer (Glu); phosphatidylethanolamine (PE); globotrihexosylceramide (Gb3); sphingomyelin (Sph); ganglioside GM1 (Cer 4).

3.4.3. Retinoic acid-induced THP-1 differentiation is associated with CD1d upregulation

RA is a potent metabolite of vitamin A, capable of promoting cell differentiation of a variety of cell lines, THP-1 cells³³⁴. Indeed, treatment of THP-1 cells with RA for 24h induced differentiation into macrophage-like cells as assessed by an increase in the expression of ICAM-1/CD54, a marker of monocyte to macrophage differentiation³³⁵, in combination with morphologic and cell arrest parameters (data not shown). Interestingly, flow cytometry determinations showed that RA-induced THP-1 differentiation was associated with a sharp increase in CD1d expression (Figure 21A). Quantitative analysis of the flow cytometry data revealed that RA treatment induced a 4-fold increase in CD1d expression in differentiated THP-1 cells, which correlated with a marked increase in CD1d mRNA levels (Figure 21B, inset). CD1d upregulation was specific since expression levels of MHC-class I and MHC-class II were not affected by RA treatment (Figure 21B). Interestingly, CBE treatment of differentiated THP-1 resulted in a further increase in CD1d cell surface expression (Figure 21A).

A



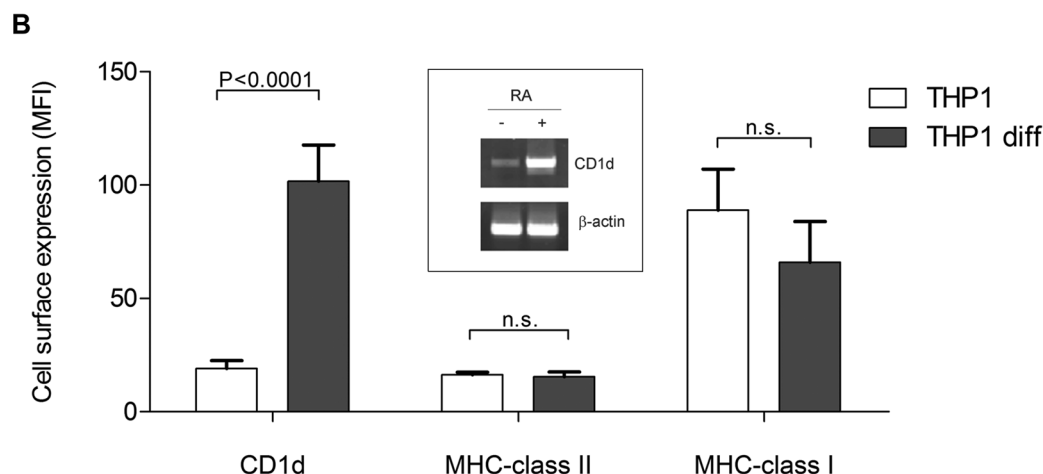
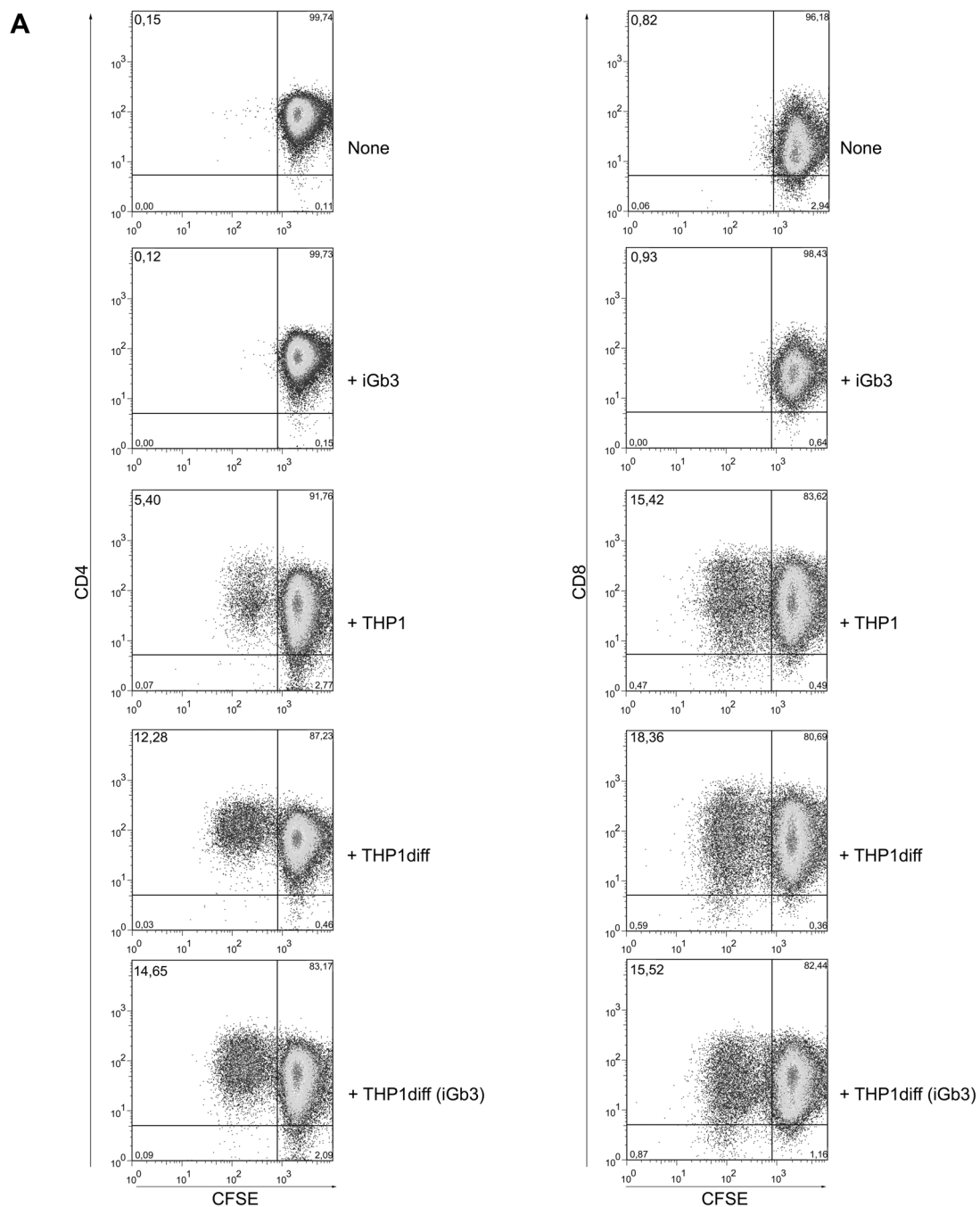


