Lipid antigen presentation and thymic selection of iNKT cells

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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Basel 2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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ABBREVIATIONS

αGalCer	alpha-Galactosylceramide
Ac_2SGLs	diacylated sulfoglycolipids
AP-2	adaptor protein complex-2
AP-3	adaptor protein complex-3
APC	antigen-presenting cell
ApoE	apolipoprotein E
β_2m	beta 2-microglobulin
BCG	bacillus Calmette-Guérin
BSA	bovine serum albumin
CDR	complementarity-determining region
DC	dendritic cell
DDMs	didehydroxymycobactins
DMSO	dimethylsulfoxide
e.g.	exempli gratia
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
GlcMM	glucose monomycolate
GM2A	GM2-activator protein
GM-CSF	granulocyte-monocyte colony stimulating factor

GPI	glycosylphosphatidyl inositol
GroMM	glycerol monomycolate
GSL-1	α-glucuronosylceramide
GSL-1’	α-galacturonosylceramide
GVHD	Graft versus host disease
hr	hour(s)
hCD1d	human CD1d
HDL	high-density lipoprotein
HLA	human leukocyte antigen
HSA	heat stable antigen
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HS	human serum
i.e.	id est
IEF	isoelectric focusing
IFNβ	interferon beta
IFNγ	interferon gamma
Ig	immunoglobulin
iGb3	isoglobotrihexosylceramide
Ii	invariant chain
IL-2	interleukin-2
IL-4	interleukin-4
IL-12	interleukin-12
IL-13	interleukin-13

IL-18	interleukin-18
i.n.	*intra nasal*
i.p.	*intra peritoneum*
i.v.	*intra venum*
iNKT	invariant natural killer T cells
kDa	kilo Dalton
KO	knock-out
LAM	lipoarabinomannan
LBP	Lipid binding protein
LC	Langerhans cell
LDL	low-density lipoprotein
LM	lipomannan
LRP	LDL-R-like protein
LTP	lipid transfer protein
mAb	monoclonal antibody
MAIT	mucosal invariant T cells
MAPK	mitogen-associated protein kinase
mCD1d	mouse CD1d
MFI	Mean Fluorescence Intensity
MHC	major histocompatibility complex
MS	multiple sclerosis
MTP	microsomal triglyceride transfer protein
MVB	multivesicular bodies
MyD88	myeloid differentiation factor 88

NOD	non-obese diabetic
NK	natural killer
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline solution
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI_3P	phosphatidylinositol 3-phosphate
PPD	purified protein derivative
PRR	pattern recognition receptor
PS	phosphatidylserine
PIMs	phosphatidylinositol mannosides
SAP	saposin
SCP-2	Sterol carrier protein-2
SGLs	sulfoglycolipids
shCD1d	soluble human CD1d
shCD1e	soluble human CD1e
TAP	transporter associated with antigen processing
TCR	T cell receptor
TG	transgenic
TGF	tumor growth factor
Th	T helper
TLR	toll-like receptor

VLDL	very-low-density lipoprotein
vs.	versus
WT	wild-type

INTRODUCTION

1.1 The immune system

The immune system is organized as a network of proteins, organs, cells and tissues that protect the body from infection with pathogenic microorganisms including bacteria, viruses and parasites. The immune response can be divided into two arms: innate immunity and adaptive immunity. Innate immunity serves as a first line of defense and lacks the ability to discriminate between pathogens, to generate memory against the pathogen and to provide protection during re-exposure. Adaptive immunity is based on selection of individual clonal populations of lymphocytes bearing highly diverse antigen-binding receptors that confers to the immune system the capacity to recognize a large variety of foreign antigens.
Invariant Natural Killer T (iNKT) cells are a unique lineage of T lymphocytes. iNKT cells recognize glycolipids, rather than peptides, in the context of CD1d molecules. The TCR responsible for the antigen recognition is composed of an invariant Vα chain paired with a restricted set of Vβ chains. Recognition of self antigens account for the peculiar functional charachteristics of iNKT cells, *i.e.* the prompt release of cytokines and the effector functions which are immediately active upon antigen challenge.

1.2 The CD1 system

In the last decade it has been demonstrated that T lymphocytes can recognize not only peptides in the context of MHC-Class I and -Class II molecules, but also foreign and endogenous lipids in association with the non-classical MHC class I-like CD1 molecules. This finding shed new light on the complexity of the immune

recognition and on its capacity to sense and respond to a broader array of molecules.

The human CD1 gene family is composed of five nonpolymorphic genes (CD1A,-B,-C,-D, and –E) located in a cluster on chromosome 1 [1], while mice express only two CD1D orthologs located on chromosome 3. It has been proposed that CD1 genes evolved by duplication and neofunctionalization of an MHC I gene before the bird-mammalian divergence. From an evolutionary point of view, the presence of an antigen presenting molecule capable of binding lipids instead of peptides offered a new weapon against bacterial infections [2]. In fact, whereas MHC I molecules specialized to present peptides derived from cytosolic antigens, CD1 trafficking pathways have evolved to acquire antigen in the endocytic pathway, and intersect those pathways utilized by a diverse range of intracellular pathogens.

Human CD1 molecules were originally classified on the basis of sequence identities into two groups: group 1 composed by CD1a, CD1b, and CD1c and group 2 composed by CD1d [1]. CD1e belongs to a third group of CD1 molecules and it is not an antigen presenting molecule, but rather a Lipid Transfer Protein (LTP).

1.2.1 Structure of CD1 molecules

CD1 are glycosylated proteins structurally similar to MHC class I molecules and this might explain the capacity of the TCRαβ to interact with both types of molecules. CD1 molecules form a stable, noncovalently associated heterodimer with beta 2-microglobulin (β_2m) of approximately 49 kDa. Like MHC class I, the ectodomains, or CD1 heavy chains, are organized into three domains, α1, α2 and α3, and anchored in the cell membrane by a transmembrane domain. The α3-domain is highly conserved among all isotypes, probably due to the necessity to

maintain the association with β_2m. α1 and α2 domains form the central lipid binding groove of each CD1 isotype and differ from one another in primary amino acid sequence and three-dimensional structure. CD1a, CD1b and CD1d binding grooves are composed of two main pockets, named A' and F' [3]. CD1b has an additional C' pocket and T' tunnel that connects the A' and F' pockets [4]. The central binding groove is surrounded by two anti-parallel α-helices (α1 and α2), which sit on top of a six-stranded β-sheet platform. The binding groove is deeper and narrower than the cleft binding peptides in classical MHC class I or II molecules [5]. The pockets and tunnels are formed by hydrophobic amino acids that interact with the lipid backbone of all CD1 antigens. The hydrophilic headgroups of lipid antigens, instead, are exposed to the cell surface and can interact with the TCR. Although the overall three-dimensional structures of individual members of the CD1 family are similar, amino acid substitutions are responsible for shaping the individual grooves and for the formation of isotype-specific pockets (**Figure 1**, left panel).

The size and depth of pockets and tunnels of the various CD1 molecules allow the binding of different lipids. For example, CD1a has the smallest binding groove among all CD1 and the A' pocket has a particular blunt-ended conformation [6]. On the contrary, human CD1b has the largest binding groove among the CD1 proteins. Thus, long chain fatty acids up to approximately C_{80} can be accommodated into CD1b molecules [7].

Recent observations suggested that endogenous short-chain fatty acid can behave as chaperones, stabilizing CD1b prior to higher-affinity ligand bindig. As predicted, a recent crystal structure revealed a CD1b binging groove stabilized by a C32-C36 endogenous phosphatidylcholine (PC) in combination with a C41-44 spacer lipid to fill the empty binding space inside the molecule [8].

The first crystal structure of mouse CD1d was obtained in 1997 and described only the two A' and F' pockets [3]. Since natively folded CD1d never appear with empty binding grooves, it seems plausible that a pocket-stabilizing factor is bound until it is replaced with another antigen during its trafficking through various intracellular compartments. More recent structures of human and mouse CD1d revealed a complex hydrogen-bonding network responsible for the orientation and the high affinity binding of lipid antigens inside the groove [9-14](**Figure 1**, right panel). As shown for CD1b, CD1d molecules produced in insect cells contain phosphatidylcholin (PC) as stabilizer lipid [12]; also in the CD1d crystal structures of Zajonc [15] and Wu [11] was identified a lipid in the A' pocket consisting of an endogenously acquired palmitic acid. This finding demonstrated that, also for CD1d, relatively short ligands can be accommodated in the A' pocket toghether with stabilizing lipids. It is interesting to speculate that these spacers could have the function of stabilizing the whole structure during protein maturation and trafficking through the different cellular compartments.

The crystal structure of CD1e is not available yet. Like other CD1 molecules, CD1e is noncovalently associated with β2m. Its α-chain is cleaved between the α_3 and the transmembrane domains in late endosomal compartments, generating soluble CD1e, which is the CD1e active form [16, 17]. A recent model suggests that CD1e binds glycolipids in a central cavity, similar to the binding of other CD1 members [16].

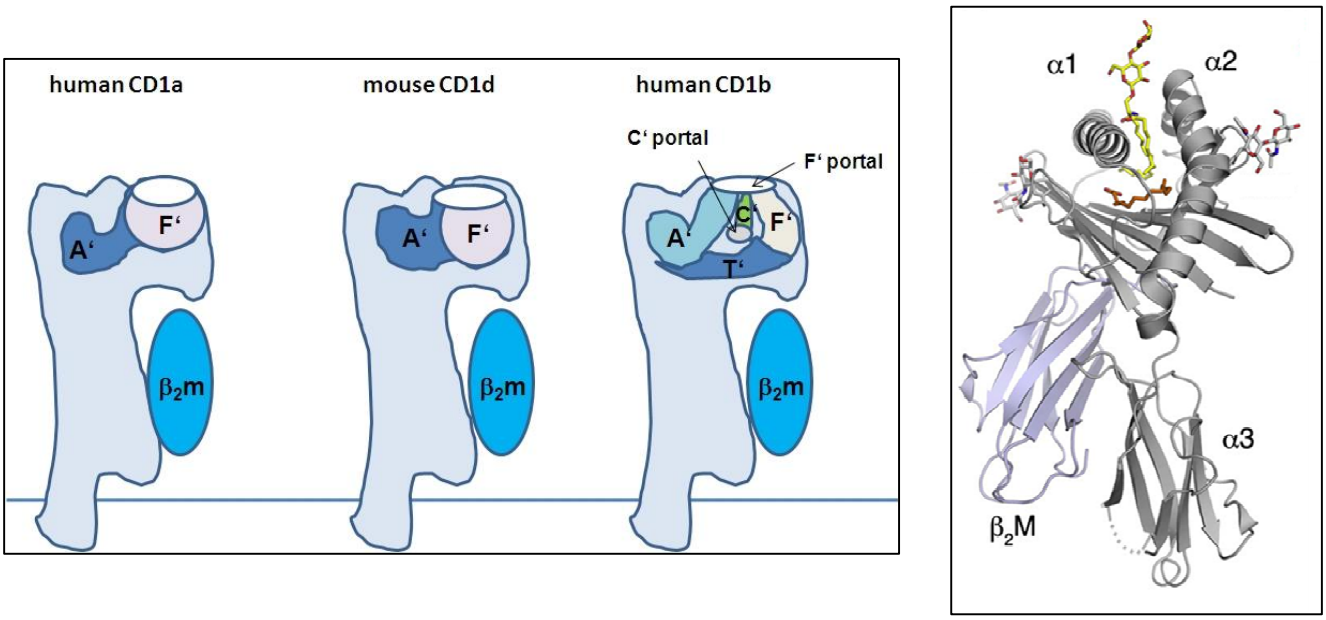


Figure 1. Schematic representation of CD1 structures (left panel). Structure of CD1d bound to αGalCer (right panel).

1.2.2 Tissue distribution of CD1 molecules

MHC Class I and MHC Class II molecules distribution among cells reflects the different effector functions of the T cells that recognize them. MHC Class I molecules are expressed on all nucleated cells, thus allowing the recognition of infected cells by cytotoxic CD8 T cells. MHC class II molecules are expressed only

by professional antigen presenting cells (APC) and by thymic stromal cells. Recognition of MHC II:peptide complexes by CD4 T cells induces the full functional activation of the APC.

CD1 molecules, although structurally similar to MHC Class I molecules, are expressed only on cells of the hematopoietic compartment, thus resembling MHC Class II molecules expression.

Group 1 CD1a-, -b and -c molecules are expressed on professional APCs and on DP $CD4^+CD8^+$ cortical thymocytes (in lower amount on SP thymocytes)[18, 19]. Langherans cells express CD1a, CD1c, but not CD1b [20]. While CD1a and CD1b peripheral expression is restricted to DCs, CD1c is also expressed by B cells (50% of peripheral blood B and marginal zone B cells)[21]. During differentiation of monocytes with GM-CSF, expression of CD1a, CD1b, and CD1c is induced and either upregulated (CD1b and c) or downregulated (CD1a) following DC maturation [22, 23]. Interestingly, group 1 CD1 molecules are not expressed on the surface of macrophages, thus rendering cross-priming and subsequent presentation by DCs essential in the case of infection by intracellular pathogens like *M. tuberculosis*.

Human and mouse CD1d molecules are expressed by most cells of hematopoietic lineage, although the highest levels of expression are found on APC, like B cells and monocytes. hCD1d is expressed by cortical thimocytes, is downregulated in medullary thymocytes and is absent on naïve peripheral T cells; hCD1d is re-expressed upon activation of peripheral T cells [24]. In mice, thymocytes, thymic stromal cells and thymic DCs express CD1d molecules [25, 26], together with monocyte-derived macrophages and DCs, and dermal DCs [25, 27-29]. In the lymph node, hCD1d is expressed on DCs in the paracortical T cell zones and on mantle zone B cells, but not on cells of the germinal center [24]. High mCD1d expression is also detected on splenic marginal zone B cells [25]. hCD1d and

mCD1d have also been detected in different cells, like epithelial cells, hepatocytes, and keratinocytes [19]. hCD1d is also expressed on several tumors[30-32].
Expression of CD1d molecules can be enhanced by cytokines, such as IFNβ, IFNγ and TNF-α and by Toll Like Receptor (TLR) 2 and TLR4 ligands [33]. The Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) controls CD1d expression by triggering retinoic acid synthesis in human DC [34]. Several pathogens can modulate CD1d expression levels. In infections with *Leishmania infantum* CD1d levels are upregulated [33], whereas Kaposi sarcoma-associated herpes virus [35] and HIV [36] downmodulate the expression of CD1d. Furthermore, herpes simplex virus-1 (HSV-1) and vesicular stomatitis virus (VSV) downregulate CD1d expression by suppressing CD1d recycling [37].
CD1e localizes to the Golgi of immature DCs, then in the lysosomes upon maturation, where it is cleaved into a soluble form [38]. CD1e never reaches the plasma membrane. Its strict intracellular localization excludes any direct interaction with T cells, thus precluding any role as antigen presenting molecule. CD1e, instead, is required for lysosomal degradation of phosphatidylinositol mannoside $(PIM)_6$ and subsequent activation of CD1b-restricted T cells [16].

2. Assembly, trafficking and loading of CD1 molecules

2.1 ER assembly

CD1 assembly and N-glycosylation takes place in the endoplasmic reticulum (ER). Newly synthesized CD1 molecules are associated to the calnexin and calreticulin chaperones [39] which bind β-2m-free CD1d heavy chains, but not β-2m-bound. The opposite occurs with MHC class I molecules. CD1d, unlike CD1b, may also traffic to the plasma membrane in the absence of β-2m [40]. Association with the thiol reductase ERp57 is important for the formation of disulfide bonds within the

CD1 heavy chain prior to assembly with β-2m [41]. The fully oxidized CD1d heavy chains dissociate from the chaperones, and the majority of CD1d heavy chains bind β-2m before exiting the ER [42]. Assembly of CD1 molecules is independent of transporter associated with antigen processing (TAP), as expression of CD1 is not affected by TAP deficiencies [43].

Before assembly, it is possible that CD1 molecules become loaded with self ER-resident lipid ligands. Human and mouse CD1d assemble in the ER with cellular phosphatidylinositol (PI)[44]; and nascent CD1d associates with phosphatidylcholine (PC)[12]. Two recent papers attempted to identify which lipids are bound to hCD1d molecules. One study described 177 lipid species and among these lipids, glycerophospholipids and sphingolipids were identified [45]. Using a different approach, namely the analysis of a soluble secreted hCD1d and of ER-retained or recycling hCD1d, a second study confirmed that phosphatidylcholine is the most abundant lipid bound to CD1d, together with sphingomyelin and lysophospholipids [46]. Microsomal triglyceride transfer protein (MTP) is an ER-resident LTP that is essential for lipidation of apolipoprotein B (apoB) and thus for assembly of very-low density lipoprotein (VLDL) and chilomicrons [47]. MTP is critical for proper assembly and antigen presentation function of CD1d, probably by facilitating the loading of the first endogenous lipid onto CD1d in the ER [19, 48, 49]. MTP is also involved in the functioning of group 1 CD1 molecules, by regulating lipid antigen presentation [50].

2.2 Trafficking

After exiting the ER, CD1 molecules traffic to the cell surface following the secretory pathway before being re-internalized into the endosomal compartments. The presence in CD1 -b, -c and -d monomers of a cytoplasmic sorting tyrosine

domain (YXXZ, where Y is a Tyr, X, any amino acid and Z a bulky hydrophobic residue) allows the binding to the specific adaptor protein (AP) complex AP-2. AP-2 is a component of clathrin-coated pits and induces endocytosis of CD1 molecules and other transmembrane cargo proteins [51].

Trafficking of the different CD1 molecules trough the endocytic compartments mirrors their capacity to sample lipid antigens which, according to their chemical properties, penetrate different endosomal compartments.

CD1a does not contain any apparent sorting motifs in its cytoplasmic tail. CD1a molecules undergo a cycle of internalization into early/sorting endosomes followed by early/recycling endosomes, where they sample distinct sets of antigens before returning to the cell surface[52, 53]. Recently it has been shown that CD1a associates with the invariant chain. This interaction facilitates CD1a recycling and seems to regulate CD1a antigen presentation [54, 55]. Also CD1c molecules have been shown to traffic to early recycling endosomes and co-localize with early recycling compartment markers [52, 56] where CD1b is almost absent [52]. Instead, CD1b is found in late endosomal/lysosomal vesicles, including the MHC class II compartments (MIIC), and colocalizes with the Lysosome-Associated Membrane Protein-1 (LAMP-1)[52].

CD1b and CD1d molecules both recycle in late endosomal/lysosomal compartments. They can both recycle via association with the MHC class II-Ii complex [57] and it has been proposed a role for the adaptor protein complex-3 (AP-3) in their recycling [58-61]. CD1c is also present in the lysosomes [62], but does not bind to AP-3 [58]. mCD1d is stable through all endocytic compartments, differently by other CD1 molecules. Probably this reflects the fact that CD1d is the only CD1 molecule present in the mouse and therefore it has evolved and acquired the capacity to sample lipids in different compartments [63](Brenner 2009, 5th NKT and CD1 meeting, Kamakura, Japan).

CD1e trafficking is different from other CD1 molecules, as it does not reach the plasma membrane, and maturation of DC modifies its localization [38]. In immature DC, membrane-bound CD1e is localized in the Golgi stalk, and upon maturation traffics to late endosomal compartments where it becomes soluble [16, 17]. The cytoplasmic domain of CD1e consists of 53 to 61 amino acids, and does not contain any specific targeting motif, but is essential to its intracellular retention and Golgi accumulation [64]. CD1e traffic to LE/lysosomes seems to be facilitated by ubiquitination of the cytoplasmic domain, a phenomenon increased upon DCs maturation [64].

2.3 Antigen processing and loading

Several events have to take place and coordinate to allow the loading of CD1 molecules with the correct lipids. These events occur mainly in intracellular compartments, where lipid metabolism and endocytic pathways intersect. Catabolic enzymes and endosomal proteins are key players in the successful loading of CD1 molecules: deficiency in one of these proteins impairs deeply CD1-mediated antigen presentation.

To be efficiently loaded onto CD1 molecules, exogenous lipid antigens must be first internalized. Lipids are insoluble in aqueous phase, therefore many lipids bind specifically to cell surface receptors. Since the majority of circulating lipids are transported as soluble complexes bound to lipoproteins, receptors that bind lipid particles such as the low-density lipoprotein receptor (LDL-R), the LDL-R-like protein (LRP) and scavenger receptors might also play an important role in the lipid uptake [65]. Cell surface receptors that bind glycans such as C-type Lectins are also involved, for example, in the uptake of LAM [66].

Another way how lipid antigens may be internalized is via incorporation into the plasma membrane, to be taken up as part of general endocytic processes. These mechanisms of internalization involve rafts, caveolae, and clathrin-coated pits [67]. Extracellular lipids are internalized by receptor-mediated pathways into clathrin-coated vesicles, while membrane-associated lipids are internalized through different routes. The membrane associated sphingomyelin and sulfatide are internalized through clathrin-coated vesicles and reach lysosomes where they are degraded. Instead other glycolipids, including lactosylceramide, globosides and the ganglioside GM1 and GM2 are internalized through a clathrin-independent mechanism, which involves also caveolae. These glycolipids rapidly merge in early sorting endosomal compartments [68], while caveolae-derived lipid domains do not mix with domains derived from clathrin-coated pits [68-70].

Lipids can be also traslocated from one leaflet of the membrane belayer to the other: enzymes like flippases, scramblases and members of the ABC transport system are involved in these processes [71]. Finally, cells can take up lipids from apoptotic bodies from infected cells. This mechanism is particularly important when infected cells do not express CD1 molecules or die as a consequence of infection: lipid antigens can be taken up by different APCs and can be efficiently and simultaneously presented with peptides [72].

Once internalized, lipid antigens sort on the basis of their physical properties. The lipid distribution across membranes results from a continuous movement of lipids between the two monolayers. For example, neutral and acidic lipids that have their charge neutralized by protonation move quickly between leaflets. By contrast, the spontaneous transbilayer movement of lipids with charged head groups is slow [71]. In some cases, localization in cholesterol or sphingolipid-rich microdomains contributes to the immunogenicity of the lipid antigens [73]. Lipids have the capacity to selectively traffic through the membranes of different cellular

compartments and to reach the same organelles where CD1 molecules are present. The length of the alkyl chain contributes to the efficient sorting of the lipids through lysosomal membranes: GlucosylMonomicolate (GMM) with long alkyl chains traffics to late endosomes where efficiently loads on CD1b, while a less immunogenic variant with a short alkyl chain loads on CD1b on the cell surface [74].

Similar to proteins, lipids undergo partial degradation in late endosomal compartments, where they are processed to smaller molecules compatible with CD1 binding and TCR interaction. However, not much is known about the enzymes involved in the generation of immunogenic lipids. To date, three glycosidases have been implicated in CD1 lipid processing: α-galactosidase, β-hexosaminidase and acidic α-mannosidase. The presence of α-galactosidase in the lysosome is important for the removal of galactose from the synthetic disaccharide galactose (α1-2)GalCer to generate αGalCer, which is recognized by iNKT cells cells [75]. Hexosaminidase-B (HexB)[76] and acidic mannosidase [16, 77] are two lysosomal hydrolases that generate immunogenic glycolipids after digestion of larger substrates. However, not all antigenic lipids require processing to become antigenic. Large glycolipid antigens containing up to eight carbohydrate moieties, like the ganglioside GQ1b, can be immunogenic without processing [78].

Lipid degradation occurs in specific localizations inside the organelles. Glycolipids can be degraded when inserted in the multivesicular membranes which have a lipid composition different from the limiting membrane. Glycolipids inserted inside the lysosomal limiting membrane are protected from digestion by a thick glycocalix. Multivesicular membranes contain the negatively charged lipids BMP (bis-(monoacylglycero)-phosphate) and phosphatidylinositol-3-phosphate [79]. The chemical properties of these lipids can favour the access of LTPs to antigenic lipids inside the membranes. Moreover, most hydrolases bind to membranes containing

negatively charged lipids and are active at a pH<5.0. In addition, the enzymes are efficient when the substrates are not membrane-associated but “lifted out” of the membranes by LTPs, thus assigning a pivotal role of LTPs in assisting lipid antigen presentation. Lysosomes (but also other organelles) are therefore the perfect environment for the functional interaction between processing enzymes and LTPs. Here the CD1 proteins and the lipids encounter and generate, at the optimal pH, functional antigenic complexes.

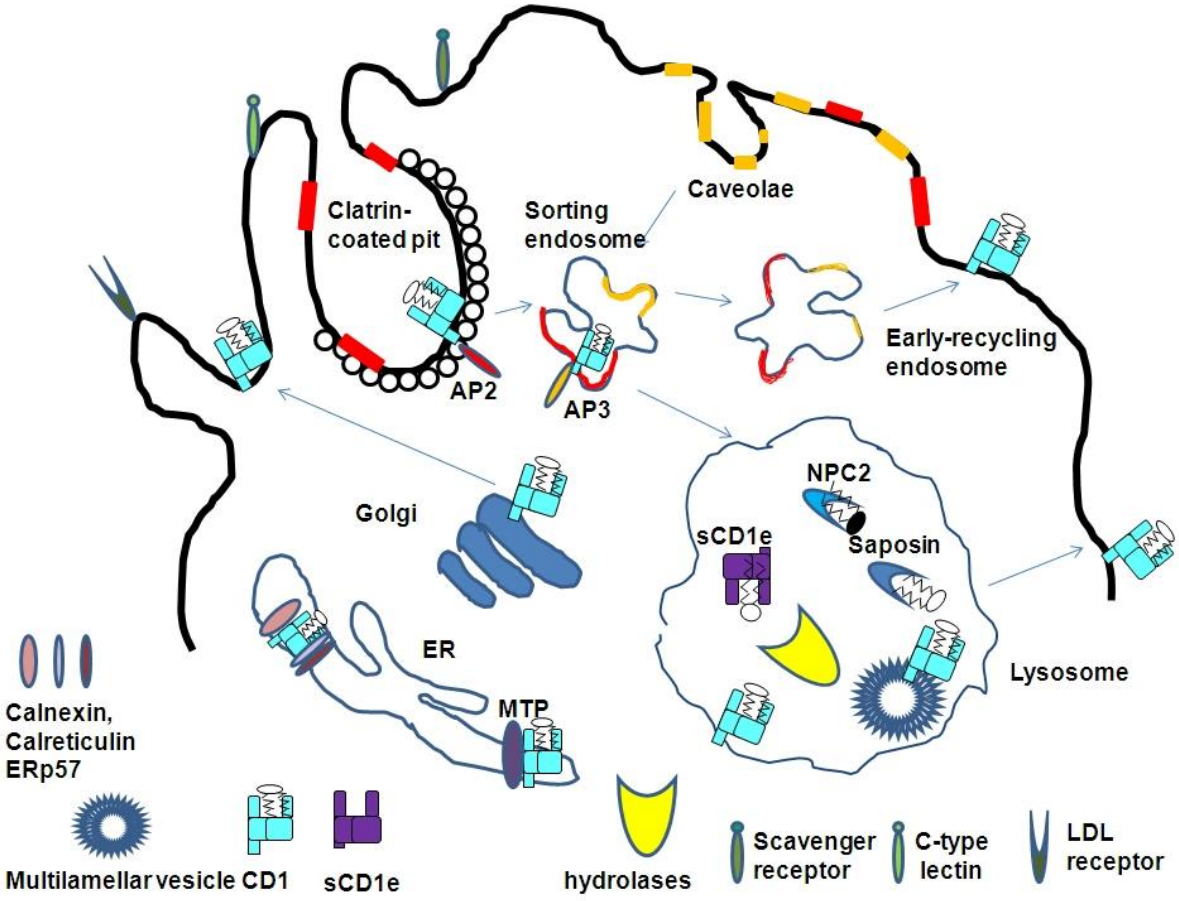


Figure 2. Schematic representation of CD1 assembly, trafficking and loading. Assembly of CD1 molecules takes place in the ER with the help of chaperones named calnexin, calreticulin, and ERp57. In the ER CD1d is loaded with self antigens with the help of MTP. CD1 associate noncovalently with β_2m, then traffic to the Golgi and to the plasma membrane. CD1b-d molecules are internalized from surface in clathrin-coated pits, associate with AP-2, and move to sorting endosomes. There, they move to late endosomes/lysosomes upon AP-3 binding. CD1a recycles only in early endosomes. In the lysosome CD1 molecules are loaded with lipids of self or microbial origin with the assistence of several LTPs such as Saposin, NPC2 and CD1e and are then exported to plasma membrane for antigen presentation.

2.3.1 Lipid Transfer Proteins

Lipids can be transported inside (and taken up from outside) the cell using different mechanisms, which can be broadly defined as vesicular and non vesicular-mechanisms. The non vesicular mechanism involves LTP. LTPs have the unique feature of being able to extract and bind a single lipid molecule from a membrane interface, thus allowing lipids to distribute among the different compartments in a fast and regulated manner.

Not all LTPs described so far showed a clear role in CD1 antigen presentation, but their function is important to maintain a proper lipid distribution inside the different compartments.

In lipid antigen presentation LTPs exert two different functions. First, they participate in CD1-lipid loading and unloading. Second, they might also facilitate extraction of exogenous lipids (generated during the degradation of microbial membranes or acquired from other cells) from the membranes and offer them to hydrolases for degradation [72, 79].

According to their cellular localization, several LTPs have been described which differently participate in transfer of the lipids through the different compartments, in assistance of processing of complex lipid antigens and in loading onto CD1 molecules. Most of the LTPs involved in CD1 presentation are localized inside lysosomes, where they colocalise with the antigen-binding site of CD1 molecules.

ER-resident LTP

Microsomal triglyceride transfer protein (MTP) is ER-resident and is involved in presentation of endogenous and exogenous iNKT cells ligands [49, 80, 81]. MTP function has been associated with transfer of triglyceride, cholesterol and some phospholipids such as PC and PI onto apoB during lipogenesis [82]. MTP

regulates the biosynthetic pathway of CD1d; in the absence [49] or silencing [80] of MTP, presentation of αGalcer is severely impaired due to a reduced CD1d surface expression. Moreover, purified MTP can transfer lipids to recombinant CD1d *in vitro* [80]. Finally, inhibition of MTP in fetal thymic organ cultures results in impaired positive selection due to the absence of properly folded self-CD1d complexes [19].

Lysosomal LTPs

Several endosomal-resident LTPs have been described, including saposins, GM2-A and NPC-2. Each LTP apparently shows a preferential binding for glycosphingolipids, although each LTP is promiscuous [83].

Saposins and GM2-A

Saposins (SAPs) A, B, C and D become enzymatically active by endosomal proteolytic cleavage of the precursor prosaposin that is secreted and internalized in late endosomes and lysosomes. The four polypeptide chains share a large degree of structural homology and have overlapping and multiple functions. All the four saposins are capable of transferring phosphatidylserine (PS) and SAP A, B and C transfer sulfatide to CD1d [84]. SAP B interacts with a variety of sphingolipids and facilitates their digestion by specific lysosomal enzymes [85]. SAP B is involved in loading of lipids onto CD1d [86]. In addition, prosaposin-deficient mice lack iNKT cells and show a defect in lipid antigen presentation [83, 87]. SAP C interacts directly with, and activates, the enzyme glucosylceramide-β-glucosidase for glucosylceramide degradation [88, 89] and is required for presentation of mycobacterial lipids by human CD1b [90].

GM2-A is ubiquitously present in endosomal compartments and is directly routed to lysosomes after synthesis. It associates with β-hexosaminidase A and assists GM2

degradation. GM2-A can also remove GT1b bound to CD1d [84]. Interestingly, GM2-A is encoded by a gene unrelated to saposins, it does not share any structural homology and its role cannot be substituted by any other SAPs.

NPC1 and NPC2

Niemann-Pick Type C-1 (NPC1) is a late endosomal/lysosomal transmembrane protein involved in the cellular transport of glycosphingolipids and cholesterol that is mutated in a majority of patients with Niemann-Pick Type C neurodegenerative disease. NPC1-deficient mice show impaired antigen presentation and iNKT cells selection [91].

The Niemann-Pick Type C-2 (NPC2) protein is a small, soluble, lysosomal protein expressed ubiquitously and secreted as a mannose-6-phosphate-tagged protein [92, 93]. NPC2 is the only LTP that has been consistently identified in the lysosomal proteome together with saposins [94]. NPC2 binds and transfers cholesterol and sphingolipids in the lysosome, but its precise function is still undefined [95]. Similar to saposins, recombinant NPC2 is able to unload lipids from and onto CD1d [96]. Deficiency of either NPC1 or NPC2 results in lysosomal storage of cholesterol, phospholipids, GM2 and GM3, glucosylceramide, lactosylceramide, sphingomyelin, and other lipids [97]. NPC2 deficiency leads to a small (-5%) complementation group of Niemann-Pick disease, which is clinically indistinguishable from NPC1 deficiency [98]. Deficiencies in NPC1 or NPC2 lead to identical phenotypes in patients, even though there are no physical relationships between the two molecules [97, 99]. However, immunological phenotypes appear to be sufficiently subtle to attribute specific functions to both NPC1 and NPC2 and separate the two complementation groups.

A detailed study on the role of NPC2 in iNKT selection and on the consequences of its absence on lipid antigens presentation is the subject of Part 1 of this thesis.

CD1e

CD1e, the fifth member of the CD1 family, has a function of LTP.

CD1e, like all the other CD1 molecules, is composed of a transmembrane α-chain that noncovalently associates with β2m. The α-chain folds in three structural α domains (α1–3), with the α1 and α2 domains delimiting a hydrophobic pocket-containing groove in which lipid ligands bind. The α-chain is cleaved in late endosomal compartments between the α3 and the transmembrane domains, and a soluble CD1e is generated, which represents the active form of this molecule [16].

So far, CD1e has been detected only in DC and CD4-CD8 DP thimocytes.

Unlike the other CD1 molecules, CD1e reaches late endosomes through sorting endosomes, without passing through the plasma membrane in either immature or maturing DC. After induction of DC maturation, CD1e disappears rapidly from the Golgi and transiently localizes in MHC-II$^+$/CD1b$^+$ vesicles [38]. Moreover, during DC maturation CD1e synthesis progressively declines and becomes undetectable after 20h post LPS maturation, while its endosomal cleavage still occurs. Complete disappearance of CD1e from Golgi compartments and relocalization in lysosomes occurs as soon as 1 hr after LPS treatment [38]. CD1e accumulates in lysosomes and persists in these compartments due to its greater stability, which has been associated with a progressive shortening of the carbohydrate side chains [38].

Although the human CD1 genes are poorly polymorphic, six different alleles have been reported for CD1E gene [100-102]. Among individuals from diverse ethnic backgrounds, alleles 1 and 2 display a frequency of 49 and 51%, respectively [100], whereas the four other alleles have been described once [102, 103]. The polymorphic nucleotides are located in exons 2 or 3 of the CD1E gene, encoding the α1 and α2 domains, respectively [100-102]. Immature CD1e displays an N-terminal propeptide ten amino acids long which is cleaved in acidic compartments

and is absent in the mature, active CD1e molecule. The role of the propeptide is to assist the association inside ER of newly synthesized CD1e with β-2 microglobulin, since its absence leads to a slower assembly of the complex [104]

The function of endosomal soluble CD1e is poorly defined. CD1e may facilitate the processing of complex glycolipids by offering them for partial degradation. This has been demonstrated by investigating the CD1b-restricted T-cell response to mycobacterial hexamannosylated phosphatidyl-*myo*-inositols (PIM_6)[16]. PIM_6 becomes antigenic and is recognized by CD1b-restricted T cells, when the six α-D-Man*p* units, are processed in the endocytic pathway into PIM_2 containing only two α-D-Man*p* units. PIM_6 degradation is exerted by lysosomal mannosidase and requires the presence of soluble CD1e to assist in the process [16].

Part 2 of this thesis describes novel and important functions of CD1e in lipid antigen presentation.

Serum LTPs

ApoE

Apolipoprotein-E (ApoE) is present as a free LTP in serum. Nevertheless, some studies implicated ApoE (and the LDL receptor) in the CD1d-dependent presentation of exogenous lipid antigens [105]. In the experimental model of (α1-2)digalactosylceramide, ApoE is necessary for its efficient delivery into lysosomal compartments where it is processed into the iNKT cell agonist αGalCer and loaded onto CD1d [105]. Uptake of ApoE-bound glycolipids is mediated by LDL receptor (LDLr), since LDLr KO mice have an impaired response to (α1-2)digalactosylceramide [105]. Further analysis revealed that ApoE renders the process that drives iNKT cells activation more efficient [105]. Finally, ApoE-deficient mice show a reduced number of iNKT cells [105].

Cytoplasmic LTPs

Lipids are not uniformly distributed inside cells, and their preferential localization is essential to many cellular functions, including membrane trafficking, cell signaling, apoptosis and mitosis. Cytoplasmic LTPs mostly facilitate and regulate transport of lipids among different organelles and concentrate (or exclude) them from specific subcellular compartments.

Glycolipid transfer proteins

Glycolipid transfer proteins (GLTPs) are small, soluble, ubiquitous proteins characterized by their ability to accelerate intermembrane transfer of glycolipids. GLTPs show specificity for both sphingoid- and glycerol-based glycolipids, but with higher specificity for lipids where the initial sugar residue is β-linked to the hydrophobic lipid backbone. GlcCer and GalCer, which are synthesized on the cytosolic side of Golgi and ER, respectively [106] are accessible to GLTPs. Moreover, GLTPs can also localize to nuclei and mitochondrial membranes, other than the lumen of the Golgi and the external face of the plasma membrane [107-109]. Crystal structures are available for human GLTPs [110], free or bound to αLacCer [110, 111], which show a two layer, α-helical topology with a single GSL binding site, composed by a surface recognition center for the sugar headgroup and an hydrophobic tunnel in which the lipid moiety is accommodated.

Phospholipids transfer proteins

Different classes of mammalian phospholipids-transfer proteins (PL-TPs) are known such as phosphatidilcoline transfer proteins (PC-TP) and phosphatidylinositol transfer protein (PI-TP). The three dimensional structure of these proteins has been elucidated, showing a hydrophobic cavity suited to accommodate lipid ligands [112, 113].

PC-PT belongs to the steroidogenic acute regulatory protein related transfer (START) domain superfamily of hydrophobic lipid binding proteins. Other LTPs are involved in the transport of cholesterol and ceramide such as MLN64 and StAR [114]. PC-TP is involved in the transport of PC from ER, the site of biosynthesis, to other subcellular organelles deficient in PC [115]. Moreover, PC-PT co-operate with plasma LTPs to remove excess of phospholipids from the plasma membrane [116]

Mammals express two PI-TP proteins, α and β, which are highly homologous. They can both transfer PI and PC across membranes, and PI-TPβ can also transfer sphingomyelin (SM). PI-TP proteins are involved in regulation of the signaling pathways in which PI is involved, transferring PI from intracellular stores to the plasma membrane as part of the receptor-controlled phosphoinositide signaling pathway [117]; PI-TPs are also involved in the activation of a PI-specific phospholipase A which is important in the generation of an eicosainod-like factor important for cell growth and survival [118].

Golgi transfer proteins

The Golgi Complex (GC) represents the central node in the biosynthesis, transport and sorting of proteins and lipids. There are several LTPs that reside in the GC, including Ceramide Transfer Protein (CERT), oxysterol binding protein (OSBP), and phosphoinositol 4-phosphate adaptor protein-2 (FAPP2), which all share a common domain organization. These LTPs bind lipids, such as ceramide, cholesterol and glycolipids, that have crucial structural and signaling roles at the interface between the late-Golgi compartments, the plasma membranes and endosomes. CERT, OSBP and FAPP2 all share a common homology domain at the N-terminus, and an additional lipid transfer domain at the C-terminus [119].

Ceramides are amides of fatty acids with long-chain (usually between 14 and 24 carbons) bases. Sphingosine is the most common base among mammals. The acyl group of ceramides is variable and is generally a saturated or monounsaturated long-chain fatty acid [71]. Sphingomyelin is found in all cell membranes, and is composed of a ceramide with additional phosphate and choline. Addition of glucose or galactose to a ceramide leads to glycosylceramides.
CERT transfer activity varies according to the chain length of the ceramide fatty acid [120], with preference for C16-18 long acyl chains ceramides. CERT mechanism of action is to extract ceramide from the cytoplasmic side of ER membranes (where it is synthesized) and deliver it to the cytoplasmic side of Golgi membranes, where it is converted into SM.
OSBP1 has a function similar to CERT. It binds with high affinity to oxysterols and cholesterol and acts at the interface between the ER and Golgi, traslocating lipids from a cytosolic compartment to the GC membranes when high levels of cholesterol and oxysterols are present [121]
FAPP2 shares an homology domain with GLTP and accumulates at the TGN-exit sites, where transport carriers destined for the plasma membrane emerge as tubular protrusions [122]. Human FAPP2 is important in the synthesis of complex glycosphingolipids and couples it with their export to the cell surface, thus determining the lipid identity and composition of the plasma membrane [122].

All these cytoplasmic LTPs bind and transfer lipids that can possibly interact with CD1 molecules. Nevertheless, none of these proteins showed an immunological function so far or a direct involvement in assistance to lipid antigen presentation.

SCP2

Sterol carrier protein-2 (SCP-2) is a ubiquitous 58 kDa protein known for more than three decades as “non specific lipid transfer protein” for its broad lipid binding capacity. SCP-2 shows a very complex gene structure, undergoes post-translational processing and is distributed in different organelles [123, 124]. The SCP-2 gene has two initiation sites coding for proteins that share a common 13 kDa C-terminus: one site encodes the 58 kDa SCP-x which is partially post-translationally cleaved to a 13 kDa SCP-2 and a 45 kDa protein; a second site codes for 15 kDa pro-SCP-2 which is post-translationally cleaved to 13 kDa SCP-2 that is identical to that derived from cleavage of SCP-x. Very little is known regarding how the relative proportions of the two transcripts are regulated [124]. Although all three proteins contain a C-terminal SKL peroxisomal targeting sequence, as much as half of total SCP-2 is localized outside the peroxisome, namely in mitochondria, endoplasmic reticulum and cytoplasm and never in lysosomes and Golgi [125-127]. Recently, a 12.9 kDa SCP-2 form has been detected in nuclear extracts of transfected cells and shown to posses DNA binding activity [128]. Increasing evidence suggests that the 58 kDa SCP-x and 45 kDa proteins are peroxisomal 3-ketoacyl-CoA-thiolases involved in the β-oxidation of branched chain fatty acids. The secondary and tertiary structure of the 13 kDa SCP-2 has been resolved [129]. The N-terminal 32 amino acids form an amphipatic α-helical region, one face of which represents a membrane-binding domain. Positively charged amino acid residues in one face of the amphipatic helices allow SCP-2 to bind to membrane surfaces containing anionic phospholipids. The hydrophobic faces of the N-terminal amphipatic α helices along with β strands 4, 5 and helix D form a ligand binding cavity able to accomodate multiple types of lipids [130]. The 13 kDa SCP-2 is the LTP capable of binding and transfering fatty acids, fatty acyl CoAs, cholesterol and phospholipids. SCP-2

mediates lipid intermembrane transfer by interacting with anionic phospholipid-containing membranes through positively charged amino acid residues in its N-terminal amphipatic helical domain. The hydrophobic faces of the N-terminal domain form a lipid-binding cavity, which can accommodate fatty acids, fatty acyl CoA, cholesterol, phospholipids and isoprenoids [124]. *In vitro* and *in vivo* studies showed that recombinant SCP-2 enhances intermembrane transfer of cholesterol, phospholipids, and glycolipids and activates enzymes involved in fatty acyl CoA transacylation [123, 124]. *Scpx/proScp2* knockout (*Scp2*$^{-/-}$) mice have been generated which completely lack *Scpx/proScp2* gene products [131]. These mice develop normally but have several marked alterations including peroxisome proliferation, hypolipidemia, decreased biliary lipid secretion, impaired hepatic lipid metabolism, and high serum levels of methyl-branched fatty acids like phytanic and pristanic acid [131]. These defects are possibly ascribed to inefficient lipid transfer and import into peroxisomes, and to defective catabolism of methyl-branched fatty acyl-CoA.

Part 3 of this thesis describes a novel immunological function of SCP-2. We show that this cytoplasmic LTP can directly influence lipid antigen presentation and iNKT cells selection by altering the correct transport of lipid antigens precursors.

2.4 Lipid Antigens

Several lipid antigens have been characterized (**Table 1**). The structures of these antigens are greatly variable, thus making very difficult to classify them. According to their origin they can be divided into two groups, namely endogenous (or eukaryotic) and exogenous (or microbial) antigens.

2.4.1 Endogenous lipid antigens

Endogenous lipids stimulating CD1 restricted T cells belong mostly to two prominent families: sphingolipids and phospholipids. Human T cell clones have been found reactive to the sphingolipids gangliosides, sphingomyelin and sulfatides [78, 132], and to the phospholipids phosphatidylcholin (PC), phosphatidyletanolamine (PE) and to phosphatidylglycerol (PG)[133]. In the mouse, T cells specific to the ganglioside GD3 are present after immunization with GD3-secreting melanoma cells [134]. Also mouse iNKT cells react to PI, PE and PG [135]. iNKT cells from human and mouse react to the self molecule iGb3 [76], which however has not been isolated from human tissues.

Glycolipids are mostly composed of a hydrophobic ceramide and a hydrophilic oligosaccharide chain. The structure of the hydrophobic lipid moiety of glycosphingolipids contributes greatly to immunogenicity: as it is shown in the model of GM1 recognition, the acyl tail buried into CD1b makes stable interactions with the CD1 molecule [78]; the length of the acyl chains, as well as their degree of saturation and rigidity, also play a role [133, 136].The polar head of lipid antigens has two important functions: it makes direct interactions with the α-helices of the CD1 molecule, thus contributing to the formation of stable CD1-lipid complexes, and directly interacts with the TCR. Thus, the specificity of recognition depends on the presence of antigen structures that make both types of interactions.

Sulfatide, a lipid abundant in the brain, binds to all human CD1 molecules [137] and also to mouse CD1d [138], thus suggesting that different CD1 molecules can bind to an overlapping lipid repertoire [137].