Figure 21. RA treatment induces upregulation of CD1d in THP-1 cells. (A) THP-1 cells were treated with RA for 24 h, and then treated with CBE for 72 hours. After treatment with CBE, CD1d expression was analyzed by flow cytometry. Histogram shows CD1d expression in untreated THP-1 cells (grey line), RA-treated THP-1 cells (THP-1diff, thin line) and RA-treated THP-1 cell treated with CBE (THP-1 diff + CBE, thick line). Background staining with mouse Igs is included (IgG, dotted line) (B) THP-1 cells were treated for 24 hours with RA, collected and cell surface expression of MHC molecules analyzed by flow cytometry. Results show MFI values (mean $\pm$ SEM, n=5-13) of CD1d, MHC-class I and II in untreated (THP-1) and RA treated THP-1 cells (THP-1 diff). Statistical significant differences between untreated and treated cells (*t*-test) are indicated. n.s., not significant. Inset: RT-PCR for CD1d and β -actin in untreated (-RA) and RA treated (+RA) THP-1 cells.

3.4.4. T cell responsiveness in co-cultures with differentiated THP-1 cells

To test the effect of differentiated THP-1 cells on T cell responsiveness, we performed co-cultures of PBL or highly pure CD4⁺ and CD8⁺ T cells, obtained using the MACS technology, with RA-treated THP-1 cells. As readout, we determined the percentage of dividing cells, by using flow cytometry. Of note, CD8⁺ T cells were more responsive than CD4⁺ T cells (by 3-fold) to untreated THP-1 cells (Figure 22A, +THP-1). However, RA treatment of THP-1 cells prior to the co-culture augmented the percentage of CD4⁺, but not CD8⁺, T cells that entered cell division (Figure 22A, +THP-1diff). Importantly, exogenous addition of iGb3 to the co-cultures resulted in an additional increase in the percentage of dividing CD4⁺ T cells (Figure 22A, +THP-1diff (iGb3)). Addition of iGb3 to cultures of CD4⁺ T cells alone had no effect on their responsiveness. As summarized in Fig 24B, CD4⁺ and CD8⁺ T cells responded differently to THP-1 cells. Importantly, flow cytometry analysis using anti-V α 24 antibodies did not detect V α 24⁺ T cells among the CD4⁺ T cells that divided (data not shown). In contrast, some dividing V α 24⁺ T cells were readily detectable when α -GC-loaded human CD1d dimmers were used (Figure 23A). In

marked contrast to iGb3, exogenous addition of α -GC to PBL preparations induced high levels of responding $V\alpha 24^+$ T cells, even in the absence of RA (Figure 23A). By using a combination of anti-CD3, CD4 and CD8 antibodies a significant increase in the percentage of dividing $CD4^+$ and DN iNKT cells, but not of $CD8^+$ iNKT cells, was observed, as summarized in Figure 25B. The level of proliferation of the different iNKT cell subsets when iGb3 was added exogenously to the co-culture was not different from that seen in co-cultures without iGb3 (Figure 23B). Similar results were observed with an iNKT cell clone (Figure 23).