Different cell types and tissues synthesize and accumulate glycosphingolipids with modified ceramide structures [139], suggesting that only some tissues express an immunogenic variant of self lipid antigens. In addition, modifications of lipid structures and the type of fatty acids used (and therefore the possibility to be or not

immunogenic) may occur during changes in lipid metabolism, for instance during ontogenesis, cell growth, viral transformation and oncogenesis [140]. It is possible therefore that, in these particular metabolic conditions, the immune system is confronted with unusual accumulated GSLs to which it is not tolerant. This mechanism could be evolved to alert the immune system and to initiate an immune response [141].

Table 1. Summary of identified CD1 lipid antigens

Antigens	**Origin**	**Restriction**
TCR -αβ		
Mycolic acid	*M. tuberculosis*	CD1b
Glucosyl monomycolate (GlcMM) and Glycerol monomycolate (GroMM)	*M. tuberculosis*	CD1b
Lipoarabinomannan	*M. tuberculosis*	CD1b
Phospatidylinositolmannoside	*M. tuberculosis*	CD1b
Diacylsulfoglycolipid	*M. tuberculosis*	CD1b
Mannosyl-b-1-phosphomycoketide	*M. tuberculosis*	CD1c
Didehydroxymycobactin	*M. tuberculosis*	CD1a
Gangliosides GM1, GD1a, GD1b, GT1b, GQ1b	Self	CD1b
Sulphatide	Self	CD1a, CD1b, CD1c mCD1d, hCD1d
Phosphoglycerolipids (PI, PE, PG)	self/bacteria/ plants	CD1a, mCD1d, hCD1d
Mannosyl-β1-phosphodolichol	Self	CD1c
Penthametyl-dihydrobenzofuran sulphonate	Chemical	hCD1d
TCR- γδ		
Unknown antigen on human APC	Self	CD1c
iNKT		
α-Galactosylceramide	*A. mauritianus*	mCD1d, hCD1d
α-Galacturonosylsilceramide and α-Glucuronosylsilceramide	*Sphingomonas spp*	mCD1d, hCD1d
BBGlI and BBGlII	*Borrelia burgdoferi*	mCD1d, hCD1d
Phosphatidylinositoltetramannoside	*M. tuberculosis*	mCD1d, hCD1d
Isoglobotrihexosylceramide	Self	mCD1d, hCD1d
Ganglioside GD3	Self	mCD1d, hCD1d

2.3.2 Exogenous lipid antigens

In 1994 it was described for the first time that a microbial lipid antigen, namely mycolic acid, could stimulate specific T cells [142]. Several lipid antigens of microbial origin, presented by all CD1 molecules, have been identified so far. Interestingly, most of the T cell lipid antigens described until now, derive from mycobacteria, even if lipids with the potential to bind to CD1 molecules are produced by many different pathogens [143]. It is not clear which are the characteristics that render a lipid immunogenic, whether lipids have to be shed by living bacteria or instead lipids included in the bacterial cell wall are more stimulatory. Moreover, it is still debated whether the response to lipids that are mostly present in virulent bacterial strains is important for the induction of protective immunity [71].

Here, lipid antigens are described according to their capacity to form stimulatory complexes with individual CD1 molecules.

The only characterized exogenous CD1a-restricted lipid antigen is Didehydroxymycobactin (DDM)[144], a mycobacterial lipopeptide. DDM is a precursor of mycobactins which are mycobacterial products involved in iron scavenge and supply to bacterial cell.

All CD1b-restricted microbial lipid antigens described so far derive from the mycobacterial cell envelope and have no close structural homologues in mammalian cells. They include lipoarabinomannan (LAM), lipomannan (LM), phosphatidylinositol mannosides (PIMs)[16, 77, 145], Glucosyl monomycolate(GlcMM)[146], and sulfoglycolipids (SGLs)[147].

Mycobacterial glycosylphosphatidylinositols are CD1b-restricted lipid antigens with a phosphatidyl inositol core [77]. One group, named PIMs, contain varying numbers of mannose residues, are produced only by actinomycetes [148, 149] and are major components of the outer leaflet of the mycobacterial plasma membrane.

LM and LAM are multi-glycosylated extensions of the PIMs. As already mentioned, CD1e contributes to immunogenicity of PIMs by trimming the non antigenic PIM_6 into the antigenic form PIM_2[16].

SGLs have recently been identified as CD1b-restricted antigens recognized by *M. tuberculosis*-specific T cells [147]. They are mycobacterial cell envelope molecules that possess a trehalose 2' sulfate core acylated by two to four fatty acids. These fatty acids can be palmitic (C_{16}), stearic (C_{18}), hydroxyphtioceranoic (C_{32}), or phtioceranoic (C_{32}). The extent of their expression in *M. tuberculosis* correlates with strain virulence in guinea pig models [150]. The active antigenic species have been shown to be diacylated sulfoglycolipids (Ac_2SGLs) containing hydroxyhtioceranoic and either palmitic or stearic acid chains. Presentation of Ac_2SGLs is efficient during infection, and induces production of IFNγ by specific human T cells and subsequent killing of intracellular bacteria *in vitro* [147, 151]

CD1c-restricted antigens described so far are also derived from mycobacteria. The first identified was shown to be closely related to mannosyl β-1-phosphomycoketides (MPMs). They are also referred to as mannosyl phosphoisoprenoids (MPIs), because of their structure containing a single fully saturated alkyl chain similar to isoprenoid lipids, with methyl branches at every four carbons. Their structure is close to mammalian mannosyl phosphodolichols (MPDs)[152].

α-galactosylceramide (αGalCer) is a glycosphingolipid derived from marine sponges that stimulates iNKT cells in a CD1d dependent manner [153](**Figure 3**). αGalCer is not present in mammals because the anomeric carbon of the sugar galactose bound to the sphingosine base has α-linkage, whereas mammalian glucosylceramides possess only a β-linkage. The broad reactivity of iNKT cells to αGalCer allowed the development of the cytofluorimetric staining procedure using dimerized (or tetramerized) CD1d molecules loaded with αGalCer [154]. Several

synthetic analogs of αGalCer with different stimulatory capacity have been described [10, 155]. Modifications of the length and degree of insaturation of the acyl chain [155] and of the sphingosine chain [156] result in a skewing of the iNKT cells response towards preferential Th1 or Th2 cytokine secretion. Recently, two structurally related bacterial antigens presented by CD1d molecules have been described, α-Glucuronosylceramide (GSL-1) and α-Galacturonosylceramide (GSL-1')[157-159], Both are sphingolipids derived from the cell wall of *Sphingomonas spp*. Bacterial lipid antigens were also identified from *Borrelia burgdoferi* [160], a pathogenic bacterium causing Lyme disease. *B. burgdoferi* expresses two abundant glycolipids, that are galactosyl diacylglycerols and were named BbGL-I and II, respectively [160].

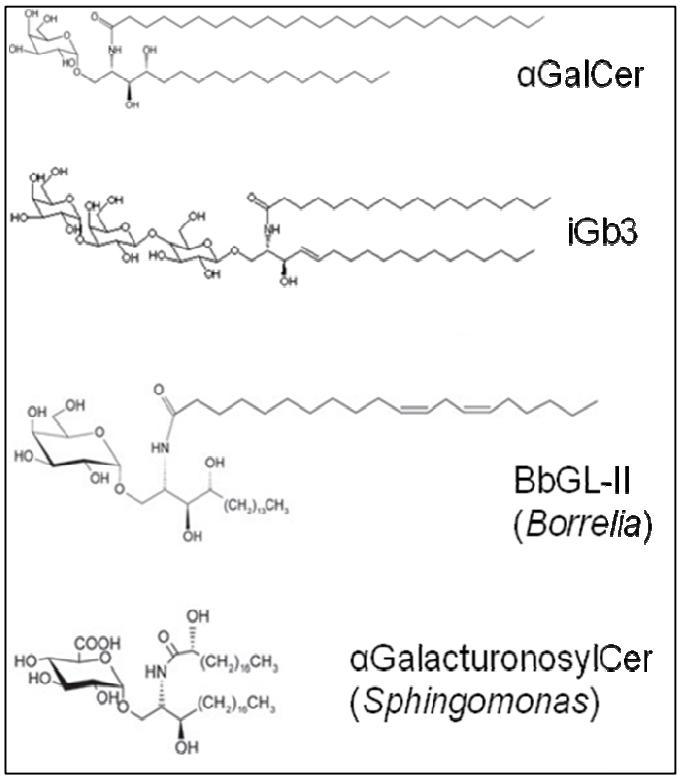


Figure 3. Structures of selected lipid antigens of self or microbial origin.

3. Lipid-specific T cells

T cells recognizing lipids in association with CD1 molecules can be divided in two groups: those restricted by group 1 CD1 molecules and those restricted by CD1d.
T cells restricted by CD1d can be further subdivided in two groups: type 1 iNKT cells, which express a semi-invariant TCR, and type 2 NKT which express a diverse TCR.
It is a matter of debate whether lipid- and peptide-specific T cells are distinct lymphocyte populations that differ in selection, priming, activation requirements and memory formation, or whether they all belong to the same lineage but differ in antigen specificity. What is known about maturation and selection of lipid-specific T cells has been almost exclusively done in mice, which express only CD1d. Therefore a detailed picture (though still incomplete) of maturation and selection is available only for Type 1 iNKT cells.

3.1 CD1-restricted (Group 1) T cells

T cells restricted by CD1a, b and c molecules play an important role in bacterial infections and autoimmune responses. TCRs of Group 1 T cells show an apparently unbiased repertoire of Vα, Vβ, Jα and Jβ genes, which are the same used by peptide-specific T cells. Very few TCRs have been sequenced, and it seems there is no particular correlation between the Vα and Vβ chains used and the type of CD1 restriction or the expression of CD4 and CD8. Analyses of the CDR3 sequences of lipid-specific clones contain template-independent N nucleotides that encode diverse sequences important for the formation of specific interactions with the lipid-CD1 complex [161, 162]. In addition, the CDR3 loops contain charged residues that can interact with acidic residues on the glycolipid antigens.

Few CD1c-restricted clones express a TCR-γδ [161, 163, 164]. Interestingly, many intraepithelial TCR-γδ recognize CD1 molecules, and the clones expressing the TCR-Vδ1 chain recognized self and exogenous lipids presented by CD1a,-c and -d transfected APC [165].

Group 1 T cells may express CD4 or CD8 co-receptors and in some cases are $CD4^-$ $CD8^-$ DN; since these coreceptors do not bind efficiently to CD1 molecules, there is not a preferential expression of CD4 or CD8.

Similarly to peptide-specific T cells, priming of lipid-specific T cells probably occurs in peripheral lymphoid organs. Dendritic cells, which express all CD1 molecules, are likely to be the best candidates for priming. After priming T cells expand, as shown in human [147, 166, 167] and guinea pigs [168, 169] mycobacterial-specific responses.

Human T cells which react to endogenous glycosphingolipids are present in the circulating blood both of healthy and MS patients [132].

Functional analyses of group 1 CD1-restricted T cell clones revealed the presence of different populations with Th1 [147, 167, 170] or Th2 [156, 171-174] cytokine profile. By using an ELISPOT assay, it was determined that in the blood of patients with multiple sclerosis the number of IFN-γ secreting lipid-specific T cells was higher than in control donors, consistent with a possible pro-inflammatory role [132]; in patients with leprosy or tuberculosis a large fraction of CD1-restricted T cells release IFN-γ, supporting an anti-bacterial role for these cells [147, 167, 170].

Lipid-specific T cells recognizing Group 1 CD1 molecules are important in bacterial infections, autoimmune responses and probably tumor immune surveillance.

Lipid-specific T cells recognize DC infected with *M. tuberculosis* and have a direct killing activity against intracellular pathogens [175]; CD1 molecules are expressed in various chronic and acute autoimmune disease. This may favor the expansion of T cells specific for self glycosphingolipids as observed in the blood of patients with

multiple sclerosis [138]. Tumors also express CD1 molecules, thus making them potential targets of tumor-specific T cells, but until now very little is known about T cells recognizing lipids expressed by tumor cells.

3.2 CD1d-restricted (Group 2) T cells

T cells restricted by CD1d can be further subdivided in two groups: Type 1 iNKT cells, which express a semi-invariant TCR, and Type 2 NKT which express a diverse TCR.

3.2.1 Type 1 iNKT cells

3.2.1.1 Phenotype and tissue distribution

Type 1 iNKT cells (also known as iNKT cells) are CD1d restricted T cells which react to the agonist αGalCer bound to CD1d and can be stained by CD1d dimers loaded with αGalCer. Mouse iNKT cells express a semi-invariant TCR, composed of the Vα14-Jα18 segments paired preferentially with Vβ8.2, Vβ7 or Vβ2 chains [176](some rare TCRs express the Vα3.2-Jα9/Vβ8 or Vα8/Vβ8 rearrangement [177]), while human iNKT cells express invariant Vα24-Jα18 rearranged chains paired with variant Vβ11 chains. Both in mouse and human, the invariant TCR-Vα rearrangement can be formed with different patterns of nucleotide deletion as well as N region additions [178]. This confirms that the iNKT-TCR selection is mediated by antigen recognition and selection.

iNKT cells in mice express the CD4 coreceptor or are CD4-CD8 DN, but never express CD8, while human iNKT cells can also express the CD8 coreceptor [177]. There are evidences that these subsets are functionally distinct [172, 179, 180], although it is not understood the role of both coreceptors, since neither CD4 nor

CD8 bind the CD1d molecule. In mouse iNKT cells, CD4 expression is probably redundant, since iNKT cells develop normally in CD4 deficient mice [181]; nevertheless, two recent studies suggested that CD4 may influence iNKT cells function [182, 183].

Phenotypically, peripheral iNKT cells show an activated/memory phenotype ($CD44^{hi}$, $CD62L^{-}CD69^{+}$)[177]. In addition, iNKT cells express inhibitory and activatory receptors typically associated with NK cell, such as NK1.1, CD122, NKG2D and members of the Ly49 family [177]. Peripheral iNKT cells display homeostatic maintenance requirements resembling those of other antigen-experienced T cells since IL-15 and IL-7, but not CD1d, are strictly required for their survival [184].

iNKT cells contribute only with a small proportion to the total lymphocyte compartment, with frequencies ranging from 0.5% to 1% in most immune organs (thymus, spleen, bone marrow, blood) and even less in lymph nodes [185], with small variations dependent on the mouse strain. Interestingly, in the mouse liver, iNKT cells can reach the 30% of the total T lymphocytes [177]. In humans iNKT cells proportions are generally lower than those of mice and can greatly vary between individuals, with percentages from less than 0.001% to over 1%, with liver and thymus the organs where NKT are more abundant [177]. This great individual variation creates difficulties in attributing a role to iNKT cells in human diseases.

3.2.1.2 Thymic development

iNKT cells development occurs in the thymus from bone marrow derived precursors. iNKT cells segregate from conventional T development at the DP thymocyte stage in the thymic cortex [186, 187]. Interestingly, expression of

particular Vβ chains appears to be important for subsequent selection of iNKT cells [188-190]. Correct rearrangement of the Vα chain allows the formation of the invariant Vα14-Jα18 iNKT cells TCR. Taken together these observations point out for a selective, rather than instructive, model for iNKT cells thymic development. Clear evidences suggest that also iNKT cells are subjected to positive and negative selection in the thymus.

Positive selection

Positive selection of conventional T cells require TCR recognition of peptide antigens presented in the context of MHC-I or -II molecules expressed by thymic cortical epithelial cells. In contrast, iNKT cells are selected by unknown self glycolipid antigens presented by CD1d on DP cortical thymocytes, as demonstrated by experiments where iNKT cell selection occurred when CD1d was expressed, under the control of the lck promoter, on DP thymocythes [191-194]. Expression of CD1d under the MHC II promoter did not rescue iNKT cells development [195], while expression of CD1d under the MHC-I promoter, although restoring the expression of CD1d on DP thymocytes, could only partially restore iNKT cells selection, suggesting that also CD1d levels play a role in selection [196]. Interestingly, epithelial cells, which mediate positive selection of conventional T cells, also express CD1d, but cannot select iNKT cells. It is possible that DP cells provide signals (and epithelial cells do not), which are uniquely required for iNKT cell development. A second non-alternative possibility is that only DP thymocytes express the correct self lipids selecting iNKT cells. Selection on DP thymocytes possibly promote some of the key features of iNKT cells; indeed, $CD4^+$ T cells selected via interaction with MHC-II on DP thymocytes rather than epithelial cells show very similar characteristics to those of iNKT cells (intermediate TCR expression, expression of CD44, NK1.1 and CD69)[197].

Furthermore, correct CD1d recycling through endocytic compartment is necessary for iNKT cell selection. CD1d has to be loaded with endogenous endosome- or lysosome- derived lipid antigen/s, and in case of altered CD1d trafficking iNKT cell selection is greatly impaired [177].

Selecting ligands

The nature of the endogenous selecting lipids remains unknown.

High affinity interactions between TCR and peptide/MHC result in deletion of developing T cells, and it has been recently determined the affinity value that defines the threshold for negative selection of cytotoxic T cells [198, 199]. Interestingly, there are some evidences that T cells subsets with regulatory capacity can be selected by high(er) affinity ligands [200]. TCR transgenic mice specific for MHC-II generate high numbers of $CD4^+CD25^+FoxP3^+$ T-regs when exposed to agonist peptides [201, 202], although this phenomenon could be due to an increased resistance to deletion by T-regs when compared to conventional $CD4^+$ cells [203]. CD8αα IELs are also generated in the presence of agonist peptide, as shown in a system of RTOC (reaggregated thymic organ culture) and TCR transgenic DP thymocytes [204]. It is not clear whether also iNKT cells can be selected by agonist ligands, and the evidence is often based on the observed memory/activated phenotype and their broad self-reactivity [177].

αGalCer, the most potent agonist for iNKT cells, was never considered a possible selecting ligand, not being present in mammalian cells; nevertheless, many possible glycosphingolipids with structural similarities with αGalCer are present in mammalian cells and were therefore carefully analyzed for their possible role in iNKT cells thymic selection. One candidate, iGb3 isoglobotrihexosylceramide, has been proposed to be the selecting ligand for iNKT cells [76], although its role is controversial.

iGb3 was identified as possible candidate in a screening of KO mice with deficiencies in enzymes responsible of the synthesis or degradation of glycosphingolipids [76]. $Hexb^{-/-}$ mice, which lack the β-hexosaminidase A and B enzymes, showed a clear block in iNKT cells development. These enzymes are responsible for the degradation of globo- and isoglobo-series of glycosphingolipids, as well as some gangliosides, in the lysosomes. The capacity to stimulate mouse and human iNKT cells and of being a relatively weak antigen when compared to αGalCer, supported the role of iGb3 as a true selecting ligand *in vivo* [76]. Several *in vivo* and *in vitro* studies supported this hypothesis [205, 206], and iGb3 also appeared to have a role in selecting the Vβ repertoire [188, 207].

Additional experiments, although not denying a role of iGb3 as iNKT cells agonist, did not support a major role of iGb3 in iNKT cells thymic selection. We (as described in detail in Part 1 of this thesis) and others [208] showed that also in other mouse models where glycosphingolipid metabolism is disrupted, iNKT cell development is impaired, even though the enzymes β-hexosaminidase have not been deliberately knocked-out and thus iGb3 metabolism is not affected. In addition, iGb3 has been very difficult if not impossible to be detected in human or mouse thymi and DC by using HPLC methods [209] or ion trap mass-spectrometry [210, 211]. Moreover, mice lacking the iGb3 synthetase gene (A3GALT2 gene) have no iNKT cell deficiency [212]. Finally, in humans iGb3 synthetase is a pseudo-gene, thus excluding that this molecule is synthesized by human cells [213]. The debate on the role of iGb3 has somehow increased the interest in other possible candidate selecting antigens, which include the weakly agonist β-glycosylceramides [214] as well as their more complex derivatives.

Negative selection

During positive selection a developing T cell recognizes a self-peptide/MHC complex with low affinity [215, 216]. Negative selection occurs when the affinity of the TCR of a developing thymocyte for a self/MHC complex is above a "safe" threshold and is therefore considered self-reactive [217, 218]. Such a high affinity interaction between TCR and self-peptide/MHC leads to distinct signals in the developing cell that trigger apoptosis and not proliferation and survival [219], thus pointing that negative selection is an active process, as opposed as the mechanism of death by neglect [217].

Although the iNKT cell TCR α-chain is invariant, there is sufficient variability in the Vβ chains to support the possibility that self-reactive cells with very high affinity for CD1d/lipid complexes arise during development [220-224]. Given the possibility that iNKT cells can be selected by agonist ligands, the question raises whether iNKT cells undergo negative selection during their development. Two studies show that the presence *in vitro* and *in vivo* of the strong agonist αGalCer during iNKT cell development completely abrogates iNKT cells development [26, 225]. These findings suggest that iNKT cells undergo negative selection (though it is possible that the strong agonist αGalCer displaced the weak selecting ligands from the complexes and therefore these models represent more a lack of positive selection than a true negative selection). Additional evidences of negative selection came from mice where CD1d was overexpressed in thymic DC in the absence of exogenously added ligands, which led to the abrogation of iNKT cells development [26]. These data suggested that the overall avidity for a natural ligand presented in the context of higher CD1d levels was so increased to induce negative selection in developing iNKT cells. Moreover, in these experiments it was also shown that DC do not mediate positive selection [191-194], and may induce negative selection, as

described for conventional T cells [226]. Interestingly, DP thymocytes can mediate both positive and negative selection [227].

It is not clear whether there is a developmental window following TCR expression during which iNKT cells can be negatively selected, as it happens for conventional T cells [228]. Recently, a transcription factor associated with negative selection of conventional T cells, Nur77 [229], has been identified on $CD24^+$ developing iNKT cells pointing out the existence of a window of susceptibility to negative selection also for iNKT cells.

Maturation of iNKT cells after positive and negative selection appears to be quite distinct from the process of conventional T cells, with iNKT cells undergoing major maturational events post-selection [230].

Thymic maturation of iNKT cells

The maturation pathway of iNKT cells can be subdivided into four different developmental stages defined by the expression of different phenotypic markers (**Figure 4**). The earliest post-DP Vα14 iNKT expresses high levels of CD24 (HSA, Heat Stable Antigen) and this maturation step is called "Stage 0". $CD24^+$ cells have a $CD44^{low/int}$ $NK1.1^-$ phenotype and some still express the CD8 coreceptor, which stops to be expressed soon after and never re-expressed. Most of these cells express CD69, suggesting that they may represent cells that have recently undergone positive selection [185]. This phenotype resembles the transitional $CD4^+$ stage of positively selected conventional $CD4^+$ and $CD8^+$ [219].

Downregulation of CD24 and CD8 allows NKT to proceed along the differentiation pathway towards the Stage 1, characterized by a $CD44^{-/dull}$ $NK1.1^-$ $DX5^-$ surface phenotype. Stage 1 iNKT cells undergo several rounds of cell division, as determined by their large size and ability to incorporate BrdU [185], and express low levels of IL-4. This expansion is followed by the expression of CD44, a marker

of T cells that have encountered the antigen, and of the T-bet mediated-expression of the β chain of the IL-15 receptor. $CD44^+$ $NK1.1^-$ $DX5^{low/int}$ iNKT cells at Stage 2 produce both IL-4 and IFN-γ. Stage 3 iNKT express CD44, DX5 and NK1.1 and produce mainly IFN-γ. Upregulation of NK1.1 expression is the hallmark of Stage 3 that correlates with a drop in proliferation. Interestingly, this upregulation can occur in the thymus or the periphery. These cells stop to divide, consistent with their entering a terminal stage of differentiation.

The precursor-progeny relationship between the different stages has not been formally demonstrated, but this developmental model fits with ontogeny data from different groups [231-233].

It has been recently proposed that, to proceed along the developmental pathway, iNKT cells have to cross two different checkpoints [230]. Checkpoint 1 corresponds to positive selection, while checkpoint 2 occurs between the Stages 2 and 3 and the successful accomplishment of this second control point is characterized by the upregulation of NK1.1. It is possible to speculate that in both these moments the TCR of the developing cells must interact with the appropriate CD1d:lipid complex, because after these checkpoints iNKT cells upregulate CD69.

During the maturation steps, after the downregulaation of the CD8 coreceptor at Stage 0, all the maturing iNKT cells express the CD4 coreceptor. In periphery, nevertheless, fully mature iNKT cells can be either $CD4^+$ or $CD4^-CD8^-$ double negative (DN). It has been demonstrated that downregulation of CD4, at least for 30-40% of maturing iNKT cells happen at the Stage 2 to 3 transition, but the meaning of this downregulation is unknown [231, 232, 234].

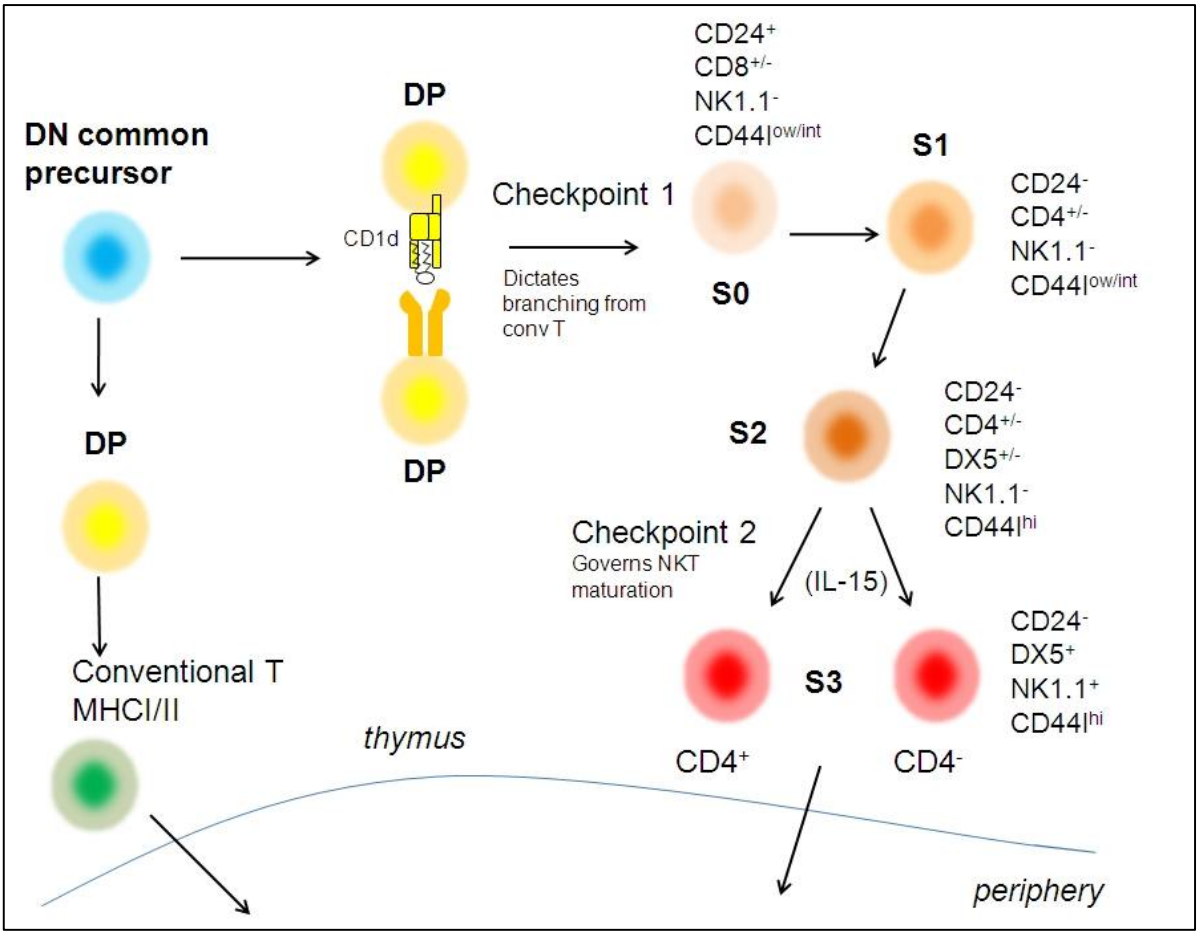


Figure 4. Schematic representation of thymic maturation of iNKT cells

Factors influencing iNKT cell development

T cell development is regulated by TCR signaling, cytokines and co-receptor signaling [219]. iNKT cell development is also regulated by these mechanisms, but also by many unique factors and in those cases where there is overlap between factors regulating T and iNKT cell development, iNKT cells often use these factors in a manner different from that of MHC-restricted T cells [185, 230].

Cytokines play a crucial role in iNKT cell development. iNKT cells upregulate the expression of the IL-2/ IL-15 receptor β-chain (CD122) during their progression from Stage 2 to Stage 3 in the thymus, indicating an important role, particularly for IL-15, in iNKT-cell maturation. Indeed, mice lacking IL-15 or CD122 are deficient in iNKT cells, whereas conventional T cells are unaffected [235, 236]. IL-15 is also important, together with IL-7, for normal iNKT-cell turnover and homeostasis [184, 237]. The transcription factor T-bet promotes the upregulation of CD122 during iNKT thymic maturation, and is therefore also important in regulation of iNKT development: T-bet$^{-/-}$ mice have a significant decrease in iNKT cell proportion and numbers, and the residual iNKT population is NK1.1^{-}, suggesting a block in NK1.1^{-} to NK1.1^{+} transition [238].

Among transcription factors, recent studies have disclosed the role of specific ones that regulate iNKT cells thymic differentiation program. PLZF (promyelocytic leukemia zinc finger, Zbtb16) is required for selection of iNKT cells [239, 240]. PLZF, a BTB-zinc finger family member, is induced after positive selection of iNKT cell precursors. Mice that are deficient in PZLF do not proceed through intrathymic expansion and effector differentiation, in periphery accumulate preferentially in lymph nodes and show marked defects in cytokine release. Also the transcription factor Erg2 (Early growth response 2), a target of the calcineurin-NFAT signaling pathway, is required for normal iNKT cells development and maturation [241]. Mice lacking Erg2 are nonviable, but Erg2$^{-/-}$ liver fetal chimeras show a strong decrease in iNKT cells. Moreover the small fraction of iNKT cells positively selected in Erg2$^{-/-}$ mice show inefficient maturation, hyper proliferate and are apoptotic either in steady state conditions or after antigen stimulation [241].

TCR signaling is also essential for iNKT cells thymic development. Successful TCR signaling requires the engagement of the Signaling Lymphocytic Activation Molecule (SLAM) family receptors and signaling through the adaptor SLAM

Associated Protein (SAP), SLAM molecules, the Src kinase *Fyn*, and downstream NF-κB [242-244].

Deficiencies in proteins involved in the TCR signaling completely abrogate iNKT cell development. Alteration of the *Fyn*-SLAM-SAP signaling pathway by deletion of one of the components selectively impairs selection of iNKT cells, but not of conventional T or NK cells. The role of SAP, which is constitutively bound to SLAM, is to recruit *Fyn* upon SLAM engagement at the cell surface, leading to at least two downstream signaling cascades: one involving NF-kB and the other involving proteins inhibiting MAPK signaling [245, 246]. Homotypic SLAM interaction, particularly SLAM1 and SLAM6, on selecting DP on one side and developing iNKT cell on the other provide unique signals that stimulate iNKT precursors[247]. Interestingly, DP thymocytes and DC from NOD mice have reduced SLAM expression, which leads to inefficient thymic selection and peripheral activation of iNKT cells [248, 249].

Alterations in proteins belonging to the NF-κB signaling pathway, both classic and alternative, also impair thymic selection of iNKT cells. The extent of iNKT cells reduction varies according to which component of the pathway is affected. T cell-targeted deletion of IKK2 (inhibitor of NF-κB Kinase 2)[250], transgenic expression of a dominant-negative form of IkBα (inhibitor of NF-κBα)[251] and RelB deficiency in stromal cells all induce a severe reduction of iNKT cells [252, 253]. On the contrary, *aly* mice which are mutated in the *Nik* kinase [252], NF-KB1$^{-/-}$, NF-KB2$^{-/-}$ mice show only a limited reduction in iNKT cells [251, 253], thus suggesting that the components of the signaling pathway contribute differently to iNKT cells selection.

3.2.1.3 Effector functions of iNKT cells

iNKT cells are functionally extremely versatile cells, having been implicated in controlling a broad array of disease conditions, such as transplants rejection, tumors, autoimmunity, atherosclerosis, allergy and infections. iNKT cells play an important role in recruiting, activating and polarizing cells of both the innate and the adaptive system by the rapid production of large amounts of cytokines together with cell-cell interactions.

Cytokine production

Due to their closeness to NK cell, iNKT cells produce molecules associated with cytotoxicity, such as perforins [172] and granzymes [254] and lyse target cells in *in vitro* assays [255]. Nevertheless, their main functional hallmark is indeed the rapid production of Th1 and Th2 cytokines, particularly IFN-γ and IL-4 [177, 256]. iNKT cells contain preformed mRNA for IL-4 and IFN-γ in their cytoplasm in steady state conditions, allowing therefore to produce and to secrete these cytokines within minutes of stimulation [257]. In addition to IL-4 and IFN-γ, iNKT cells can secrete different arrays of cytokines, including IL-2, TNF-α, TGF-β, IL-5, IL-10, IL-13, IL-21 and IL-17 [172, 258-262]. Upon stimulation, iNKT cells secrete IL-3 and GM-CSF [263]. Although it is clear that the cytokines produced by iNKT cells do influence the outcome of a diverse range of diseases, it is still unclear how iNKT cells, which simultaneously produce Th1 and Th2 cytokines, can modulate their cytokine profile to mediate the appropriate immune response [177, 264]. Current theories suggest that a combination of factors, including microenvironment, interaction with the APC, antigen structure, co-receptor signaling and differential function by iNKT cells all play a role in determining the type of secreted cytokines and the overall iNKT cell response [177].

Effect of iNKT cells on recruitment, activation and differentiation of myeloid lineage cells and activation of NK and MHC-restricted T cells

Secretion of IL-3 and GM-CSF by activated iNKT cells causes the mobilization of myeloid progenitor cells and recruitment of neutrophils to the periphery [265]. Furthermore, injection of αGalCer causes an efflux of granulocytes (mostly neutrophils) in the circulation and transiently in the liver [263] and *intranasal* (*i.n.)* administration of αGalCer leads to accumulation of neutrophils in the lungs [266]. Therefore, iNKT cell mediated recruitment of neutrophils in early innate phases of antimicrobial immune response appears to be a crucial process.

Through cognate interaction and cytokine secretion iNKT cells can promote differentiation and activation of APC. For instance, human iNKT cells can drive the differentiation of autologous PBMCs into immature DCs by secreting IL-13 and GM-CSF (following recognition of self-lipid loaded CD1d)[267]. Activated iNKT cells induce upregulation of MHC-II, costimulatory molecules and sustain the production of IL-12 in mouse and human DC [268-270]. For these effects on APC αGalCer has been proposed as adjuvant to enhance the immune response to co-administered protein antigens [271, 272].

Transactivation of NK cells by iNKT cells is rapid and potent. Ninety minutes post *intra venous* (*i.v.)* αGalCer injection in WT but not in $CD1d^{-/-}$ mice, splenic NK cells upregulate CD69 and start to produce large quantities of IFN-γ [273]. After 24 hours liver NK undergo extensive proliferation and exhibit maximal cytotoxic activity [274]. IFN-γ, IL-2 and IL-12 production generates an activation loop between NK and iNKT cells [273, 274].

iNKT cells contribute to expansion and activation of MHC-restricted conventional T cells via secretion of cytokines and cell-cell interaction. After *i.v.* αGalCer injection $CD44^{hi}$ $CD4^{+}$ or $CD8^{+}$ memory T cells in the liver and in the spleen undergo extensive bystander proliferation [274].

B cell help

Like $CD4^+$ MHC-restricted T cells, activated iNKT cells can provide both non-cognate and cognate help to B cells presenting lipid antigens onto CD1d. Within hours following *i.v.* αGalCer injection, splenic B cells strongly upregulate CD69, CD86 and MHC-Class II molecules in WT but not in iNKT cells-deficient mice [273, 275]. In both human and murine systems, iNKT cells-derived Th2 cytokines contribute to early B cell activation [275, 276]. Moreover, iNKT cells activity may help to sustain serological memory following primary immunization with protein antigens [277].

Role of iNKT on immune suppression and autoimmune diseases

iNKT cells have been implicated in a range of disease states in experimental models and in clinical settings. At present many reports don't provide mechanistic understanding and are therefore based merely on the analysis of iNKT cells-deficient mice. Indeed, several controversial outcomes arise by the use of $CD1d^{-/-}$ or $J\alpha18^{-/-}$ mice, possibly due to how gene-deficient mice are matched with the littermate controls with respect to genetic background and environmental factors. This is particular important in studies of complex multigenetic diseases such as diabetes, lupus, cancer or asthma. In addition, the administration of αGalCer in gain-of-function experiments must be carefully interpreted due to the massive release of cytokines which hardly resemble chronic diseases. Said so, several lines of evidence suggest a direct association between iNKT cell deficiencies and Type 1 Diabetes susceptibility [278, 279]; restoration of iNKT cells in NOD mice via adoptive transfer or introduction of a Vα14 TCR transgene protects from diabetes [280-282] as well as activation of iNKT cells through administration of αGalCer or upregulation of CD1d in the pancreatic beta islets [283-285]. Inefficient iNKT cell

function in NOD mice can be due to problems with signaling through SLAM molecules during development [248] and activation [249]. Recently, it has been described a congenic NOD mouse strain (NOD.*Nkrp1*^{b}.*Nkt2*^{b}) with increased thymic and peripheral iNKT cells and decreased Type 1 diabetes incidence. These mice showed differential expression in several genes on chromosomes 1 and 2, including the gene encoding for the peroxisomal protein Pxmp4 [286].

iNKT cells are also capable of protecting during EAE (a mouse model of brain inflammation), although iNKT cells role is controversial. Some models show that transgenically increasing iNKT cell numbers significantly suppresses EAE [287] and CD1d$^{-/-}$ mice show worse EAE as compared to WT mice [288]. However, others have reported no difference in disease severity [289, 290].

iNKT cells involvement in other autoimmune diseases comparing iNKT cells-deficient mice with WT or following αGalCer treatment include lupus[291], scleroderma [292], colitis [293, 294] as well as suppression of Graft-versus-host-disease (GVHD) [295, 296] and promotion of graft survival [297-299].

Role of iNKT cells in immune potentiation and protection from pathogens

Several studies have been carried out to define the role of iNKT cells in immunity to microbial infections. These studies often rely on the outcome of the infection in CD1d$^{-/-}$ or Jα14$^{-/-}$ mice, or on the impact of iNKT cell activation on infection after administration of αGalCer. By all these methods it was shown that iNKT cells participate in the response to various microbial pathogens including bacteria, fungi, parasites and viruses [300, 301]. Interestingly, the effect of iNKT cells was not always protective for the host, like in the case of *Chlamydia trachomatis* infection [302].

There are several ways how iNKT cells are activated during bacterial infections, which can be broadly divided in “direct” and “indirect” activation pathways.

The *direct activation pathway* postulates that iNKT cells recognize a bacterial lipid antigen presented by CD1d on the infected APC. TLR-mediated DC activation, inflammatory cytokines like IL-12 or recognition of endogenous antigens are not involved in this response. In 2005 for the first time were identified lipid antigens from *Sphingomonas spp.* that were able to activate iNKT cells [157-159]. *Sphingomonas* are Gram-negative α-proteobacteria lacking LPS, are ubiquitously present in the environment and are not considered pathogenic. The antigens identified in the cell walls of these bacteria, α-Galacturonosylceramide and α-Glucuronosylceramide, structurally resemble αGalCer, with whom they share the α-anomeric linkage attaching the glycan headgroup to the sphingosine base. During *Sphingomonas* infection, recognition of self lipids or stimulation of TLR receptors is not required to activate iNKT cells, since these bacteria produce the stimulatory GSL [157, 158]. iNKT cell-deficient mice show a reduced clearance of *Sphingomonas* bacteria shortly (1-3 days) after infection in the liver and in the lungs [157, 158], thus suggesting that these cells exert a protective effect.

Bacterial lipid antigens were also identified from *Borrelia burgdoferi* [160] pathogenic bacteria, which are the causing agents of Lyme disease. *B. burgdoferi* expresses galactosyl diacylglycerols containing a mixture of C14:0, C16:0, C18:0, C18:1 and C18:2 fatty acids with C16:0 and C18:1 the most abundant. The synthetic lipid antigen containing an oleic acid (C16) in *sn1* and a palmitic acid (C18:1) in *sn2* was the most potent in activating iNKT cells [160]. By using MyD88$^{-/-}$ mice it was shown that activation of iNKT cells was TLR-independent and it was also possible to load these antigens on CD1d-tetramers and stain hepatic iNKT cells [160]. In a recent congress it was shown that also pathogenic Gram-positive bacteria like *Streptococcus pneumoniae* contain lipid antigens recognized by iNKT cells [63](Kinjo 2009, 5th NKT and CD1 meeting, Kamakura, Japan).

The *indirect activation pathway* does not depend on recognition by the iNKT-TCR of a microbial antigen, but depends on cytokines released by activated DC and/or recognition of endogenous lipids. During *Salmonella typhimurium* infection [303], LPS stimulates TLRs on DC and induces IL-12 release. iNKT cells are activated by the combination of IL-12 produced by LPS-stimulated DC and by recognition of endogenous lipids presented on CD1d. iNKT cells fail to activate during infection with *Salmonella typhimurium* when anti-IL-12 (or anti CD1d) mAbs are co-administered [157, 303]. Moreover, iNKT cells are not activated in MyD88$^{-/-}$ mice infected with *Salmonella typhimurium* [303]. It is not clear whether LPS can directly induce the upregulation of the endogenous lipids recognized by iNKT cells [301].

Schistosoma mansoni-egg-sensitized DC induce IFN-γ and IL-4 production by iNKT cells [304]. In this response, TLR-mediated activation of DC or IL-12 is not involved. However, recognition of endogenous antigen is required, which could eventually be iGb3 since Hexb$^{-/-}$ DC do not stimulate iNKT cells after infection, even though the disruption of lysosomal vesicles could account for this failure [305]. It is not clear whether *S. mansoni* eggs can directly induce the upregulation of the endogenous lipids recognized by iNKT cells.

E.coli-LPS-stimulated DC secrete IL-12 and IL-18 and both cytokines are sufficient for IFN-γ production by iNKT cells, and recognition of endogenous antigens presented by CD1d is not necessary for iNKT cells activation [306]. The mechanism involved seems to be the achievement of a critical cytokine concentration, produced by LPS-stimulated APC, which is sufficient for induction of iNKT cells activation.

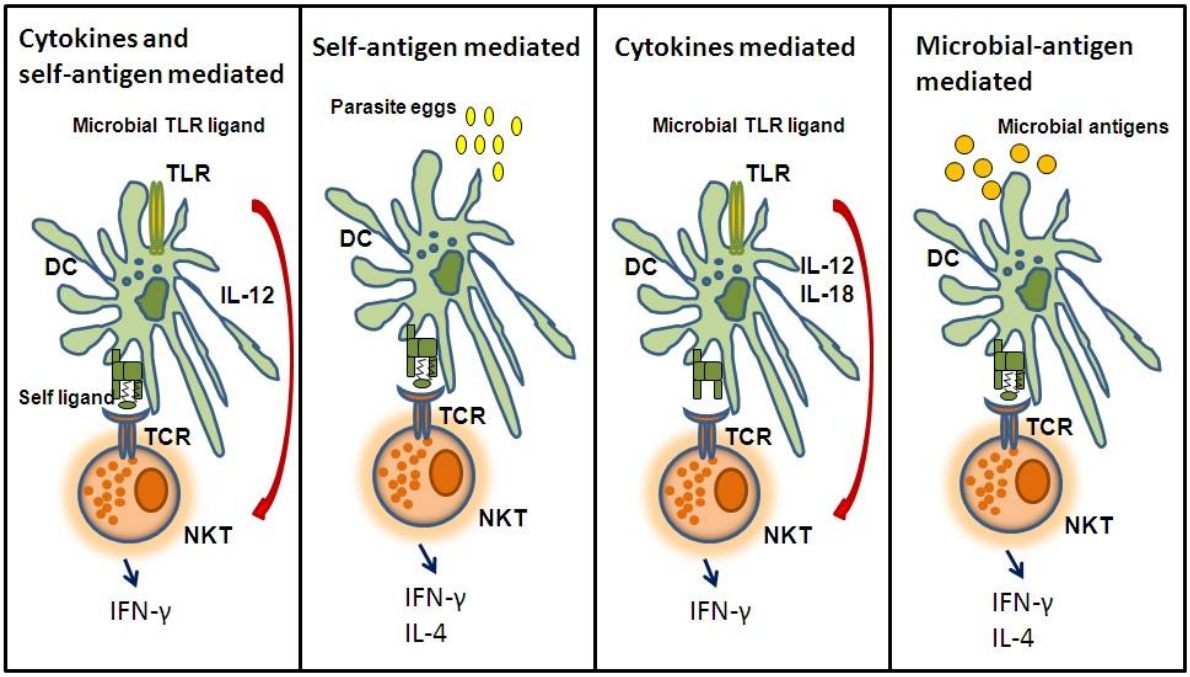


Figure 5. Schematic representation of iNKT cells activation during microbial infection

3.2.2 Type 2 NKT cells

Type 2 NKT are CD1d-restricted T cells that express a diverse TCR [307]. Very little is known about these cells that are present both in human and mouse [138, 308-310], Since Type 2 NKT do not react to αGalCer and therefore cannot be stained with αGalcer:CD1d dimers, it has not been possible to precisely enumerate these cells in mixed populations of human and mouse. They have been identified among clones self-reactive to the CD1d-transfected T cell lymphoma RMAS [310]. In addition, Type 2 NKT cells, unlike type 1 iNKT, are not dependent on the ability of CD1d to traffic to endosomes for their autoreactivity [311].

Type 2 NKT probably recognize diverse antigens. A population of non-Vα14 T cells is specific for CD1d:sulfatide complexes [308, 312] and can be stained with CD1d:sulfatide tetramers [13, 138].

Recently, it has been described that a population of murine Type 2 NKT cells recognize the self lipid Lyso-sulfatide and which express preferentially the Vβ3 and Vβ8 TCR rearrangements together with the Vα14 chain [313].

Moreover, functional studies comparing the response to Hepatitis Virus (HV) [314] and suppression of tumor rejection [315] in $CD1d^{-/-}$ mice (that lack Type 1 and Type 2 NKT) and in $Jα18^{-/-}$ mice (that lack only Type 2) have shown that type 2 NKT are functionally distinct from type 1 iNKT cells.

SUMMARY AND AIMS

The previous introductory part has reviewed the current knowledge on CD1 and lipid immunology. Although the last 20 years witnessed a dramatic expansion of the field, many aspects remain elusive and require additional studies.

Little is known about the mechanisms by which stimulatory CD1:lipid antigen complexes are generated and how is the influence of CD1d lipid antigen presentation on thymic selection and peripheral activation of iNKT cells.
The aim of this study is to investigate the mechanisms contributing to generation of lipid-specific complexes. In particular, I aim to investigate the impact of LTP residing in different cellular compartments on lipid antigen generation, loading on CD1 molecules, presentation to T cells, positive selection in the thymus as well as stimulation of peripheral CD1-restricted T cells.

These studies are divided in three parts:

1. **Impact of the absence of the lysosomal LTP NPC2 or of the enzyme β-Galactosidase on lipid antigen presentation and on thymic selection of iNKT cells.**

Recent studies demonstrate that different lysosomal lipid transfer proteins, like saposins and GM2-A, are implicated in loading of endogenous and exogenous lipid antigens onto CD1d and thus in CD1d-restricted presentation of lipid antigens important for iNKT cells thymic selection [84, 87].
Development of iNKT cells is also completely abolished in mice deficient in saposins [84]. Deficiencies in the lysosomal enzyme Hexb, which is involved in the lipid degradation pathway, or in saposins cause severe imbalances in lipid

metabolism. It has never been addressed whether lipid metabolism alteration, and thus accumulation of storage lipids, could contribute to the impairment of lipid antigen presentation and iNKT cell thymic selection.

This study has directly assessed the contribution of imbalance in lipid metabolism, consequence of ablation of the LTP NPC2 and of the enzyme β-Galactosidase, on generation of stimulatory CD1d:lipid antigen complexes, on their presentation to iNKT cells, and thus on thymic selection of iNKT cells.

2. Investigation of the functional interaction of CD1e with the other CD1 molecules

CD1e is the fifth member of the CD1 family. It is the only member of the CD1 family that does not reach the plasma membrane and is soluble in lysosomes, therefore it might have functions going beyond presentation of lipid antigen to T cells. CD1e binds lipid molecules in the lysosomal compartment and facilitates processing of complex glycolipid antigens, thus participating in lipid editing of CD1b-presented antigens [16].
Nothing is known about the role of CD1e in editing CD1 restricted lipids, either of self or of microbial origin.

This study has demonstrated the capacity of CD1e to interact with all lysosomal CD1 molecules and to influence the response of lipid specific-T cells, including that of iNKT cells. Generation of transgenic mice expressing CD1e in professional APC allowed us to study the contribution of CD1e in the formation of antigenic CD1d:lipid complexes and their capacity to activate iNKT cells.

3. **Impact of the absence of the peroxisomal and cytoplasmic LTP SCP-2 on lipid antigen presentation and on thymic selection of iNKT cells.**

All the LTP influencing lipid antigenicity and thus iNKT cell thymic selection that have been investigated so far are localised within the lysosome. The role of LTP residing in other subcellular compartments, like the cytoplasm and the peroxisomes, on presentation of lipid antigens has never been investigated. Furthermore, whether cytoplasmic LTP are involved in generation and intracellular trafficking of endogenous-lipid antigens remains unknown.

This study has directly investigated the role of a cytoplasmic/peroxisomal LTP in the generation of the repertoire of selecting lipids, and its impact on thymic selection of iNKT cells.

MATHERIAL AND METHODS

Bacteria

Sphingomonas paucimobilis (P1 strain) bacteria were used in infection experiments. Bacterial cultures in the logarithmic phase were obtained by growing *S. paucimobilis* in Mueller-Hinton broth (Difco) at 37℃. Series of 1/10 dilution were prepared and bacteria cell number was determined by CFU count after overnight cultures on Blood-agar plates (Blutspendezentrum Basel).

Cell culture reagents

RPMI 1640 medium (LuBio) was supplemented with 1 mM sodium-pyruvate (Gibco), 50 μM non-essential amino acid (Gibco), 2 mM UltraGlutamine II solution (Gibco) and 100 μg/ml Kanamycin (Gibco) and used as general cell culture medium. RPMI medium was supplemented with 10% fetal calf serum (FCS, LuBio, Europe) for tumor cell line cultures or with 5% human serum (HS, from healthy, blood group AB+ donors, Blutspendezentrum Basel) and 100 U/ml human recombinant IL-2 for T cell cultures. D-MEM medium (Gibco) was used to culture mouse cells and supplemented with 100 μM β-Mercaptoethanol.

All the cell culture media were filtered through Stericup GP Express PLUS 0.22 μM membrane filters (Millipore).

Cells

The following cell lines were used: THP-1, a monocytic leukemia; C1R, an EBV transformed human B cell line, J558, a mouse myeloma cell line, all obtained from American Type Culture Collection (ATCC). THP-1 cells transfected with CD1B gene were generated as described below [147].

The following human iNKT cell clones were used: VM-D5, JS-7, JS-63, JS-2, BGA-10, BGA-15, BGA-30; the following human Type 2 NKT cell clones were used: BGA-26, K36-B101, K46-C1, K46-B29, K46-B39, K36-A12, S56d, K46-B7 obtained as described below.

The FF13 Vα14 iNKT hybridoma was obtained as described below.

The following human self- and microbial-reactive CD1b-restricted T cell clones were used: GG33a (GM1), DS1C9b (Sulfatide), Z4B27 (Ac_2SGL, Diacylated Sulfoglycolpid), Z5B71 (GroMM, Glycerol Monomycolate)

The following self-and microbial-reactive CD1c-restricted T cell clones were used: DN4.99; K34-A27f, DS1B9c (Sulfatide), DL15A31 (unknown *M. tuberculosis* lipid fraction) obtained as described below.

Freezing and thawing of primary cells and cell lines

Cells were washed once with PBS (LuBio), then resuspended in freezing medium containing 90% FCS and 10% dimethylsulfoxide (DMSO, Sigma-Aldrich). Samples were stored in liquid nitrogen containing tanks. In order to thaw cells, frozen vials were incubated at 37°C until they were thawed, first diluted with 500 µl of PBS and then transferred to 15 ml tubes containing PBS and washed twice.

Generation of human T cell clones

CD1d-restricted human Vα24 Type 1 iNKT clones were established from PBMC of healthy donors. PBMCs were expanded *in vitro* by stimulation with 100 ng/ml α-GalCer. After 14 days, αGalCer expanded lines were cloned by limiting dilution in Terasaki plates. The cells were incubated in the presence of 5 x 10^5/ml irradiated PBMC (1500 RAD), in 20 μl of RPMI medium supplemented with 5% HS, 1000 U/ml IL-2 and 1 μg/ml phytohemaglutinin (PHA; Murex, Dartford). After 10-15 days growing cultures were transferred to 96 well-plates, expanded and tested for antigen specificity.

CD1b- and CD1c-restricted human T cell clones were established from PBMC of healthy donors and cloned by limiting dilutions in Terasaki plates. The cells were incubated in the presence of 5 x 10^5/ml irradiated PBMC (1500 RAD), in 20 μl of base medium supplied with 5% HS, 1000 U/ml IL-2 and 1 μg/ml PHA (Murex, Dartford). After 10-15 days growing cultures were transferred to 96 well-plates, expanded and tested for CD1 restriction and, where possible, for antigen specificity.

Maintenance of human T cell clones

Human T cell clones were cultured in RPMI cell culture medium containing 5% HS and IL-2 at 100 U/ml and expanded by restimulation every 25-35 days. Restimulation was performed in the presence of irradiated (1500 RAD) PBMC (1:1 ratio) in the same cell culture medium supplied with 1 μg/ml PHA. For antigen dependent activation experiments, T cell clones were used 12-18 days after restimulation.

Generation of murine Vα14 hybridomas

C57BL/6 mice were injected *i.p.* with 5 µg αGalCer. 3 days after injection mice were suppressed and splenocytes, thymocytes and liver cells were isolated. Cells were kept overnight in culture with medium supplemented with 10% FCS and 100 µM β-mercaptoethanol. Cells were then sorted according to binding to hCD1d:αGalCer dimer and mouse anti-TCRCβ.

Sorted cells were rested overnight and then fused with mouse BW-5147 thymoma cells using standard procedures. Briefly, sorted cells and thymoma cells were mixed 1:5 in serum free RPMI medium containing 1% Hepes (Gibco). Cells were washed once, supernatant removed until pellet was dry. Meanwhile a PEG solution (2g PEG 4000 (Merck), 2.2 ml GKN 3M, 0.8 ml distilled water and 0.04 ml NaOH 0.1N) was prepared in a sterile glass tube. This solution was dissolved under shaking in boiling water and kept it at 37°C.

1 ml of PEG solution was added slowly in 1-2 minutes to the cells in a 37°C water bath with gentle shaking. Cells were left 1 minute before diluting in RPMI-Hepes over 10 minutes. The cells were kept at 37°C for2 minutes and then centrifuged 10 minutes at 1000 rpm.

Cells were resuspended in RPMI-15% FCS containing 100 µM β-Mercaptoethanol before seeding in flat bottom 96 wells plates at $1x10^5$ cells/ 100 µl/well.

After overnight incubation 100 µl of RPMI-15% FCS containing 2% HAT-supplement and 0.01 mM β-Mercaptoethanol were added to each well. After 7 days growing wells were supplemented with HT medium and then screened for specificity by hCD1d:αGalCer dimer staining as described below.

Generation of stable transfectants

Human promyelocytic THP-1 cells were transfected with cDNA of human CD1B, or CD1C, or CD1D genes only using BCMGS-Neo vectors by electroporation as described. Stable transfectant were subsequently transfected with the CD1E gene, using a BCMGS-Hygro vector by electroporation as described. Full-length cDNA was amplified by RT-PCR from total RNA of DC using Superscript-Rnase H-RT (Life technologies) and *Pfu* DNA Polymerse (Promega) using the following primers:

CD1B: for (5'SallXC1b_f): 5' TAT AGT CGA CAT GCT GCT GCT GCC ATT TCA AC-3'

rev (3'ClaXC1b_r) 5'' CCA TCG ATG GGG TTT CTC CAG TAG AGG A-3'

CD1C: for (hCD1c XhoI) 5' CTC GAG GAC ATG CTG TTT CTG CAG TTT C-3'

rev (hCD1cNotI) 5' GCG GCC GCT CAC AGG ATG TCC TGA TAT GAG C-3'

CD1D: for (hCD1d XhoI) 5' CTC GAG ATA TGG GGT GCC TGC TG-3'

rev (3'hCD1d) 5' GAG TCA CAG GAC GCC CTG ATA GG-3'

CD1E: for (CD1eXhoI_for) 5' CCT CGA GCT CAA GCT TCG AAT TCT GC-3'

rev (CD1eNotI_rev) 5' CGC CGG CGA ATG TCA CCA GAG TTG G-3'

The amplified genes were sequenced after cloning into the pBluescriptII KS+ (Stratagene). For eukaryotic expression, the genes were subcloned into BCMGS. Stable transfectants were generated by using Effectene Transfection Reagent (Qiagen) with 0.2 µg DNA in 60 µl EC buffer, 1.6 µl Enhancer, 5 µl Effectene, 350 µl RPMI-10% FCS on $8x10^4$ cells/well. 24 hr after transfection, G418 sulphate (0.5 mg/ml, Calbiochem) or Hygromycin B (0.15 mg/ml, Sigma) were added for

selection and maintained for a minimum of 3 weeks. Expression of the proteins was assessed by Flow cytometry.
Cells were grown in RPMI-1640 supplemented with 10% FCS, 10 mM HEPES, 2 mM UltraGlutamine II, MEM nonessential amino acids, 1 mM Na-pyruvate and 100 µg/mL kanamycin (all from Invitrogen, Basel, Switzerland).
The same protocol was used to generate stable C1R CD1D single and CD1D/ CD1E double transfectants.

Fixation of APCs

Human cell lines and mouse thymocytes or mDC were washed and resuspended in PBS (3 x 10^6/ml) containing 0.05% glutaraldehyde for 20 s. Additional fixation was blocked with 0.2 M Lysine/PBS. Cells were washed twice with RPMI-medium containing 10% FCS.

T cell stimulation assays

Experiments with endogenous antigens

Human THP1 and C1R cells stably transfected with CD1B or CD1C or CD1D genes or with CD1B and CD1E, CD1C and CD1E or CD1D and CD1E ($5x10^4$ /well) were incubated with individual self reactive, CD1-restricted T cells clones(10^5/well). After 24 hr T cell activation was estimated by measuring cytokine release using ELISA assays.

mDC ($5x10^4$ cells/well) or thymocytes ($4x10^5$ cells/well) were plated with the cross-reactive human clone JS-63 ($1x10^5$ cells/well) or with the murine iNKT

hybridoma FF13 ($1x10^5$ cells/well). After 24 hr T cell activation was estimated by measuring cytokine release using ELISA assays.

Experiments with exogenous antigens

APC were pulsed 2 hr at 37° with serial dilutions of sonicated αGalCer, αLacCer, iGB3, Ac_2SGL, GroMM, GM1, Sulfatide, mycobacterial lipid extracts before plating ($5x10^4$ cells/well) and subsequent addition of the specific T cell clones ($1x10^5$ cells/well). After 24-48 hr T cell activation was estimated by measuring cytokine release using ELISA assays.

To study the kinetics of activation induced by CD1e presence, THP-1 D, THP1-D+E, mDC WT and Eα-DC were pulsed with 10 ng/ml αGalCer for selected periods (1-24 hr). The cells were then washed and suspended in PBS ($2x10^6$/ml) containing glutarhaldeide 0.05% for 20 s for fixation. Additional fixation was blocked with 0.2 M Lysine/PBS. APC were plated 10^5/well in 1:1 ratio with the human iNKT clone VM-D5 or with the murine iNKT hybridoma FF13 for 24 hr. T cell activation was estimated by measuring cytokine release using ELISA assays.

In some experiments mDC were pulsed for different times with heat-inactivated *S. paucimobilis* at a DC:Bacteria ratio of 1:200.

Chasing experiments

mDC Eα-CD1e tg or WT or THP1 transfected with CD1d or CD1d and CD1e were pulsed with 1ng/ml of αGalCer for 2 hr and chased for different periods of time before addition of FF13 iNKT hybridoma cells or the human VMD5 NKT clone.

The amounts of realeased cytokines decreased with longer chases. This allowed identification of persistence of stimulatory CD1d:antigen complexes.

CD1d-plate bound experiments

MaxiSorp Plates (Nunc) were coated overnight at room temperature with 10 µg/ml Bir1.4 mAb [316]. Human sCD1d purified by isoelectric focusing (IEF)[8], was coated overnight at room temperature at 5 µg/ml on 96 well plate. For experiments of αGalCer loading onto CD1d, sonicated αGalCer (2 µg/ml) was added in the presence or absence of different soluble recombinant LTP (SAP-A,-B,-C and BSA) (4 µg/ml) or of soluble recombinant CD1e (10 µg/ml). For experiments of αGalCer unloading from CD1d, sCD1d dimers, coated to the plastic as previously described, were loaded with sonicated αGalCer (2 µg/ml). After overnight incubation, wells were washed in order to remove unbound αGalCer.Different soluble recombinant LTP (SAP-A,-B,-C and BSA) (4 µg/ml) or soluble recombinant CD1e (10 µg/ml) were then added to the wells and incubated overnight at room temperature. After extensive washing, human iNKT cell clones (VM-D5 and JS-63) ($1.5x10^5$/well) were plated in RPMI-1640 medium containing 5% human serum and 100 U/ml human IL-2. Activation was detected by ELISA measuring cytokines released in cell culture supernatants.

Blocking experiments

mDC from $Scp2^{-/-}$ or WT mice were used to stimulate the murine FF13 iNKT hybridoma in the absence of exogenously added lipid antigens and in the presence

or absence of 20 µg/ml anti-mouse CD1d mAb (clone 1B1) or of irrelevant isotype matched mAb. Activation of iNKT cells was measured by cytokine release.

Bacterial infection and stimulation experiments

mDC from wt or Eα-CD1e tg mice were incubated for indicated time periods at 37°C with different MOI (range from 0.3 to 100 bacteria: 1 APC), after 3-4 hr gentamycin (15 µg/ml, Invitrogen) was applied. 2hr later iNKT cells were added and incubated for 24hr.

In some experiments mDC were incubated with heat-inactivated (72°C, 1 hr) bacteria for 2 hr before addition of FF13 iNKT hybridoma cells at a ratio DC:Bacteria of 1:100.

Experiments with drugs inhibiting glycolipids accumulation

Thymocytes were pre-incubated for 24 hr with 50 µM *N*-butyldeoxynojirimycin (*N*B-DNJ) (Calbiochem, Merck Biosciences, Nottingham, UK), while BM-derived DCs were differentiated from bone marrow progenitors in the presence of 50 µM *N*B-DNJ. T cells were then added to the plates, in the presence of 50 µM*N*B-DNJ. Specific inhibition was expressed as percentage of iNKT cell activation in the absence of drug (100% of stimulation).

Activation was detected by measuring cytokines released in cell culture supernatants by ELISA.

Cytokine determination by Enzyme Linked Immunosorbent Assay (ELISA)

Released cytokines from T cell activation assays were measured by ELISA. ELISA plates (Maxisorp, Nunc) were coated with the capture antibodies for 12 hr at 4℃, dissolved in PBS at a concentration from 1-10 μg/ml, containing 0.02% NaN_3 (Fluka). The incubation of the coating antibody was done using 50 μl of antibody solution per well. After coating, the ELISA plates were washed once with washing buffer containing PBS and 0.05% Tween 20 (Fluka) on a plate washer (ELX50 autostrip washer, Polygon Science) and wells were blocked with 200 μl of blocking buffer containing PBS, 0.05% Tween 20 and 1% bovine serum albumin (BSA, Sigma-Aldrich) for at least 1 hr at room temperature. After blocking, plates were washed and incubated for 2 hr with 70 μl of sample or the standard recombinant cytokines. Then plates were washed three times with washing buffer and incubated for at least 1hr with 70 μl of the detection antibody, diluted in blocking buffer at 1-5 μg/ml. After incubation plates were washed again three times and incubated with 70 μl of Streptavidin-HRP (Zymax Streptavidin-HRP conjugate, Zymed) diluted in blocking buffer 1:4000. After 1hr incubation, plates were washed five times and incubated with 90 μl of the substrate solution (Fast o-phenylenediamine dihydrochloride tablet, Sigma-Aldrich). The substrate incubation was done for 2-5 min then stopped by addition of 10% H_2SO_4 (50 μl/well, Fluka). The absorption was read at 490 nm, with an ELISA reader (Spectra Max 190, Molecular Devices). The samples were expressed as duplicates or triplicates and concentration of the cytokine was calculated with a standard curve made by serial dilutions of the appropriate recombinant cytokine (Pharmingen).

The following coating antibodies were used: HB 8700 (ATCC) for human IFN-γ; 8D48 antibody (BD Biosciences) for human IL-4; BVD2-23B6 (BioLegend) for

hGM-CSF; JES6-1A12 (BD Biosciences) for mouse IL-2; 11B11 (BD Biosciences) for mouse IL-4 and R4-6A2 (BD Biosciences) for mouse IFN-γ.

The following revealing biotinylated antibodies were used: γ69-2GV for human IFN-γ; MP4-25D2 (BD Biosciences) for human IL-4; BVD2-21C11 (BioLegend) for hGM-CSF; JES6-5H4 (BD Biosciences) for mouse IL-2; 11B11 (BD Biosciences) for mouse IL-4 and XMG1.2 (BD Biosciences) for mouse IFN-γ.

Production of recombinant cytokines

Human cytokines IL-4, IL-6, IL-2, GM-CSF and mouse GM-CSF were obtained by culturing J558 cells transfected with plasmid containing the appropriate cytokine cDNA in base cell culture medium supplemented with 10% FCS. Cell-free culture supernatants were harvested from cell cultures containing 1-2x10^7cells/ml. The concentration of hGM-CSF, hIL-4, hIL-6 and mGM-CSF were determined by ELISA. The active units of human IL-2 were measured using the standard IL-2-dependent cytotoxic T cell line (CTLL-2) proliferation assay.

Thymidine proliferation assay

APC (5x10^4/well) and responder cells (2x10^5/well) were cultivated in 96 well plates in the presence of serial dilution of antigen for 96 hr. In the last 18 hr 1 μCi of [^{3}H] Thymidine was added to the cells. Incorporation of radioactivity was measured by liquid scintillation counting using a Bekton Dickinson counter.

Mice

To generate Eα-CD1e tg mice (B6.Cg-Tg(H2-Eα-CD1E)1Dfim, shortly named Eα-CD1e tg), full length human CD1E cDNA was cloned into the EcoRI site of the pDOI5 expression vector under the control of the mouse H-2 Eα promoter[317]. The construct, devoid of vector sequences, was used for microinjection into B6x($B6D2F_1$) fertilized oocytes.
Primers used to clone the human gene into the pDOI5 vector were:

5'CD1e: 5' CGA GGG TCT CTG CTG TCC TGG-3'

3'CD1e: 5' AGA CTG CTG TGT TTC ACC CGA C-3'

Two transgenic founders transmitting the transgene to the progeny were obtained and from them two B6 congenic tg mouse lines were derived by backcrossing at least 6 times to C57BL/6. In addition, Eα-CD1e tg mice were bred to DBA/2 or BALB/c to generate F_1 mice.

β-$Gal^{-/-}$ and $NPC2^{-/-}$ mice were backcrossed to C57BL/6 and BALB/c mice, respectively. β-$Gal^{-/-}$ mice were obtained from J. Matsuda (Osaka, Japan), $NPC2^{-/-}$ were obtained from P. Lobel (Piscataway, NJ, USA); $CD1d^{-/-}$ mice were backcrossed to C57BL/6 and were obtained from R. McDonald, (Epalinges Switzerland); D011 mice were backcrossed to BALB/c and were kindly provided by U.Guenthert (Basel, Switzerland). $Scp2^{-/-}$ mice were backcrossed to C57BL/6 and were obtained from U.Seedorf (Münster, Germany)

BALB/c and C57BL/6 mice were maintained and bred in the animal facility at our Institute. All animals used for experiments were sacrificed at 3 -10 weeks of age. These studies were approved by the "Kantonales Veterinaeramt Basel-Stadt".

Screening of transgenic and KO mice

Mice were marked for long time identification and toe/tail or ear clip biopsy was performed according to the rules of "Kantonales Veterinaramt Basel-Stadt". Tissue samples were placed in Eppendorf tubes (1.5 ml) and incubated over night at 56°C in 500 µl of lysis buffer containing 100 mM Tris HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 0.4 mg/ml Proteinase K (Axon Lab). Lysates were centrifuged for 10 min at maximal speed and transferred to fresh tubes. DNA was precipitated with one volume of isopropanol (Fluka) and recovered using glass capillaries. After placing the capillaries for 5 min in 70% ethanol, DNA was dried and resuspended in 400 µl of TE (10 mM Tris HCl, 1 mM EDTA, pH 7.5). Obtained DNA samples were diluted in TE buffer to reach a concentration of 30 µg/ml and 1 µl was used for PCR.

PCR was preformed according to the protocol using Taq Polimerase (NEB).

Primers used for identification of $NPC2^{-/-}$ mice:

5'_NPC2PCR3: 5'_CAT TCT CAG TAT TGT TTT GCC AAG_3'

5'_NPC2PCR2: 5'_TTT CCT CCC TAG TCA AAC TCA ACT_3'

3'_NPC2PCR2-3: 5'_AGT GAG AAT TAT GGA CCC AGA CTC_3'

5'_NPC2PCR1: 5'_GCA CAC GTA GAG CTC AGA GAA TAA_3'

3'_NPC2PCR1: 5'_ TTC TCA CCA CCA CTA CTG CTG TGT TTT_3'

Primers used for identification of β-Galactosidase$^{-/-}$ mice:

T-Neo732F: 5'_CTC GCG CCA GCC GAA CTG TT_3'

T-Neo1351R:5'_GTT CGA GGC CAC ACG CGT CA_3'

Wild_3: 5'_CAT TCC TGC CAA GAC AGT AG_3'

BK1415_1R: 5'_ATG GCC TCA GTG TTC AGT GGG_3'

Primers used for identification of CD1d$^{-/-}$ mice:

3'mCD1d_scre: 5'_AGG AGA CCA CGG ACA AAT AGG G_3'

5'mCD1d_scre: 5'_GGG CAA GTT GAG TAA CCA GCA G_3'

Primers used for identification of CD1e tg mice:

3'CD1e_A3_rev: 5'_AGA CTG CTG TGT TTC ACC CGA C_3'

5'CD1e_for(ex2): 5'_CGA GGG TCT CTG CTG TCC TGG_3'

Primers used for identification of Scp2$^{-/-}$ mice:

3'rev: 5'_ACC AAG GTC AGG TTG GAG TG_3'

5'for: 5'_CAC GTC ACT TCT GCT TCC AG_3'

PCR conditions were adjusted according to primer sequences and size of the generated products.

Real-time quantitative PCR of mouse CD1e transgenic founders

Expression levels of the transgenic constructs in founders mice were checked by real-time quantitative PCR. A total of 20 ng cDNA was used as a template for real-time quantitative PCR. Primers were mixed with SYBR-Green, a highly specific, double-stranded DNA binding dye, to detect PCR product as it accumulates during PCR cycles. To standardize the amount of sample cDNA added to the reaction the calculated amount of the gene of interest was compared with the amount of constitutively expressed β-actin gene.

The following primers were used:

for CD1e:

Forward (CD1e_Q2_FOR)_ACA CAG TGA GGG CTC AGG AT;
Reverse(CD1e_Q2_REV)_CAA GCT GAA ATT GAC CAG CA;

β-actin:

Forward (1637) _ CCA TAG GCT TCA CAC CTT CCT G;
Reverse (1638) _ GCA CTA ACA CTA CCT TCC TCA ACC G;

PCR conditions were adjusted according to the sequence of primers and the length of generated products. Bands were scanned, CD1e products were compared with β-actin level for all time points and dilutions.

Preparation of mouse lymphoid cells

The organs of interest were isolated from sacrificed mice under sterile conditions and placed in D-MEM (Gibco) medium supplied with 10% FCS, 100 μg/ml Kanamycin and 10 mM HEPES buffer. Cells were isolated by meshing the organs on metal sieves using 2 ml syringe plunges. Isolated cells were transferred to 15 ml conical bottom tubes and incubated for 5 min at room temperature. Clear suspensions were transferred to new tubes excluding tissue debris. In case of cell preparation from spleen, red blood cells were lysed by adding 5 ml of ice-cold Guy's solution to the cell pellet. Cells were incubated on ice for 2-3 min then lysis was stopped by addition of 10 ml medium containing 10% FCS. Additional debrises were removed and cells were washed once with fresh medium. Isolated cells were

either directly used for staining or were cultivated in D-MEM medium with 10% FCS.

For further analysis of Vα14 iNKT cells, thymocytes were depleted of heat-stable Ag (HSA, CD24)$^+$ and CD8$^+$ cells by treatment with rat IgM mAb B2A2 and rat IgM mAb 3.168.8.1 *plus* rabbit complement. Viable recovered cells were purified on a lympholyte M gradient (Cedarlane Laboratories, Hornby, Ontario, Canada).

Liver mononuclear cells (LMC) were isolated by meshing the organ on metal sieves using 2 ml syringe plunges. Parenchimal cells were separated by centrifugation at 300 g for 5 minutes. Red blood cells were lysed by adding 5 ml of ice cold Guy's solution to the cell pellet. The cells were then purified on a Percoll 30%-70% gradient (Amersham Biosciences). In brief, liver suspension was diluted in PBS containing 30% of Percoll and slowly applied on a solution of RPMI-10% FCS containing 70% of Percoll. LMC were separated by centrifugation at 1000 rpm, room temperature for 20 min with slow acceleration and slow breaking rate. The ring containing liver mononuclear cells was collected and washed twice in medium containing 10% FCS. Additional debrises were eliminated by filtration through a 0.22 μM nylon mesh.

To recover peritoneal macrophages, mice were injected *i.p.* with 1 ml tioglycollate solution (Casein hydrolysate, 17 g/L; soy peptone, 110 g/L; L-Cysteine, 0.25 g/L; glucose, 6 g/L; sodium chloride, 2.5 g/L; sodium thioglycollate, 0.5 g/L; sodium sulfite, 0.1 g/L; agar, 0.7 g/L). 72 hr later mice were sacrificed and peritoneum was gently washed with 2 ml of PBS. The peritoneal solution was then collected, washed twice with PBS and the cells used for subsequent cytofluorimetric analysis.

Isolation of DO11 OVA specific cells

Spleen from D011 tg mice was collected and cells were isolated by meshing the organs on metal sieves using 2 ml syringe plunges. Red blood cells were lysed by adding 5 ml of ice-cold Guy's solution to the cell pellet. After washing $CD4^+$ Ova- specific tg cells were sorted from total splenocytes using anti-CD4-labeled magnetic beads (MACS, Miltenyi) according to manufacturer's protocol. Purity (>98%) was checked by cytofluorimetric analysis.

Generation of PPD specific murine cell lines

Mice were injected with 100 μg of Mycobacterial purified protein derivative (PPD). After 14 days mice received a boost of 50 μg PPD. 3 days after boost mice were sacrificed and the draining lymph nodes were collected. Cells were then restimulated *in vitro* for additional 2 weeks with 20 μg/ml of PPD in the presence of 5U/ml recombinant IL-2.

Preparation of mouse bone-marrow derived dendritic cells

Bone marrows were isolated from mouse femurs and tibias. After the removal of muscles both ends of bones were cut and marrows were flushed out with 2 ml of RPMI-5% FCS culture medium using a syringe. Cells were resuspended by pipetting and then washed. In order to generate the DC, bone marrow progenitors were cultivated for 8 days using RPMI culture medium supplemented with 5% FCS and 1000 U/ml of mGM-SCF. DC differentiation was checked by FACS staining of CD11c, CD1d and MHC class II surface markers.

Cell surface markers staining

Cells were washed with FACS-buffer (0.5% human albumin, 0.02% NaN_3 in PBS) and incubated with the primary mAbs or with the appropriate isotype-matched irrelevant mAb for background determination. After washing with FACS buffer cells were stained with fluorochrome-conjugated secondary reagents. All incubations and washings were done at 4°C in FACS-buffer.

Intracellular staining

Cells were washed three times with PBS, fixed with 2% paraformaldehyde (PFA, Merk) solution in PBS for 15 min at room temperature and washed three times with FACS-buffer. In order to permeabilize cell membrane, cells were incubated in FACS-buffer containing 0.1% saponin (Sigma-Aldrich) for 5 min at room temperature. Cells were stained with primary antibodies or isotype matching control antibodies, then washed three times and incubated with fluorochrome conjugated secondary reagents. All incubation steps for staining were done at room temperature for 30 min and all the reagents were prepared in FACS-buffer containing 0.1% saponin.

Production of soluble hCD1d protein and generation of hCD1d:αGalCer dimers

The pCD1D Bluescript II plasmid containing the full-length cDNA of human CD1D, coding for the extracellular soluble domain of the human protein, was used as template for amplification by using the following primers:

3’ BamH1CD1dsol_AAG GAT CCA CGC CAG TAG AGG ACG ATG;
5’ Nde1CD1d_GCC ATA TGG TCC CGC AAA GGC TTT TC;

The PCR product was cloned into the pBluescript-BirA vector, which contains the sequence for the BirA peptide tag (GGGLNDIFEAQKIEWHE)[8]. The DNA fragment encoding soluble hCD1d fused in frame with the C-terminal BirA tag sequence was subcloned into BCMGS-Neo and supertransfected into murine J558 cells previously trasfected with human b2m cDNA [318].
Cells were cultured and expanded in medium containing 10% FCS and then adapted to 5% FCS medium. For final protein production cells were cultivated in flasks (175 cm^2, BD Falcon) with medium containing 3% low IgG serum (Gibco). Soluble hCD1d was purified from supernatants by IEF (iso electric focusing). Purity of the separation was checked by Western Blot (WB) using anti-Bir 1.4 mAbs and Silver staining. This preparation was incubated for 45 minutes with αGalCer (12 μg/ml) at room temperature.
shCD1d and mouse anti-BirA monoclonal antibody of IgG2b isotype were incubated at a molar ratio of 2:1 for 15 minutes at room temperature and then used for staining. Dimers were revealed with fluorochrome-labelled Goat anti-Mouse IgG2b (10 μg/ml). Dimers were stable at +4°C for more than 6 months.

Flow cytometry

The following antibodies specific for human determinants were used: hCD1d: αGalCer dimers (generated as described above), anti-Vα24 (C15), anti-Vβ11 (C21), anti-CD3ε (UCHT1).
The following antibodies specific for mouse determinants were used: mouse CD1d:DimerX, anti-CD3ε (145-2C11), anti-TCR Cβ (H57/597), anti-CD4 (clones

GK1.5 and RM4-5), anti-CD8α (53-6.7), anti-CD11c (HL-3), anti-TCR γδ-biotin (GL3), anti-CD122 (TM-Beta1), anti-CD132 (4G3), anti CD1d (1B1) anti-CD11b (M1/70), anti-CD45R (RA3-6B2) from BD Biosciences; anti-CD69 (H1.2F3), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-NK1.1 (CD161b/c) (PK136), anti-CD49b (DX5), anti-CD24 (M1/69), anti-CD45R (RA3-6B2), anti-CD11b (M1/70), anti-TCRVβ 7 (TR310) from Biolegends. Anti-CD1d (HB322) and anti-MHC class II (m5 114), anti-TCR-Cβ (H57-597), anti-TCR-Vβ 8.2 (F23.2) purified from hybridoma culture supernatants in our laboratory The anti-mCD1d:αGalCer complexes mAb L363 was kindly obtained from S.Porcelli.

The following secondary reagents were used: Goat Anti-Mouse (GAM) IgG Alexa 647 (Molecular Probes), GAM IgG FITC (SBA), GAM IgG PE (SBA), Streptavidin (SA) RPE (SBA), SA APC, SA Alexa-750 and SA PE-Texas Red (Caltag) were used as revealing secondary reagents when the mAbs were not directly labeled to fluorochromes.

Samples were analyzed using CyAn ADP flow cytometer (DakoCytomation, Baar, Switzerland). Nonviable cells were excluded from the analysis using light scatter and incorporation of propidium iodide (Fluka). Data were analyzed using Summit 4.2 (DakoCytomation) or FlowJo software (TriStar).

Mouse CD1d:αGalCer dimer staining

Cells were washed with FACS-buffer (0.5% human albumin, 0.02% NaN_3 in PBS) and Fcγ-receptors were blocked with anti-CD16/32 mAb (10μg/ml). After washing with FACS buffer cells were incubated with 15μg/ml mCD1d dimers (mouse DimerX, BD Pharmingen) pre-loaded overnight with 50 ng αGalCer. After washing, cells were incubated with 5 μg/ml GAM IgG1-PE. Aspecific binding was

blocked with addition of irrelevant mIgGs and rIgGs (25 μg/ml).

Magnetic Microbeads separation by MACS® technology

Magnetically labeled cells were separated over a MACS® column (Miltenyi Biotech) placed in an autoMACS® Separator (Miltenyi Biotech). Thymocytes labelled with PE-conjugated CD1d:αGalCer dimers bound to anti-PE magnetic beads were separated by positive selection. The positive fraction was further stained for additional surface markers.

Apoptosis determination by Annexin-V staining

Enriched thymic iNKT cells, stained with CD1d:αGalCer dimers and antibodies specific for cell surface markers, were resuspended in Annexin V Binding Buffer (BD Pharmingen), containing 0.1 M Hepes, 1.4 M NaCl and 25 mM $CaCl_2$ at pH 7.4. Cells were stained with 50 μl Annexin V-FITC solution (1 μg/ml) for 15 minutes at room temperature and then resuspended in 150 μl of 7-AAD (3 μg/ml) (BD Pharmingen).

Cell-Cycle measurement with EdU staining

Mice were injected intravenously with 1 mg of EdU (5-ethynyl-2'-deoxyuridine, Invitrogen) 3 hr before analysis according to manufacturer protocol of Click-it EdU staining kit (Invitrogen). Thymi were collected, meshed with tea strainers and washed with PBS as described. Thymocytes were stained with mCD1d:αGalcer dimers and MACS enriched as described. After staining of

additional surface markers, cells were washed with PBS 1% BSA once and then fixed with 4% PFA (paraformaldehyde) 15 minutes at RT. Cells were then permeabilized with Saponin. EdU-Click-it reaction cocktail, containing $CuS0_4$ and the AF-488-azide dye, was prepared according to manufacturer protocol and was applied to the cells for 30 minutes at RT. To determine DNA content, after washing cells were incubated with Ribonuclease A and 7AAD for 30 minutes before samples were passed on a CyAn ADP flow cytometer (DakoCytomation).

Production of monoclonal antibodies from hybridomas

Hybridoma cells producing monoclonal antibodies (mAb) were cultured and expanded in medium containing 10% FCS and then adapted to 5% FCS medium. For final antibody production cells were cultivated in flasks (175 cm^2, BD Falcon) in medium containing 3% low IgG serum (Gibco). mAb were purified from collected supernatants using 5 ml protein G column (HiTrap ProteinG, Pharmacia). The column was connected with a peristaltic pump (2132 Mikroperpex Peristaltic Pump, LKB Bromma) and equilibrated with PBS, before the hybridoma supernatant was loaded at a flow rate of approximately 1 ml/min at 4°C. Each time 500 to 1000 ml of supernatant were used for antibody purification. After supernatant loading, the column was connected with an UV-spectrophotometer (2238 UVICORD SII, LKB Bromma) set at 280 nm and washed with PBS until all contaminating serum components were removed. Then the antibody was eluted with a solution of 100 mM glycine dissolved in water at pH 3.0. The antibody was collected in 15 ml tubes and immediately neutralized with 500 µl of a Tris solution (1M, pH 7.4). The antibody was filtered, NaN_3 was added at a final concentration of 0.02% and the protein concentration was determined with an UV-spectrophotometer (V-530,

Jasco) at 280 nm wavelength. An OD of 1.3 was estimated to correspond to 1 mg/ml of antibody concentration.

Biotinylation of purified antibodies

The purified antibody solution was dialyzed against borate buffer (pH 8.5) containing 0.2 M H_3BO_3 (Fluka), 0.1 M KCl (Fluka), in order to remove all small organic molecules containing free amino groups. Dialysis was performed in dialysis tubes (3.5 kDa cutoff, Spectra/Por CE Float A Lyzer, Spectrum). Antibody concentration was adjusted to at least 1 mg/ml. Biotinylation was performed in the borate buffer pH 8.5 in small glass vials containing a magnetic stirrer. During stirring at room temperature, biotin-NHS ester (Sigma-Aldrich) was added stepwise (in 4 steps) at 5 min interval between each addition step. The amount of biotin was calculated as 20 times molar excess to antibody molecules, so that ratio between biotin-NHS ester:antibody was 20:1.
Biotin-NHS ester was dissolved in DMSO under nitrogen atmosphere at a concentration of 10 mg/ml. After the last addition of biotin-NHS ester, the solution was further stirred for 30 min then dialyzed against PBS. The concentration of the biotinylated antibody was determined by UV-absorption as described and success of biotinylation was tested by either FACS staining or by ELISA.

Conjugation of mAb with AF-488 dye

After purification as described, antibody concentration was determined by Bradford methods and adjusted to at least 5 mg/ml. Alexa Fluor 488 powder diluted in 10 µl DMSO (Molecular Biology grade) was added to 2 ml antibody solution in

water drop by drop in small glass vials containing a magnetic stirrer and let shake at room temperature for 1 hr. The solution was then dialyzed against PBS to eliminate the unbound material. The concentration of the conjugated antibody was determined by UV-absorption as described and success of conjugation was tested by either FACS staining or by ELISA.

CFSE labeling

Cells were washed extensively with PBS before CFSE (Carboxyfluorescein succinimidyl ester) labeling. Cells were resuspended at $2x10^6$ /ml in a solution of PBS and CFSE (0.5 μM, Molecular Probes) and then incubated 10 minutes at 37° C. Additional incorporation was blocked by addition of 5% human Albumin. Cells were then washed in PBS and resuspended in RPMI-10% FCS. CFSE dilution was assessed 3-5 days after staining.

Synthesis of C8 αGalCer

αGalCer containing a C8 acyl chain was prepared following, in part, literature procedures. Briefly, glycosylation of properly protected azidophytosphingosine, obtained according to Schmidt and Maier [319], with tetra-O-benzyl-a-D galactopyranosyl bromide [320] using the “in situ anomerization” [321] protocol gave the corresponding α-glycoside. Reduction of the azide by hydrogenation with Lindlar catalyst [321] and conventional acylation with octanoyl chloride gave the protected C8 αGalCer. Removal of the isopropylidene group by acidic hydrolysis and of the benzyl groups from galactosyl moiety by hydrogenolysis catalyzed by Pd(OH)2/C gave the desired C8 αGalCer.

Immunofluorescence and biochemical analyses

BM-derived DC from WT and Eα-CD1e tg mice were fixed, permeabilized and labelled with the mAb 20.6, revealed using FITC-conjugated polyclonal goat anti-mouse Abs (Jackson Laboratories) as previously described [16]. After washing, cells were incubated with 5% non immune mouse serum, and then stained with anti-TGN38 mAb, specific for trans-Golgi Network compartments and with anti MHCII mAbs specific for MHCII associated compartments.

The biochemical maturation of CD1e was analyzed by pulse-chase labelling experiments, followed by immunoprecipitation, and performed as previously described[16]. DC were metabolically labeled with [^{35}S] methionine and cysteine in the presence of HRP–transferrin conjugates for 30 min. Cells were incubated with hydrogen peroxide at 0 °C and lysed either directly or following chase at 37 °C for 2 h in normal culture medium. CD1e was immunoprecipitated with the 20.6 mAb, deglycosylated with EndoH (H) or EndoF (F) or left untreated, separated on denaturing electrophoresis gels and analyzed by autofluorography.

Lipid extraction

Total lipids were extracted from thymi of WT and *Scp2*$^{-/-}$ mice according to the Folch method [322]. Briefly, 3.5 ml of methanol were added to cell suspension of thimocytes (1-2 x10^8 cells); after 90 seconds of sonication on ice at 50 Hz 7 ml of Chloroform were added. The mixture was incubated 2 hours at room temperature shaking and then centrifuged 5 minutes at 3000 rpm, 4°C. The organic phase was dried under nitrogen flow and stored at 4 °C. A second extraction O/N with 10 ml of chloroform was performed from the inorganic phase and after centrifugation (5 minutes, 3000 rpm, 4°C) the organic phase was mixed with the

previous extraction and dried under nitrogen flow. Dry lipids were dissolved in chloroform:methanol 2:1, washed with 3.5 ml of PBS pH 5.6 and after centrifugation, separated from the aqueous phase and dried. 1 ml of chloroform:methanol 1:1 was added, and after filtration through a Sep-Pak Classix C18 cartridge (Waters) lipids were dried and stored at -20°C until analysis.

LC-MS analysis of *Scp2*$^{-/-}$ and WT lipids

Lipid extracts were spiked with appropriate internal standards and analyzed by liquid chromatography-mass spectrometry (LC-MS).

The internal standards used included 1,2-dioctanoyl-glycero-3-phosphoinositol **(**PI 16:0) (Echelon Biosciences Inc), 1,2-dimyristoyl-glycero-phosphoserine (PS 28:0), 1,2-dimyristoyl-glycero-3-phosphoethanolamine (PE 28:0), 1,2-dimyristoyl-glycero-3-phosphocholine (PC 28:0), lauroyl sphingomyelin (C12-SM), N-heptadecanoyl-d-*erythro*-sphingosine (C17-Ceramide) and d-glucosyl-ß1-1'-N-octanoyl-d-*erythro*-sphingosine (C8-Glucosyl Ceramide) which allowed the measurement of PI, PS, PE and pPE, PC and ePC, SM, Cer and Glu-Cer respectively. Polar glycerophospholipids and sphingolipids were separated using normal phase HPLC. Briefly, lipids were separated on a Luna silica column (3µm, 2mm x 150mm; Phenomenex) with gradient elution of 100% chloroform/methanol/water/ammonia solution (90:9.5:0.5:0.32, by vol.) changing to 100% chloroform/methanol/water/ammonia solution (50:48:2:0.32, by vol.) over 50 min at 0.35ml/min. Quantification was done using a triple quadrupole instrument (API 3200; Applied Biosystems) operated in multiple reactions monitoring (MRM) mode. PE/pPE, GM3, PI, PS and PA were detected in negative ionization while Cer, GluCer, PC/ePC, SM were detected in positive ionization. The various lipid classes

were measured using previously reported MRM transition pairs and instrument settings.

Electrophoresis, transfer and western blotting

To analyze the production of soluble recombinant proteins, Western Blot analysis was performed. 40 µl of 2x concentrated reducing sample buffer containing 60 mM Tris HCl pH 6.8 (Fluka), 30% glycerol (Fluka), 10% sodium dodecyl sulfatide (SDS, Sigma-Aldrich), 4 mM Na_2EDTA (Fluka), 4% 2-mercaptoethanol (Sigma-Aldrich) and 0.005% bromophenol blue (Fluka) were added to the samples, which were then incubated at 95°C for 5 min.