B

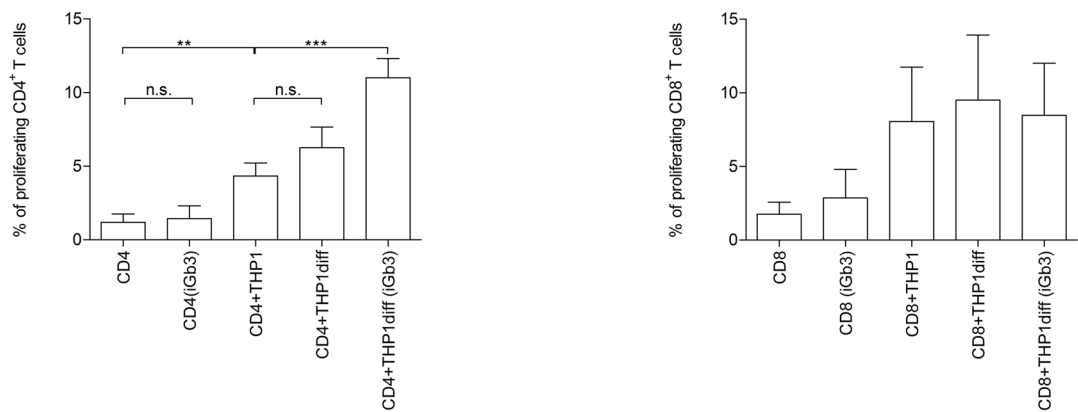
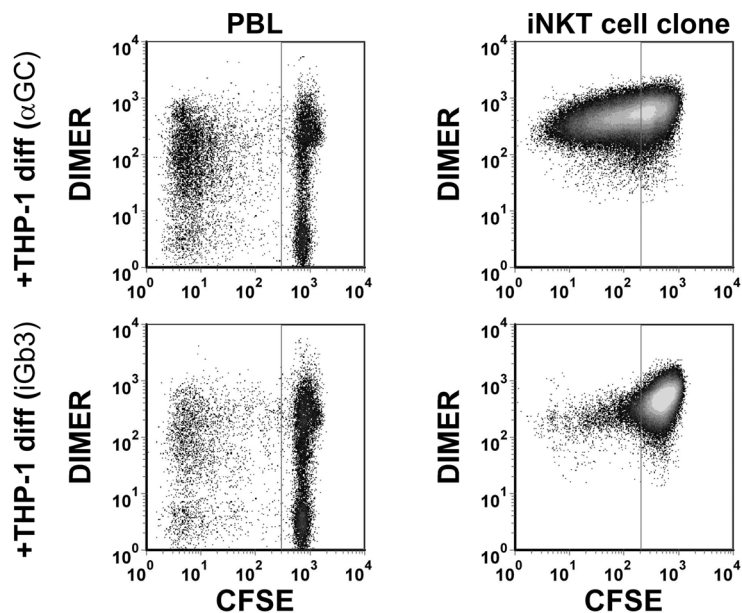


Figure 22. T cell proliferation induced by RA treated THP-1 cells. CD4⁺ and CD8⁺ T cells were isolated as indicated in Material and Methods, labeled with CFSE and cultured for seven days with untreated THP-1 cells, RA-treated THP-1 cells and RA-treated THP-1 cells incubated with 200 ng/mL of iGb3 or with 200 ng/mL of iGb3 alone. (A). Dot blots show CFSE fluorescence vs CD4 or CD8 expression in CD4⁺ (left dot-plots) and CD8⁺ (right dot-plots) T cells under the different culture conditions after gating in live cells. The percentages of cells in each quadrant are indicated. (B) Graphs show the percentages of CD4⁺ (left graph) and CD8⁺ (right graph) T cells that divided during the seven days in culture. Statistical significant differences between conditions (*t*-test) are indicated. **P<0.01; ***P<0.001. n.s., not significant.