20 µl from each sample were applied with Hamilton syringes (Hamilton Bonaduz) in the slots of a stacking polyacrylamide gel (5% polyacrylamide content, 0.75 mm thickness), overlaid above a separating acrylamide gel (8.5% polyacrylamide content, 0.75 mm thickness) in an electrophoresis device (Mini Protean II™). After application of the samples and a molecular weight marker (Rainbow, Biorad), the gel electrophoresis apparatus was connected with a power supply (Model 1000/500, Biorad) and voltage was applied at 20 Volt during run in the stacking gel and 40 Volt during the run in the separating gel.

Composition of running buffer for electrophoresis (pH 8.8): 3 g of Tris, 14.4 g of glycine (Fluka), 1 g of SDS per 1 l; 5% stacking gel: 2.3 ml of Tris (0.5 M in water, pH 6.8), 100 µl of SDS (10% in water), 1.5 ml acrylamide (30%, Biorad), 100 µl APS (10% in water, Fluka), 10 µl of TEMED (Fluka) and water up to 9 ml; 8.5% separating gel: 2.5 ml of Tris (1.5 M in water, pH 8.8), 100 µl of SDS (10% in water), 2.9 ml acrylamide (30%), 50 µl APS (10% in water), 5 µl of TEMED and water up to 10 ml. After separation proteins were transferred to a nitrocellulose

membrane. The separating gel was removed from the apparatus and preequilibrated in transfer buffer for 15 – 30 min. Then the gel was attached to a nitrocellulose membrane (0.2 μM Hybond-C extra, Amersham) of identical size and inserted in a transblot apparatus (Mini protean II, Biorad) supported by filter papers (GB 002 Gel-Blotting-Paper, Schleicher & Schuell) and sponges. The transfer apparatus was filled with transfer buffer, connected to a power supply (Model 1000/500, Biorad) and transferred under constant current conditions (200 mM) for 4 h at 4°C. Buffer was cooled by ice and stirred with a magnetic stirrer during transfer. When transfer was finished, nitrocellulose membrane was removed, washed in PBS and immersed in a solution of 5% gelatin (Top Block, Juro) dissolved in PBST (PBS, 0.05% Tween 20) at 4°C overnight for blocking.

Composition of transfer buffer: 3 g of Tris, 14.4 g of glycine, 20 ml of CH_3OH (Fluka) per 1l.

After blocking, the membrane was washed three times for 5 min with an excess of PBST, then incubated with the appropriate mAb dissolved in PBST, 2.5% gelatin at a concentration of 5 μg/ml for 3 h under shaking at room temperature. Then the membrane was again washed three times in PBST and incubated with a Streptavidin-HRP (SBA), diluted 1:2500 in PBST + 2.5% gelatin for 1 hr. The membrane was washed three times with PBST, 2 times with PBS, finally the enhanced chemoluminescence reaction (ECL-reaction) was performed. The ECL-reaction was done with a supersignal kit (Pierce). After 5 min incubation the excess of liquid was removed, membrane was wrapped and exposed to X-ray film (Biomax MR, Kodak) for various times. The film was developed using X-ray film developer (Curix 60, AGFA).

Statistical analysis

In cytokine release assays, data are expressed as mean ± standard deviation (s.d.) and analysed with unpaired Student's *t*-test two-tailed, with Welch correction. In staining assays, median ± s.d. are analysed using Mann-Whitney-Willcoxon multiple comparison test. *P* values ≤ 0.05 are considered statistically significant.

CHAPTER 1

Differential alteration of lipid antigen presentation to iNKT cells due to imbalances in lipid metabolism

(These results have been published in the *European Journal of Immunology*, **2007**, *37*, 1431-1441)

SUMMARY

Defects in lysosomal lipid trafficking or degradation result in a severe imbalance of lipid metabolism, but little is known about the consequences of such an imbalance on the presentation of lipid antigens to lipid-reactive T cells, like the iNKT cell subset. As described before, unlike conventional MHC-dependent T cells, iNKT cells are positively selected by CD1d expressing DP cortical thimocytes [194]. The key event deciding for selection of iNKT cells is the appropriate presentation of endogenous thymic lipid antigen(s) by CD1d. Many steps are involved in this process, including proper conformation of the CD1d molecule, correct intracellular trafficking of lipids and CD1d, generation of lipid antigen(s), and loading of the lipid antigen(s) onto CD1d in late endosomes/lysosomes [323-326].
Different lysosomal lipid transfer proteins, like saposins and GM2-A, have been implicated in loading of endogenous and exogenous lipid antigens onto CD1d and thus in CD1d-restricted presentation of selection-relevant lipid antigens to iNKT cells [84, 87]. Indeed, development of iNKT cells is completely abolished in mice deficient in saposins [84]. However, deficiency in Hexb or saposins causes severe imbalances in lipid metabolism, and in more recent studies it has been discussed that lipids accumulating in lysosomes might impair presentation of lipid antigens by CD1d, and thus thymic selection of iNKT cells. Presentation of lipid antigens to iNKT and their thymic development are also deficient in mouse models of different other types of lysosomal lipid storage diseases, i.e., in mice deficient in β-Hexosaminidases A and S ($Hexa^{-/-}$), α-Galactosidase ($\alpha\text{-}Gal^{-/-}$), β-Galactosidase ($\beta\text{-}Gal^{-/-}$), or the lysosomal NPC1 protein [91, 208].

In this part of my thesis I describe the impact of accumulating lipids in lysosomes on generation of lipid antigens and their presentation to iNKT cells, and the mechanism by which lipid antigen presentation is disturbed in this context.

To gain a better understanding of the influence of disturbed lipid metabolism and lysosomal lipid storage on the presentation of lipid antigens and the development of iNKT cells, we analyzed two mouse models of imbalanced lipid metabolism: mice deficient in the lysosomal enzyme β-Galactosidase (EC 3.2.1.23), which is involved in the glycosphingolipid degradation pathway (**Figure 6**), and mice deficient in the lysosomal lipid transfer protein NPC2, whose precise functions are still largely unknown.

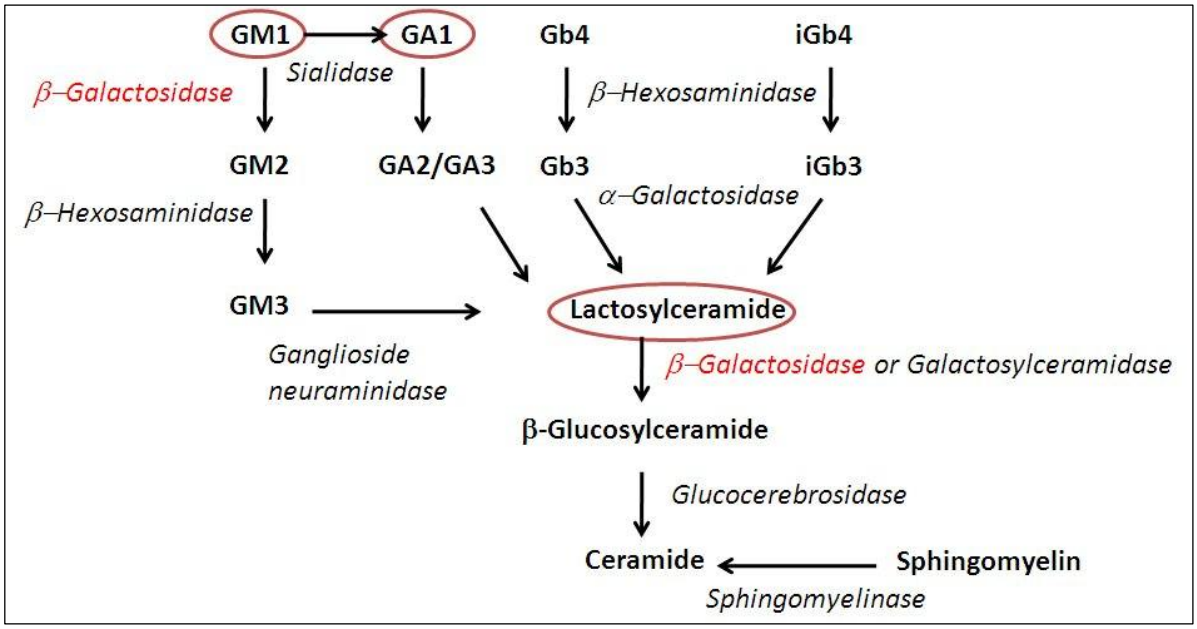


Figure 6. Schematic representation of glycosphingolipids degradation pathway. In red circles are defined the lipids which are accumulated in absence of the β-Galactosidase enzyme.

RESULTS

Thymic selection of iNKT cells is impaired in β-Galactosidase$^{-/-}$ mice

To test the effect of the absence of the enzyme β-Galactosidase (β-Gal) on thymic development of iNKT cells a complete flowcytometric analysis of thymocytes isolated from β-Gal$^{-/-}$ and WT mice was performed. The analysis of the complete thymus (**Figure 7A**) showed a strong reduction in the number of iNKT cells in KO mice as compared to WT. This reduction was better visualized when the thymocytes were depleted from CD24^{+} (HSA^{+}) and CD8^{+} cells, a procedure that allows an enrichment in iNKT cells [188] (**Figure 7B**).

The residual population of iNKT cells that was selected in the absence of β-Gal bound CD1d:α-Galactosylceramide (CD1d:αGalCer) dimers with remarkably high avidity, as shown by the MFI (Mean Fluorescence Intensity) of dimer staining.

Thymocytes frequencies of conventional TCR-αβ (defined by the percentage of TCR-Cβ^{+} in **Figure 7A**) as well as DN, DP, CD4^{+}, and CD8^{+} cells (**Figure 8A**) and TCR-γδ T cell (**Figure 8C**) were normal.

CD1d levels on the surface of DP thymocytes (the cell type driving positive selection of iNKT cells) remained unchanged between WT and KO thymocytes, thus excluding that the reduction in iNKT cells in the thymus was due to a lower CD1d expression (**Figure 8B**).

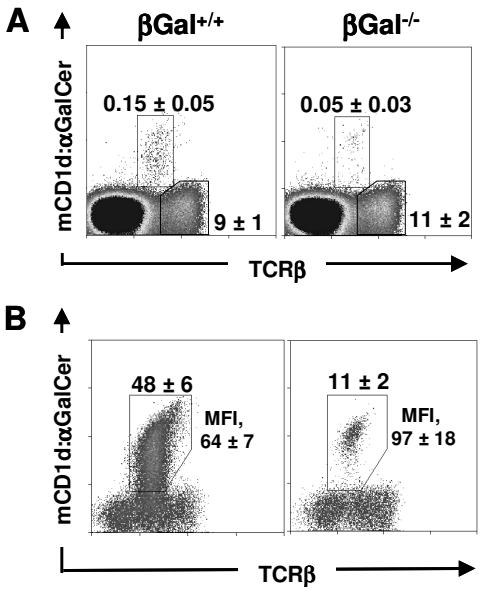


Figure 7. iNKT are reduced in thymi of β-Gal$^{-/-}$ mice. Staining of complete (A) or of CD24^{+} and CD8^{+} depleted (B) thymi of WT and β-Gal$^{-/-}$ mice with mCD1d:aGalCer dimer and TCRβ. Numbers in gate represent the percent of iNKT cells ± s.d. or of conventional T cells ± s.d MFI: mean fluorescent intensity of dimer staining ± s.d.

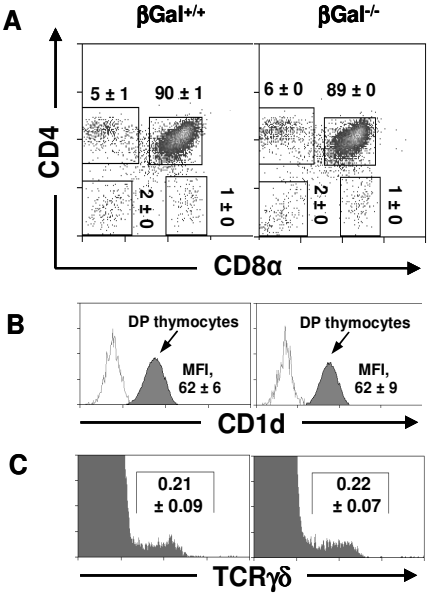


Figure 8. Conventional TCR-αβ cells and TCR-γδ cells are normal in thymi of β-Gal$^{-/-}$ mice. (**A**) Double staining for CD4 and CD8 markers of thymocytes from WT and β-Gal$^{-/-}$ mice. (**B**) CD1d expression levels on CD4-CD8 DP thymocytes (gray area); white area, isotype control. (**C**) Staining of thymocytes with mAb against TCR-γδ. Numbers indicate percentage of cells in the defined gates ± s.d.

Liver and spleen are the major organs to which thymus-derived iNKT cells home. The frequencies of iNKT cells were reduced in livers and spleens of βGal$^{-/-}$ mice to a similar extent as in the thymus (**Figure 9**).

The numbers of mononuclear cells and the frequencies of conventional TCR-αβ T cells were normal in livers and spleens of β-Gal$^{-/-}$ mice, as defined by the percentage of TCRβ^{+} cells in **Figures 9A** and **9B**. As in the thymus, the residual population of iNKT cells bound CD1d:αGalCer dimers with high avidity.

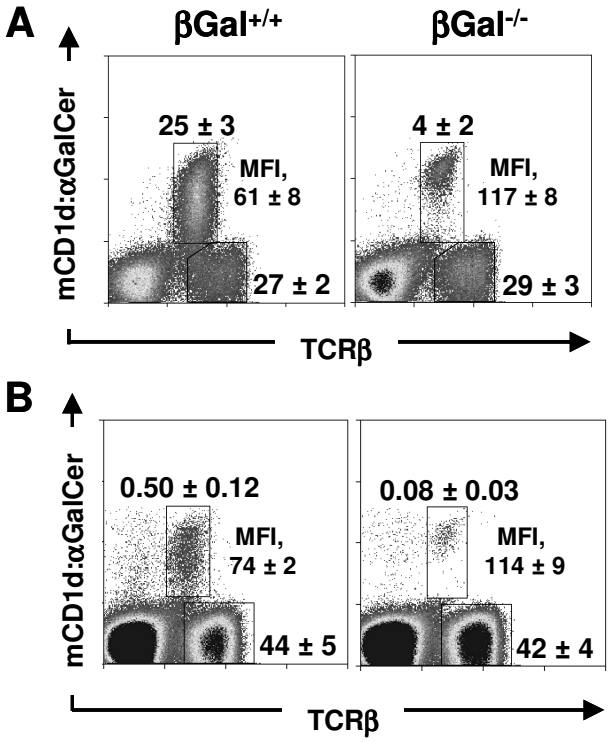


Figure 9. iNKT are reduced in liver and spleen of β-Gal$^{-/-}$ mice. Staining of liver (**A**) and spleen (**B**) of WT and β-Gal$^{-/-}$ mice with mCD1d:aGalCer dimer and TCRβ. Numbers in gate represent the percent of iNKT cells ± s.d. or of conventional T cells ± s.d MFI: mean fluorescent intensity of dimer staining ± s.d.

The Vβ domain is known to influence the avidity of CD1d:glycolipid binding by iNKT cells [188-190, 207, 327, 328]. We therefore analyzed the expression of the two major Vβ chains, Vβ8.2 and Vβ7, which mostly pair with the invariant TCR-α chain of iNKT cells, in the residual iNKT cell populations. In addition, since mouse iNKT cells are either $CD4^+$ or DN, we analyzed the expression of the CD4 co-receptor in the residual NKT population. Interestingly, the iNKT cell population that was selected in the absence of β-Galactosidase showed a remarkable shift in the Vβ8.2/Vβ7 ratio towards Vβ7 expression (**Figure 10A** and **B**), whereas CD4 was expressed at normal frequency and intensity (**Figure 10C**). Frequencies of $Vβ8.2^+$, $Vβ7^+$ and $CD4^+$ cells were not changed among conventional TCR-αβ T cells in β-Galactosidase-deficient mice (**Figure 10A, B, C** lower panels).

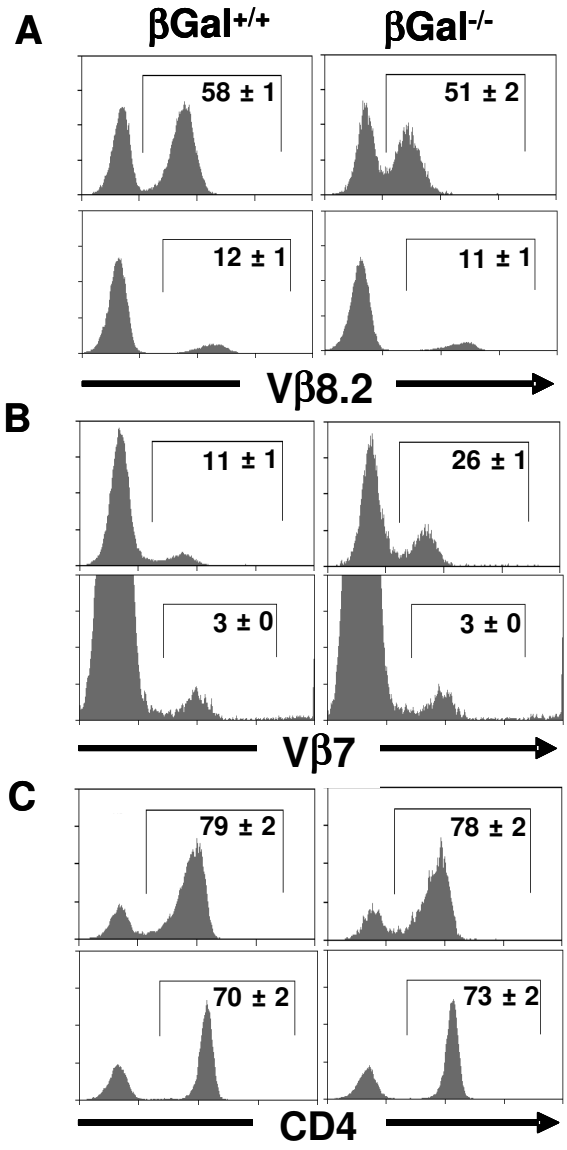


Figure 10. Residual iNKT cell population in β-Gal$^{-/-}$ mice is skewed towards the Vβ7 usage. Staining of hepatocytes with anti-TCR-Vβ8.2 (**A**), anti-TCR-Vβ7 (**B**) or anti-CD4 (**C**). Upper panels, gating on iNKT cells; lower panels, gating on conventional TCR-αβ cells. Numbers indicate percentage of positive cells in the indicated gates ± s.d.

Presentation of lipid antigens is impaired in βGal$^{-/-}$ thymocytes

Since thymic development of iNKT cells was impaired in β-Gal$^{-/-}$ mice, we wondered whether presentation of lipid antigens was also impaired if the enzyme β-Galactosidase is missing. We utilized an *in vitro* system in which a human Vα24 iNKT clone is stimulated by mouse thymocytes or DC in the absence of additional lipid antigens. Under these conditions, APC from WT mice caused the release of human IL-4 by the Vα24 iNKT clone (**Figure 11**). This stimulation was completely abolished when β-Gal$^{-/-}$ thymocytes were used as APC (**Figure 11** upper panel). DC were also able to induce the secretion of human IL-4 by the iNKT clone (**Figure 11** lower panel), but deficiency of β-Galactosidase in DC did not impair the cytokine release.

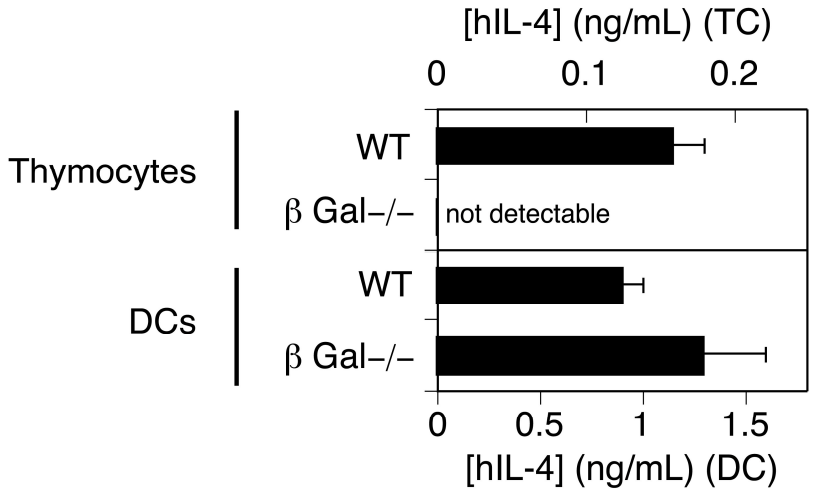


Figure 11. Presentation of endogenous lipid antigens is impaired in β-Gal$^{-/-}$ mice. Thymocytes, TC, upper panel, and DC, lower panel, from WT or β-Gal$^{-/-}$ mice were used to stimulate a human iNKT clone in the absence of exogenously added lipid antigens. Release of human IL- 4 was determined in the cell culture supernatants after 24 hr.

This finding allowed us to speculate that a certain degree of cell type-specific difference is possible and indicates that β-Galactosidase *per se* is not involved in the stimulation of the human iNKT clone in the described *in vitro* system.

To check whether presentation of exogenously given lipid antigens is also disturbed in the absence of β-Galactosidase, a mouse iNKT hybridoma was stimulated with β-Gal$^{-/-}$ or WT thymocytes in the presence of the strong glycolipid agonist αGalCer or of iGb3. Both αGalCer and iGb3 were less efficiently presented by β-Gal$^{-/-}$ as compared to WT thymocytes (**Figure 12**).

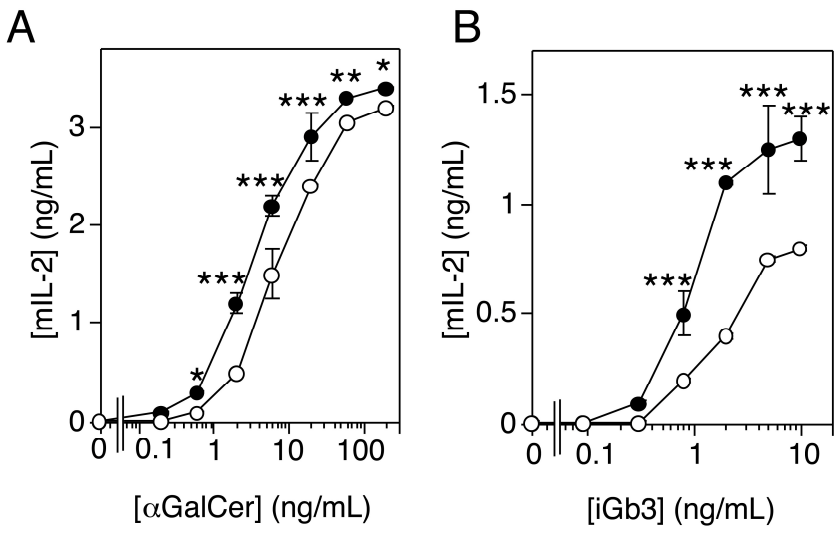


Figure 12. Presentation of exogenous lipid antigens is impaired in β-Gal$^{-/-}$ mice thymocytes. Thymocytes from WT (●) or β-Gal$^{-/-}$ (○) mice were used to stimulate a mouse iNKT hybridoma in the presence of different concentrations of αGalCer (**A**) or iGb3 (**B**). Release of mouse IL-2 was determined in the cell culture supernatants after 24 hr. * $p<0.05$, ** $p<0.01$,*** $p<0.001$ comparing WT and β-Gal$^{-/-}$.

In contrast, DC from β-Gal$^{-/-}$ mice were not impaired in presenting αGalCer (A), iGb3 (B) and αLacCer (C)(**Figure 13**).

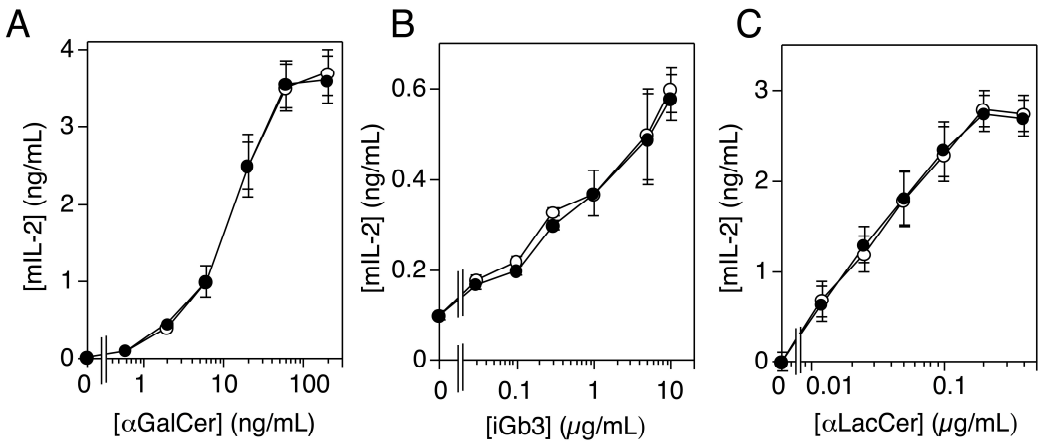


Figure 13. Presentation of exogenous lipid antigens is not impaired in β-Gal$^{-/-}$ mice DC. DC from WT (●) or β-Gal$^{-/-}$ (○) mice were used to stimulate a mouse iNKT hybridoma in the presence of different concentrations of (**A**) αGalCer, (**B**) iGb3 or (**C**) αLacCer. Release of mouse IL-2 was determined in the cell culture supernatants after 24 hr. * $p<0.05$, ** $p<0.01$,*** $p<0.001$ comparing WT and β-Gal$^{-/-}$.

Since lipids are loaded onto CD1d molecules inside the lysosomes, we wondered whether lysosome function was generally impaired in β-Gal$^{-/-}$ APC.

Presentation of peptides via MHC class II was not affected by the lack of β-Galactosidase, since β-Gal$^{-/-}$ splenocytes perfectly presented Mycobacterial purified protein derivative (PPD) to a specific murine T cell line (**Figure 14**).

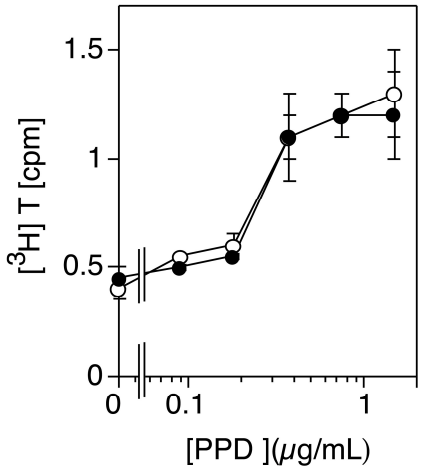


Figure 14. β-Galactosidase deficient splenocytes have normal lysosomal function. Irradiated splenocytes from WT (●) or β-Gal$^{-/-}$ (○) mice were incubated with different concentrations of purified protein derivative (PPD) and a PPD-specific murine T cell line for 4 days. $^{[3H]}$thymidine was added for 8 h, and $^{[3H]}$thymidine incorporation was determined.

These results show that presentation of lipid antigens, and not of peptides, is disturbed in β-Gal$^{-/-}$ thymocytes, and this might explain the impairment of thymic selection of iNKT cells in β-Gal$^{-/-}$ mice.

Pharmacological reduction of lipid accumulation in β-Gal$^{-/-}$ thymocytes improves lipid antigen presentation

The enzyme β-Galactosidase hydrolyses the terminal β-galactosidic residues of different glycoconjugates (**Figure 6**), including the gangliosides GM1 and GA1, and

lactosylceramide [329]. A deficiency in this enzyme could therefore cause the lack of a lipid antigen involved in thymic selection of iNKT cells.

However, our findings that β-Gal$^{-/-}$ DC were able to stimulate human iNKT cells in the absence of additional lipid antigen and that presentation of exogenously given lipid antigens was impaired in β-Gal$^{-/-}$ thymocytes suggest that an indirect rather than a direct mechanism is responsible for impaired selection of iNKT cells in β-Gal$^{-/-}$ mice. To test for the hypothesis that the accumulation of lipids negatively influences lipid antigen presentation in β-Gal$^{-/-}$ mice, we took advantage of an inhibitor of glycosphingolipid biosynthesis, N-butyldeoxynojirimycin (NB-DNJ), which reduces pathological lipid storage in vitro and in vivo [330]. Incubation of β-Gal$^{-/-}$ thymocytes with NB-DNJ significantly improved their capacity to present αGalCer to iNKT hybridoma cells, thus indicating that storage lipids disturb lipid antigen presentation in β-Gal$^{-/-}$ thymocytes (**Figure 15**). This mechanism might also explain why thymic selection of iNKT cells is impaired in β-Gal$^{-/-}$ mice.

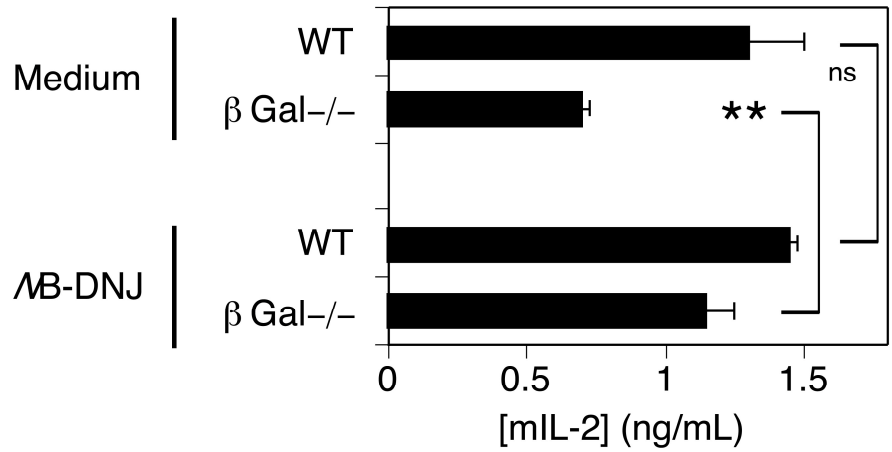


Figure 15. Accumulation of lysosomal storage lipids causes the impairment in presentation of lipids in β-Gal$^{-/-}$ thymocytes. Thymocytes from WT or β-Gal$^{-/-}$ mice were incubated with NB- DNJ or medium before αGalCer (1 ng/mL) and murine iNKT hybridoma cells were added. IL-2 release to cell culture supernatant was determined. The bar chart shows the mean release of IL-2 (±SD; n=3). ** $p<0.01$; ns, not significant.

Intracellular defects account for impairment of lipid antigen presentation in β-Gal$^{-/-}$ cells

Accumulating lipids might disturb lipid antigen presentation in several ways. Storage lipids could directly block or modify CD1d molecules, could change membrane structures, and/or could disturb proteins involved in the transport of lipid antigens, in the unloading of non-antigenic lipids from CD1d, and/or in the loading of lipid antigens onto CD1d. To investigate whether the formation of functional CD1d:lipid antigen complexes on the cell surface is impaired in β-Gal$^{-/-}$ thymocytes, these cells were fixed with glutaraldehyde and tested for their capacity to present αGalCer to iNKT hybridoma cells [155]. Interestingly, upon fixation β-Gal$^{-/-}$ and WT thymocytes presented αGalCer equally to iNKT hybridoma cells (**Figure 16**), showing that the formation of functional CD1d:αGalCer complexes directly on the cell surface is not disturbed in β-Galactosidase-deficient thymocytes.

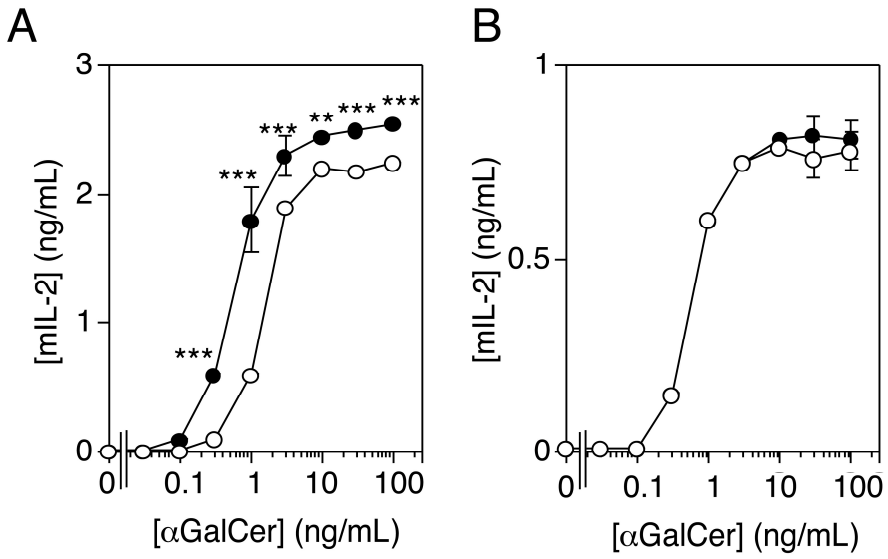


Figure 16. Intracellular defects with no disturbance of the CD1d molecule account for the impairment in presentation of lipids in β-Gal$^{-/-}$ thymocytes. Living (**A**) or fixed (**B**) thymocytes from WT (●) or β-Gal$^{-/-}$ (○) mice were incubated with various concentrations of αGalCer before the addition of mouse iNKT hybridoma cells. IL-2 release to cell culture supernatant was determined. Diagrams show the mean release of IL-2 (±SD; n=3). ** $p<0.01$, *** $p<0.001$ comparing WT and β-Gal$^{-/-}$.

Thymic selection of iNKT cells is impaired in NPC2$^{-/-}$ mice

To test the effect of the absence of the LTP NPC2 on thymic development of iNKT cells, the same series of experiments done for the β-Gal$^{-/-}$ mice were performed. Thymocytes isolated from NPC2$^{-/-}$ and WT mice were therefore first analysed by flowcytometry.

The absence of NPC2 inhibited thymic development of iNKT cells by 60–70%. This was determined by flowcytometric analysis of thymocytes from NPC2$^{-/-}$ and WT mice (**Figure 17**), as described for β-Gal$^{-/-}$ mice.

Thymocytes numbers and frequencies of conventional TCR-αβ, as defined by the percentage of TCRβ^{+} cells in **Figure 17A** and TCR-γδ T cells (**Figure 18C**), as well as DN, DP, CD4^{+}, and CD8^{+} cells were normal (**Figure 18A**).

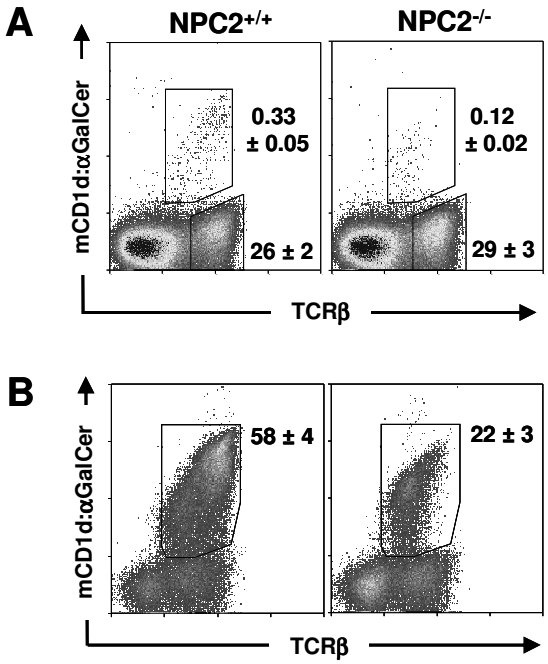


Figure 17. iNKT are reduced in thymi of NPC2$^{-/-}$ mice. Staining of complete (A) or of CD24^{+} and CD8^{+} depleted (B) thymi of WT and NPC2$^{-/-}$ mice with mCD1d:αGalCer dimer and TCRβ. Numbers in gate represent the percent of iNKT cells ± s.d. or of conventional T cells ± s.d MFI: mean fluorescent intensity of dimer staining ± s.d.

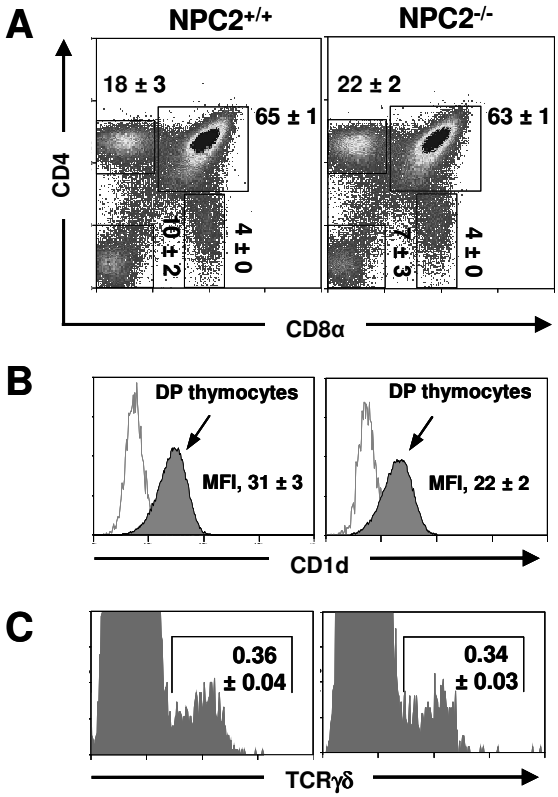


Figure 18. Conventional TCR-αβ cells and TCR-γδ cells are normal in thymi of NPC2$^{-/-}$ mice. (**A**) Double staining for CD4 and CD8 markers of thymocytes from WT and NPC2$^{-/-}$ mice. (**B**) CD1d expression levels on CD4-CD8 DP thymocytes (gray area); white area, isotype control. (**C**) Staining of thymocytes with mAb against TCR-γδ. Numbers indicate percentage of cells in the defined gates ± s.d.

CD1d surface expression was somewhat lower on DP thymocytes in the absence of NPC2 (**Figure 18B**). However, this decreased surface expression is not sufficient to explain the low numbers of iNKT cells selected in NPC2$^{-/-}$ mice since

$CD1d^{+/-}$ mice, expressing half the amount of CD1d as compared to $CD1d^{+/+}$ mice, have normal numbers of iNKT cells [188, 195].

Also in the periphery, in liver and spleen, frequencies and numbers of iNKT cells were greatly reduced in $NPC2^{-/-}$ mice (**Figure 19**), although total mononuclear cell numbers were significantly increased, especially in the liver. Further analysis revealed that this increase is mainly due to elevated numbers of B cells. (**Figure 20**)

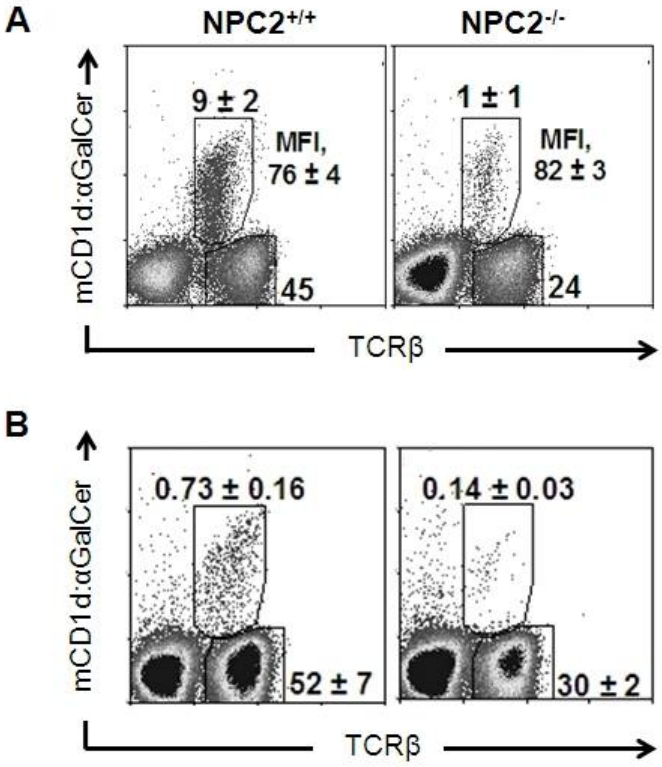


Figure 19. iNKT are reduced in liver and spleen of $NPC2^{-/-}$ mice. Staining of liver (**A**) and spleen (**B**) of WT and $NPC2^{-/-}$ mice with mCD1d:αGalCer dimer and TCRβ. Numbers in gate represent the percent of iNKT cells ± s.d. or of conventional T cells ± s.d MFI: mean fluorescent intensity of dimer staining ± s.d.

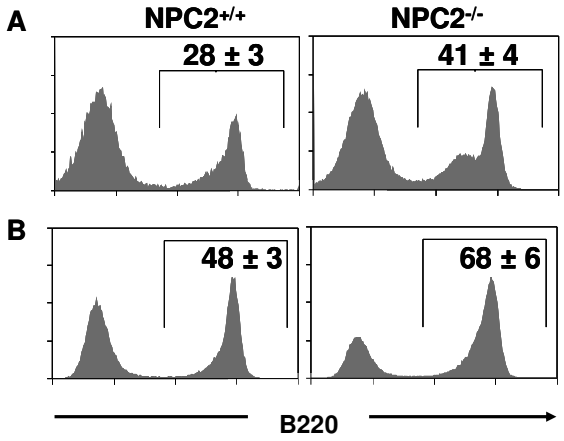


Figure 20. B cells are increased in liver and spleen of NPC2$^{-/-}$ mice. Staining of liver (**A**) and spleen (**B**) of WT and NPC2$^{-/-}$ mice with anti-CD45RB (B220). Numbers in gate represent the percent of B cells ± s.d. or of conventional T cells ± s.d

We analyzed the expression of Vβ8.2, Vβ7 and CD4 in the residual iNKT cell populations in NPC2$^{-/-}$ mice. The iNKT cell population that was selected in the absence of NPC2 did not show a preferential Vβ usage (**Figure 21A** and **B**), whereas CD4 was expressed at higher frequency in NPC2$^{-/-}$ iNKT (**Figure 21C**). Frequencies of Vβ8.2^{+}, Vβ7^{+}, and CD4^{+} cells were not changed among conventional TCR-αβ T cells in NPC2-deficient mice.

Despite the fact that both β-Gal$^{-/-}$ and NPC2$^{-/-}$ mice are deficient in iNKT cells, there are some differences regarding the residual populations of iNKT cells: (i) the small population of iNKT cells selected in the absence of NPC2 bound CD1d:αGalCer dimers with similar avidity as iNKT cells from WT mice, whereas in β-Gal$^{-/-}$ mice only NKT cells binding these dimers with high avidity could be detected (**Figures 7, 9, 17 and 19**). (ii) The ratio of CD4^{-}/CD4^{+} cells among iNKT (but not among conventional TCR-αβ T) cells in NPC2$^{-/-}$ mice was significantly increased by a factor of 3.5, whereas CD4 expression was normal among iNKT cells in β-Gal$^{-/-}$ mice (**Figures 10C and 21C**). (iii) The ratio of Vβ8.2/Vβ7 among iNKT cells in

NPC2$^{-/-}$ mice was only slightly decreased, whereas in β-Gal$^{-/-}$ mice these cells clearly expressed Vβ7 at higher frequency (**Figures 10A,B and 21A,B**). These repertoire differences suggest that different mechanisms account for NKT deficiency in the two models.

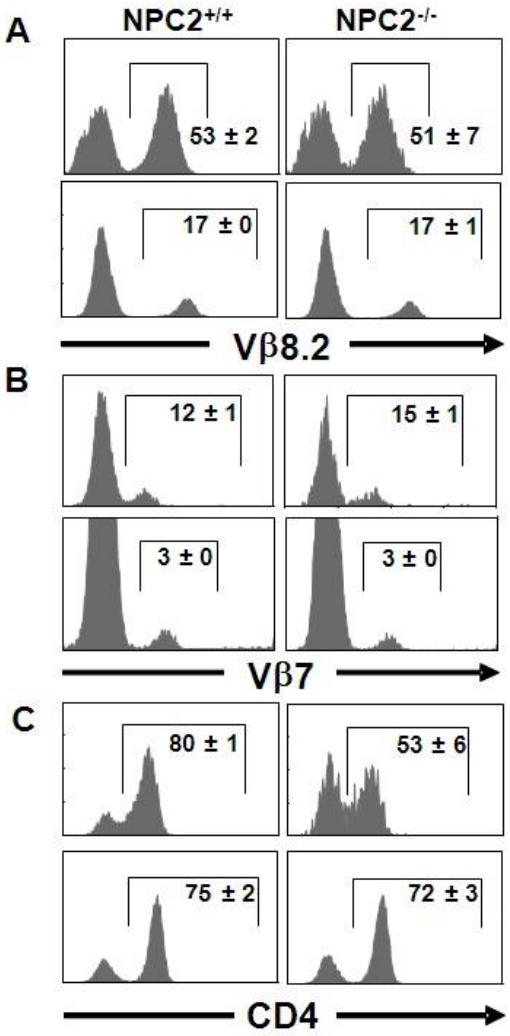


Figure 21. Residual iNKT cell population in NPC2$^{-/-}$ mice expresses more CD4 than WT. Staining of hepatocytes with anti-TCR-Vβ8.2 (**A**), anti-TCR-Vβ7 (**B**) or anti-CD4 (**C**). Upper panels, gating on iNKT cells; lower panels, gating on conventional TCR-αβ cells. Numbers indicate percentage of positive cells in the indicated gates ± s.d.

Presentation of lipid antigens is impaired in NPC2$^{-/-}$ APC

To investigate whether presentation of lipid antigens is impaired in NPC2$^{-/-}$ APC, we first checked for the capability of NPC2$^{-/-}$ thymocytes or DC to stimulate a human Vα24 iNKT clone in the absence of additional lipid antigens, as described above. Thymocytes or DC from WT mice stimulated the human iNKT clone to release human IL-4, whereas this stimulation was strongly impaired if cells were deficient in NPC2 (**Figure 22**).