A



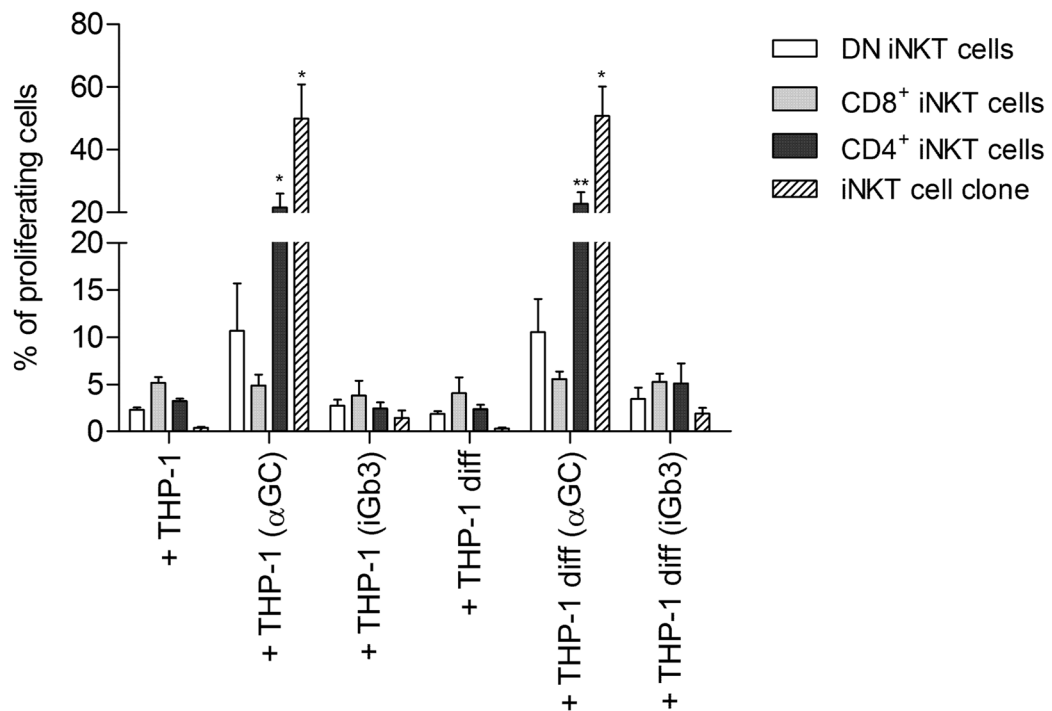
B

Figure 23. iNKT cell expansion after stimulation with lipid antigens. PBL, or an iNKT cells clone, were labeled with CFSE, and cultured for 5 days with untreated THP-1 cells, untreated THP-1 cells incubated with α -GC or with iGb3, RA-treated THP-1 cells and RA-treated THP-1 cells incubated with α -GC or with iGb3. (A) Dot-plots show α -GC-loaded human CD1d dimmers (DIMER) vs CFSE labeling in cultures using PBL and an iNKT cell clone. Figure shows one representative example of cells expanded in the presence of RA-treated THP-1 and either α -GC or iGb3. (B) Graph shows the percentage of every iNKT cell subset and the iNKT cell clone that proliferated during the co-culture period (mean $\pm$ SEM). Statistical significant differences when compared with untreated THP-1 cells (*t*-test) are indicated. **P*<0.05; ***P*<0.01.

3.5. Discussion

Despite recent advances, our understanding of the molecular mechanisms controlling CD1d and MHC-class II expression on the surface of antigen-presenting cells is still limited. Although CD1d and MHC-class II cytoplasmic domains contain conserved motifs directing them through similar intracellular trafficking pathways, their expression at the cell surface appears to be under the control of different molecular cues. Indeed, we have shown that sphingolipid accumulation constitute a novel regulatory element for CD1d expression in monocytes, which appears to be sphingolipid-specific. An association between lipid accumulation and CD1d upregulation has previously been reported in

macrophages from atherosclerotic lesions¹⁹⁸ and iron overloaded liver epithelial cells³²². In the latter case, the CD1d form that was upregulated was the β_2m -free form. Interestingly, we have found that β_2m -free CD1d molecules as well as β_2m -associated CD1d molecules are upregulated in GD patients. In addition, we have shown that MHC-class II cell surface expression observed under conditions of sphingolipid accumulation probably reflects the inflamed state of the monocytes (Research Work 1 and 2).

To further corroborate these findings, we decided to undertake a comparative analysis between chitotriosidase activity in GD patients undergoing ERT and the expression of MHC-class II molecules. Chitotriosidase is an enzyme whose activity reflects the inflammatory status of monocytes and macrophages. Therefore, by replacing the defective enzyme with an active recombinant form of GluCerase, ERT aims at reducing the GluCer storage burden that can be monitored by measuring chitotriosidase activity. In this study, we have shown a positive correlation between the decrease in chitotriosidase activity observed along the ERT and the decrease in surface expression of MHC-class II molecules by *ex vivo* monocytes. These results constitute a strong argument in favor of the view that upregulation of MHC-class II expression by *ex vivo* monocytes of Gaucher patients is related to the inflammatory status of these cells (Research Work 1) and are in agreement with reports showing high levels of chitotriosidase activity in a number of chronic inflammatory diseases³³⁶. Although the affected tissues in Gaucher patients frequently present high levels of GluCer, this is not sufficient to account for the dramatic alterations that takes place in tissues pathology and suggest that other biochemical pathways must be contribute to the pathological alterations²⁹⁴. In this context, it has been recently suggested that accumulation of sphingolipids in a macrophage model of GD could interfere with other biochemical pathways outside the lysosome, resulting in macrophage dysfunction³³⁷. The correlation between chitotriosidase activity and MHC-class II expression observed in *ex vivo* monocytes of GD patients with longer periods of ERT could probably indicate the correction of secondary alterations. These results point to MHC-class II expression as a putative biomarker of ERT efficiency.