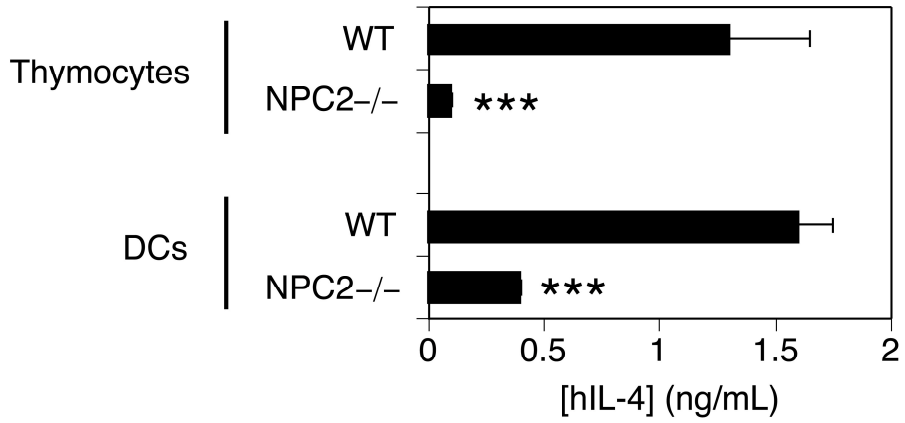


Figure 22. Presentation of endogenous lipid antigens is impaired in NPC2$^{-/-}$ mice. Thymocytes, TC upper panel, and DC, lower panel, from WT or NPC2$^{-/-}$ mice were used to stimulate a human iNKT clone in the absence of exogenously added lipid antigens. Release of human IL- 4 was determined in the cell culture supernatants after 24 hr. *** $p<0.001$ comparing WT and NPC2$^{-/-}$.

An interesting point is that NPC2$^{-/-}$ DC, in contrast to thymocytes (**Figure 18B**), had normal expression levels of CD1d on the surface (data not shown) and nevertheless were weak APC.

When thymocytes (**Figure 23**) or DC (**Figure 24**) were deficient in NPC2, the stimulation of mouse iNKT hybridoma cells in the presence of αGalCer (A), iGb3 (B) or αLacCer (C) was also impaired.

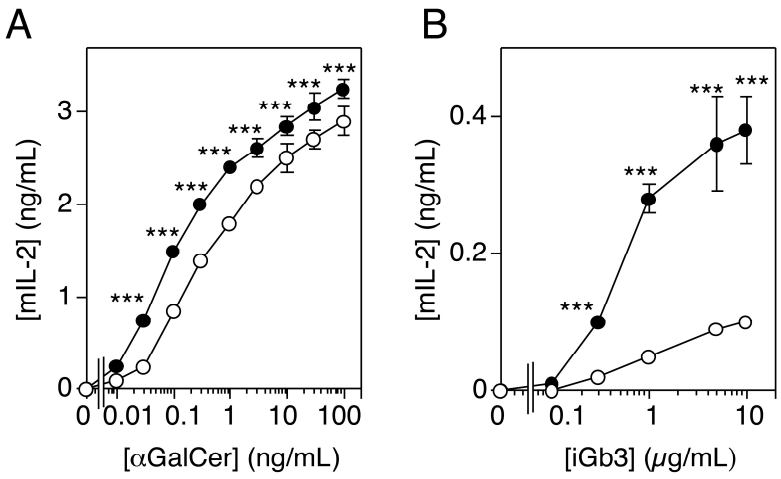


Figure 23. Presentation of exogenous lipid antigens is impaired in NPC2$^{-/-}$ mice thymocytes. Thymocytes from WT (●) or NPC2$^{-/-}$ (○) mice were used to stimulate mouse iNKT hybridoma cells in the presence of different concentrations of (**A**) αGalCer or (**B**) iGb3. Release of mouse IL- 2 was determined in the cell culture supernatants after 24 hr. * $p<0.05$, ** $p<0.01$,*** $p<0.001$ comparing WT and NPC2$^{-/-}$.

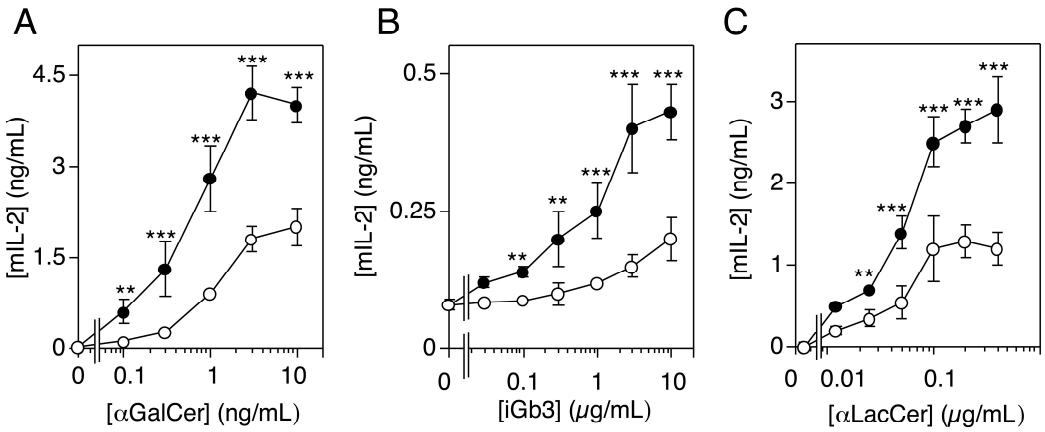


Figure 24. Presentation of exogenous lipid antigens is not impaired in NPC2$^{-/-}$ mice DC. DC from WT (●) or NPC2$^{-/-}$ (○) mice were used to stimulate mouse iNKT hybridoma cells in the presence of different concentrations of αGalCer (A), iGb3 (B) or αLacCer (C). Release of mouse IL- 2 was determined in the cell culture supernatants after 24 hr. * p<0.05, ** p<0.01,*** p<0.001 comparing WT and NPC2$^{-/-}$.

Presentation of peptides via MHC class II was not influenced by the lack of NPC2 since NPC2$^{-/-}$ DC perfectly presented ovalbumin to specific T cells derived from OT-II transgenic mice (**Figure 25**)

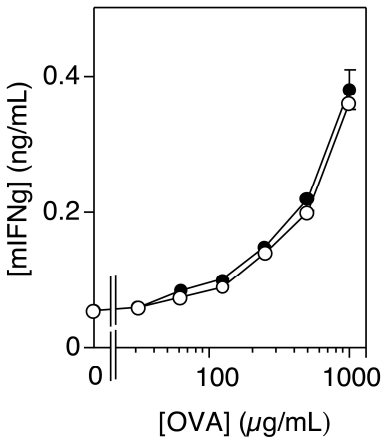


Figure 25. NPC2 deficient DC have normal lysosomal function. Irradiated DC from WT (●) or NPC2$^{-/-}$ (○) mice were incubated with different concentrations of ovalbumin (OVA) and CD4^{+} T cells from OT-II transgenic (DO11.10) mice for 4 days. IFN-γ release to cell culture supernatant was determined. Diagrams show the mean IFN-γ release (± SD).

Since NPC2 is thought to be involved in lipid transport [95, 97, 331], we wondered whether the lower efficacy of lipid antigens in NPC2$^{-/-}$ cells is due to a changed half life of functional CD1d:lipid antigen complexes. To test it, NPC2$^{-/-}$ or WT DC were pulsed with αGalCer for 2 hours and chased for different periods of time before addition of iNKT hybridoma cells. The kinetics by which the stimulatory capacity of DC decreased, and thus the half-life of functional CD1d:αGalCer complexes, were identical between NPC2$^{-/-}$ and WT cells (t1/2 14 hr). (**Figure 26**)

Therefore, the formation of stimulatory CD1d:αGalCer complexes, rather than their maintenance, is affected in NPC2$^{-/-}$ cells. Thus, the low numbers of iNKT cells selected in NPC2$^{-/-}$ mice can be explained with the deficiency in lipid antigen presentation.

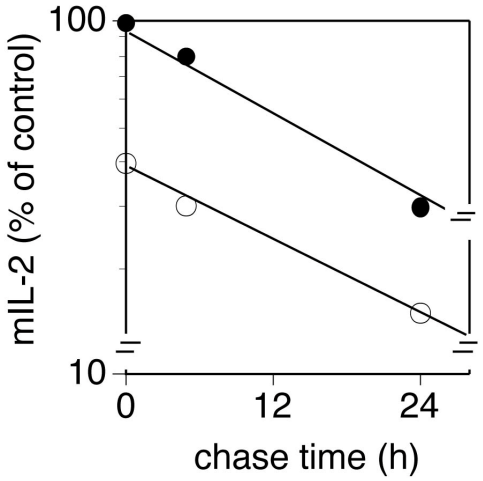


Figure 26. Formation of stimulatory CD1d**:**αGalCer complexes is altered in NPC2$^{-/-}$ DC. DC from WT (●) or NPC2$^{-/-}$ (○) or mice were pulsed with αGalCer (100 ng/mL) for 2 hr and chased for different periods of time before addition of murine iNKT hybridoma cells.

Pharmacological reduction of lipid accumulation in NPC2$^{-/-}$ thymocytes improves lipid antigen presentation

To better understand the mechanism of impaired lipid antigen presentation in NPC2$^{-/-}$ APC, we tested whether accumulation of lipids might play a role. Glycosphingolipid biosynthesis was pharmacologically inhibited in thymocytes with NB-DNJ, as described above. Analogous experiments with DC could not be performed because of toxicity of NB-DNJ at pharmacological concentrations, similar to observations described for the analogous drug N-butyldeoxygalactonojirimycin [91]. Incubation of NPC2$^{-/-}$ thymocytes with NB-DNJ significantly improved their capacity to present αGalCer to iNKT hybridoma cells (**Figure 27**), indicating that accumulating lipids contribute to the impairment of lipid antigen presentation in NPC2$^{-/-}$ thymocytes. This mechanism might also explain why thymic selection of iNKT cells is impaired in NPC2$^{-/-}$ mice.

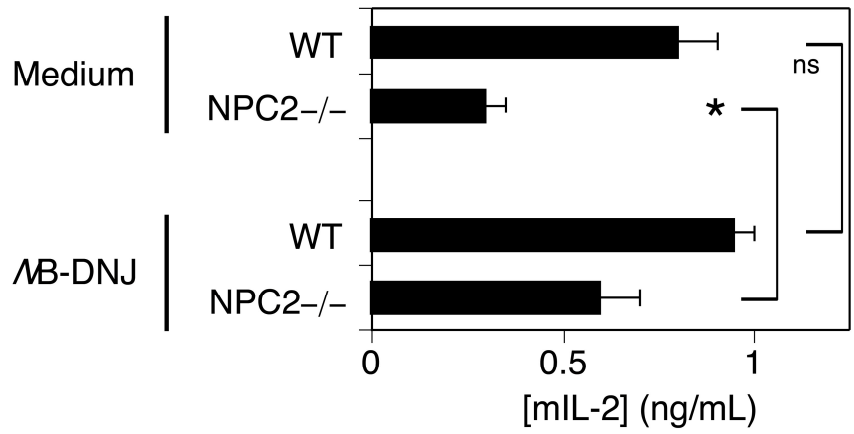


Figure 27. Accumulation of lysosomal storage lipids accounts for the impairment of lipid antigen presentation in NPC2$^{-/-}$ cells. Thymocytes from NPC2$^{-/-}$ or WT mice were incubated with NB-DNJ or medium before αGalCer (1 ng/mL) and murine iNKT hybridoma cells were added. IL-2 release to cell culture supernatant was determined. The bar chart shows the mean release of IL-2 (±SD; n=3). * $p<0.05$; ns, not significant.

The formation of stimulatory CD1d:lipid antigen complexes is impaired in NPC2$^{-/-}$ APC

To investigate whether the formation of functional CD1d:lipid antigen complexes on the cell surface is impaired in NPC2$^{-/-}$ APC, DC from NPC2$^{-/-}$ and WT mice were fixed with glutaraldehyde and then incubated with αGalCer to test their presentation capacity to iNKT hybridoma cells. For this experiment we chose DC rather than thymocytes because surface CD1d levels were not reduced in the case of NPC2$^{-/-}$ DC, as mentioned above. Fixation did not improve the efficacy of αGalCer stimulation by NPC2-deficient APC (**Figure 28A**, right panel). Increasing concentrations of αGalCer could not overcome this defect, suggesting that the CD1d:αGalCer complexes formed on NPC2$^{-/-}$ DC might have intrinsic differences limiting the stimulation of iNKT cells. These differences could depend on the presence of spacer molecules disturbing proper binding of stimulatory lipid antigens or, alternatively, could be ascribed to a reduced number of CD1d molecules freely available for binding αGalCer.

To test for the first possibility, lipid antigens with short alkyl chains were used together with NPC2$^{-/-}$ DC (**Figure 28B**). Indeed, αGalCer containing a C8 alkyl chain binds to CD1d also in the presence of spacer molecules inserted in the A' pocket [15]. We compared therefore the stimulatory capacity of two αGalCer molecules containing a C8 and a C26 alkyl chain, respectively (**Figure 28**).

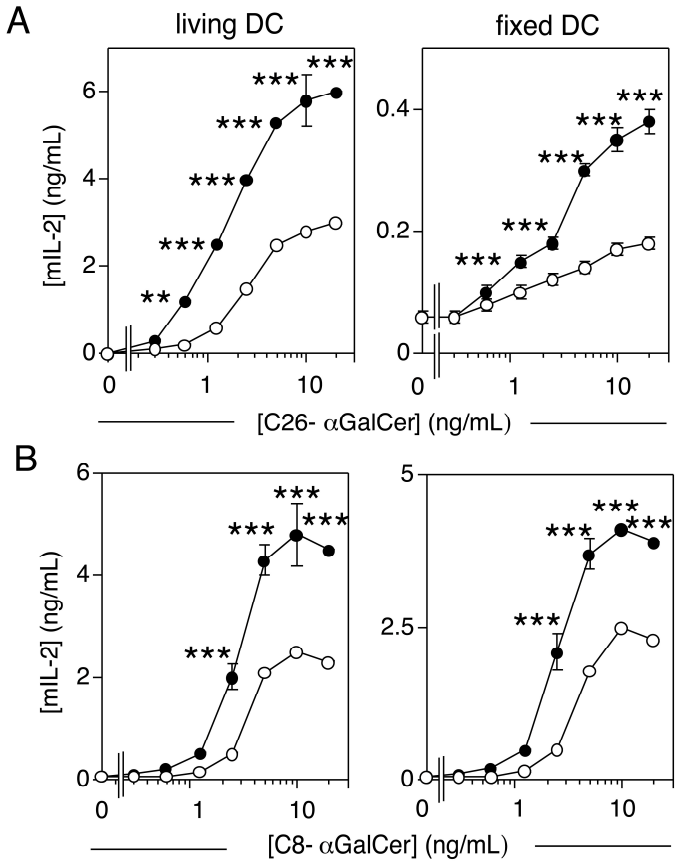


Figure 28. Disturbance of CD1d in forming functional complexes with lipid antigen accounts for the impairment of lipid antigen presentation in NPC2$^{-/-}$ cells. Living (left panel) or fixed (right panel) DC from WT (●) or NPC2$^{-/-}$ (○) or mice were incubated with various concentrations of "classical" C26 αGalCer (**A**) or a C8 αGalCer analogue (**B**) before the addition of iNKT hybridoma cells. IL-2 release to cell culture supernatant was determined. Diagrams show the mean release of IL-2 (±SD; n=3). ** $p<0.01$, *** $p<0.001$ comparing WT and NPC2$^{-/-}$

These experiments revealed three main findings: (i) the reduction of the alkyl chain length from C26 to C8 did not improve the efficacy of αGalCer stimulation by living or fixed NPC2$^{-/-}$ DC. This suggests that a smaller number of stimulatory CD1d:αGalCer complexes is formed on NPC2$^{-/-}$ cells regardless of the alkyl chain

length. (ii) When fixed DC were used, about 100 times more C8 αGalCer was required for maximal T cell stimulation as compared to living DC. Instead, C26 αGalCer was equally potent with both fixed and living DC. This indicates that the presence of short alkyl chains affects the formation of stable complexes with CD1d molecules modified by fixation. (iii) The maximal cytokine release induced by C8 αGalCer was similar when living or fixed DC were used, thus indicating that the CD1d:αGalCer complexes formed with this analogue stimulate iNKT cells very efficiently.

DISCUSSION

Deficiencies in enzymes of the lysosomal glycosphingolipid degradation pathway or in lysosomal lipid transfer proteins cause imbalances in lipid metabolism. Recently, mouse models of lipid storage diseases have been studied with respect to the relevance of lysosomal enzymes and lipid transfer proteins for stimulation and selection of iNKT cells, which recognize glycolipid antigens in the context of CD1d antigen-presenting molecules. These studies revealed that Hexb-[76, 208], Hexa-[208], α-Gal-[208], β-Gal-[208], saposins-[84], or NPC1-[91, 208] deficient mice have reduced numbers of iNKT cells and, in the cases of $Hexb^{-/-}$, $\beta\text{-Gal}^{-/-}$, $saposin^{-/-}$, and $NPC1^{-/-}$ mice, defects in lipid antigen presentation. iNKT cell deficiency has originally been explained as a result of lacking iGb3 in the case of $Hexb^{-/-}$, and of impaired saposin-mediated lipid transfer onto CD1d molecules in the case of $saposin^{-/-}$ mice. Recently it has been instead hypothesized that lysosomal lipid storage might have an independent, nonspecific negative effect on lipid antigen presentation and thymic selection of iNKT cells [91, 208]. However,

the contribution of lysosomal storage lipids to an impairment of lipid antigen presentation has not been carefully investigated, so far. Here we investigated two mouse models of imbalanced lipid metabolism, β-Gal$^{-/-}$mice (a model for GM1 gangliosidosis) and NPC2$^{-/-}$ mice (a model for Niemann Pick C2 disease). In both models, development of iNKT but not of other T cells was impaired by 70%. The simplest explanation for iNKT cell deficiency in these mice would be a specific involvement of β-Gal in the generation of iNKT cell-selecting lipid antigen(s) in the thymus and a specific role of NPC2 as a lipid transfer protein for iNKT cell-selecting lipid antigen(s) or their precursors. However, our data suggest that it is rather indirect mechanisms, particularly the accumulation of different types of lipids, which are responsible for impaired selection of iNKT cells in the two mouse models. The enzyme β-Gal is involved in the hydrolyzation of terminal β-galactosidic residues of glycoconjugates, including the gangliosides GM1 and GA1, and lactosylceramide [329](**Figure 6**). GM1/GA1 can no longer be degraded to GM2/GA2 and GM3/GA3 and represent the most prominent glycolipids accumulating in patients and mice deficient in β-Gal [332-334]. However, the lack of gangliosides such as GM2/GA2 or GM3/GA3 probably does not explain deficient development of iNKT cells in β-Gal$^{-/-}$ mice since mice lacking ganglio-series glycolipids as a result of genetic deficiency of GM2 or GM3 synthases, the key enzymes for their synthesis, displayed no apparent defect in development of NKT cells [76]. Lactosylceramide does not accumulate in β-Gal$^{-/-}$ mice because its degradation, involving the removal of terminal β-galactose residues, can be exerted by an alternative enzyme, Galactosylceramidase (EC 3.2.1.46)[335, 336]. Hence, assuming that accumulating lipids in lysosomes would not interfere with the glycosphingolipid degradation pathways, the degradation products of lactosylceramide would be generated normally in these mice. iGb3, which for some time was considered a selecting lipid antigen, is also generated normally because

β-Gal is not involved in the globoside degradation pathway. Overall, we therefore considered the possibility that deficiency of β-Gal indirectly, rather than directly, causes the impairment of iNKT cell development. This consideration was supported by our observations that β-Gal$^{-/-}$ DC, in contrast to β-Gal$^{-/-}$ thymocytes, were able to stimulate human Vα24 iNKT cells in the absence of additional lipid antigens and that presentation of exogenous lipid antigens (αGalCer or iGb3) by β-Gal$^{-/-}$ thymocytes was impaired. Similarly, Gadola et al. [208] showed impaired presentation of αGalCer by β-Gal$^{-/-}$ splenocytes. Furthermore, we found that presentation of exogenous lipid antigen is improved by pre-treatment of β-Gal$^{-/-}$ thymocytes with NB-DNJ, an inhibitor of glycosphingolipid biosynthesis known to reduce pathological lipid storage in vitro and in vivo [330]. This indicates that accumulating lipids disturb lipid antigen presentation in β-Gal$^{-/-}$ thymocytes. GM1, the major glycolipid accumulating in β-Galactosidase-deficient patients and mice, competes with other glycolipids for binding to CD1d [337]. This mechanism would affect the formation of stimulatory CD1d:lipid antigen complexes on the cell surface. However, since upon fixation β-Gal$^{-/-}$ and WT thymocytes presented lipid antigen equally, it is intracellular defects that account for the impairment of lipid antigen presentation in β-Gal$^{-/-}$ cells. Disturbance of proteins involved in the transport of lipid antigens, in the unloading of non-antigenic lipids from CD1d, in the loading of lipid antigens onto CD1d, and/or in the maintenance of functional CD1d:lipid antigen complexes might occur. The effect of β-Galactosidase deficiency on lipid antigen presentation could be observed with thymocytes and splenocytes, but not with DC. Different quantities or types of accumulating lipids might explain why lipid antigen presentation is impaired in fixation β-Gal$^{-/-}$ thymocytes but not DC. Therefore, this demands caution with the cell type used for lipid antigen-presentation experiments to avoid misinterpretations, for example regarding thymic selection.

The proteins NPC1 and NPC2 are thought to be involved in the transport of different lipids [97, 331]. Deficiency of either NPC1 or NPC2 results in lysosomal storage of cholesterol, phospholipids, GM2 and GM3, glucosylceramide, lactosylceramide, sphingomyelin, and other lipids [97]. The precise mechanisms of action or the substrates of these putative transporters have not been fully defined, yet. However, it is inferred that NPC1 and NPC2 have nonredundant functions in a common pathway of lipid transport [97] with a particular role for NPC2 as a lysosomal cholesterol transfer protein [95]. Recently, impaired glycosphingolipid trafficking, lipid antigen presentation, and development of iNKT cells in NPC1$^{-/-}$ mice have been described [91, 208]. Glycosphingolipid trafficking was partly restored by pre-treatment of NPC1-deficient cells with N-butyldeoxygalactonojirimycin *in vitro* [91]. The impact of this partial restoration on lipid antigen presentation to iNKT cells has not been studied. However, since accumulating lipids disturb glycosphingolipid trafficking in NPC1$^{-/-}$ cells [91, 331], they might also contribute to impaired lipid antigen presentation if NPC1 is absent.

In the present study, we demonstrated that lipid antigen presentation is impaired in NPC2$^{-/-}$ APC, including thymocytes, and that thymic selection of iNKT cells is disturbed in NPC2$^{-/-}$ mice. Impaired presentation of lipid antigens was not due to a change in the half-life of CD1d:lipid antigen complexes thus excluding faster degradation of CDd:lipid antigen complexes. Importantly, lipid antigen presentation could be improved by pre-treatment of NPC2$^{-/-}$ APC with NB-DNJ, directly demonstrating that accumulating glycosphingolipids disturb lipid presentation in NPC2$^{-/-}$ APC. Two mechanisms describing how the accumulating lipids might inhibit CD1d antigen presentation can be envisaged. First, they could hinder unloading of the spacer molecules that are associated with CD1d [15]. However, this possibility is not supported by the results obtained with the C8 αGalCer analogue. This lipid antigen can effectively bind to CD1d in the presence of

spacers [15]. Nevertheless, it does not restore the presentation capacity of NPC2$^{-/-}$ APC. A second mechanism is that accumulating glycosphingolipids could form very stable complexes with CD1d and prevent the formation of complexes containing the stimulatory lipid antigens. Indeed, a number of lipids are able to bind to CD1d without being antigenic for iNKT cells, including phospholipids [328, 337] and glucosylceramide [328] which accumulate in NPC2$^{-/-}$ cells. Although this mechanism is difficult to prove experimentally, it is in agreement with our findings showing that pre-treatment of NPC2$^{-/-}$ DC with NB-DNJ partially restores lipid antigen presentation and that even high concentrations of αGalCer could not overcome the presentation defect. Although the accumulation of lipids impairs lipid antigen presentation in both β-Gal$^{-/-}$ and NPC2$^{-/-}$ cells, the underlying mechanisms are different. We cannot exclude that also the mouse strain background (C57BL/6 in the case of β-Gal$^{-/-}$ and BALB/c in the case of NPC2$^{-/-}$ mice) may influence the effect of accumulating lipids on the phenotype of iNKT cells. However, because of the characteristic types of lipids accumulating in β-Gal$^{-/-}$ *vs*. NPC2$^{-/-}$ mice, we favour the hypothesis that it is mainly a change in the quantity and type(s) of presented lipid antigens that influences the phenotype and TCR repertoire among the residual iNKT cell populations in β-Gal$^{-/-}$ *vs*. NPC2$^{-/-}$ mice. This includes a remarkable decrease of the Vβ8.2/Vβ7 ratio in β-Gal$^{-/-}$ but not NPC2$^{-/-}$ and an increase of the CD4^{-}/CD4^{+} ratio in NPC2$^{-/-}$, but not β-Gal$^{-/-}$ mice. In β-Gal$^{-/-}$ mice, limiting quantities of available lipids responsible for positive selection might favour development of iNKT cells expressing Vβ7, which confers high avidity binding to CD1d in the context of endogenous selecting lipid antigen(s)[188, 207]. In NPC2$^{-/-}$ mice, changes in the types of selecting lipid antigens might affect the strength of TCR signalling and cause more frequent downregulation of CD4, resembling down-regulation of CD4 and CD8 on DP thymocytes caused by stimulation of the TCR [338].

In summary, disregulation of lipid metabolism and accumulation of lipids cause defective presentation of lipid antigens and impaired thymic selection of iNKT cells. The exact underlying mechanisms are diverse, depending on the cause of imbalance, and, as a consequence, different subsets of iNKT cells are selected in thymi of mice suffering from different lipid storage diseases.

CHAPTER 2

CD1e participates in shaping the lipid antigen repertoire

SUMMARY

CD1 proteins are expressed by a limited number of cell types, including dendritic cells. Only some mammals express CD1e, a CD1 family member with important differences as compared to the other CD1 proteins. CD1e has functions going beyond the presentation of lipid antigen to T cells. It binds lipid molecules in the lysosomal compartment and facilitates processing of complex glycolipid antigens, thus participating in lipid editing of CD1b-presented antigens.

Nothing is known about the role of CD1e in editing CD1d or CD1c restricted lipids, either of self or of microbial origin.

Here we describe that the presence of CD1e on APC expressing other CD1 molecules influences the response of T cells, including that of iNKT cells. APC from human CD1E-transgenic mice are more efficient than wild-type APC in the stimulation of T cells with microbial antigens as well as of autoreactive iNKT cells. The function of CD1e is therefore broad and the mechanism how it modulates lipid-specific T cell responses involves the generation of lipid antigens both of endogenous and exogenous origin, possibly by endosomal editing of lipid molecules and by facilitating their loading and unloading on other CD1 molecules.

RESULTS

CD1e participates in the stimulation of CD1b- and CD1c-restricted T cells.

To study in detail the role of CD1e in tuning the lipid-specific T cell response to CD1c- and CD1b-dependent antigens, we took advantage of our collection of lipid-specific T cell clones with various CD1 restriction and specific for self and non-self antigen. THP-1 cells expressing CD1b or CD1c, alone or in combination with CD1e, were generated by transfection and were used as APC in T cell activation assays. Expression levels of CD1b and CD1c, CD1e and MHC Class I molecules of THP1-CD1c and THP1-CD1b single or double transfectants are illustrated in **Figure 29**.

CD1b- or CD1c-dependent T cell clones reactive to self antigens were variously affected in their response by the presence of CD1e in APC (**Figure 30A-C**). When APC expressed CD1e, the autoreactive response of CD1b-restricted clone GG123b was inhibited (**Figure 30A**), that of the CD1c-autoreactive clone DN4.99 was increased (**Figure 30B**), and that of the CD1c- autoreactive clone K34B27.F was unaffected (**Figure 30C**). The response to exogenously added antigens presented by CD1b was also differently influenced by CD1e. The CD1b-restricted response of the GG33a clone to ganglioside GM1 was impaired in the presence of CD1e (**Figure 30D**). The response of the CD1b-restricted clone Z4B27 to the SGL2 synthetic analog of the mycobacterial diacyl sulfoglycolipid [147, 151, 339] was equal irrespective of CD1e (**Figure 30E**). The response of the Z5B71 clone to GroMM was instead increased in the presence of CD1e-expressing APC (**Figure 30F**) as well as increased was the response of the CD1b-restricted clone DS1C9b to sulfatide (**Figure 30G**).

The presence of CD1e in APC co-expressing CD1c gave similar results also in the case of exogenously added antigens. The response of the clone DL15A31 to a

semi-purified preparation of *M. tuberculosis* lipid antigens was not affected (**Figure 30I**), while the clone DS1B9c responded slightly better to sulfatide when APC expressed CD1e. The effect was visible at low but not at high antigen doses (**Figure 30H**).

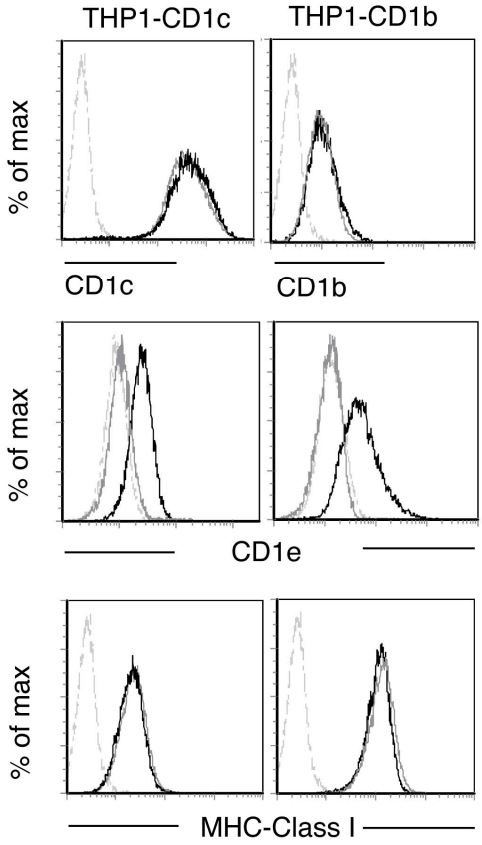


Figure 29: CD1 levels are identical in THP1 single and double transfectants. THP1-CD1c (left side) and THP1-CD1b (right side) single (gray line) and double transfected (black line) where analysed for expression levels of CD1 molecules (upper panel), CD1e (middle panel) and MHC-Class I (lower panel).

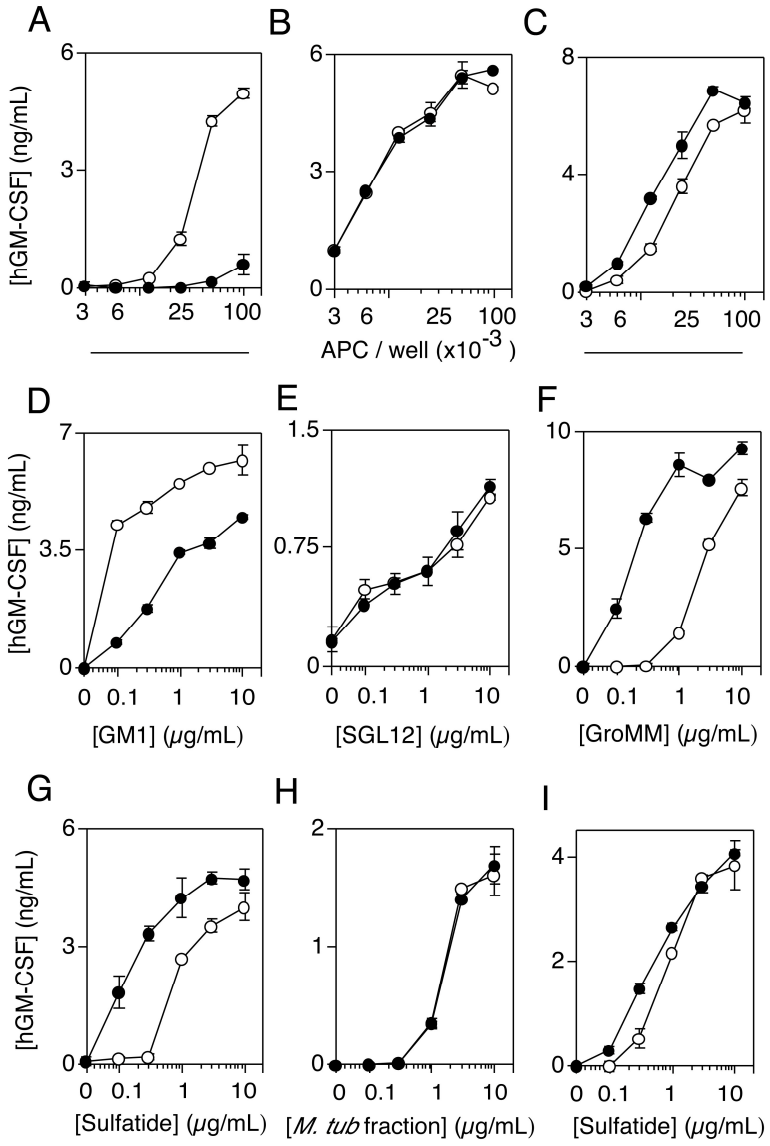


Figure 30. CD1e participates in the presentation of CD1b- and CD1c-restricted lipid antigens (**A-C**) Self antigens. (**A**) CD1b-restricted clone GG123b response to THP-1 CD1B and CD1E (●) and THP-1 CD1B only (○). (**B,C**) CD1c-restricted clones K34B27.F (**B**) and DN4.99 (**C**) response to THP-1 CD1C and CD1E (●) and to THP-1 CD1C only (○). (**D-G**) CD1b-dependent exogenous antigens. (**D**) GG33a clone response to GM1, (**E**) Z4B27 clone response to SGL12, (**F**) Z5B71 clone response to GroMM and (**G**) DS1c9b clone response to sulfatide presented by THP-1 CD1B and CD1E (●) than THP-1 CD1B only (○). (**H,I**) CD1c-dependent exogenous antigens. (**H**) DL15A31 clone response to a semi-purified fraction of mycobacterial lipids, and (**I**) DS1b9c clone response to sulfatide presented by THP-1 CD1C and CD1E (●) and THP-1 CD1C only (○). Human GM-CSF release was determined by ELISA and expressed as mean ng/ml ± s.d. of duplicate wells.

Taken together these findings suggest that CD1e differently influences the presentation of endogenous and exogenous lipid antigens, by favoring or limiting presentation by CD1b and CD1c molecules. In some cases, though, CD1e is irrelevant. We did not observe any apparent correlation with CD1-restriction, and probably the nature of the lipid antigen is important.

Type 1 iNKT and type 2 NKT clones respond differently to self lipids in the presence of CD1e

To investigate whether the presence of CD1e in APC influences also the response of CD1d self-reactive clones, THP-1 were transfected with CD1D cDNA or with both CD1D and CD1E cDNAs and were used as APC in the absence of additional lipid antigens. Several type 1 iNKT and type 2 NKT clones were selected according to their autoreactivity and to different TCRβ CDR3 sequences (data not shown).
The presence of CD1e influenced differently the response of distinct self-reactive clones evaluated as cytokine release. For all the type 1 iNKT clones that we tested, the presence of CD1e in THP1 cells resulted in an increased GM-CSF production (**Figure 31, left panel).** Type 2 NKT clones, instead, showed a more variable responsiveness in the presence of CD1e (**Figure 31, right panel**). Similar results were obtained using C1R cells (a human B cell line) transfected with CD1D or both CD1D and CD1E cDNAs, thus excluding any effect related to the cell type used as APC (**Figure 31B**).

When we analysed the effect of CD1e by titrating the APC, some clones were positively influenced (A), some others were negatively influenced (B) and only few clones were unaffected by CD1e expression (C) (**Figure 32**).

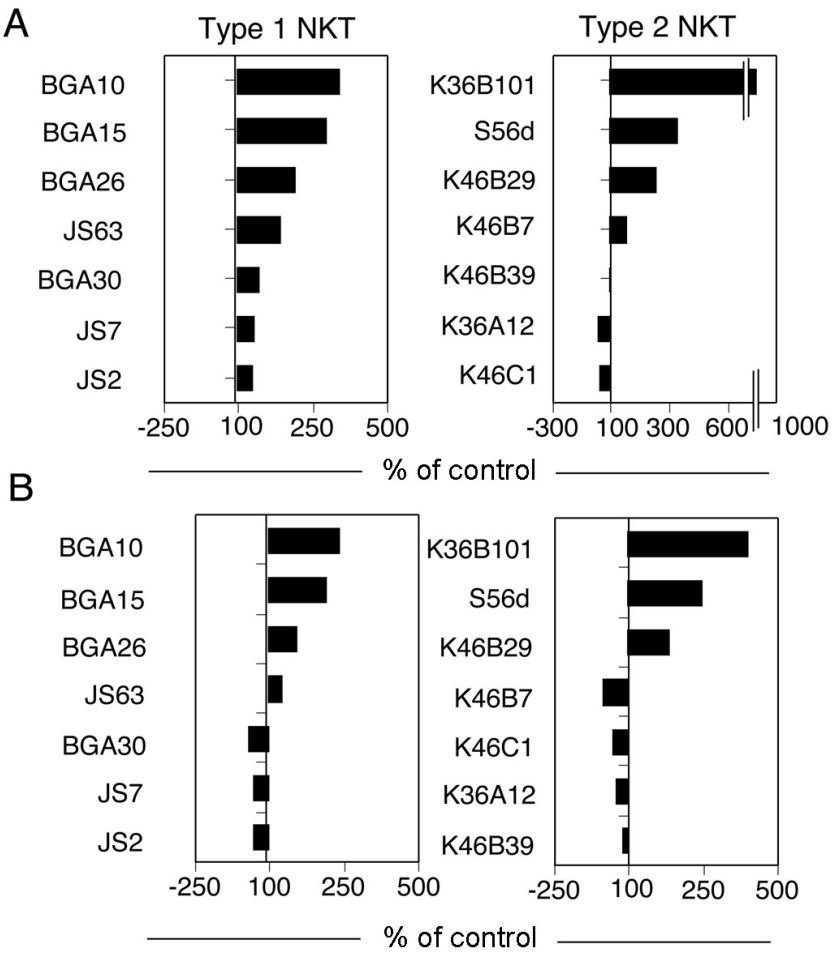


Figure 31. CD1d dependent clones (type 1 and type 2) respond differently to self lipids in the presence of CD1e. A panel of type 1 iNKT cells (left panel) and type 2 (right panel) NKT cells was incubated with single and double transfected THP-1 (**A**) and C1R (**B**) and the difference in activation is measured as % of change of hGM-CSF released after stimulation with hCD1D+E transfectants as compared to hGM-CSF released to hCD1d transfectants only (100%).

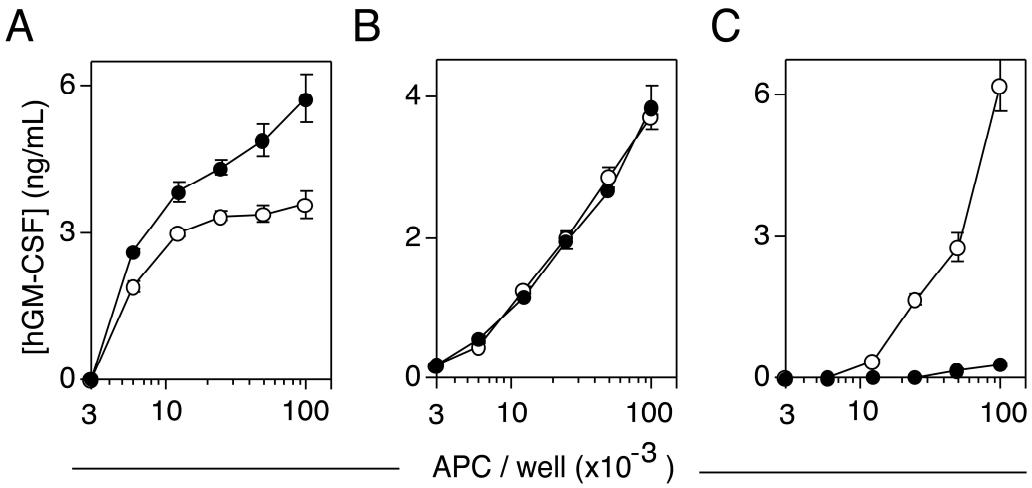


Figure 32. (**A**) Human Type 1 iNKT cells or (**B,C**) Type 2 NKT cells were incubated with decreasing number of THP-1 cells transfected either with CD1D gene (○) or with CD1D and CD1E genes (●). After 24 hr hGM-CSF release (ng/ml ± s.d.) to cell culture supernatant was determined by ELISA.

We also excluded any aspecific effect due to differences in CD1d expression levels among single and double transfectants since CD1d (left panel), as well as MHC-I (right panel), was equally expressed on both types of transfectants. CD1e expression was detected only in double transfected cells (middle panel) (**Figure 33**). Similar CD1d expression levels among single and double transfectants were also observed on C1R cells (data not shown).

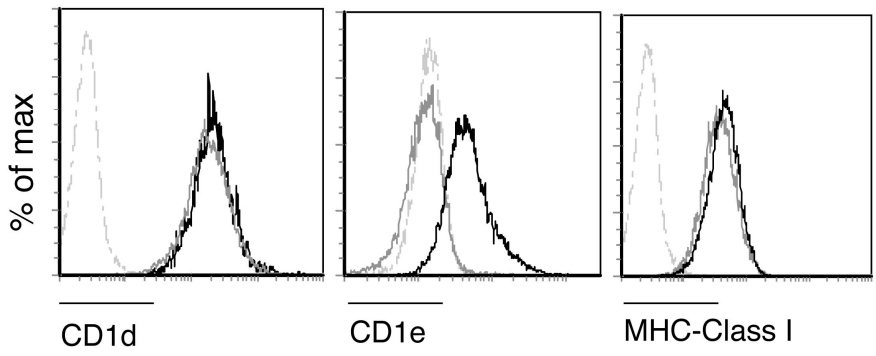


Figure 33. CD1d levels are identical in THP1 CD1D single (grey line) and CD1D, CD1E double transfectants (black line). CD1d (left panel), CD1e (middle panel) and MHC-I expression levels (right panel) detected on both types of transfectants. Dotted grey line, irrelevant mAb.

These results suggest that CD1e expression in APC can influence the response of CD1d self-reactive T cells to endogenous antigens. The function of CD1e probably depends on the nature of the lipid antigen, since different clones are influenced to different extent or are not at all influenced in their reactivity by the CD1e presence in APC.

CD1e influences the types of cytokines secreted in response to the presentation of exogenous lipid

We next studied whether the presentation of exogenous lipid antigens known to be potent iNKT TCR agonists is also affected by CD1e presence. A nonself-reactive Type 1 iNKT human clone was stimulated with APC transfected with CD1D or CD1D and CD1E in the presence of glycolipid agonists αGalCer, αLacCer or the *Sphingomonas*-derived antigen GSL'. In this set of experiments the antigens were

kept in the well during the entire period of stimulation. It has been recently shown that the types of cytokines released after TCR triggering varies hierarchically according to the strength of the signal [340]. We therefore measured the release of GM-CSF, IL-4 and IFN-γ in response to lipid antigen presentation to the human iNKT cell clone. We observed a common hierarchy of cytokine secretion for the three antigen tested. GM-CSF secretion was not influenced by CD1e presence (**Figure 34 A,D,G**) which, instead, inhibited the secretion of IL-4 (**Figure 34 B,E,H**) and IFN-γ (**Figure 34 C,F,I**) for all the antigens tested. The threshold of activation required for secretion of GM-CSF is known to be lower than the threshold required for IL-4 and IFN-γ secretion. These findings suggest that, in the presence of CD1e, an equilibrium may exist between generation of new CD1d:lipid antigen complexes and unloading of re-internalized complexes. As a consequence, in the presence of CD1e fewer CD1d:antigen complexes may be available on the surface of APC. Secretion of cytokines which require a higher threshold of activation and an higher number of CD1d:antigen complexes on the surface may be therefore decreased in the presence of CD1e.