In this study we also wanted to ascertain whether the changes in CD1d expression in monocytes that have been induced to accumulate sphingolipids *in vitro* correlated with changes on T cell responsiveness. These changes could eventually help understanding the anomalies found previously on T cell populations in Gaucher patients (Research Work 1; ¹³²). Since the most affected cell line in GD patients is the macrophage, we decided to use the myelomonocytic cell line THP-1, which upon RA treatment enters cell cycle arrest and differentiation into macrophage-like cells³³⁴. Indeed, we have shown that RA treatment induced THP-1 differentiation and a marked increase in cell surface expression of CD1d, and both effects correlated with an increase in mRNA expression. In contrast,

neither MHC-class I or MHC-class II were significantly altered. The use of the GluCerase inhibitor CBE on differentiated THP-1 cells resulted in a further increase in CD1d expression, though without statistical significance, indicating the existence of control mechanisms that restrain CD1d upregulation that occurs upon GluCerase inhibition on *ex vivo* monocytes (Research Work 1). These results indicate that differentiation of the monocytic cell line THP-1 into macrophage-like cells is associated with upregulation of cell surface CD1d. They are in agreement with recent studies showing that RA is linked with CD1d upregulation in DCs and monocytic cell lines through the activation of the RA receptor^{338,339}. In this regard, it is plausible to think that similar changes in CD1d expression may take place during differentiation of monocytes into tissue macrophages *in vivo*. Considering that RA signals can be elicited by lipids³⁴⁰, these results strongly indicate that anomalies in lipid metabolism, such as the observed in Gaucher patients and other LSD, may be sensed by cells of the monocytic lineage and result in changes in CD1d expression.

Due to the described role of CD1d in the activation of iNKT cells, we wanted to ascertain whether differentiated THP-1 cells expressing high levels of CD1d would be capable of activating iNKT cells when co-cultured with *ex vivo* T cells. Although previous studies indicating that THP-1 are slightly stimulatory for human T cells^{341,342}, to our knowledge this is the first report showing that pure CD4⁺ and CD8⁺ T cells respond to THP-1 cells. Interestingly, the lower capacity of CD4⁺ T cells to respond to THP-1 cells, when compared to CD8⁺ T cells was counteracted when RA treated THP-1 cells were used, suggesting the existence of CD4⁺ T cell-specific activation signals driven by RA-treated THP-1 cells. Also, addition of iGb3 to the co-cultures significantly increased the percentage of dividing CD4⁺ T cells, but not of dividing CD8⁺ T cells. To our surprise, we could not detect any V α 24⁺ T cell among the dividing CD4⁺ T cells. These results are interesting for several reasons. First, CD1d:iGb3 complexes are thought to selectively activate iNKT *in vivo* and *in vitro*¹⁶¹. Second, a number of reports have provided experimental evidence on the role played by cell surface CD1d in eliciting strong proliferative responses of iNKT cells when complexed with α -GC, a non-physiologic CD1d ligand from the marine sponge *Agelas mauritianus* thought to act as an alloantigen⁹⁵. Third, based on data from the literature including our own, the frequency of iNKT cells is about 1% whereof roughly half are CD4⁺ (Research work 1; ³⁴³). Assuming that V α 24⁺CD4⁺ T cells are activated by CD1d:iGb3 complexes and undergo three cycles of cell division (see Fig 24A, lower left dot-plot) without undergoing apoptosis, one would expect to have about 8% of V α 24⁺CD4⁺ T cells by the end of the culture, which is not the case. However, as expected, addition of α -GC to the co-cultures resulted in the expansion of V α 24⁺CD4⁺ T cells.