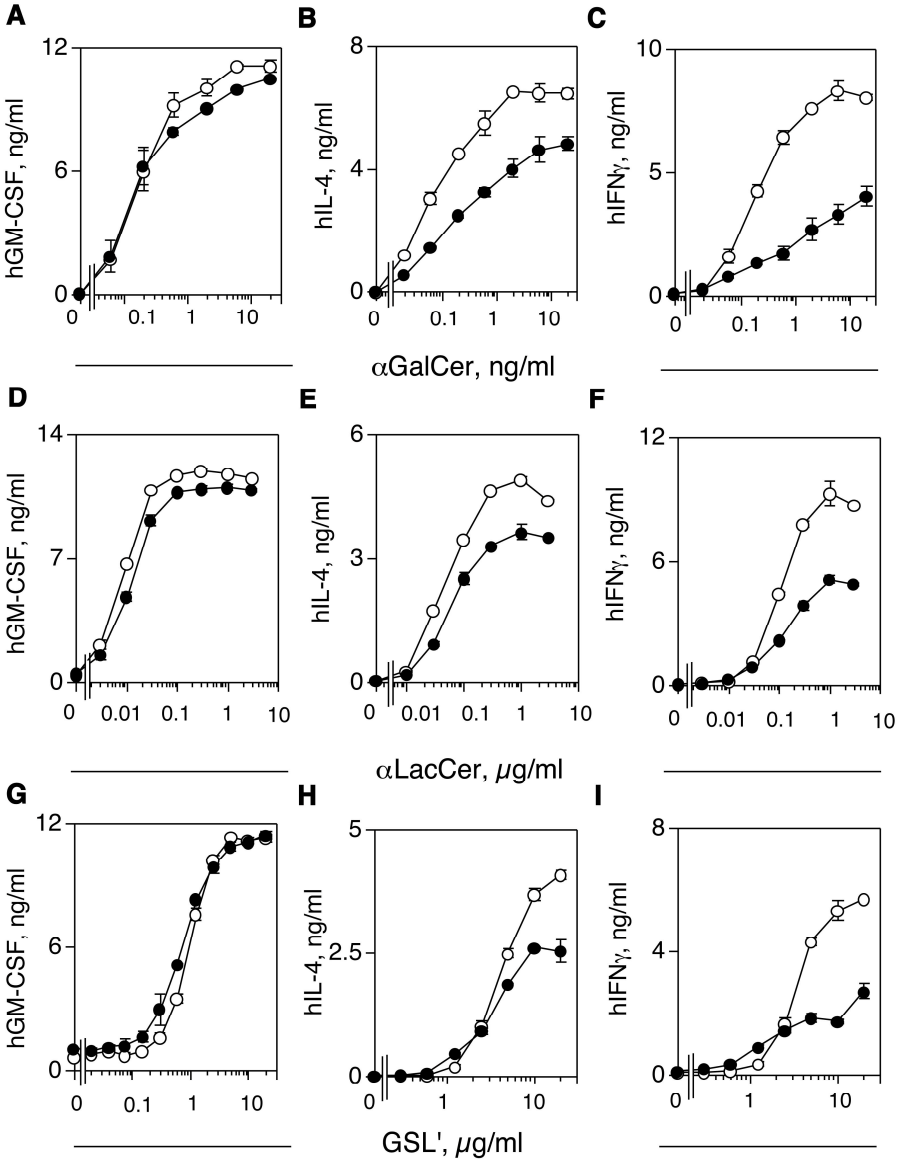


Figure 34. CD1e influences the type of cytokines secreted in response to the presentation of exogenously added lipid antigen.. Human monocyte-derived THP-1 cells transfected with CD1D gene (○) or with CD1D and CD1E genes (●) were incubated for 2 hr with different concentrations of αGalCer (**A, B, C**), or of αLacCer (**D,E,F**) or of Sphingomonas-derived GSL' (G,H,I) before addition of the human iNKT clone VM-D5.,hGM-CSF and hIL-4 (ng/ml ± SD) release was determined by ELISA after 24 hr, while hIFN-γ release was determined after 48 hr by ELISA.

We then analysed the effect of CD1e on iNKT cell response when the antigen is not constantly available, but is pulsed only for short time. We chose αGalCer as reference agonist. When αGalCer was pulsed for short time, CD1e presence allowed a faster response detectable as early as 4 hr after pulsing by measuring IL-4 (**Figure 35A**) or GM-CSF release (data not shown). In the absence of CD1e the kinetic of the response was slower and at later time points the CD1e effect was reduced. These data suggest that, at early time points after the antigen becomes available, CD1e may facilitate the formation of CD1d:lipid complexes, thus allowing prompt iNKT cells activation.

Next, antigen chasing experiments were performed. APC were pulsed with αGalCer for 2 hr, then washed and chased at 37°C for different times before fixation and addition of iNKT cells. APC expressing CD1e showed a faster reduction of iNKT cells stimulation, suggesting a fast decay of the stimulatory CD1d: αGalCer complexes (**Figure 35B**).

These finding support the hypothesis that CD1e may orchestrate the formation of CD1d complexes, by facilitating the turnover of lipids onto CD1d molecules. CD1e may facilitate loading of lipid antigens at early time points, while facilitating the unloading of CD1d:antigen complexes at later time points, thus participating in the fine tuning of the immunological response.

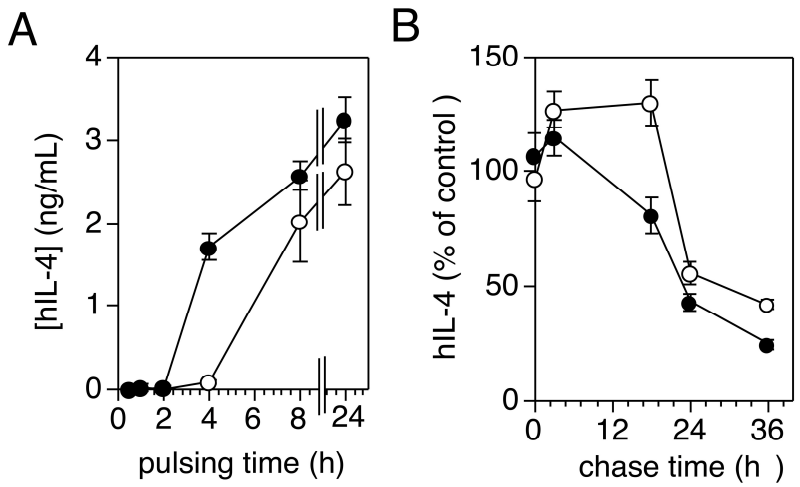


Figure 35. The presentation of αGalCer is increased when the antigen is pulsed for short time. (**A**) THP1 cells transfected with hCD1D gene (○) or with CD1D and CD1E genes (●) were incubated for different time with αGalCer (10 ng/ml) and then fixed before the addition of the human Vα24 iNKT clone VM-D5. (**B**) CD1e facilitates the degradation of CD1d-αGalCer complexes. THP-1 cells transfected with hCD1D gene (○) or with hCD1D and hCD1E genes (●) were pulsed for 2 hr with αGalCer (1 ng/ml) and chased for different periods of time before the addition of the human iNKT clone VM-D5. Diagrams show the mean release of cytokines (± SD) (n=3).

CD1e facilitates loading and unloading of α-GalCer onto and from CD1d

These findings suggested that CD1e might facilitate loading and unloading of antigens onto and from other CD1 family members, thus acting as a lipid transfer protein.

To investigate whether CD1e facilitates lipid binding to CD1d, we performed a plate-bound CD1d-based lipid presentation assay [316]. Soluble human CD1d (sCD1d) was coated onto 96-well plates and αGalCer was added in the presence or in the absence of recombinant CD1e (sCD1e) at pH 4.5 to mimic the acidic environment of lysosomes where CD1d and CD1e co-localize. iNKT cells were

then added and their response was determined by measuring GM-CSF release 18 hr later. Recombinant soluble saposins A, B, or C were used as controls due to their role in assisting CD1d loading [84, 341]. The addition of CD1e significantly enhanced the response of iNKT cells. Furthermore, CD1e was more efficient than saposins B and C in facilitating the formation of stimulatory complexes (**Figure 36A**).

Next we tested the possibility that CD1e may also unload αGalCer from CD1d.

CD1e can also efficiently unload αGalCer from CD1d (**Figure 36B**). sCD1d was coated on the plastic and then loaded with αGalCer. The excess of antigen was washed before the addition of sCD1e or of saposins A, B or C as control LTP. After 24 hr iNKT cells were added and their response was measured by cytokine release assay. CD1e significantly decreased the response of iNKT cells, indicating that it can also unload αGalCer from sCD1d, while saposins were not effective in this type of CD1d unloading experiments.

Taken together these results show that CD1e behaves as a LTP and facilitates transfer of lipids to and from CD1d.

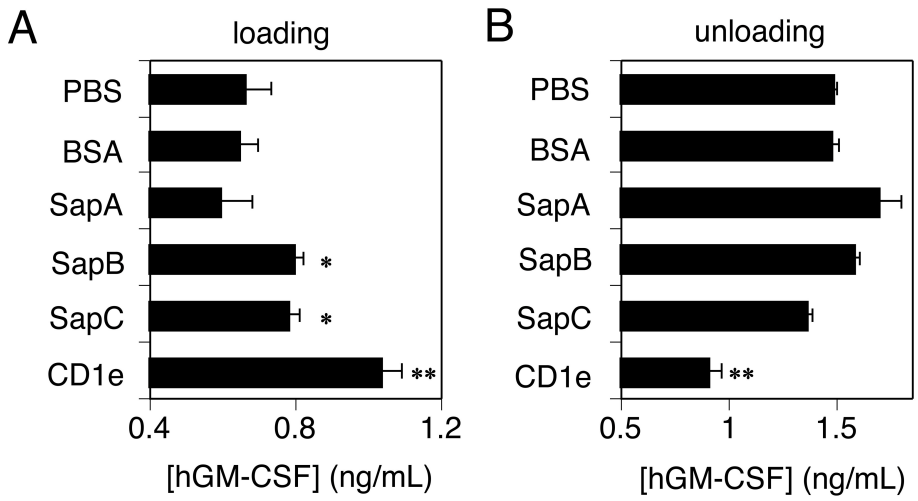


Figure 36. CD1e facilitates loading and unloading of plate bound CD1d with αGalCer (**A**). Plate bound CD1d was pulsed with 1 μg/ml of αGalCer in the presence of different soluble recombinant lipid transfer proteins (SapA, B, C and BSA) or of soluble recombinant CD1e or with PBS only and then a human iNKT clone was added to the plate. After 24 hr the release of hGM-CSF was measured by ELISA. (**B**). Plate bound CD1d was pulsed overnight with 1 μg/ml of αGalCer. Different soluble recombinant lipid transfer proteins or PBS only or soluble recombinant CD1e were added to the wells overnight and then a human iNKT clone was added to the plate. After 24 hr the release of hGM-CSF was measured by ELISA. Diagrams show the mean release of cytokines (± SD) (n=3).
** $p<0.01$ comparing loading and unloading in the presence of PBS or of soluble recombinant lipid transfer proteins.

Generation and characterization of CD1e transgenic mice

To investigate whether CD1e also participates in CD1d-mediated antigen presentation *in vivo*, we generated transgenic mice expressing the human CD1e gene. The Eα-CD1e tg mouse model was established, in which the human CD1E cDNA was expressed under the H-2 Eα promoter directing the expression on APC including thymic epithelial cells [317](**Figure 37**). Several lines of transgenic mice were obtained and in two of them the transgene was correctly expressed.

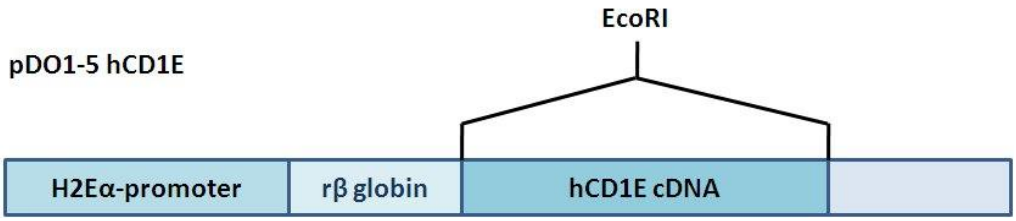


Figure 37. Schematic representation of the pDO1-5 hCD1E vector used for microinjection to generate Eα-CD1e transgenic mice.

In Eα-CD1e transgenic mice, the expression of CD1e was found in B cells, peritoneal macrophages, BM-derived DC, at levels comparable to what observed in human cells (data not shown), but not in T lymphocytes (**Figure 38**).

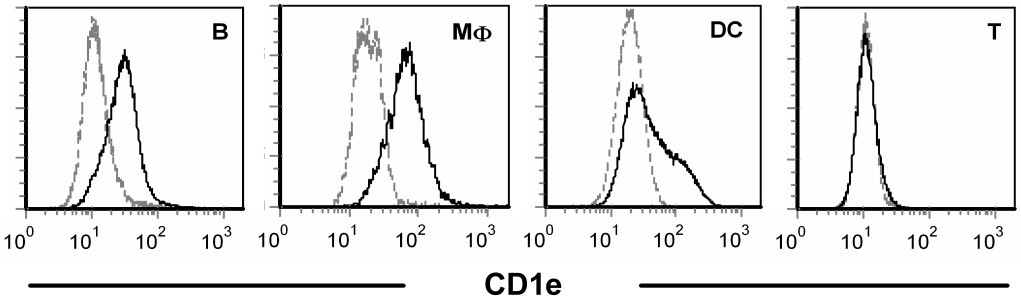


Figure 38. CD1e is expressed by B cells, Macrophages and Bone Marrow-derived DC of Eα- CD1e transgenic mice but not by T cells. Splenocytes from WT and Eα-CD1e tg mice were analyzed for expression of CD1e in B cells ($B220^+$ cells) and T cells ($CD3^+$). Peritoneal macrophages were obtained by *i.p.* injection of tThioglycollate and $Mac1^+$ cells were analyzed for CD1e expression. Bone marrows were differentiated for 8 days with mGM-CSF and $CD11c^+$ cells were analyzed for CD1e expression.

In human cells, CD1e is targeted to the lysosomes where it is cleaved into an active soluble form. Therefore, we investigated whether in transgenic mouse cells CD1e protein is correctly processed.

DC from WT or Eα-CD1e tg mice were metabolically labeled for 30 minutes with ^{35}S and chased for 2 or 4 hours. Membrane proteins were immunoprecipitated with the 20.6 anti-CD1e mAb and the glycosylation of the immunoprecipitated proteins was examined by treatment with Endoglycosidase H or F. Both enzymes cleave between two N-acetylglucosamine residues linked to asparagines in oligomannose, but not complex oligosaccharides from glycoproteins. These enzymes are used to monitor post-translational modifications in the Golgi apparatus. Between 2 and 4 hr after syntesis, CD1e molecules become Endo H resistent, suggesting that cleavage occurs late in its biosynthesis. 4 hr after chasing, only a 28 kDa protein was immunoprecipitated, corresponds to the CD1e soluble form. These findings confirm that also in mouse Eα-CD1e DC CD1e is processed in the soluble active form (**Figure 39**).

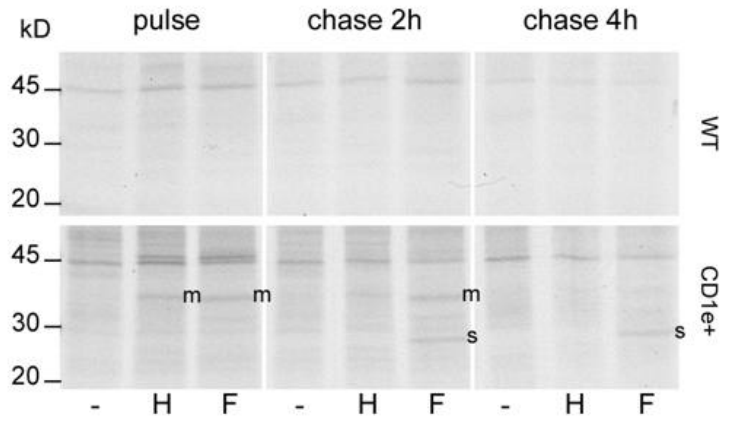


Figure 39. CD1e in DC from transgenic mice is cleaved in endosomal compartments. DC were metabolically labeled with [^{35}S] methionine and cysteine for 30 min. Cells were lysed either directly or following chase at 37 °C for 2 h in normal culture medium. CD1e was immunoprecipitated with the 20.6 mAb, deglycosylated with EndoH (H) or EndoF (F) or left untreated(-), separated on denaturing electrophoresis gels and analyzed by autofluorography. Letters indicate the position of cleaved CD1e, m: membrane CD1e, s: soluble CD1e.

To investigate the intracellular localization of tg CD1e, DC from Eα-CD1e and non tg littermates were stained with the anti-CD1e mAb 20.6, with mAb specific for TGN-38, a marker of Golgi, or for I-A^b. Confocal microscopy showed that CD1e partially co-localizes with TGN-38 (**Figure 40** upper panels) as well as with MHC-II associated compartments, (**Figure 40** lower panels), thus showing the same pattern of distribution as in human cells [17].

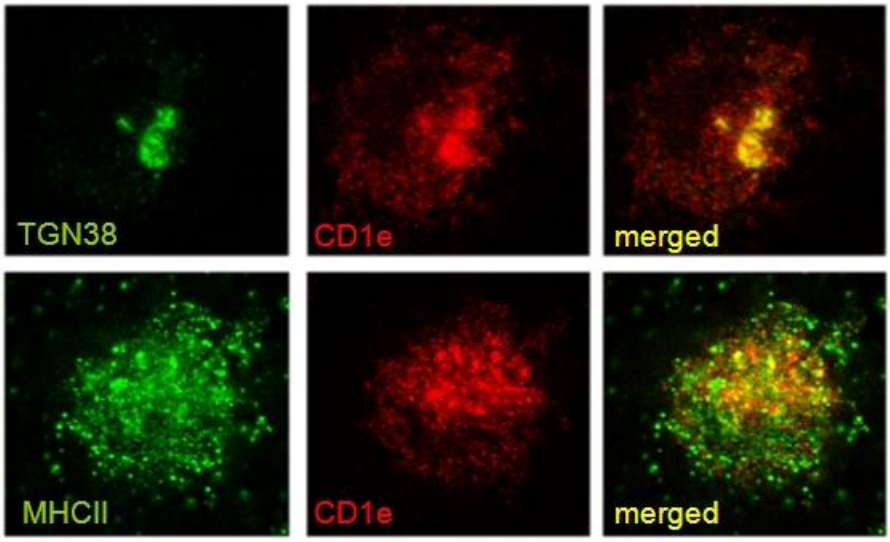


Figure 40. CD1e protein in Eα-CD1e transgenic mice traffics to late endosomes and becomes soluble. Confocal analysis of DC from Eα -CD1e transgenic mice confirms the colocalization of the protein with TGN associated proteins (upper panels) and with MHC II in MHC II compartments (lower panels).

Next, we investigated by flow cytometry whether tg CD1e expression influenced the development of iNKT cells, or of other cells of the hematopoietic compartment.

iNKT cells were identified by staining with anti-TCRβ mAb and mouse CD1d:αGalCer dimers. The analysis of iNKT cells in the thymus (**Figure 41A**), liver and spleen (**Figure 41B**) showed no significant difference in the total numbers (data not shown) and percentages between Eα-CD1e tg mice and non tg littermates.

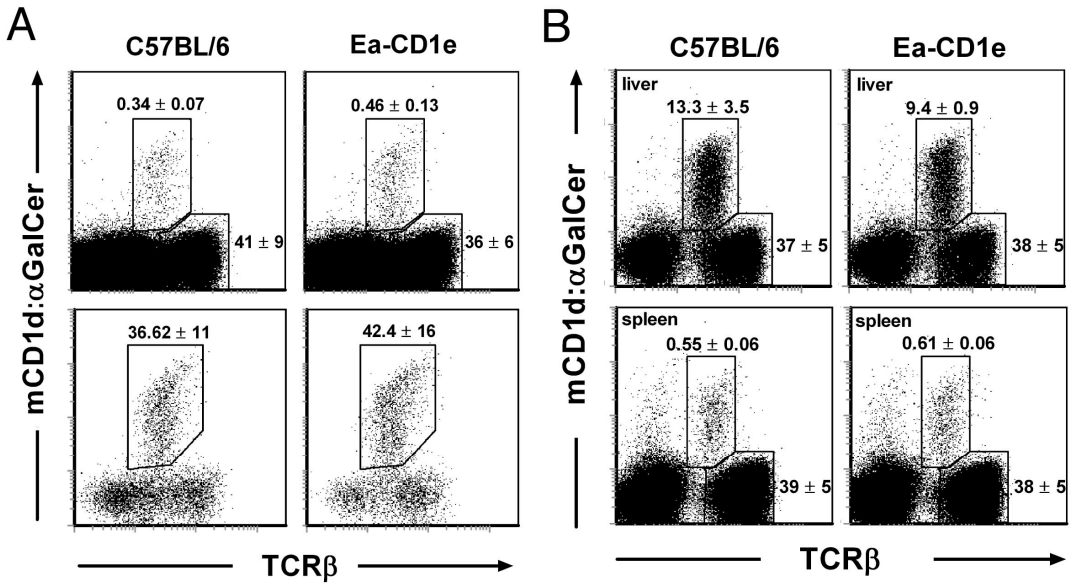


Figure 41. Eα-CD1e tg mice show a normal phenotype. (**A**) Staining of complete (upper panel) or of $CD24^+$ and $CD8^+$ depleted (lower panel) thymi of WT and Eα-CD1e mice with mCD1d:αGalCer dimer and TCRβ. Numbers in gate represent the percent of iNKT cells ± s.d. or of conventional T cells ± s.d.(**B**) Liver mononuclear cells (upper panel) or splenocytes (lower panel) from Eα-CD1e or WT mice were stained with mouse CD1d:αGalCer dimers and mAb against TCRβ and analyzed by flow cytometry. The cytograms in (**B**) depict the gates defining iNKT and conventional TCRαβ T cells, as in (**A**).

The Vβ domain is known to influence the avidity of binding of iNKT cells to CD1d:αGalCer complexes [188]. We analysed therefore the TCR Vβ repertoire of iNKT cells (left panels) as well as of conventional T cells (right panel), focusing on the expression of the Vβ8.2 and Vβ7 chains, which are the two major Vβ chains pairing with the invariant TCR-α chain of iNKT cells. In addition, since iNKT are either $CD4^+$ or DN, we analysed the expression of the CD4 co-receptor on iNKT cells. We found that Vβ usage as well as CD4 expression was unaltered in Eα-

CD1e tg mice as compared to non transgenic littermates, both in iNKT cells and in conventional T cells (**Figure 42**).

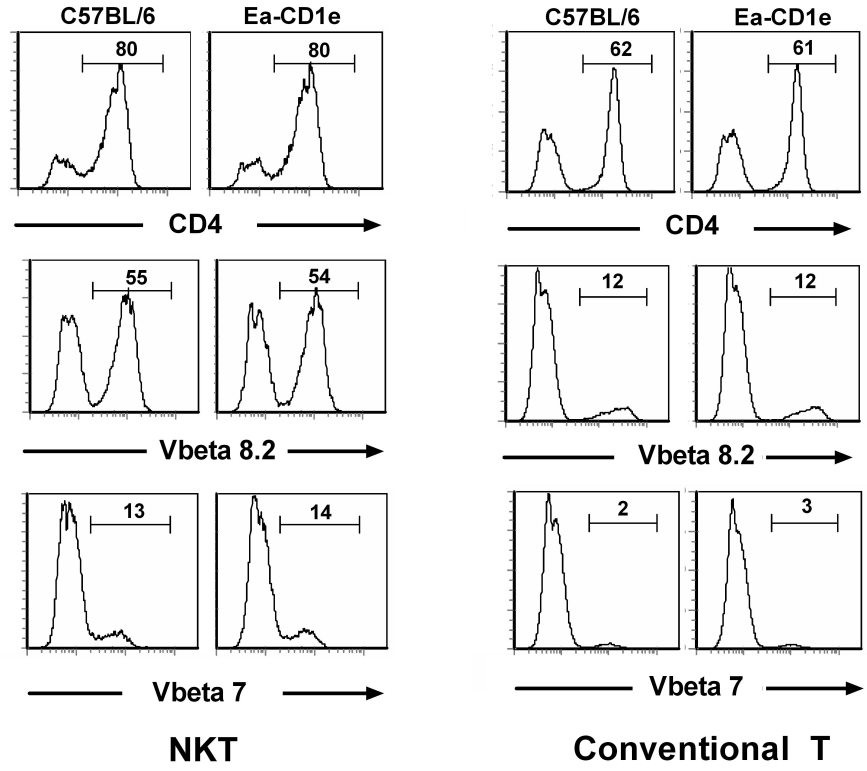


Figure 42. iNKT cell population in Eα-CD1e mice expresses normal levels of CD4 and shows normal TCR-Vβ repertoire. Hepatocytes were stained with anti-CD4 (upper panel) anti-TCR-Vβ8.2 (middle panel), anti-TCR-Vβ7 (lower panel). Left panels show iNKT cells (gated as CD1d:αGalCer dimer^{+} and TCR-Cβ^{+}); right panels, show conventional TCR-αβ cells (gated as CD1d:αGalCer dimer^{-} and TCR-Cβ^{+}). Numbers indicate percentage of positive cells in the indicated gates.

We next analysed numbers and percentages of conventional TCRαβ cells, TCRγδ cells as well as B cells and macrophages in thymus and spleen. Conventional TCRαβ cell percentages were similar in WT and Eα-CD1e in thymus (**Figure 43**, upper panel) and spleen (**Figure 44**, upper panel), as well as thymic TCRγδ (**Figure 43**, lower panel). Splenic B cells, CD11b^{int} and CD11b^{hi} were also similar in percentage both in WT and tg mice (**Figure 44**, lower panel).

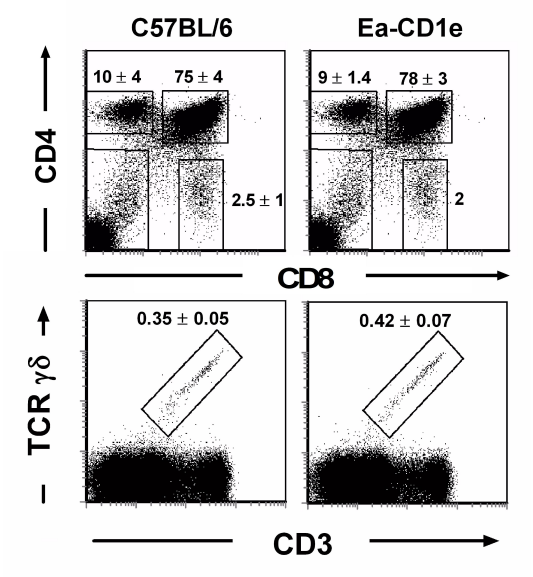


Figure 43. Expression of CD4, CD8α and TCR-γδ cells is normal in thymi from Eα-CD1e mice. Thymocytes from Eα-CD1e or WT mice were analyzed for expression of CD4 and CD8α (upper panel) and TCR-γδ (lower panels). Numbers represent the mean percentage of positive cells (± s.d.) in the indicated gates.

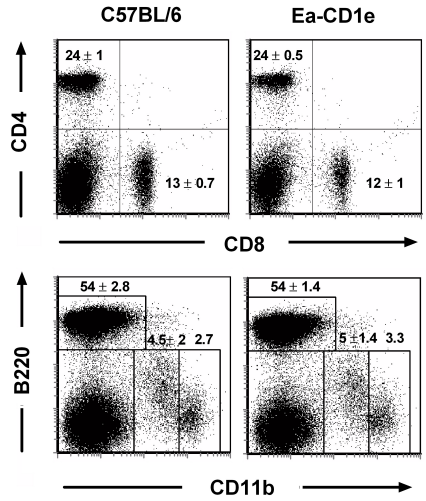


Figure 44. Expression of CD4, CD8α, B220 and CD11b on splenocytes from Eα-CD1e or WT mice is normal. Splenocytes from Eα-CD1e or WT mice were analysed for expression of CD4 and CD8α (upper panel) or B220 and CD11b (lower panel). Numbers represent the mean percentage of positive cells (± s.d.) in the indicated gates.

CD1d levels on DP thymocytes (**Figure 45**, left panel) and on DC were also normal (**Figure 45**, right panel)

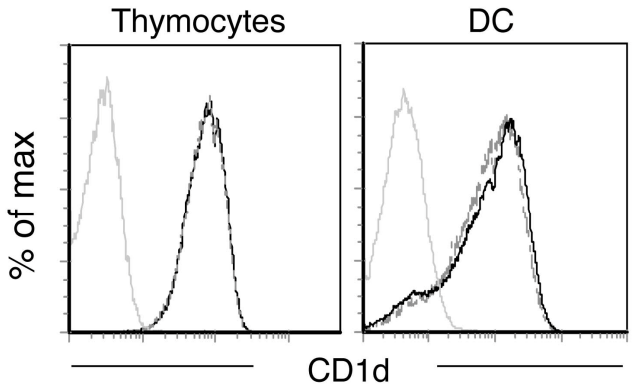


Figure 45. Histograms show the expression of CD1d on DP thymocytes (left panel) and on BM-derived DC (right panel) of Eα-CD1e (black line) or WT mice (dotted gray line).

The genetic background does not have any influence on the expression of CD1e and iNKT cell generation, as demonstrated by the analysis of F_1 mice derived from breeding the original Eα-CD1e tg mice (C57BL/6) to DBA/2 or BALB/c mice (**Figure 46**).

Eα-CD1e transgenic mice have therefore a normal immunological phenotype and represent a new animal model to study the role of CD1e in CD1d-restricted antigen presentation *in vivo*.

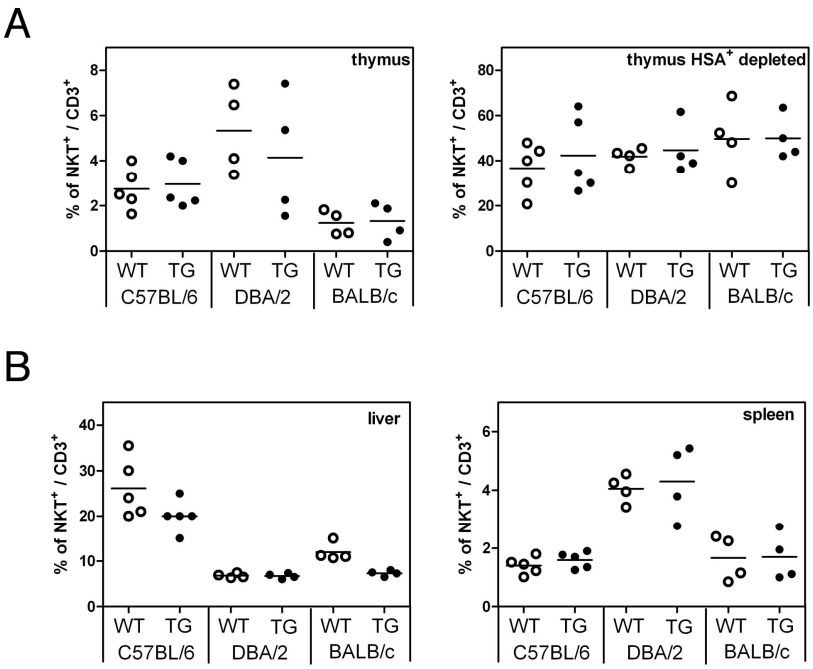


Figure 46. Genetic background has no influence on CD1e expression. Analysis of thymi (A, left panel), thimy CD24+ depleted (A, right panel), liver (B; left panel) and spleen (B, right panel) of F_1 mice bred on DBA/2 and BalB/c. (○), WT mice; (●), Eα CD1e mice. Every dot corresponds to one mouse.

Presentation of endogenous lipid antigens to iNKT cells is facilitated in CD1e tg mice

To investigate whether CD1e also facilitates the presentation of mouse endogenous lipid antigens to iNKT cells, we used APC from CD1e tg mice to stimulate a human iNKT clone, which is reactive to self antigens in the context of mouse CD1d [342]. CD1e expressing DC were able to stimulate the human iNKT cells more efficiently then DC from non tg littermates (**Figure 47A**). Thymocytes from Eα-CD1e mice, which do not express CD1e, presented self lipids to the same extent as WT thymocytes (**Figure 47B**).

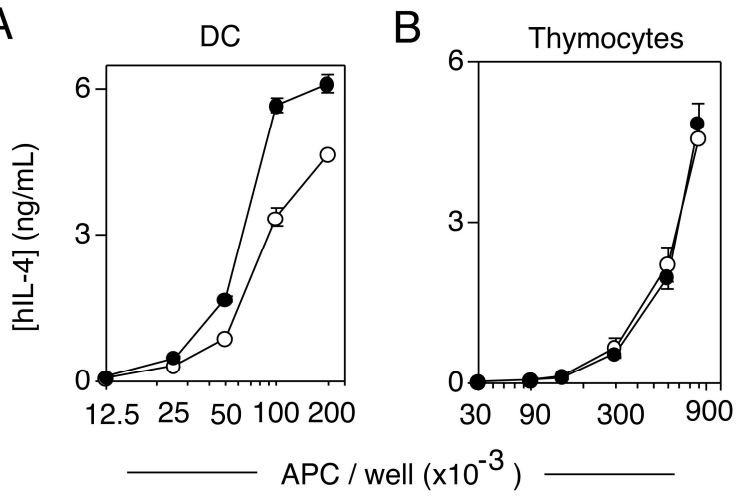


Figure 47. Self lipids are better presented by murine CD1e^{+} APC. (**A**) BM-derived DC or (**B**) thymocytes from Eα-CD1e tg mice (●) or from non-tg littermates (○) were incubated with the human self-reactive iNKT clone JS63. After 24 hr, IL-4 (ng/ml ± SD) release was determined by ELISA.

We then analyzed how tg CD1e influenced the presentation of exogenous lipids using a mouse iNKT hybridoma stimulated with αGalCer presented by DC (**Figure**

48A) or thymocytes (**Figure 48B**) from Eα-CD1e tg mice. CD1e$^+$ APC presented αGalCer with the same efficiency as wild type APC when the antigen was kept in the wells during the entire time of the experiment.

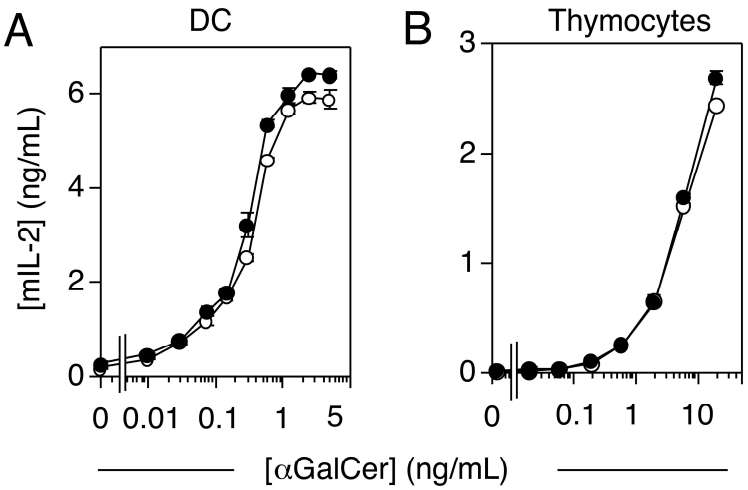


Figure 48. Exogenous lipids are normally presented when kept in the well. (**A**) BM-derived DC or (**B**) thymocytes from Eα -CD1e tg mice (●) or from non-tg littermates (○) were incubated for 2 hr with different concentrations of αGalCer, as indicated, and the antigen kept in the well before the addition of the iNKT mouse hybridoma FF13. After 24 hr IL-2 release (ng/ml ± s.d.) was determined by ELISA.

Instead, as previously observed with human APC, CD1e facilitated the presentation of αGalCer when the lipid was pulsed for short times and then washed out (**Figure 49**). Mouse iNKT hybridoma cells released more IL-2 when DC were incubated 1-8 hr with αGalCer before being washed, fixed and co-cultivated with T cells (**Figure 49**). This effect might be the consequence of surface display by CD1e$^+$ DC of more stimulatory complexes than wild-type DC. These experiments

also showed that the CD1d-αGalCer complexes appeared on the cell surface with the same kinetics in transgenic and wild-type DC, thus suggesting that, in these experimental conditions, CD1e does not accelerate CD1d loading.

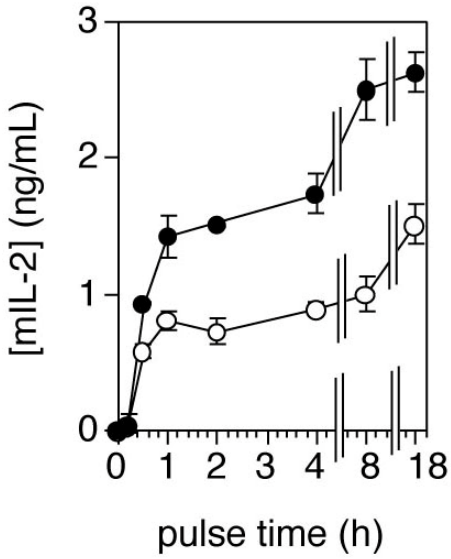


Figure 49. The presentation of αGalCer is increased when the antigen is pulsed for short time. (**A**) BM-derived DC from Eα-CD1e tg mice (●) or from non-tg littermates (○) were incubated for different time with αGalCer (10 ng/ml) and then fixed before the addition of the iNKT mouse hybridoma FF13. After 24 hr IL-2 release (ng/ml ± s.d.) release was determined by ELISA.

In another series of experiments we investigated whether DC from Ea-CD1e tg mice were also capable of presenting lipid antigens derived from heat-killed *Spingomonas paucimobilis*. When heat killed bacteria were added to the cell

cultures during the entire period of the experiments, CD1e+ and wild-type DC showed identical presentation capacity (**Figure 50A**), confirming the data obtained with αGalCer. However, when bacteria were added for a short period and then washed before APC fixation, $CD1e^+$ DC were more stimulatory than wild-type DC (**Figure 50B**). Also with this antigen the total release of IL-2 was higher without a change in the kinetics of surface appearance of CD1d-αGalCer complexes.

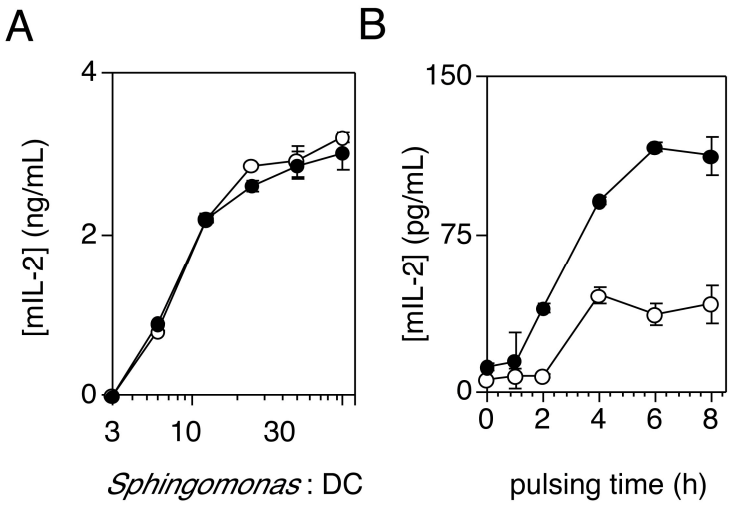


Figure 50. DC from Eα-CD1e tg mice induce a better response after stimulation with *hi S. paucimobilis.* (**A**) DC from Eα-CD1e tg mice (●) or from non-tg littermates (○) were incubated at a different ratio of *hi S.Paucimobilis* before the addition of the iNKT mouse hybridoma FF13. (**B**) DC from Eα-CD1e tg mice (●) or from non-tg littermates (○) were incubated for different time with *hi S.Paucimobilis* (bacteria:APC 200:1) and then fixed before the addition of the iNKT mouse hybridoma FF13. After 24 hr IL-2 release (ng/ml ± s.d.) release was determined by ELISA.

In a next series of experiments, it was investigated whether also in mouse DC CD1e participates in unloading CD1d from lipid antigens as previously found with human APC. Mouse DC were pulsed with αGalCer then washed and incubated at 37° for different times before fixation and addition of iNKT cells. These chase experiments showed that in $CD1e^+$ DC the half-life of the stimulatory complexes was shorter than in wild type DC. Indeed, in $CD1e^+$ DC iNKT cell response was reduced to 50% after 20 hr of chase, whereas in wild type DC the same reduction was observed only after 32 hr **(Figure 51)**. Thise resulte confirm those obtained with CD1e-transfected human APC and support the hypothesis that CD1e accelerates the exchange of CD1d bound lipids.

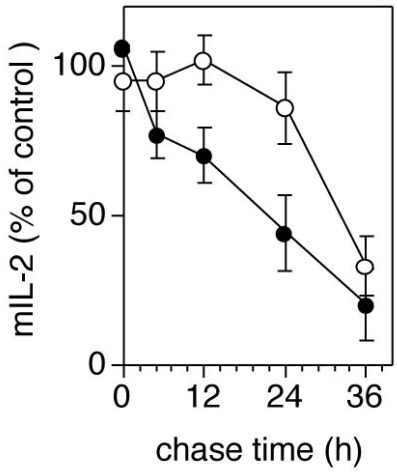


Figure 51. Half life of CD1d:αGalCer complexes is reduced in BM derived DC from Eα-CD1e transgenic mice. BM-derived DC from Eα-CD1e tg mice (●) or from non-tg littermates (○) were pulsed for 2 hr with αGalCer (2 ng/ml) and chased for different periods of time before the addition of the iNKT mouse hybridoma FF13. Diagrams show the mean release of cytokines (± s.d.) (n=3).

Since CD1e participates in the generation of CD1d:aGalCer complexes, we next compared the expression levels of antigenic complexes on the surface of DC from WT and Eα-CD1e mice using flow-cytometry. We took advantage of the L363 mAb, which specifically recognizes mouse CD1d molecules loaded with αGalCer [343]. DC from WT and Eα-CD1e mice were incubated for 24 hr with αGalCer and then analyzed by flow cytometry with the L363 mAb (**Figure 52A**). Unexpectedly, $CD1e^+$ DC showed lower staining than WT DC, which is indicative of lower levels of CD1d:αGalCer complexes expressed on the plasma membrane (**Figure 52A, B**). Similar lower staining was observed when the antigen was incubated for 96 h, indicating a constant lower expression of loaded complexes in CD1e-tg DC than in wild type DC. A titration of αGalCer showed less staining in CD1e-tg DC at all tested doses (**Figure 52C**), confirming that CD1e reduces surface appearance of CD1d-αGalCer complexes as detected by the L363 mAb. Taken together these findings show an important role of CD1e in limiting the number of complexes detected by the L363 mAb.

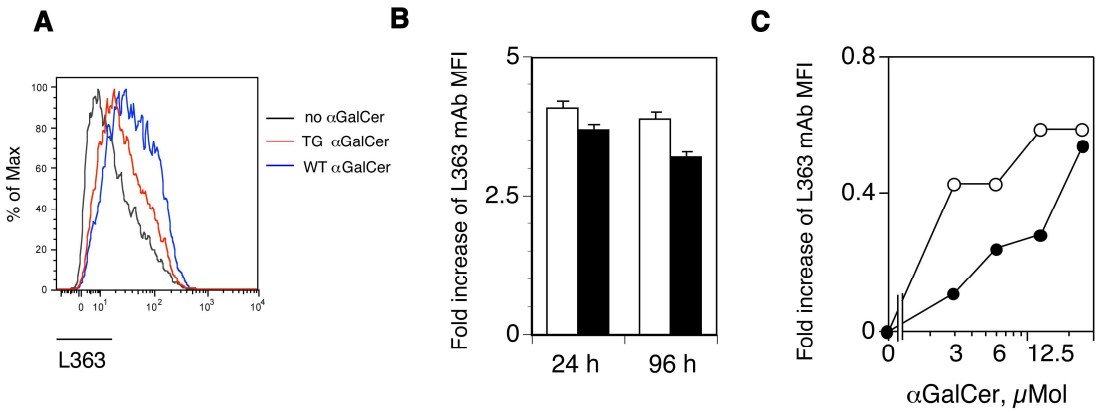


Figure 52. CD1e facilitates unloading of CD1d:aGalcer complexes. (**A**) Detection of CD1d:αGalCer complexes by L363 antibiody staining on the surface of DC from Eα-CD1e DC (red line) or WT DC (blue line) pulsed 24 h with 50 μMol αGalCer. Black line, background of unpulsed DC. (**B**) Detection of CD1d:αGalCer complexes by L363 antibiody staining on the surface of DC from Eα-CD1e DC (black bars) or WT DC (white bars) pulsed 24 h with 50 μMol αGalCer and stained 24 or 96 hr later. (**C**) Detection of CD1d:αGalCer complexes by L363 antibiody staining on the surface of DC from Eα-CD1e DC (●) or WT DC (○) pulsed 24 h with different doses of αGalCer and stained 24 hr after pulse.

DISCUSSION

Among the CD1 family, CD1e is the only soluble protein, thus supporting a chaperone-like rather than an antigen-presenting function. Furthermore, CD1e is never present at the cell surface and therefore cannot engage the TCR. The cellular localization [17] and the intracellular trafficking [38] of CD1e have been extensively studied. The immunological function of CD1e reported until now is to assist processing of complex glycolipid antigens presented by other CD1 molecules [16]. Our findings indicate that CD1e may also exert other important functions in presentation of lipid antigens.

The model of the CD1e structure [16] predicts important differences with other CD1 molecules. Other CD1 molecules have at least 2 hydrophobic pockets, whereas CD1e appears to contain only one large pocket positioned between the α1- and α2-helices and above the six β-strands. The calculated size of the CD1e binding groove is smaller than that of CD1b. The C'-F' channels of CD1e combine to form a large hydrophobic binding pocket, which might allow the accommodation of a large variety of lipid compounds differing in lipid tail length, unsaturation and likely also in lateral substituted groups. Another important difference is found in the global distribution of charged amino acids in the α1 helix, in the region that corresponds to the TCR-recognition face. Charged amino acids, which are critical for the induction of T cell response, are almost absent in the α1 helix of CD1e, whereas they are always present in other antigen-presenting molecules. This important difference supports the conclusion that CD1e is not involved in direct interaction with the TCR.

CD1e is cleaved in a soluble active form in lysosomes and co-localizes with LE and MIIC compartments. This localization suggests that CD1e is present in the same compartments as CD1b, CD1c and CD1d and thus might participate in the presentation of lipid antigens by these CD1 molecules. Probably CD1e does not influence lipid antigen presentation by CD1a that recycles in early endosomes.
To study the possible interaction of CD1e with the other CD1 family members, we initially studied whether CD1e could influence CD1b- and CD1c-restricted antigen presentation. We took advantage of our collection of CD1b- and CD1c-restricted T cell clones, which recognize either self or microbial lipid antigens. CD1e presence in APC influences the response to both endogenous and exogenous lipid antigens in several ways. CD1e can increase, decrease or leave unchanged the response to lipid antigens, presented by either CD1b or CD1c.

The different influence that CD1e has on presentation of tested lipid antigens is independent from the presenting CD1 molecule. Probably it depends on whether lipids traffic to the same compartments where also CD1e is present and also whether they bind to CD1e. CD1e might influence presentation of exogenous and endogenous lipids with several not mutually exclusive mechanisms: i) CD1e may increase the solubility of lipid antigens, by extracting them from membranes or micelles thus making them more available for presentation; ii) CD1e may participate in processing of lipids, thus facilitating the generation of smaller molecules with CD1-binding capacity and T cell stimulatory activity. With the same mechanisms CD1e may accelerate the degradation of stimulatory lipid antigens; iii) CD1e may facilitate loading of bound lipid antigens on other CD1 molecules with a chaperone function, and iv) it may facilitate unloading of non-stimulatory lipids from other CD1 molecules, thus accelerating lipid exchange.