When analyzed within the aforementioned conceptual framework, one can propose three main scenarios to try to explain the lack of iNKT responsiveness to CD1d upregulation when iGb3, but not α -GC, was added exogenously. First, $V\alpha 24^+CD4^+$ T cells did not survive to the 7-day culture period after being activated by differentiated THP-1 cells expressing CD1d:iGb3 complexes. We think this possibility is unlikely because there is no reason for $V\alpha 24^+CD4^+$ T cells being more prone to apoptosis. Furthermore, in co-cultures where iGb3 was added there was a two-fold reduction in the percentage T cell apoptosis when compared to cultures of T cells alone (unpublished results). Second, $V\alpha 24^+CD4^+$ T cells have down-regulated the conserved T cell receptor during the expansion phase. This is not unlikely since down-regulation of cell surface markers identifying T cells subsets is a normal event during cell proliferation and as has been shown for the iNKT marker NK1.1^{225,226}. Third, the stimulus provided by the CD1d:iGb3 complex is not strong enough to drive $V\alpha 24^+CD4^+$ T cells into the cell cycle. The latter scenario seems the more likely one if we consider that the presence of α -GC induced a strong $V\alpha 24^+CD4^+$ T cell expansion. In this context, Szatmari *et al* also failed in eliciting iNKT cell expansion with CD1d-overexpressing dendritic cells unless exogenous α -GC was added³³⁹. Therefore, the mechanism whereby CD1d-overexpressing differentiated THP-1 cells elicit the expansion of conventional $CD4^+$ T cells when exogenous iGb3 is added remains to be elucidated, but is unlikely to be related to MHC-class II molecules since their expression is very low and was not affected by RA. Extrapolating these results to the *in vivo* situation, it is plausible to think that chronic activation of $CD4^+$ T cells by lipid-laden monocytes/macrophages could lead to a progressive $CD4^+$ T cells exhaustion and loss. This scenario would agree with our previous studies showing low numbers of peripheral blood $CD4^+$ T cells in GD patients, an anomaly that is partially corrected by ERT (Research Work 1).

In summary, hereby we have provided experimental evidence for the existence of a close relationship between cell differentiation, lipid metabolism and CD1d expression in monocyte-like cells and the impact of these biological events on T cell responsiveness *in vitro*. Such an interrelationship may also exist in other cell types^{108,322} and have implications under pathological chronic inflammatory conditions¹⁹⁸.

IV – Concluding Remarks

Immunology faced a new challenge when it became evident that T lymphocytes recognize not only peptides presented by MHC-class I and II molecules, but also lipid antigens presented by CD1d molecules. Due to the role of GSL as endogenous ligands for CD1d¹⁹⁹, sphingolipidosis emerged as models to study the impact of lipid storage in the immune system. Moreover, sphingolipidosis are characterized by a wide clinical and genetic heterogeneity. In general, a phenotype/genotype correlation cannot be established, suggesting that other mechanisms must be implicated in the pathophysiology of these disorders. In sphingolipidosis, GLS accumulate to massive levels in lysosomes, leading to pathology. Among these disorders, two caught our attention for different reasons: Gaucher disease and Fabry disease. Gaucher disease was studied not only due to its incidence, being the most frequent LSD in Portugal¹⁵, but also because the affected cell is the macrophage. In fact, these macrophages, the so called Gaucher cells are the hallmark of the disease, and are the main storage cell in this pathology. In the case of Fabry disease, the main reason for the study of its immunological phenotype relates to the description of Zhou *et al* in 2004 that suggested iGb3 as the putative endogenous ligand required for iNKT cells thymic selection¹⁶¹. iGb3 is an isomeric form of Gb3 the GSL stored in Fabry disease. Both GSL differ in the galactose link, with Gb3 having an α 1-4-linked galactose in and iGb3 an α 1-3-linked galactose (reviewed in¹⁷²), leading us to hypothesized that alterations in CD1d and iNKT cells could be found in FD patients. Furthermore, the availability of the Fabry knockout mice in our laboratory reinforced the idea of the studying the immunological alterations in FD.

Despite the existing evidence for a link between lipids and the immunological system, few studies addressed the role of immunological cells and molecules in LSD. Early studies in GD reported anomalies of B cells, as B cell lymphocytosis^{119,120} and benign monoclonal gammopathies³⁰³, as well as some cases of lymphocytic leukaemia¹¹⁹; abnormalities of cellular immunity and NK cells¹³⁵; lysosomal vacuolar and non-vacuolar inclusions in T and NK cells¹³⁶, and an early study performed in our laboratory showed a relationship between anomalies in lymphocyte numbers and bone disease¹³². However, studies of T cell phenotype and function were surprisingly absent up until the beginning of the work presented in this thesis. The absence of immunological studies on human sphingolipidosis is even more shocking if we consider that: i) subsets of regulatory T lymphocytes, as NKT cells, can recognize lipids in the context of CD1d molecules expressed by monocytes and macrophages¹¹⁰; ii) cell surface expression of CD1d depends on trafficking through MHC-class II enriched compartments, such as endosomes and lysosomes, where the simultaneous editing and binding of lipids to CD1d occurs and where GSL accumulate in

LSD; iii) cytokines secreted by NKT cells may regulate the function of monocytes, macrophages and others surrounding cells.