The response to exogenously added GM1 is decreased in the presence of CD1e. GM1 could have higher affinity for CD1e than for CD1b and thus it could become sequestered by CD1e and less available for presentation to T cells. In the case of PIM_6, CD1e is required to process this large antigen into the smaller PIM_2 by the lysosomal α-mannosidase. In a similar manner CD1e might facilitate processing of GM1 by lysosomal β-Galactosidase, with the consequence of rendering it not immunogenic [78].

When we tested the effects of CD1e on the presentation of another self-glycosphingolipid such as sulfatide, we found that CD1e facilitates sulfatide presentation by both CD1b and CD1c. Sulfatide is a component of all cellular membranes and is composed by a galactose, modified in position 3 by a sulfate, connected with a β-glycosidic bond to ceramide. CD1e could increase the solubility of sulfatide, facilitate its extraction from membranes or assist its loading onto CD1b or CD1c.

We also found that in certain instances the presence of CD1e is irrelevant for T cell stimulation. This is the case of SGL12 presentation. This compound is a semi-synthetic analog of di-acylated-sulfoglycolipid (Ac_2SGL), a lipid antigen produced by virulent *M. tuberculosis* composed of a 2'-O-sulfate-α-D-trehalose core acylated at position 2 by a palmitic (or stearic) acid and at position 3 by a hydroxyphtioceranoic acid. The semi-synthetic SGL12 is acylated at position 2 by a palmitic acid, and at position 3 by a C26 carbon-long fatty acid containing 4 branched methyl groups. Since CD1e does not affect the presentation of SGL12 at low as well as high doses, it is likely that it does not bind SGL12.

Another important effect of CD1e is on the type of cytokines secreted by lipid-specific T cells. Upon stimulation T cells release different cytokines according to the strength of the antigenic stimulus [340]. GM-CSF secretion is already detected when the TCR signal is weak, while IFN-γ and IL-4 production occur only upon stronger TCR signaling.

To study *in vitro* the possible interaction between CD1e and CD1d, we investigated the response of many autoreactive type 1 and type 2 CD1d-restricted NKT cell clones.

Type 1 NKT cells (also defined iNKT according to the expression of the invariant TCR α chain) react to a series of lipids including αGalCer, glycosphingolipids and glycodiacylglycerols of microbial origin and to unknown self lipids, whereas type 2 NKT cells recognize diverse antigens that remain unknown [138, 308-310, 313].

In the presence of CD1e all tested autoreactive type 1 iNKT cells showed increased reactivity to endogenous lipid antigens. On the contrary, type 2 NKT cells were differently influenced. CD1e presence was irrelevant for some type 2 NKT cells, it increased the autoreactivity of other clones and was detrimental for the response of a small number of clones. The naturally produced endogenous lipid antigens stimulating iNKT and type 2 NKT cells remain unknown and therefore

it is not possible to clearly ascribe these different effects to the recognition of different lipid antigens by type 1 and type 2 NKT cells. It is tempted to speculate that the tested iNKT cell clones, which are all positively influenced by the presence of CD1e, recognize endogenous lipids sharing common characteristics and which allow CD1e binding and transfer to CD1d. On the contrary, type 2 NKT cells recognize different types of lipids whose presentation is influenced by CD1e according to their nature, cellular localization and capacity to interact with CD1e.

An unexpected finding was that CD1e also influences the response to exogenous antigens by directly affecting individual cytokine release. We observed a hierarchy in the secretion of cytokines, being GM-CSF secretion not influenced by CD1e, while secretion of IL-4 was poorly affected and that of IFN-γ was greatly diminished. Probably CD1e, by participating in the turnover of lipids binding to CD1d, decreases the number of antigenic complexes remaining on the cell surface and thus it influences more effectively the release of cytokines requiring a higher threshold of TCR signalling. These findings suggest that CD1e has a direct effect on the quality of the response of iNKT cells. This effect may have important influences on the immunoregulatory role of these cells *in vivo*.

CD1e also increased the stimulatory capacity of APC pulsed for short time and then fixed, thus implying that it facilitates the formation of more stimulatory complexes when the time of CD1d loading is limiting. Four hours were sufficient to generate stimulatory complexes with α-GalCer on the surface of fixed APC. A careful analysis of the kinetics of the response showed that CD1e does not accelerate the formation of the complexes. Instead, it induces a stronger T cell response, probably ascribed to an increased number of antigen-loaded complexes. Alternatively, the total number of complexes is not affected and those that are

formed are more stimulatory. Recently, it has been described that complexes formed by CD1d and α-GalCer within lysosomes are more efficient in inducing IFN-γ release, whereas those formed on the cell surface induce less IFN-γ without reducing the amounts of IL-4 [344]. Since CD1e is present only intracellularly, it is likely that it only influences complexes formed within lysosomes.
An additional function that can be ascribed to CD1e is its capacity to unload CD1d molecules. This effect is more pronounced when the antigen is limiting and leads to a faster disappearance of the T cell-stimulatory capacity. In other words, CD1e facilitates the formation of more active complexes as soon as the antigen becomes available and, at later time points, also participates in the fast decay of APC stimulatory capacity. These temporal effects result in early activation of iNKT cells, but also in a "switch off" of the response at later times. Thus, CD1e may also contribute to a temporal focusing of the response, thus avoiding a possible detrimental prolonged activation of iNKT cells.

The chaperone function of CD1e seems different from that of other LTPs that influence loading of lipid antigens on CD1 molecules. Several LTPs, including saposins, GM2A and NPC-2 [84, 91, 208], directly participate in loading of CD1d molecules inside late endosomes and lysosomes. Our data show that CD1e can also directly participate in loading of lipids onto CD1d. Indeed, CD1e is more efficient than Saposins B and C to assist loading of α-GalCer onto CD1d *in vitro*. Moreover, CD1e facilitates unloading of α-GalCer from CD1d, while saposins are inefficient in performing this task [86]. Whether the unique structure of CD1e is important and how it influences lipid exchange from other CD1 molecules remains poorly defined. Site-directed mutagenesis studies may contribute to clarify this issue.

In conclusion, CD1e may assist loading and unloading of lipids, thus increasing the turnover of CD1d:lipid complexes. How this influences the *in vivo* response was a matter of initial studies performed in CD1e transgenic mice.

We generated transgenic mice expressing CD1e in APC, thus mimicking the expression in human cells. The construct was made with the MHC Ea promoter driving the expression of CD1e cDNA. In these transgenic animals the total numbers of thymic and peripheral leukocytes were normal, as well as the size and apparent structure of lymphoid organs. Thus, the hematopoietic compartment was normal excluding the possibility that the transgene insertion site and expression might have influenced normal immune system development. The expression of the protein was investigated using CD1e-specific mAbs that showed proper intracellular localization. Western blot analysis also showed that CD1e matures in the Golgi compartment and when it reaches lysosomes is cleaved into a smaller molecule. These post-translational modifications closely resemble those in human cells, thus showing the presence of a protein with the physiological characteristics of CD1e present in human cells.

When iNKT cells were investigated, their numbers in the thymus and periphery were normal. In addition, their differentiation stages in the thymus and phenotype were normal. iNKT cells from Ea-CD1e mice also showed normal response to relevant glycolipid antigens. Thus, expression of CD1e in antigen-presenting cells *in vivo* does not induce major detectable alterations in iNKT cells.

In a series of experiments we investigated the functions of CDe expressed in mouse APC. We first investigated the capacity of CD1e to assist CD1d-mediated antigen presentation. In fully accordance with the human system, CD1e expressed in mouse DC facilitated presentation of endogenous lipids to autoreactive iNKT cells and assisted the generation of stimulatory complexes during early phases of

antigen encounter. Moreover, also in mouse DC, CD1e participated in unloading of CD1d:aGalCer complexes, as demonstrated by chasing experiments. CD1e facilitated stimulation of iNKT cells by *Sphingomonas* antigens, particularly when bacteria were pulsed only for short periods of time. This finding might be of relevance during bacterial infections since by facilitating formation of stimulatory complexes, CD1e might participate in ready activation of iNKT cells.

The pleiotropic immunological functions of CD1e suggest a co-ordinated evolution of different molecules involved in presentation of lipid antigens. The presence of an intracellular chaperone resembling other CD1 molecules, might be instrumental to select, among a variety of lipids with different structures, those with the capacity to fit the CD1 binding grooves and thus with the highest probability to be immunogenic. This property may focus the immune response on defined classes of lipids by facilitating their processing, their loading and unloading and by generating complexes with higher stimulatory capacity. CD1e thus may be considered an intracellular controller of lipid antigen presentation with increased importance whenever antigen availability is limiting.

When presentation of lipid antigens is compared to that of protein antigens, it appears that the immune system has exploited the chaperone/assisting function of intracellular molecules in both types of antigen response. Indeed, in the case of peptide antigens HLA-DM, an intracellular molecule that does not reach the plasma membrane, participates in optimization of presentation. HLA-DM closely resembles the structure of other MHC class II molecules, which form peptide complexes recognized by T cells. It is also localized in the MIIC compartments where peptide antigens are loaded on MHC class II molecules, and forms transient complexes with loading MHC class II molecules. The main functions attributed to HLA-DM are the facilitation of binding and release of peptides. Importantly, while the on rate of binding is accelerated with all peptides tested, the release of bound peptides is

different according to the affinity of interaction between the peptide and MHC class II, *i.e.* weak peptides are readily released, whereas the release of strong peptide binders is not affected. This discriminatory effect confers HLA-DM an editing function, which focuses the immune response on antigens forming more stable stimulatory complexes with MHC class II molecules.
CD1 e also facilitates loading and unloading of lipid antigens on other CD1 molecules. It is also colocalized within the same compartments where other CD1 molecules form stimulatory complexes with lipids and it also never reaches the plasma membrane. It is unclear whether CD1e mostly affects the turnover of CD1 weakly binding antigens and thus promotes persistence of stable complexes within APC. This is difficult to investigate due to the intrinsic difficulties to evaluate the binding affinity of lipid that are water insoluble. Novel biophysical methods should be developed in order to investigate this important issue.

In conclusion, CD1e optimizes lipid-specific T cell responses by participating in lipid antigen editing and by influencing presentation by CD1b, CD1c and CD1d molecules in multiple ways. CD1e also facilitates the response of iNKT cells to endogenous lipid antigens possibly by facilitating their loading onto CD1d and/or by promoting generation of more stimulatory CD1d-lipid complexes. All these pleiotropic functions of CD1e optimize presentation of lipid antigens and thus the lipid specific T cell response.

CHAPTER 3

Sterol carrier protein 2 (SCP-2) is required for the maturation of iNKT cells and their stimulation by endogenous lipids

SUMMARY

Studies in genetically manipulated mice have demonstrated the importance of several enzymatic machineries in generating antigenic lipids and of LTP in their incorporation in CD1d-glycolipid complexes.

All the LTP influencing lipid antigenicity and iNKT cell numbers that have been investigated so far are localised within the same compartments as the lipid-binding side of CD1 molecules. This topological concordance is the obvious necessity if a LTP has to transfer lipids to CD1. The role of LTP residing in the cytoplasm on presentation of lipid antigens has never been investigated. Furthermore, whether cytoplasmic LTP are involved in generation and intracellular trafficking of self-lipid antigens remains unknown.

In this study we have investigated SCP-2 which is a LTP with broad substrate specificity mainly present in the cytosol and in peroxisomes [124].

Scpx/proScp2 knockout (*Scp2*$^{-/-}$) mice have been generated which completely lack *Scpx/proScp2* gene products [131]. These mice develop normally but have several marked alterations in lipid metabolism.

Here we show that *Scp2*$^{-/-}$ mice have a reduction of iNKT cells. While the residual iNKT population is functionally normal as well as CD1d-restricted lipid presentation of exogenous lipids by CD1d, the stimulation of autoreactive T cells by endogenous lipid antigens is altered. *Scp2*$^{-/-}$ mice show a different lipid repertoire in the thymus to wild-type (WT) mice. In addition, thymic iNKT selection in *Scp2*$^{-/-}$ mice is inefficient and results in a relative accumulation of immature iNKT cells.

RESULTS

SCP-2 influences iNKT cell numbers

Deletion of the protein SCP-2 results in a significantly reduced number of iNKT cells (**Figure 53**). When compared to WT, *Scp2*$^{-/-}$ mice at 4-6 weeks of age showed a reduced percentage of iNKT cells in thymus (~50%) as well as in liver and spleen (both ~40%) (**Figure 54A**).

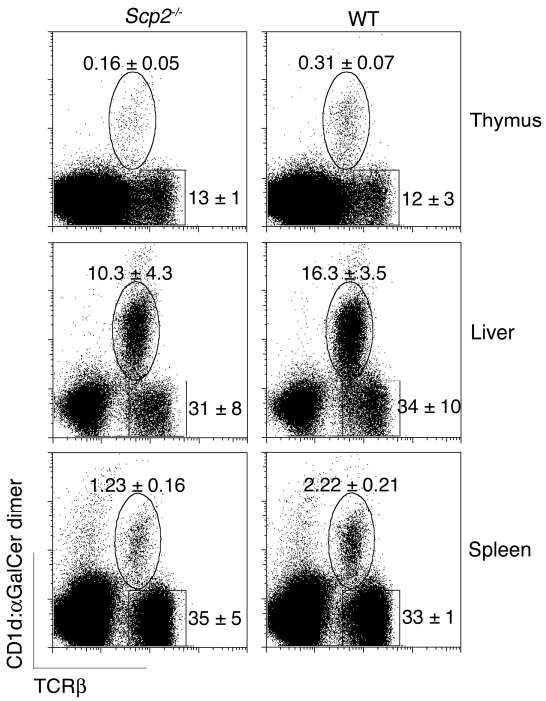


Figure 53. iNKT cells are reduced in *Scp2*$^{-/-}$ mice. Flow cytometry of thymocytes, liver mononuclear cells and splenocytes from *Scp2*$^{-/-}$ and WT mice stained with anti-TCRβ mAb and CD1d:αGalCer dimers. Numbers represent the median of percentages ± s.d. of cells in the gated areas.

The absolute number of iNKT cells was also significantly reduced in the thymus and the liver, in which normal cellularity was observed. On the contrary, in the spleen the total numbers of iNKT cells were comparable to that of WT animals because young animals (3-5 weeks old) show a slight increase in organ size and total cellularity (**Figure 54B**). The splenomegaly disappears in older animals, which show a reduction of iNKT cells also in the spleen.

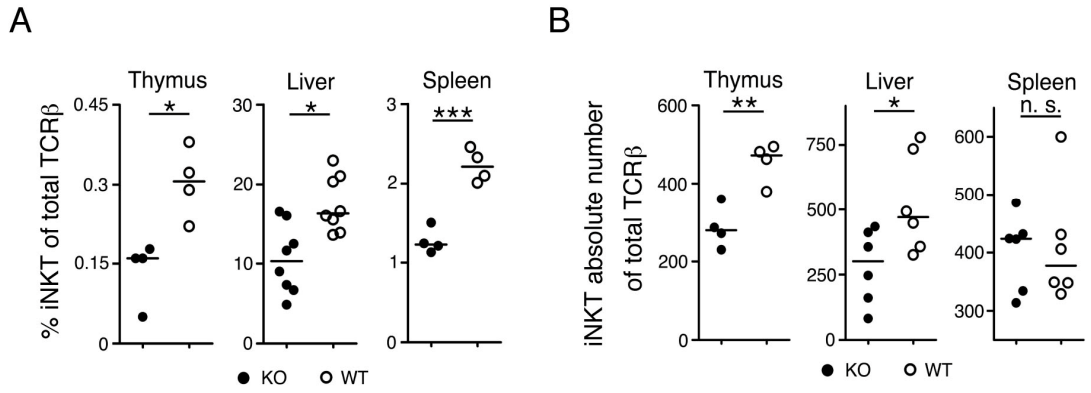


Figure 54. iNKT cells are reduced in *Scp2*$^{-/-}$ mice. (**A**) iNKT cells quantified as percentage of total TCRβ^{+}, and (**B**) absolute numbers in thymus, liver and spleen of *Scp2*$^{-/-}$ (●) and WT mice (○). Each circle indicates an individual mouse. Horizontal bars represent medians. *, P ≤ 0.05; **, P≤ 0.01; ***, P≤ 0.001; n.s., not significant.

The alterations seem to exclusively affect iNKT cells, because the phenotype, percentages, and total numbers of conventional T cells were normal in the thymus and liver (**Figure 55A and B**). In the spleen, while percentages were normal, total numbers were increased and this increase was significant for both CD4 and CD8 populations (**Figure 55D**). B cells and monocytes in liver and spleen were normal (**Figure 55C and E**).

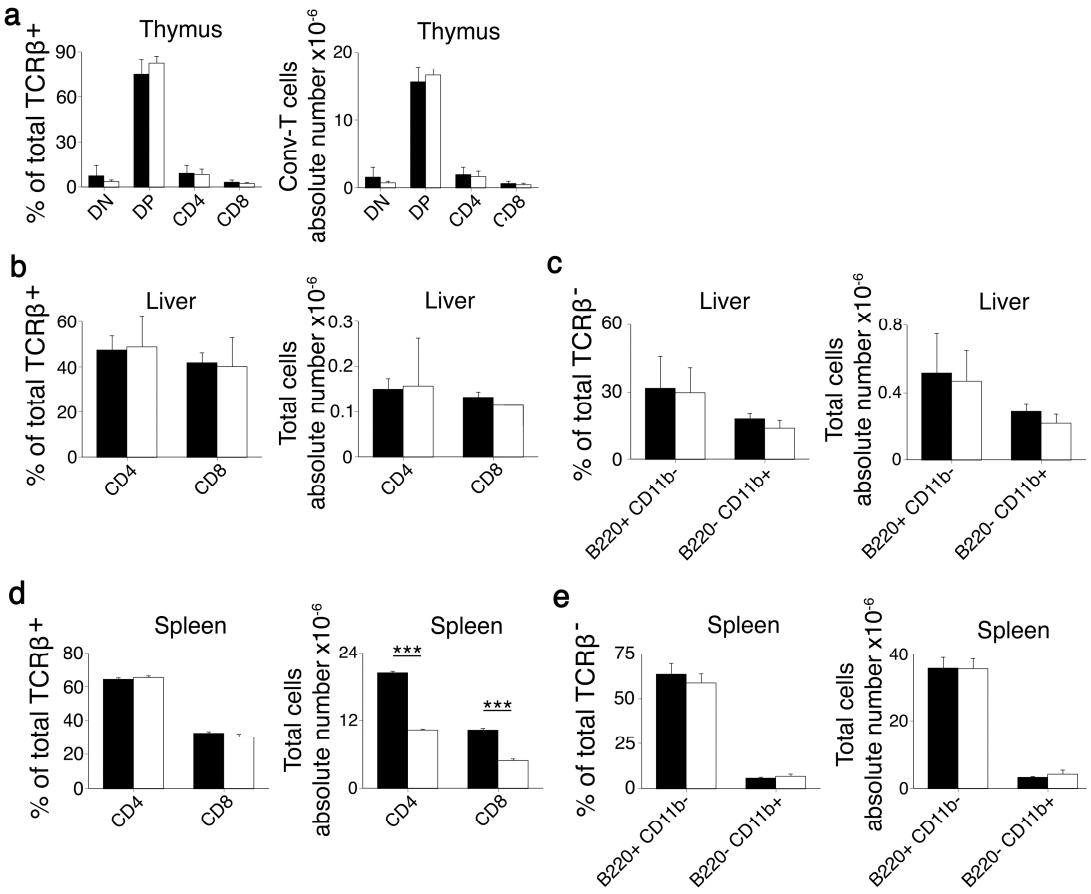


Figure 55. Phenotype, percentages and absolute numbers of conventional T and B cell populations in *Scp2*$^{-/-}$ mice. (**A**) Percentage and total number of conventional T cells in thymus with DN, DP, single CD4, and single CD8 phenotype in *Scp2*$^{-/-}$ (black columns) and WT (white columns) mice. Percentage and total number of conventional T cells in (**B**) liver or (**D**) spleen with single CD4, and single CD8 phenotype in *Scp2*$^{-/-}$ (black columns) and WT (white columns) mice. Percentages and total numbers of B cells (B220^{+} CD11b^{-}) and monocytes (B220^{-} CD11b^{+}) in (**C**) liver or (**E**) spleen of *Scp2*$^{-/-}$ (black columns) and WT (white columns) mice. All results are expressed as average ± s.d. of groups of 4 mice.

The analysis of the expression of the two most frequently used Vβ chains (Vβ8.2 and Vβ7) showed a normal representation in thymus, spleen and liver (**Figure 56A**), suggesting no major alterations in the TCR Vβ repertoire in these mice. The intensity of staining of iNKT cells with CD1d:αGalCer dimers was similar in WT and *Scp2*$^{-/-}$ mice, possibly excluding different capacity of the remaining iNKT cells to interact with this CD1 complex [327]. The expression of CD4 was reduced in iNKT cells in thymus and spleen of *Scp2*$^{-/-}$ mice and was associated with a relative increase of CD4^{-}CD8^{-} DN iNKT cells as compared to WT mice (**Figure 56B**).

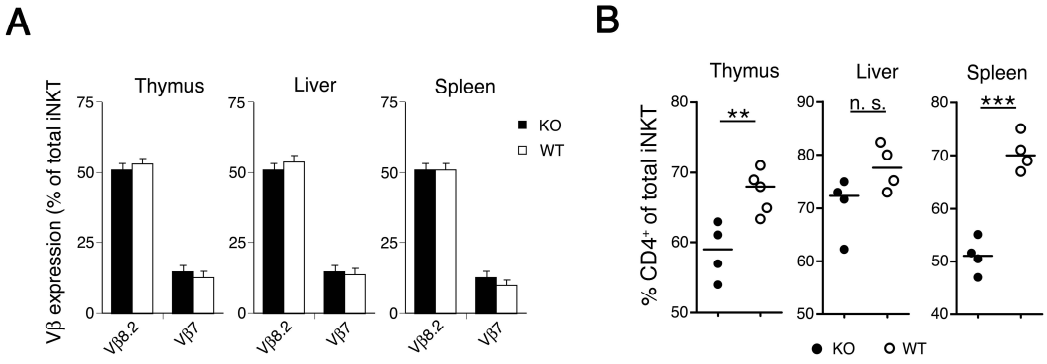


Figure 56. CD4 expression is reduced in iNKT cells from *Scp2*$^{-/-}$ mice (**A**) iNKT cells expressing TCR Vβ8.2 or Vβ7 in thymus, liver and spleen of *Scp2*$^{-/-}$ (black columns) and WT (white columns) mice. Results are expressed as mean percentages of total CD1d:αGalCer dimer^{+} cells ± s.d. of groups of 2 mice. (**B**) Percentages of iNKT cells expressing CD4 in thymus, liver and spleen of *Scp2*$^{-/-}$ (●) and WT mice (○). Each circle indicates an individual mouse. Horizontal bars represent medians. **, P≤ 0.01; ***, P≤ 0.001; n.s., not significant.

Residual NKT cells in *Scp2*$^{-/-}$ mice are functionally normal

We investigated whether the residual iNKT cells in *Scp2*$^{-/-}$ mice show differences in terms of phenotype and function. By using a combination of mAb specific for CD44, a marker of antigen-experienced T cells, for the homing receptor CD62L, CD4, NK1.1, and for the activation marker CD69, most of iNKT cells in liver and spleen showed markers of effector cells (CD44^{hi} CD62L^{-} CD69^{+}), both in *Scp2*$^{-/-}$ and WT mice (**Figure 57**).

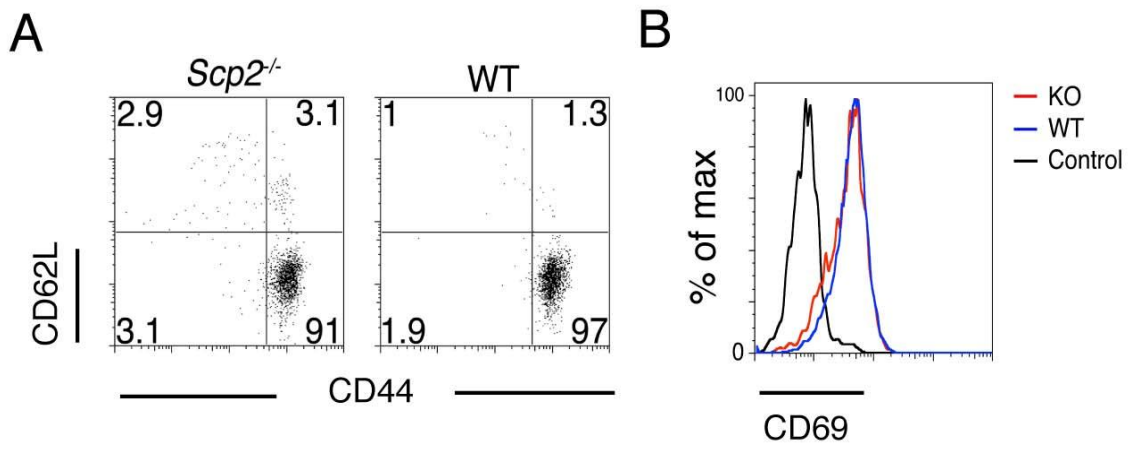


Figure 57. Residual iNKT cells of *Scp2*$^{-/-}$ are phenotipically normal in periphery. (**A**) Flow cytometry of liver iNKT cells (gated on anti-TCRβ^{+} and CD1d:αGalCer dimer^{+}) from *Scp2*$^{-/-}$ and WT mice stained with anti-CD62L and CD44, (**B**) and with anti-CD69. Numbers indicate percentages of cells in the quadrants and are representative of 4 mice/group.

Interestingly, the $CD4^-$ subset of iNKT cells expressing NK1.1 was significantly increased in *Scp2*$^{-/-}$ mice (**Figure 58**).

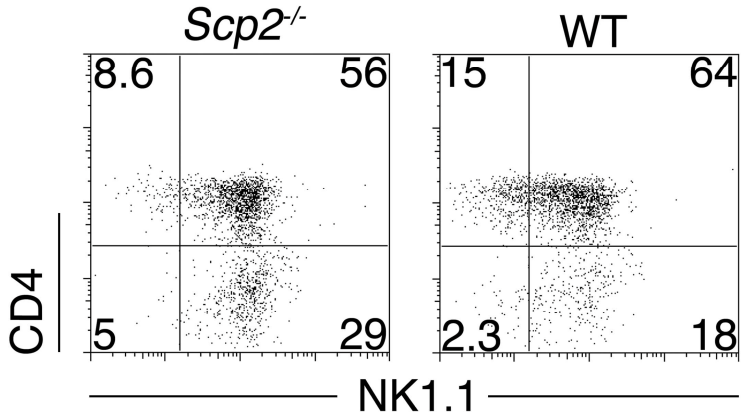


Figure 58. Residual iNKT cells of *Scp2*$^{-/-}$ have higher levels of NK1.1 in periphery. (A) Flow cytometry of liver iNKT cells (gated on anti-TCRβ$^+$ and CD1d:αGalCer dimer$^+$) from *Scp2*$^{-/-}$ and WT mice stained with anti-CD4 and NK1.1 mAb. Numbers indicate percentages of cells in the quadrants and are representative of 4 mice/group

Peripheral iNKT cells from *Scp2*$^{-/-}$ mice were functionally normal because after stimulation of splenic iNKT cells with DC pulsed with heat-inactivated *Sphingomonas paucimobilis* or iGb3, the upregulation of CD69 was comparable to that of WT iNKT cells (**Figure 59**).

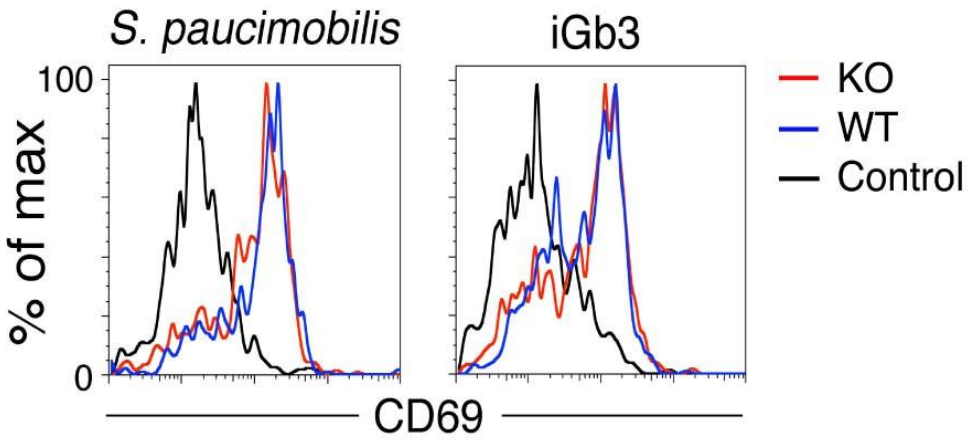


Figure 59. Residual iNKT cells of *Scp2*$^{-/-}$ are functionally normal in periphery. Upregulation of CD69 on spleen iNKT cells (gated on anti-TCRβ^{+} and CD1d:αGalCer dimer^{+}) from *Scp2*$^{-/-}$ (red line) or WT (blue line) mice, after stimulation with WT-DC pulsed with (A) heat-inactivated *Sphingomonas paucimobilis* (200:1 - bacteria:DC) or (B) iGb3 (10 µg/ml). Black line histograms depict CD69 basal levels of unstimulated spleen iNKT from WT mice (median fluorescence intensity, MFI: *Scp2*$^{-/-}$ 115 ± 20, WT 150 ± 15 for *S. paucimobilis* stimulation, *Scp2*$^{-/-}$ 74 ± 10, WT 61 ± 15 for iGb3 stimulation).

In addition, iNKT cells were capable of releasing cytokines *in vivo* in response to injection of αGalCer, although due to their reduced number, the amounts of IL-4 and IFNγ in the serum were significantly reduced as compared to that of WT mice (**Figure 60**).

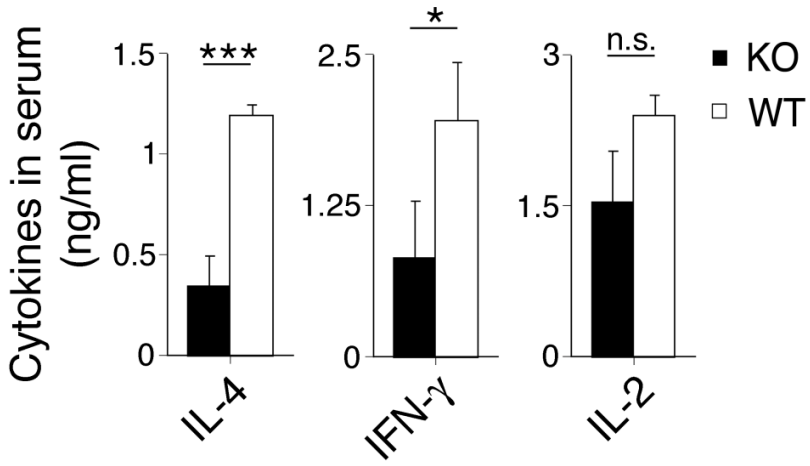


Figure 60. Residual iNKT cells of *Scp2*$^{-/-}$ secrete cytokines in periphery Cytokine in the serum of *Scp2*$^{-/-}$ (black column) or WT (white column) mice, 24 h after intraperitoneal injection of 2 µg of αGalCer. Cytokine release is expressed as average ± s.d. of groups of 2 mice. *, P ≤ 0.05; ***, P≤ 0.001; n.s., not significant.

APC from *Scp2*$^{-/-}$ mice present exogenous lipids normally

In *Scp2*$^{-/-}$ mice, DP thymocytes, which mediate positive selection of iNKT cells, and bone-marrow derived DC showed normal CD1d plasma membrane levels (**Figure 61**), thus excluding that reduced CD1d level may account for iNKT cells reduction in *Scp2*$^{-/-}$ mice.

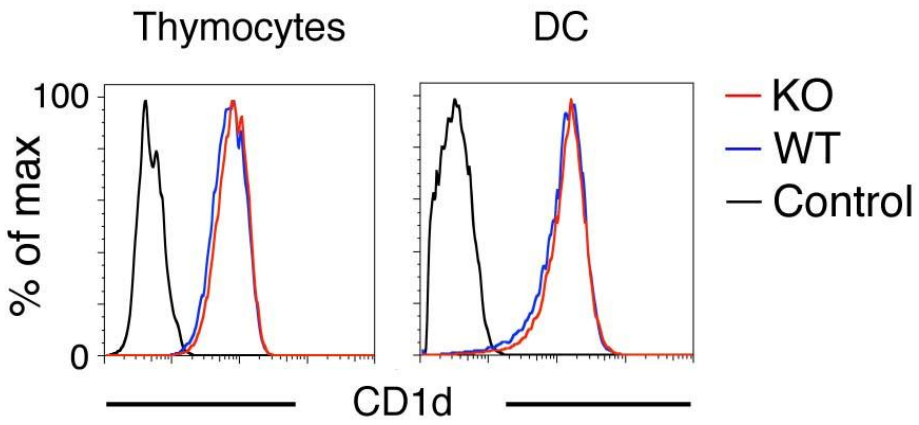


Figure 61. CD1d expression in *Scp2*$^{-/-}$ mice is normal. (A) Flow cytometry of DP thymocytes and DC from *Scp2*$^{-/-}$ mice (red lines) and WT mice (blue lines) stained with anti-CD1d mAb. (MFI: *Scp2*$^{-/-}$ 80 ± 5, WT 72 ± 10 for thymocytes, *Scp2*$^{-/-}$ 150 ± 10, WT 140 ± 15 for DC). Black line histograms correspond to staining with irrelevant isotype-matched mAb. Data are representative of 2 independent experiments.

The decreased number of iNKT cells observed in *Scp2*$^{-/-}$ mice could be ascribed to a defect in the presentation capacity of APC, as found in APC of mice lacking other lipid-binding proteins like saposins [80, 84]. We therefore tested whether *Scp2*$^{-/-}$-APC have a defect in the presentation of exogenous antigens. Mouse iNKT hybridomas were efficiently and equally stimulated by thymocytes (left panel) or DC (right panel) from either *Scp2*$^{-/-}$ or WT mice presenting αGalCer **(Figure 62A)** and iGb3 (**Figure 62B**), thus excluding a defect in antigen presentation capacity of *Scp2*$^{-/-}$-APC.

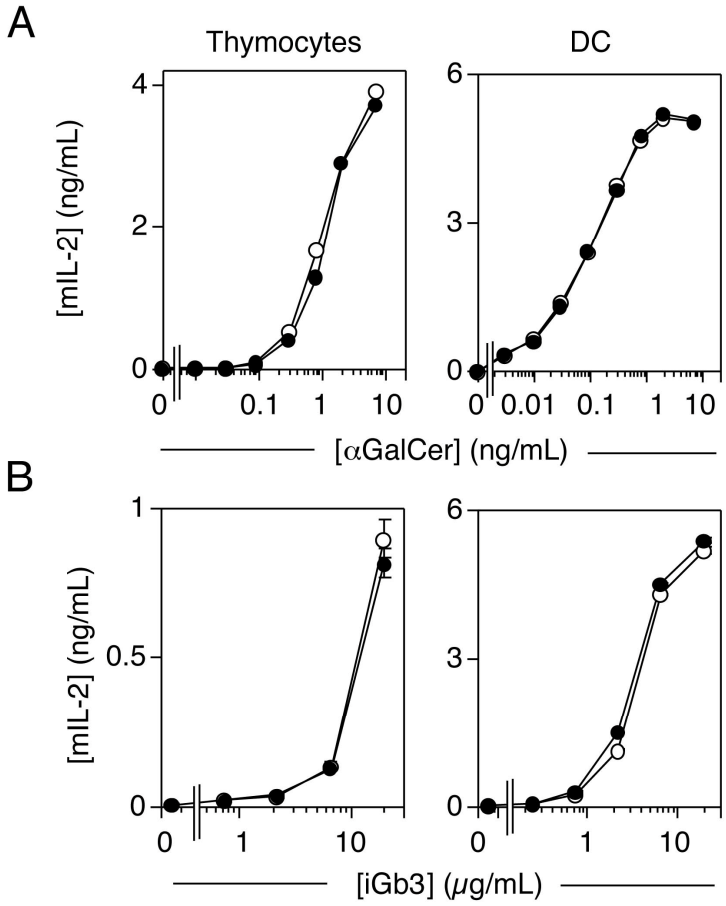


Figure 62. Exogenous lipid antigen presentation capacity in *Scp2*$^{-/-}$ mice is normal. Stimulation of the mouse NKT hybridoma FF13 by various doses of (**A**) αGalCer and (**B**) iGb3 presented by thymocytes or DC from *Scp2*$^{-/-}$ mice (●) and WT mice (○). Results are expressed as mean release of IL-2 ± s.d. of duplicates, and are representative of 2 independent experiments.

To further ascertain whether APC from *Scp2*$^{-/-}$ mice are capable of normal antigen presentation, DC were infected with *S. paucimobilis* and used to stimulate iNKT cells from WT spleen cells. DC from *Scp2*$^{-/-}$ and WT mice induced similar upregulation of CD69 (**Figure 63**) on responding iNKT cells, demonstrating that all the series of events which begin with infection and lead to the generation of CD1d-lipid stimulatory complexes are normal in *Scp2*$^{-/-}$ mice. Importantly, cells incubated with DC without bacteria also upregulated CD69 and the extent of this upregulation by *Scp2*$^{-/-}$-DC was smaller than the one induced by WT-DC. These findings suggest that *Scp2*$^{-/-}$-APC might be defective in presentation of endogenous lipid antigens.

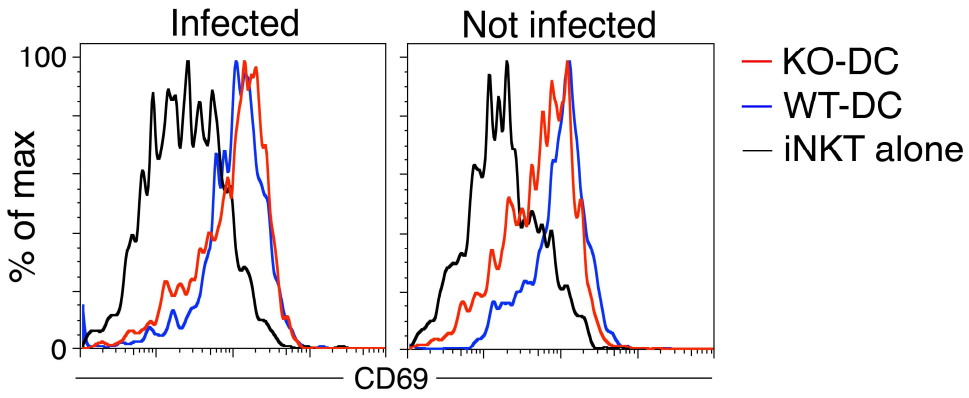


Figure 63. Exogenous lipid antigen presentation capacity in *Scp2*$^{-/-}$ mice is normal. Upregulation of CD69 on spleen iNKT cells (gated on anti-TCRβ^{+} and CD1d:αGalCer dimer^{+}) from WT mice after stimulation with *Scp2*$^{-/-}$-DC (red lines) or WT-DC (blue lines) infected or not with *S. paucimobilis* (1:1). Black line histograms depict CD69 basal levels of CD69 in unstimulated spleen iNKT cells. (MFI: basal level 21 ± 6, with infected *Scp2*$^{-/-}$-DC 119 ± 10, with infected WT-DC 116 ± 12; basal level 15 ± 5, with not infected *Scp2*$^{-/-}$-DC 58 ± 8, with not infected WT-DC 105 ± 5). Data illustrated are representative of one out of 4 independent experiments.

Presentation of endogenous lipids by *Scp2*$^{-/-}$ mice is altered

The presentation of endogenous lipid antigens was tested by using mouse autoreactive iNKT hybridoma cells and DC from *Scp2*$^{-/-}$ or WT mice as APC. FF13 cells released much less IL-2 after stimulation with *Scp2*$^{-/-}$-DC (**Figure 64)** as compared to WT APC. When the number of APC per well was increased, a plateau was reached with both APC, but a large difference remained in the total cytokine release, indicating a qualitative and not a quantitative alteration of the self-lipid-CD1d stimulatory complexes. As expected, APC from CD1d$^{-/-}$ mice were unable to stimulate the iNKT hybridoma (**Figure 64 triangle symbol**).

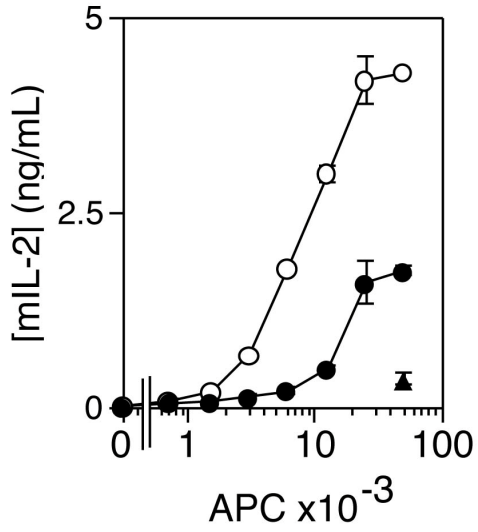


Figure 64. Endogenous lipid antigen presentation capacity in *Scp2*$^{-/-}$ mice is altered. Various amounts of DC (A) or thymocytes (B) from *Scp2*$^{-/-}$ (●), WT (○), or CD1d$^{-/-}$ (▲) mice were used to stimulate the mouse iNKT hybridoma FF13 in the absence of any exogenously added lipid antigen. Results are expressed as mean release of IL-2 ± s.d.

The response of the iNKT hybridoma cells to DC was blocked by addition of anti-CD1d mAb (**Figure 65**), confirming the CD1d restriction of autoreactive cells.

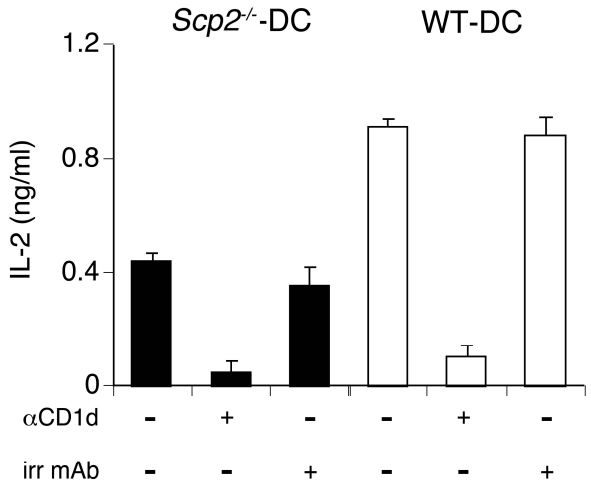


Figure 65. Endogenous lipid antigen presentation is CD1d-dependent. DC (2.5 x 10^4/well) from *Scp2*$^{-/-}$ (black columns), WT (white columns) were used to stimulate the mouse iNKT hybridoma FF13 in the presence of anti-CD1d mAb 1B1 (20 µg/ml) or the same concentration of irrelevant isotype-matched mAb. Results are expressed as mean release of IL-2 ± s.d. of duplicates, and are representative of 2 independent experiments.

Taken together, these findings suggest that the reduced numbers of iNKT cells in *Scp2*$^{-/-}$ mice are not the consequence of impaired CD1d expression or of altered generalised lipid antigen presentation. The finding that *Scp2*$^{-/-}$-APC are less efficient in activating peripheral NKT cells with endogenous ligands suggests that these APC might have alterations in the synthesis and/or presentation of self-lipid antigens.

Scp2$^{-/-}$ mice have an altered repertoire of self-lipids

Since Scp2 is a lipid transfer protein with important functions in lipid metabolism, we made the hypothesis that *Scp2*$^{-/-}$ mice have an altered lipid repertoire that might result in inadequate stimulation and maturation of iNKT cells. Particular attention was put on isolation of thymocytes in order to minimise their contamination with other cell populations possibly not involved in thymic selection of iNKT cells.

Lipids were extracted from *Scp2*$^{-/-}$ and WT thymocytes and analysed by electrospray ionisation mass spectrometry (ESI-MS) operated in multiple reactions monitoring mode. A total of 167 different lipid species spanning 13 different lipid families were quantified (**Figure 66**). Except for 6 lipid species that were enriched >140% and 11 lipid species that were diminished <60% in *Scp2*$^{-/-}$ mice as compared to WT mice (**Figure 66A**), most of the lipid species measured did not vary significantly.

Overall, *Scp2*$^{-/-}$ thymic extracts exhibited decreased amounts of glucosylceramides (GlcCer), phosphatidic acids (PA), plasmalogens of phosphatidylethanolamine (pPE), phosphatidylethanolamines (PE) and phosphatidylserines (PS) while phosphatidylcholines (PC) were increased (**Figure 66B**). When individual lipid species were compared, almost all GlcCer and pPE were diminished in *Scp2*$^{-/-}$ thymocytes (**Figure 66C**). No preferential reduction was observed in GlcCer species regarding their incorporation of dehydrosphingosine (d18:0) or sphingosine (d18:1) base and fatty acids. When the relative abundance of the individual GlcCer species was analysed again no difference was detected (data not shown), suggesting that in *Scp2*$^{-/-}$ mice GlcCer are diminished independently from the availability of individual bases and fatty acids. Likewise, most of the pPE species measured were diminished, probably as a consequence of defective SCP-2 function in lipid transport and export from peroxisomes, where the ether bonds of plasmalogen precursors are generated. pPE also showed a relative increase of

lipid species containing long and unsaturated fatty acids (**Figure 66D**), possibly caused by reduced fatty acid β-oxidation in peroxisomes. PE species, which are not synthesised within peroxisomes, were only slightly decreased as compared to PE in WT thymocytes and their fatty acid composition was normal (**Figure 66D**), thus excluding a general defect in fatty acid usage.