Work described in this thesis clearly showed that GSL accumulation impact the immunological system both at the molecular and cellular level. We started this work by showing that alterations in intracellular GSL metabolism, in particular in GluCer, is closely linked to the regulation of CD1d, the non-classical MHC-family molecule involved in the cross-talk with iNKT cells and was followed by the finding that the sphingolipid-mediated CD1d upregulation may be lipid-specific highlighting differences between humans and mice models of LSD, since this upregulation was not observed in FD patients and different results for conventional T and iNKT cells were found in FD patients and FD mice model.

The pathways of CD1d and MHC-class II molecules regulation are not yet completely understood. Both molecules are regulated by external factors and share intracellular trafficking routes¹⁷³. Here we demonstrated that sphingolipid accumulation represents a novel regulatory component for CD1d and MHC-class II expression in monocytes. Initially, we hypothesized that upregulation of CD1d and MHC-class II resulted from the increased transcription of encoding genes as a consequence of cytokines present in patients. This hypothesis was ruled out by comparing RNA levels of these molecules, with no differences being observed between GD patients and healthy controls. A second possibility was that intracellular trafficking of CD1d and MHC-class II was impaired as a result of GluCer accumulation in lysosomes of GD patients. The role of GluCer on the endocytic sorting has been described by Sillence *et al*, who showed that altered levels of GluCer were responsible for changes in the endocytic sorting of Bodipy-Lactosylceramide, impairing the traffic from the Golgi apparatus to lysosomes, and induced changes in cholesterol levels and cellular distribution³¹⁴. In fact, a slight increase in GluCer levels, substantially less than the levels present in GD patients, was reported to be sufficient to alter lactosylceramide trafficking³¹⁴. GluCer storage was also described as resulting in the increased expression levels of Sap C, cathepsin D, LAMP-1 and LAMP-2³¹⁵.

Having characterized the cell surface expression of MHC-family molecules in peripheral blood monocytes, we expanded our study, to determine if the therapy, which tends to normalize GluCer levels, impacted the levels of CD1d and MHC-class II expression, we studied GD patients along the treatment. These follow-up studies showed that MHC-class II expression tends to decrease with therapy, while no alterations were observed in CD1d expression, suggesting that indeed CD1d expression is altered by GSL, or at least by GluCer accumulation, while the altered MHC-class II expression results from the inflammatory status of the patients. By performing a series of experiments where we

induced GSL accumulation by CBE, the irreversible inhibitor of GluCerase, both in *ex vivo* PBMC and in the monocytic cell line THP-1, we showed that in fact GSL accumulation impacts CD1d expression without having any effect on MHC-class II expression. These results supported the association of MHC-class II with inflammation, which is further sustained by the correlation between MHC-class II expression and chitotriosidase activity during treatment in GD patients. Jeyakumar *et al* questions if inflammation, a feature common to a number of LSD³²⁸, results directly from intracellular GSL accumulation or from the appearance of apoptotic or injured cells²². In view of our results, both from the *in vitro* model of GD, where no alterations in MHC-class II expression is observed in lipid-loaded cells, and the results obtained with FD and GD monocytes, where MHC-class II is upregulated we may hypothesize that increased expression of MHC-class II molecules, a normal occurrence in the presence of inflammatory stimuli^{344,345}, results from signals triggered by apoptotic or injured cells *in vivo*.

Whether imbalances in T cell and iNKT cell populations observed in GD patients result directly from alterations in CD1d and MHC-class II cell surface expression remains uncertain. However, if upregulation of CD1d and MHC-class II impacts iNKT cells and regulatory T cells respectively, this could contribute to the modulation of the clinical symptoms. This appears to be supported by the results obtained with GD patients, since CD1d and MHC-class II upregulation is accompanied by decreased percentages of CD4⁺ T cells and increased percentages of CD8⁺ T cells. The reduced number of CD4⁺ T cells probably resulted from apoptotic signals triggered by an increased T cell activation induced by MHC-class II upregulation in lipid-laden monocytes. On the other hand, the study performed with PBL from FD patients, revealed that despite the upregulation of MHC-class II, no imbalances could be observed on T and iNKT cells, which contradicts that hypothesis. This was also valid in the case of FD mice. We and Gadola *et al*, showed that Fabry mice presented decreased numbers of hepatic iNKT cells, and Gadola *et al* also showed that CD1d expression was not altered¹⁶⁶. Our studies in T cell responsiveness to CD1d upregulated cells showed that purified CD8⁺ T cells have a higher capacity to respond to THP-1 cells than purified CD4⁺ T cells when cocultured. However, no alterations in CD8⁺ T cell response are observed when CD1d upregulation is induced in THP-1 cells. On the other hand, magnetically isolated CD4⁺ T cells, when cocultured with RA-treated THP-1 cells increased their division, suggesting that RA-treated THP-1 deliver cell activation signals to CD4⁺ T cells, which are further increased when iGb3 is added to the culture. Surprisingly, we could not detect Vα24⁺ T cells among the proliferating CD4⁺ T cells.

It can be argued that CD1d:iGb3 complexes do not represent a stimulus sufficiently strong to drive an iNKT cell response that can be detected after seven days in culture. Apparently, this is the most probable scenario, because when α -GC, instead of iGb3, was added to the culture, a strong proliferation of V α 24⁺CD4⁺ T cells was observed. Our results are further supported by the work of Szatmari and colleagues that also failed to induce iNKT cell proliferation when in the co-culture assays CD1d-overexpressing dendritic cells were not previously incubated with α -GC³³⁹. Indeed, it is worth mentioning, that the majority of studies of iNKT responsiveness and function have used α -GC as CD1d ligand. α -GC that acts as an alloantigen was derived from the marine sponge and found during a screen for reagents with anti-tumoral properties¹⁵⁸. The use of α -GC in iNKT cell studies provided a considerable amount of information on the biology of iNKT cell. However, some of the results obtained are debatable in the sense that they may not reflect the truly behavior of iNKT cells *in vivo*. Instead, we favour the view where CD1d complexed with self-lipids delivers survival signals to iNKT cells and also to conventional T cells.

Chemokines are expressed in sites of inflammation in order to attract and amplify leukocyte responses, but are also release from those sites and can be detected in body fluids³⁴⁶. *In vivo*, iNKT cells are rare in the blood, but accumulate at higher levels in liver, bone marrow and spleen. Differential expression of chemokines and their receptors regulates iNKT cells distribution in the periphery and migration to inflamed tissues (reviewed in^{347,348}) early in inflammation where they influence and promote the conventional T cells response induce maturation of APCs and recruit inflammatory homing cells through the chemokine production³⁴⁹. iNKT cells display robust migration to the inflamed tissues through chemokines like CXCL9, CXCR10³⁴⁷ and TNF- α ²⁹². Considering this, and knowing the role of CD1d molecules on the homeostasis of iNKT cells, with a positive correlation between CD1d expression and iNKT cells activation³⁵⁰, we must also consider the role of cytokines and chemokines in the homing process of iNKT cells to affected tissues on LSD. In GD, van Breemer *et al* described that plasma levels of macrophage inflammatory protein (MIP)-1 α (CCL3) and MIP-1 β (CCL4), two chemokines known to be involved in osteoclastic bone resorption in multiple myeloma³⁵¹, were markedly increased, interfering in the equilibrium between bone resorption and formation¹³³. It is important to refer that in GD, this delicate balance is clearly disturbed, being bone loss clearly favoured²⁴. MIP-1 α and MIP-1 β , in GD, are not produced by Gaucher cells but by surrounding cells¹³³. MIP-1 α , the ligand of CCR1, a chemokine receptor expressed by most of CD8⁺ and DN iNKT cells, induces chemotaxis of these cells²⁴⁰ what can justify the decreased numbers of peripheral CD8⁺ T cells observed in GD patients with bone

involvement¹³². Increased levels of chemokines in serum have been described in other disorders, like MIP-1 β and MCP-1 in systemic sclerosis³⁵² and CCL22 in atopic dermatitis³⁵³. The role of soluble factors in pathogenesis of bone disease in GD is further supported by the increased levels of IL-1 β and IL-6^{127,128}, described as having strong bone resorption activity (reviewed in³⁵⁴), which are also upregulated in multiple myeloma³⁵⁵⁻³⁵⁷.

Taken together results obtained by us and other authors^{154,166}, argue in favour that is the storage of GSL above a certain threshold level in endosomes/lysosomes and not the nature of the stored GSL that result in iNKT cell deficiency. Schumann and colleagues, besides reporting the impairment of lipid-reactive CD1d-restricted T cells in mice models of LSD, namely KO mice for β -galactosidase (β -gal^{-/-}) and KO for the lysosomal lipid transfer protein NPC2, showed that in β -gal^{-/-} thymocytes previously treated with NB-DNJ, an inhibitor of GSL biosynthesis known to reduce pathological lipid storage³⁵⁸, the presentation of exogenous lipids was improved³⁵⁹. These results supported the view that indeed is the lipid accumulation the responsible for iNKT cells impairment in LSD. However, if it is the entrapment of endogenous ligands within membranous cytoplasmic storage bodies or if the ligands are out-competed by storage lipids for binding to CD1d the responsible for the deficient numbers of iNKT cells remained uncertain¹⁶⁶.

In our opinion, these series of studies constitute an important piece of information that shed light on the complex molecular mechanism regulating the expression of CD1d in monocytes and may turn to be important to understand the clinical heterogeneity of patients with sphingolipids.

V – References

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