PC was the only increased lipid family. Most of the PC species were increased (**Figure 66C**), while their relative abundance remained constant (data not shown), suggesting that in *Scp2*$^{-/-}$ thymocytes a compensatory mechanism is operating that contributes to PC accumulation independently of used fatty acids. Taken together these findings show that lack of SCP-2 induces a subtle yet preferential alteration of only a few lipid species, some of which require intact peroxisomal transport.

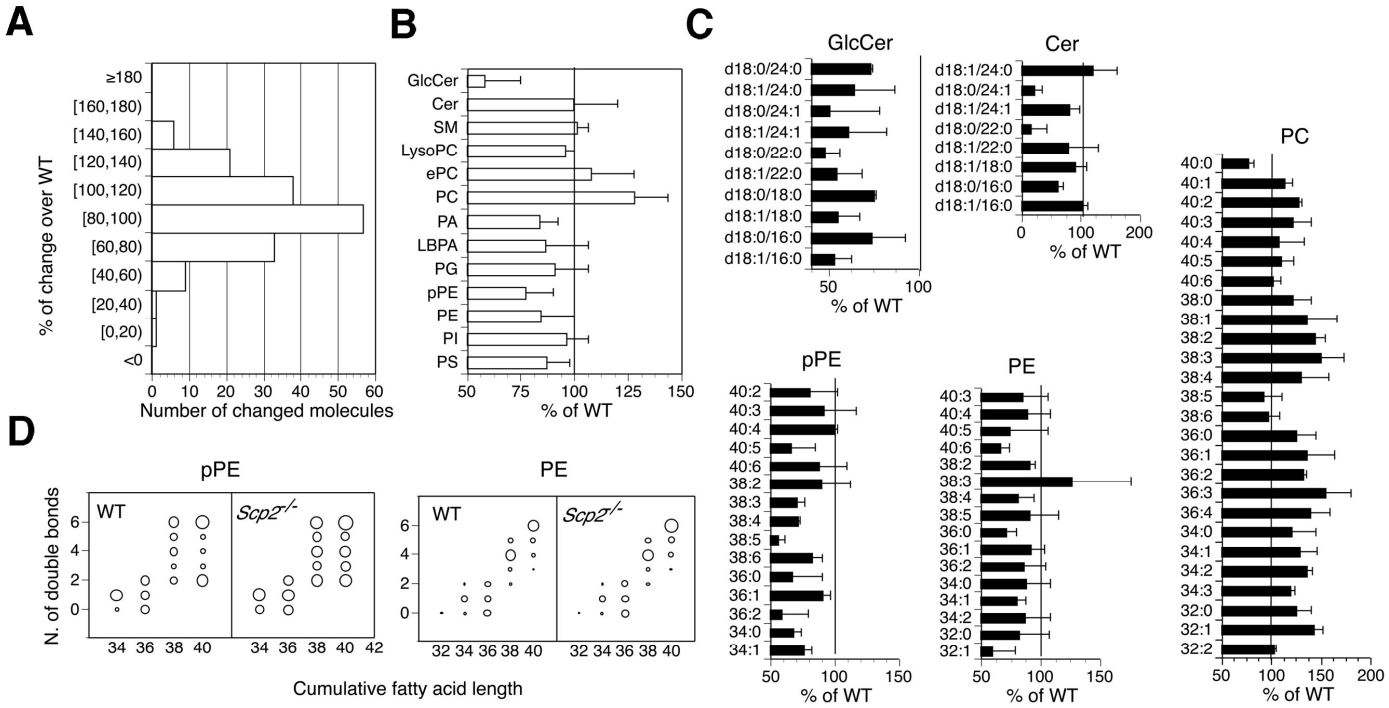


Figure 66. Comparison of the lipid repertoire between *Scp2*$^{-/-}$ and WT thymocytes. Thymocyte total lipid extracts were spiked with appropriate internal standards and analysed by LC-ESI-MS. (**A**) Histograms represent the number of changed lipids in *Scp2*$^{-/-}$ mice distributed into categories according to the % difference as compared to WT. (**B**) Thymocyte lipids in *Scp2*$^{-/-}$ mice as compared to WT: GlcCer, glucosylceramide; Cer, ceramide; SM, sphingomyelin; LysoPC, lysophosphatidylcholine; ePC, ether phosphatidylcholine; PC, phosphatidylcholine; PA, phosphatidic acid; LBPA, lysobisphosphatidic acid; PG, phosphatidylglycerol; pPE, plasmalogens of phosphatidyletanolamine; PE, phosphatidyletanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. (**C**) Analysis of the lipid species differing in fatty acid chain length and degree of unsaturation. Results are expressed as percentages of mean ± s.d. as compared to WT. Lipids were extracted from two thymi per group and subjected to at least two runs of analyses. (**D**) pPE and PE lipid species are plotted according to fatty acid chain length and degree of unsaturation. The circle sizes are proportional to the relative abundance of lipid species at each set of coordinates. Results are representative of extracts derived from two thymi per group and from at least two runs of analyses.

iNKT cell maturation is blocked in *Scp2*$^{-/-}$ thymus

The lack of Scpx/pro-Scp2 gene leads to major alterations in the lipid repertoire of *Scp2*$^{-/-}$ mice and selectively impairs the number of iNKT cells (**Figure 53**) and affects response to self-lipids (**Figure 64**); therefore, we performed further studies to characterise the type of alteration in the thymus.

iNKT thymocyes were MACS-enriched with CD1d:αGalCer dimers [233] and analysed by multi-parametric flow cytometry. Two types of partial blocks in iNKT cell maturation were detected. The first one was characterized by the accumulation of cells at Stage 0 (defined as CD24^{+}, CD1d:αGalCer dimer^{+}, CD44^{-} and NK1.1^{-}) (**Figure 67A**). The difference between *Scp2*$^{-/-}$ and WT mice is highlighted by the significant increase in the ratio of CD24^{+}:CD24^{-} cells observed in *Scp2*$^{-/-}$ thymic iNKT cells when compared to WT ones (**Figure 67B**).

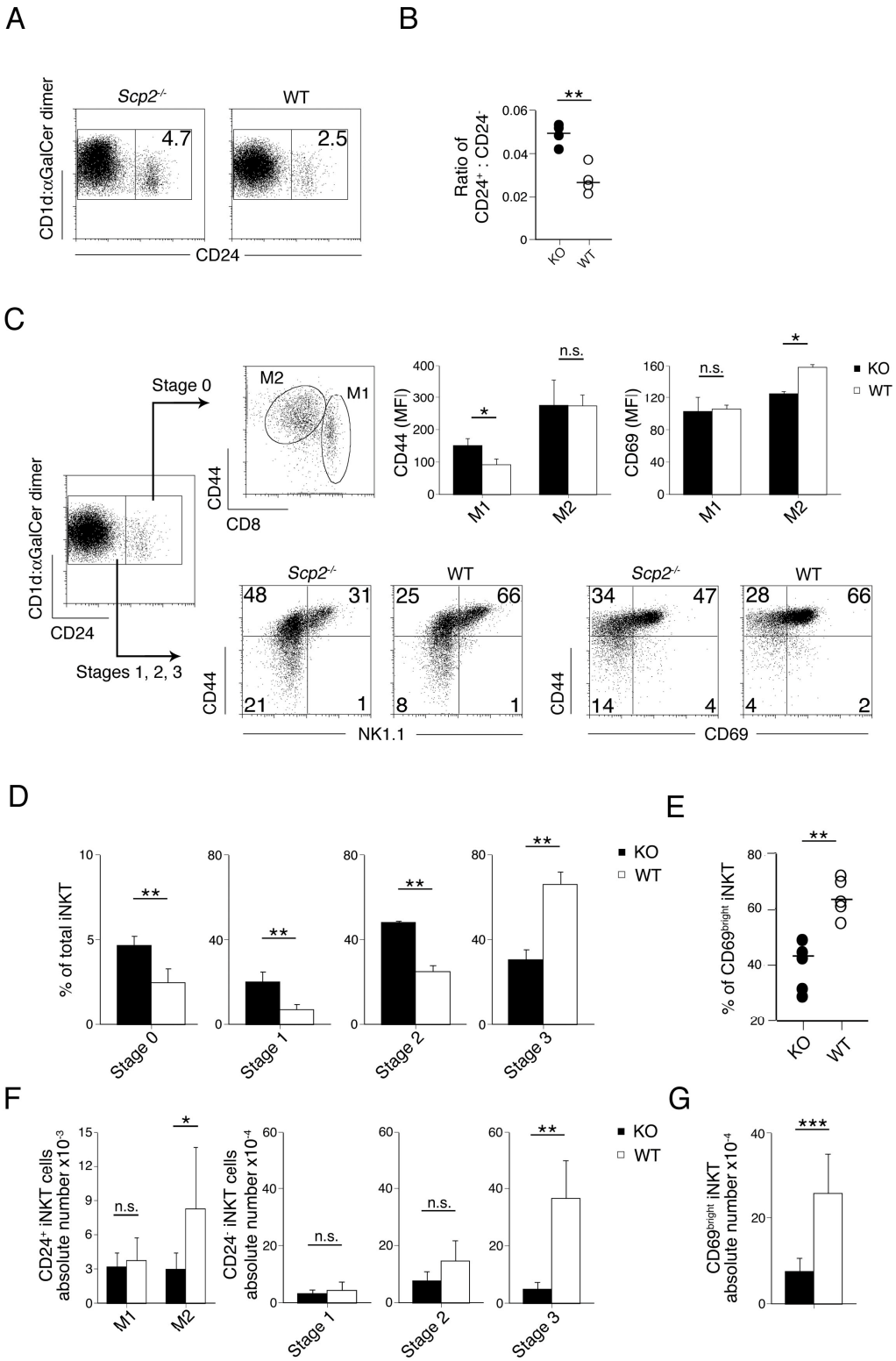
The phenotype of iNKT cells accumulating at Stage 0 was further characterised by analysing co-expression of CD8, CD62L, CD44 and CD69 using 7 colour flow cytometric analysis. Within Stage 0 two populations were detected, indicated as M1 and M2. M1 iNKT cells express markers of more immature cells (CD8^{hi}, CD44$^{low\text{-}int}$, CD62L^{int}) than M2 cells (CD8^{int}, CD44$^{int\text{-}hi}$, CD62L$^{int\text{-}hi}$) (**Figure 67C** and **Figure 68**). M1 cells also express less CD69 than M2 cells, thus indicating that they represent cells not yet positively selected.

In *Scp2*$^{-/-}$ mice M1 cells expressed higher levels of CD44 and M2 cells expressed lower levels of CD69 than in WT mice (**Figure 67C**). In addition, total *Scp2*$^{-/-}$ M2 cells were less abundant than WT M2 cells (**Figure 67F**), suggesting a reduced capacity of *Scp2*$^{-/-}$ iNKT cells to progress into more mature stages.

The second thymic block was at Stage 2 (TCR Cβ^{+}, CD1d:αGalCer dimer^{+}, CD24^{-}, CD44^{+}, NK1.1^{-}). Scp2$^{-/-}$ iNKT thymocytes at stage 1 and 2 were increased in percentage and were not significantly different in total numbers from WT cells. On the contrary, Stage 3 cells (TCR Cβ^{+}, CD1d:αGalCer dimer^{+}, CD24^{-}, CD44^{+},

NK1.1^{+}) were decreased either in percentage or in total number as compared to WT cells (**Figure 67C, D and F).** Importantly, in *Scp2*$^{-/-}$ mice a marked reduction of the total number and percentage of cells expressing CD69 was also observed (**Figure 67C, E, G**). Finally, the expression levels of CD49b (DX5), another marker defining more mature iNKT cells [230, 233] was decreased in *Scp2*$^{-/-}$ iNKT thymocytes as compared to WT ones (**Figure 69**).

Figure 67. iNKT cells development is blocked in *Scp2*$^{-/-}$ thymus. (**A**) Flow cytometry of CD1d:αGalCer dimer-MACS-enriched thymic iNKT cells from *Scp2*$^{-/-}$ and WT mice stained with anti-CD24 mAb. Numbers represent percentages of CD24^{+} iNKT thymocytes. (**B**) Ratio of CD24^{+}:CD24^{-} iNKT cells of *Scp2*$^{-/-}$ (●and WT (○) mice. **, P = 0.0012. (**C**) Analysis of iNKT developmental subsets. Within the CD24^{+} thymocytes (Stage 0) two discrete subpopulations (M1 and M2) can be further identified according to the expression of CD8 and CD44 (upper panels). Columns represent expression (MFI ± s.d.) of CD44 (middle panel) or of CD69 (right panel) in M1 and M2 subpopulations in *Scp2*$^{-/-}$ (black columns) or WT (white columns) mice. *, $P \leq 0.05$; n.s., not significant. CD24^{-} thymocytes (Stages 1, 2, 3) are further divided according to their expression of CD44 and NK1.1 or CD44 and CD69 (lower panels). Numbers represent the percentages of cells in the quadrants. (**D,F**) iNKT cell distribution among different developmental subsets. Results are expressed as (D) percentages after CD1d:αGalCer dimer-MACS-enrichment or (F) as absolute numbers of total thymic iNKT cells from *Scp2*$^{-/-}$ (black columns) and WT (white columns) mice. *, $P \leq 0.05$; **, $P \leq 0.01$; n.s., not significant. (**E**) Percentages and (**G**) absolute numbers of CD69^{bright} thymic iNKT cells in CD24^{-} thymocytes (Stages 1, 2, 3) of *Scp2*$^{-/-}$ (●) and WT (○) mice. *, $P \leq 0.05$; ***, $P \leq 0.001$. All depicted experiments were performed on at least 5 mice per group.


A
B
C
D
E
F
G
Scp2-/-
WT
4.7
2.5
CD1d:αGalCer dimer
CD24
Ratio of CD24+ : CD24-
KO
WT
Stage 0
Stages 1, 2, 3
M2
M1
CD44
CD8
CD44 (MFI)
CD69 (MFI)
n.s.
48
31
21
1
25
66
8
1
34
47
14
4
28
66
4
2
NK1.1
CD69
% of total iNKT
Stage 0
Stage 1
Stage 2
Stage 3
% of CD69bright iNKT
CD24+ iNKT cells absolute number x10-3
CD24- iNKT cells absolute number x10-4
CD69bright iNKT absolute number x10-4

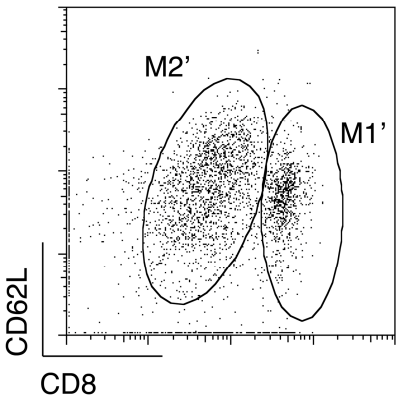


Figure 68. iNKT Stage 0 thymocyte subpopulations. Within the CD24^{+} thymocytes (Stage 0) two discrete subpopulations (M1' and M2') can be further identified according to the expression of CD8 and CD62L, similarly to the ones illustrated in Figure 60 in which CD44 is plotted on the y axis. Plot illustrates WT iNKT thymocytes and is representative of at least 4 independent experiments.

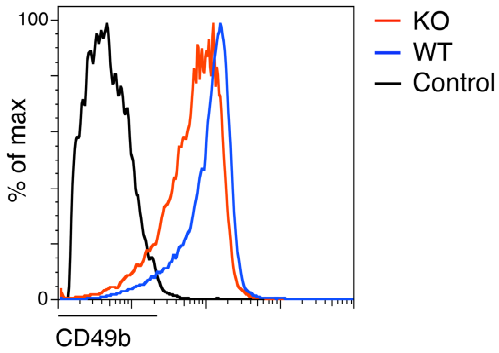


Figure 69. *Scp2*$^{-/-}$ iNKT thymocytes have reduced expression of CD49b (DX5). Thymic iNKT from *Scp2*$^{-/-}$ (red lines) or WT (blue lines) mice were CD1d:αGalCer dimer-MACS-enriched and stained with anti-CD49b. Histograms represent expression levels of CD49b on CD24^{-} iNKT cells. Black histogram represents staining with isotype-matched irrelevant mAb.

We then investigated the expression of CD132 (common γ chain) and CD122 (IL-2Rβ and IL-15Rβ chain) because the transition from Stage 2 to Stage 3 is strictly IL-15 dependent [184]. Both receptors were expressed at the same levels in iNKT thymocytes from the two mice (**Figure 70**), excluding that a defect in these receptors leads to reduction of thymic iNKT cells.

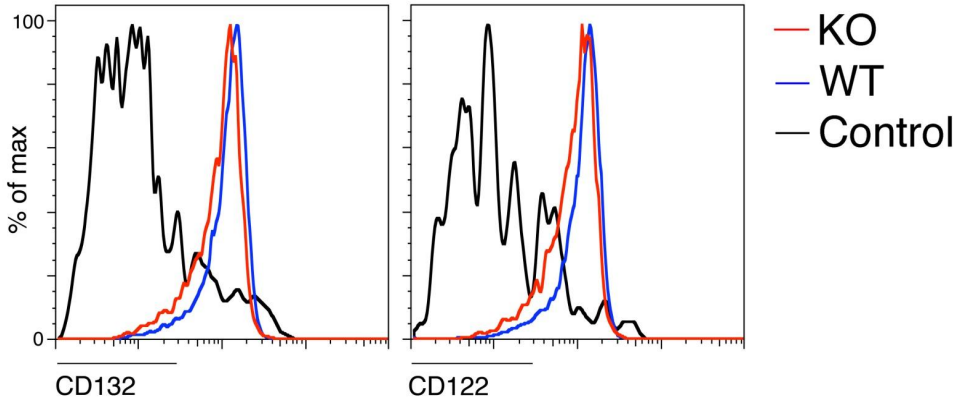


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Figure 70. *Scp2*$^{-/-}$ iNKT thymocytes have normal expression of CD132 and CD122. Thymic iNKT from *Scp2*$^{-/-}$ (red lines) or WT (blue lines) mice were CD1d:αGalCer dimer-MACS-enriched and stained with anti-CD123 (left panel) or anti-CD122 mAb (right panel). Histograms represent expression levels of Stage 2-gated cells.

To distinguish among increased cell death and reduced proliferation of *Scp2*$^{-/-}$ thymocytes, both apoptotic cells enumeration and cell cycle analyses were performed. Apoptotic iNKT cells were not increased in Stages 0, 1 and 2 in *Scp2*$^{-/-}$ as compared to WT mice, but rather decreased (**Figure 71**). Instead, a slight increase of apoptotic cells was observed in Stage 3.

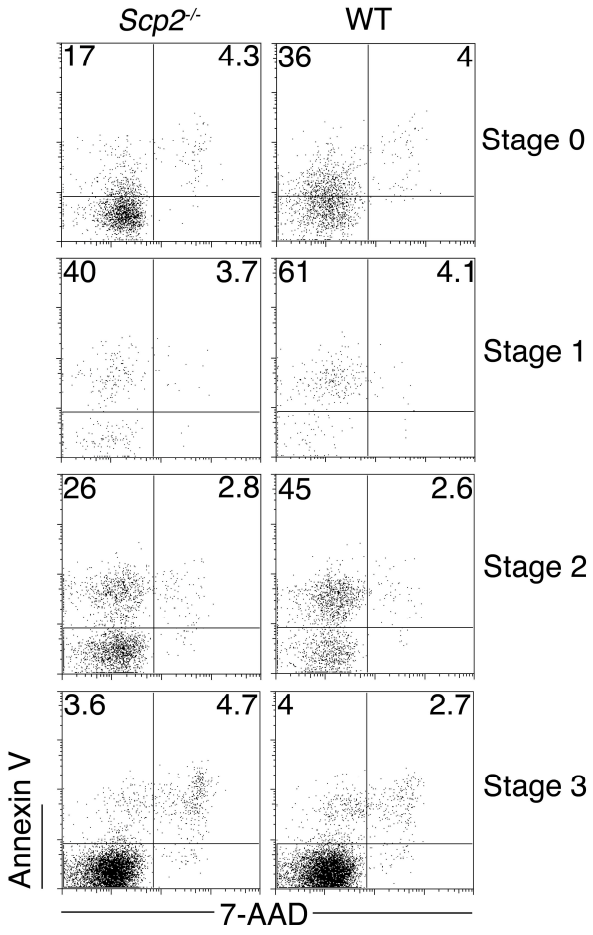

Figure 71. *Scp2*$^{-/-}$ iNKT thymocytes don't show a higher mortality rate. To evaluate apoptosis, CD1d:αGalCer dimer-MACS-enriched thymic iNKT were stained with Annexin V and 7-AAD. Numbers represent the median of percentages of cells in the quadrants.

Cell cycle analyses revealed that *Scp2*$^{-/-}$ iNKT cells in S phase were largely decreased in Stage 3, moderately decreased in Stages 1-2 and slightly decreased in Stage 0 (**Figure 72**).

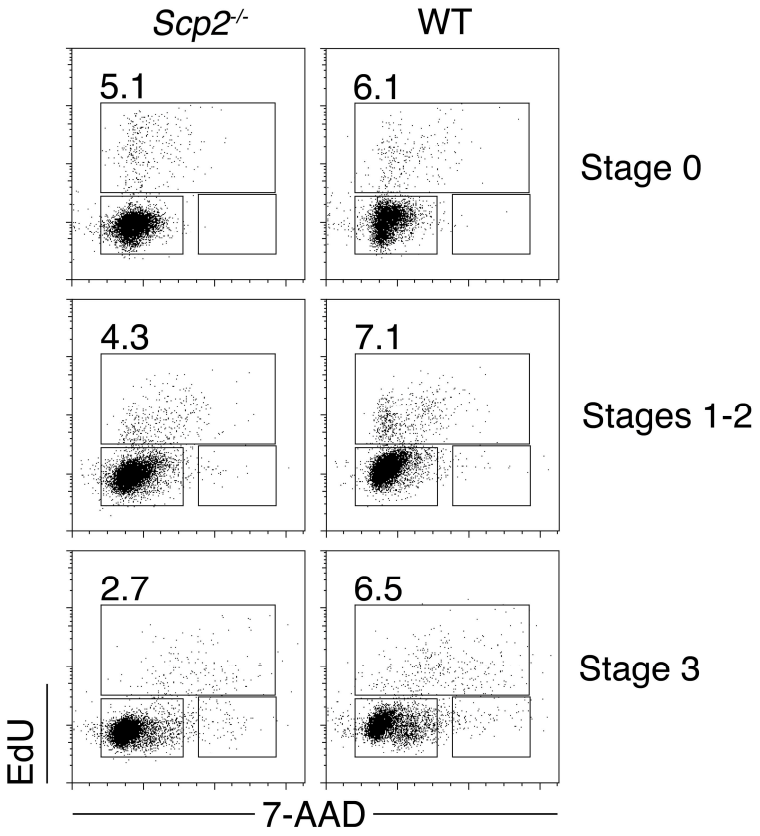

Figure 72. *Scp2*$^{-/-}$ NKT thymocytes have reduced capacity to proliferate. Cell cycle analysis of thymic iNKT from *Scp2*$^{-/-}$ and WT mice injected intravenously with 1 mg EdU. After CD1d:αGalCer dimer-MACS-enrichment of cells, incorporation of EdU and 7-AAD were detected by intracellular flow cytometry. Numbers represent the median of percentages of cells in the gated areas.

Collectively, these data show that the absence of SCP-2 causes a reduction of iNKT thymocyte numbers associated with delayed maturation, low CD69 expression, reduced proliferation and altered cell death. The balance between these events results in a block of immature iNKT cells in Stage 0, and diminished capacity to expand and mature to Stage 3. Thus, SCP-2 deficiency prevents iNKT cells to remain activated and expand before exiting the thymus.

DISCUSSION

Lipid metabolism involves a complex network of enzymes and transfer proteins, which are interconnected in different organelles and membranes inside the cell. Synthesis and degradation of lipids take place in defined subcellular compartments as a consequence of the physiological association of enzymes with specific cellular membranes and the topological characteristics of individual lipids.

The different localisation of synthetic and catabolic enzymes has an important impact on lipid-specific immune response, since it influences the presence of lipid antigens in cellular compartments wherein CD1 molecules traffic and become loaded.

Some lipids require transport by LTP, which distribute lipid monomers across aqueous phases among organelles [345]. LTP may transport precursors of complex lipids and thus optimise their synthesis, or may extract lipids from membranes and offer them to catabolic enzymes.

Here we find that the LTP SCP-2 may regulate the thymic development and peripheral numbers of iNKT cells. SCP-2 is associated with the cytoplasmic leaflet of the ER, mitochondria and peroxisomes, and has been ascribed the important

function of distributing lipids among these membranes [124]. Due to its cellular distribution, SCP-2 does not co-localise with the antigen-binding domains of CD1 molecules, and therefore cannot participate in CD1 antigen loading. Since SCP-2 contains a peroxisomal targeting motif, it is also imported into peroxisomes [126]. Moreover, it was detected in the nucleus where it may act as a transcription factor [128]. Important consequences of the SCP-2 deficiency are reduced peroxisomal beta-oxidation of fatty acids, leading to accumulation of branched- and very long chain fatty acids, and alterations in membrane lipid composition and lipid biliary secretion [346].

The alterations involved in the generation of normal peripheral iNKT cell repertoire can be classified into five classes: i) alterations of transcription factors essential for iNKT cell development and maturation [239-241, 347-349], ii) alterations of proteins involved in iNKT TCR signal transduction [242, 244, 350, 351], or iii) in interactions between developing iNKT thymocytes and selecting cells [307, 352, 353], iv) modifications of the lysosomal environment affecting correct lipid trafficking, disposal and loading on CD1d [76, 84, 91, 96, 208, 342], and v) alterations of the ER-located LTP MTP, which may affect CD1d assembly and recycling [49, 80, 91].

The alterations induced by SCP-2 deficiency demonstrate a novel type of mechanism affecting normal development and expansion of iNKT cells. In *Scp2*$^{-/-}$ mice, iNKT cells are reduced both in percentage and in absolute numbers, whereas other cell populations including conventional T cells, B cells and monocytes are normal. The residual iNKT cells show normal numbers of Vβ8.2- and Vβ7-expressing cells, indicating no major alterations in the Vβ repertoire. iNKT cells also show normal representation of memory/activated markers, reflecting normal peripheral stimulation. A reduction of CD4^{+} iNKT cells is instead observed in the thymus, spleen and liver, resembling a defect previously described in iNKT

cells from NPC2$^{-/-}$ mice (Chapter 1 of this thesis, **Figure 21**). The role of CD4 co-receptor in iNKT thymic selection has not been reported, although a direct interaction between human CD1d and CD4 may occur [181, 183]. It remains unknown why these two LTP-deficient mice share this unique characteristic. In MHC-restricted T cells, strong and persisting selective signals during thymic development facilitate CD4 expression [354, 355]. It is tempting to speculate that both *Scp2*$^{-/-}$ and NPC2$^{-/-}$ mice are unable to generate CD1d-lipid complexes inducing prolonged signalling in maturing iNKT cells and therefore, inefficiently maintain the CD4 co-receptor expression.

In *Scp2*$^{-/-}$ mice, the NK1.1 marker differently from CD4, is expressed by a large number of peripheral iNKT cells. This observation is difficult to explain, since NK1.1 upregulation is considered as the final maturation step of iNKT cells [231, 232, 234]. Therefore, the residual iNKT cells belong to a fully mature CD4^{-} population.

Despite alterations in the number and phenotype, residual iNKT cells appear functionally normal. They react to common iNKT cell stimulatory antigens including αGalCer, iGb3 and *S. paucimobilis* glycolipids. iNKT cells from *Scp2*$^{-/-}$ mice produce IFN-γ, IL-4 and IL-2 upon stimulation, thus revealing normal effector functions.

The deficiency of several LTP located in lysosomes leads to important alterations in lipid antigen presentation, and consequently to reduced numbers of thymic and peripheral iNKT cells. Since SCP-2 is a LTP, which is clearly absent from lysosomes, we carefully investigated the antigen-presentation capacity of *Scp-2*$^{-/-}$-APC. Thymocytes and DC, both relevant APC *in vivo*, present αGalCer, iGb3 and *S. paucimobilis* glycolipids with the same efficiency as WT-APC. Interestingly, we noticed that they have instead a reduced capacity to present endogenous lipids to autoreactive iNKT cells. This was confirmed by weak upregulation of CD69 on freshly isolated iNKT cells and reduced IL-2 release by autoreactive iNKT

hybridoma cells. The known functions of SCP-2, together with our observation that the stimulation with endogenous lipids is reduced, suggested that the lipid composition of *Scp2*$^{-/-}$-APC is modified. Comparative lipidomic analysis revealed quantitative and subtle qualitative differences between *Scp2*$^{-/-}$- and WT-thymocytes. Several phospholipids such as pPE, PE, PS and PA were diminished while PC levels were increased in *Scp2*$^{-/-}$ cells (**Figure 66B**). PE, PS, PA and PC did not show any relative change in the used fatty acids, thus excluding general acylation defects. A relative increased use of long chain fatty acids was instead found in PE plasmalogens (**Figure 66D**). Two non-alternative mechanisms might explain these pPE changes, namely a reduced fatty acid β-oxidation within peroxisomes and a reduced availability of plasmanyls, which are also generated within these organelles [356] and are the precursors of plasmalogens. Since SCP-2 contributes to trafficking of peroxisomal lipids, its deficiency might affect the repertoire of lipids generated within this organelle.

A second finding was that the total amount of GlcCer was diminished whereas its precursor Cer was unchanged. Therefore, GlcCer reduction might be ascribed to inefficient synthesis, reduced transport of Cer to Golgi where GlcCer is synthesised, or to an increased utilisation of GlcCer as substrate for the synthesis of more complex glycosphingolipids. Further studies will clarify which mechanisms induce GlcCer reduction and whether SCP-2 is involved in the transport of Cer used for GlcCer synthesis.

The observed alterations of the lipid repertoire in the thymus might have direct immunological relevance and affect the selection steps of iNKT thymocytes. The nature of iNKT selecting ligands remains unclear and several lipid classes probably participate in these events.

In *Scp2*$^{-/-}$ mice the reduction of iNKT cells in the thymus is associated with two maturation blocks. The first block occurs at Stage 0, which identifies iNKT cells

during and immediately after positive selection. We characterised two subpopulations within Stage 0 cells, which are also present in WT mice, and we defined them as M1 and M2. M1 cells are more immature than M2 cells according to the expression of CD24, CD44, CD62L, CD8 and CD69 markers. Whether M1 cells are the immediate precursors of M2 cells or instead the two populations represent independent maturation stages remains to be investigated. In *Scp2*$^{-/-}$ mice, the total number and phenotype of M1 cells are normal, whereas M2 cells are diminished. In addition, M2 cells also show a significant reduction of the CD69 activation marker, thus suggesting a reduced stimulation by endogenous ligands.

The second block occurs at Stage 2. *Scp2*$^{-/-}$ thymocytes present an accumulation of Stage 1 and 2 cells and a significant reduction of the total number of Stage 3 cells. Stage 3 cells also show slightly increased apoptosis and decreased cycling capacity. Reduced expression of CD69 and CD49b (DX5) was observed in Stage 2 and 3 iNKT cells, possibly consequence of weak stimulation. All together these phenomena may concur in poor expansion of iNKT thymocytes leading to the observed reduced NKT cell numbers in the thymus and in the periphery.

The two stages where iNKT thymocytes are blocked in *Scp2*$^{-/-}$ mice have been associated with antigen stimulation necessary for continuous activation of iNKT thymocytes preceding their final maturation and exit to the periphery [230]. SCP-2 might affect these selection events with different mechanisms. It might be involved in the synthesis of selecting ligands. This possibility is difficult to investigate because the nature of the self-antigens inducing positive selection and thymic expansion is not known. A second mechanism is alteration of lipid traffic within *Scp2*$^{-/-}$ cells, such as altered lipid import into peroxisomes and mitochondria, export from peroxisomes, or reduced traffic of Cer to Golgi. This latter mechanism would be relevant if SCP-2-transported Cer is converted to GlcCer and to more complex glycosphingolipids, which have been indicated as possible selecting ligands [76,

357]. However, so far SCP-2 has not been identified as a Cer transporter and has not been found associated with Golgi membranes [124, 358], where GlcCer is synthesised.

In conclusion, these studies have revealed that SCP-2, a cytoplasmic and peroxisomal LTP, is involved in the regulation of iNKT cell maturation and expansion. This adds a novel level of regulation that involves lipid transport within cytoplasm and organelles, generation of complex lipids, and accumulation of stimulatory selecting lipids.

The unique localisation and shuttling functions of SCP-2 suggest that lipids, which are modified within peroxisomes and mitochondria might be relevant in thymic selection of iNKT cells. Future studies will investigate whether these organelles participate in immunity to lipid antigens.

CONCLUSIONS

The work presented in this thesis aimed to characterize the mechanisms by which stimulatory CD1d:lipid antigen complexes are generated and which are the contributions to this process of lipid transfer proteins and of enzymes of the lipid degradation pathway. This work also aimed to study how thymic selection and peripheral activation of lipid specific T cells are affected when stimulatory CD1:antigen complexes generation is impaired.
To study these mechanisms I took advantage of different knock-out and transgenic mouse models, in which particular LTPs (or enzymes of the lipid degradation pathway), localized in different cellular compartments, were either knocked-out or expressed as transgenes.

Formation of the CD1:antigen complex is the last of a series of events taking place in different cellular compartments, where CD1 molecules are assembled and traffic, and where antigenic lipids are internalized, processed or synthesized.
Since CD1d loading with antigenic lipids takes place mostly in lysosomes, lysosomal LTPs or enzymes of the lipid degradation pathway were the first molecules studied in order to understand how stimulatory CD1:lipid complexes and thymic selection of iNKT take place. By the analysis of mice knocked-out for particular LTPs or enzymes it has been possible to identify some of the key players on the generation of stimulatory CD1:antigen complexes, like saposins [84] and Hex-b [76]. However, deficiencies in enzymes of the lysosomal glycosphingolipid degradation pathway or in lysosomal lipid transfer proteins cause imbalances in lipid metabolism [208]. As a consequence of these defects, lipids accumulate inside lysosomal compartments, where CD1:lipid antigen complexes are generated. In a first study, I analysed two mouse models of imbalanced lipid metabolism, β-Gal$^{-/-}$ mice (a model for GM1 gangliosidosis) and NPC-2$^{-/-}$ mice (a

model for Niemann Pick C2 disease). The genes knocked-out in these two models encode proteins that localize inside lysosomes. In both models generation of stimulatory CD1d:lipid complexes was impaired and development of iNKT, but not of other T cells, was defective.

The data suggest that the accumulation of storage lipids is the main cause of impaired thymic selection of iNKT cells. Indeed, accumulation of storage lipids in both models disturbed lipid antigen presentation to iNKT and affected the formation of stimulatory CD1d:lipid antigen complexes on the cell surface. Presentation of exogenous lipid antigen to iNKT cells was improved by pre-treatment of APC with NB-DNJ, an inhibitor of glycosphingolipid biosynthesis known to reduce pathological lipid storage *in vitro* and *in vivo* [330].
Although the accumulation of lipids impairs lipid antigen presentation in both β-Gal$^{-/-}$ and NPC-2$^{-/-}$ cells, the underlying mechanisms are different. Different types of lipids accumulate in β-Gal$^{-/-}$ *vs*. NPC2$^{-/-}$ mice. The phenotype and TCR repertoire among the residual iNKT cell populations in β-Gal$^{-/-}$ *vs*. NPC2$^{-/-}$ mice is therefore differently influenced by the quantity and type(s) of lipid antigens that can form stimulatory CD1d:antigen complexes.

In a second study, I analysed whether CD1e functionally interacts with other CD1 family members, thus participating in the generation of CD1:antigen complexes.
Mature CD1e is a soluble protein, and the immunological function reported until now is to assist the processing of complex glycolipid antigens presented by CD1b molecules [16].
By using human transfectants co-expressing CD1e and CD1b, or CD1c or CD1d, I observed that CD1e presence in APC influences in several ways the response of specific clones to endogenous and exogenous lipid antigens. CD1e can directly

participate in loading and unloading of lipids onto and from CD1d. I performed these studies both in the human system and in a transgenic mouse model. I observed that the nature of the antigen is critical for interaction with CD1e. Moreover, the data showed that, at least for CD1d-mediated antigen presentation, CD1e influences the type of cytokines released by human iNKT cell clones by altering the number of stimulatory complexes on the surface.

CD1e could influence lipid antigen presentation by several mechanisms: i) increasing solubility of lipid antigen by extracting them from membranes in order to facilitate their association with CD1 molecules and thus their presentation to T cells; ii) participating in lipid processing; iii) facilitating loading of lipid antigens on other CD1 molecules, and iv) facilitating unloading of non-stimulatory lipids from CD1.

Kinetic studies demonstrated that CD1e participates in the formation of CD1d:lipid antigen complexes at early time points, while it facilitates the unloading of the complexes at later time points, thus contributing to the fine tuning of the immunological response of iNKT cells. This function might be important during infections, thus limiting antigen presentation only when bacteria are present and produce antigenic lipids. This hypothesis is supported by the fact that CD1e facilitates presentation of *Sphingomonas* antigens to iNKT cells, particularly when the bacteria are pulsed only for a short period of time. Importantly CD1e also accelerates the “switch off” of the response at later time points, thus avoiding prolonged iNKT cell activation.

In these studies I also found that LTPs residing in cellular compartments different from lysosomes are also relevant for iNKT cell stimulation. ApoE, a serum protein involved in transport of lipid inside the cell and MTP, an ER-resident LTP involved in correct folding of CD1 molecules are two examples of non-lysosomal LTPs

influencing CD1d-mediated selection and peripheral activation iNKT cells. However, it was never investigated whether LTPs resident in the cytoplasm, which is one of the most important cellular compartments where lipids are generated, modified and distributed to different membranes, are also involved in iNKT cell development and stimulation.

In a third study I analysed the role of a non-lysosomal LTP, SCP-2, in regulating thymic development, peripheral numbers and functional activation of iNKT cells. SCP-2 is associated with the cytoplasmic leaflet of the ER, mitochondria and peroxisomes, and has been ascribed the important function of distributing lipids among these membranes [124]. SCP-2 does not co-localise with the antigen-binding domains of CD1 molecules, and therefore cannot participate in CD1 antigen loading.

Scp-2$^{-/-}$-APC were defective in presenting endogenous lipids to autoreactive iNKT cells and showed an altered lipid composition. Indeed, in *Scp-2*$^{-/-}$ mice iNKT cells thymic development is partially blocked. This arrest occurs at two maturation checkpoints, which have been associated with intrathymic antigen stimulation necessary for expansion and terminal maturation of iNKT thimocytes [230]. SCP-2 contributes to lipid trafficking within peroxisomes, and we found that its deficiency affects the repertoire of lipids of peroxisomal origin. SCP-2 might be involved in the synthesis of iNKT cell selecting ligands, or in lipid traffic within cells, relevant for iNKT cell stimulation.

In conclusion, this study has revealed a novel level of regulation that involves lipid transport within cytoplasm and cellular organelles, generation of complex lipids, and accumulation of stimulatory selecting lipids.

The increasing knowledge of the CD1 system shows that CD1 and MHC systems share several similarities. The two systems evolved to react to different kinds of

challenges, while common features have been maintained. It is interesting to note that in both systems a stringent quality control mechanism exists that disciplines the correct loading of antigens. In both cases this function is exerted by chaperone proteins, that are located in different cellular compartments.

The evolution of the CD1 system in primates indicates that recognition of lipid antigens is beneficial to the organism and suggests that this class of antigens might be considered for therapeutic approaches. The lack of polymorphism of CD1 molecules is instead an important difference with the MHC system and its physiological implications remain unclear.

Lipid recognition has been considered as a “simpler” version of peptide recognition. A more complex picture is, instead, emerging showing that lipid antigen presentation is complex and is involved in several physiological and pathological immune responses. Studies in this field in a near future will clarify how the immune system has selected the most appropriate strategies to enlarge the repertoire of recognized antigens and cope with different pathogens.

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RESEARCH AND PROFESSIONAL EXPERIENCE

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2002-2003:

MSc student, Immunology of Diabetes Unit, Scientific Institute San Raffaele, laboratory of Dr. M. Falcone, Milan,Italy

LANGUAGE SKILLS

English: fluent

German: basic

Italian: native speaker

TRAINING COURSES

2007 -International ENII-Mugen Immunology Summer School, Capo Caccia, Sardinia, Italy

2005 -LTK module 1: Introductory course in Laboratory Animal Science, Universitaet Zurich, Institute fuer Labortierkunde

2004 -"Basic Course Flow Cytometry", Scientific Institute H.San Raffaele, Milan

2003 -"Corso di Formazione sulla sperimentazione animale", Institutional Animal e Commettee (IACUC), Scientific Institute H.San Raffaele, Milan

PRACTICAL EXPERIENCE

Computer skills: Microsoft Office, Internet, Delta Graph, GraphPad Prism, Photoshop, InStat, Summit, FCS Express, FlowJo

Cellular biology and immunology techniques: FACS (up to 9 colors), ELISA, cell culture (tumor and primary cells), isolation of primary cells from body fluids, T cell activation assays, mouse hybridoma generation

In vivo experiments (in mice): anesthesia, intra peritoneal, intra venous, subcutaneous injections, immunization

Molecular biology and biochemical techinques: PCR, expression of recombinant proteins in mammalian and insect system, protein purification and quantification, antibodies labelling

PUBLICATIONS

1. M.Falcone, **F.Facciotti**, N.Ghidoli, P.Monti, S.Olivieri, L.Zaccagnino, E.Bonifacio, G.Casorati, F.Sanvito, N.Sarvetnick "Upregulation of CD1d molecules

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5. **F. Facciotti**, M.Cavallari, J. Schümann, D. Nebenius-Oosthuizen, C. Angénieux, F. Signorino-Gello, H. De la Salle, L. Mori and G. De Libero "CD1e participates in generation of iNKT cell ligands" *Manuscript in preparation*

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COMMUNICATIONS

International:

-5th International Symposium on CD1/NKT cells, Kamakura, Japan, March 2009
"Pleiotropic functions of CD1e" **F.Facciotti**, M.Cavallari, J.Schuemann, C. Angénieux, F. Signorino-Gelo, H. De la Salle, L. Mori and G. De Libero

-4th NKT cell and CD1 workshop, Abbazia di Spineto, Italy, September 2006
"NKT development is impaired in the absence of aGalactosidase or NPC2 protein" **F.Facciotti**, J.Schuemann, A.Collmann, J. Matsuda, P. Lobel, L. Mori, G. De Libero

-1st TONECA Symposium Coordination Action on the Aetiology, Pathology and prediction of type 1 diabetes in Europe, Hannover, July 2004
"CD1d Transgenic NOD as a model to study NKT cell-DC interaction in autoimmune diabetes" **F.Facciotti**, E.Bonifacio

National:

-Biovalley Science Week, Basel, October 2007
"CD1e participates in generation of iNKT cell ligands" **F. Facciotti**, M.Cavallari, J. Schümann, D. Nebenius-Oosthuizen, C. Angénieux, F. Signorino-Gello, H. De la Salle, L. Mori and G. De Libero

-SGAI meeting, Basel, April 2007
"CD1e participates in generation of iNKT cell ligands" **F. Facciotti**, J. Schümann, D. Nebenius-Oosthuizen, C. Angénieux, F. Signorino-Gello, H. De la Salle, L. Mori and G. De Libero

PRIZES

-4th NKT cell and CD1 workshop, Abbazia di Spineto, Italy, 2006, **POSTER PRIZE** awarded by Alexis Corporation

"NKT development is impaired in the absence of aGalactosidase or NPC2 protein" **F.Facciotti**, J.Schuemann, A.Collmann, J. Matsuda, P. Lobel, L. Mori, G. De Libero

-Biovalley Science Day, Basel, 2007 **POSTER PRIZE** awarded by Roche

"CD1e participates in generation of iNKT cell ligands" **F. Facciotti**, M.Cavallari, J. Schümann, D. Nebenius-Oosthuizen, C. Angénieux, F. Signorino-Gello, H. De la Salle, L. Mori and G. De Libero

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AKNOWLEDGMENTS

I would like to thank my supervisor Prof. Gennaro De Libero for giving me the opportunity to work in his laboratory. Thank for the guidance and support throughout my thesis work. He had a fundamental role in my scientific and personal development and always pushed me to improve.

I would like to thank Prof. Antonius Rolink for being my Fakultaetverantwortlicher, Prof. Ed Palmer and Prof. Kurt Ballmer-Hofer for having accepted to be members of my Thesis Commettee.

I thank past and present members of the Experimental Immunology lab which shared these years with me. I thank particularly Lucia for the support in all the mice experiments, both scientifically and technically. I thank Lena for her excellent molecular biology work and Nino for his unvaluable work in extracting and purifying lipids for my studies. A thank goes to the collegues of the lab Marco Cavallari, Anthony, Marco Lepore, Ramu, Huseyin, Manu, Simon and Melissa: we spent good and bad times together.

I thank particularly all the people of the Animal Facility: Ueli, Nicole and Tamara and all the past and present members of the facility for the incredible and costant help they gave me in these years.

A special gratitude goes to Jens Schuemann, who thought me so much and always believed in me.

I thank Prof. Panza for the synthesis of αGalcer analogs and Kirin Brewery for providing αGalCer.

I would like to thank the Foundations that supported my participations to Congresses: “Reisefonds fuer den akademischen Nachwuchs der Universitaet Basel”, ZMG (Schweizerische Gesellschaft fuer Zellbiologie, Moleckularbiologie und Genetik), FAG Freiwillige Akademischen Gesellschaft), SGAI (Schweizerische Gesellschaft fuer Allergologie und Immunologie).

I also thank Alexis Corporation and Roche which awarded me with Poster Prizes.

A special thank goes to The Girls, which have been not only collegues, but more than friends: Magda, Sami (and all the Arangio's), Maria, Sabri and Hati. You girls only know how much I owe you and there are no words that can express it.

Il ringraziamento piu' grande va alla mia famiglia e a Marco. Non avrei potuto fare tutto questo senza il vostro sostegno costante in questi anni. Siete e sarete sempre il porto sicuro della mia vita.