

Activation and immunoregulatory function of type II natural killer T lymphocytes

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Natural killer T (NKT) lymphocytes make up a potent immunomodulatory subset of innate-like lymphocytes. NKT cells are activated by self-lipids presented by the unconventional MHC class I-like molecule CD1d, resulting in the rapid production of a range of different cytokines, that modulate innate and adaptive immunity. NKT cells possess regulatory properties in several immune settings such as autoimmunity, infection and cancer. However, the activation of NKT cells is not fully understood. In this thesis, we have addressed the role of self-lipids for type II NKT cell activation and autoreactivity, and employed self-lipids to investigate the immunoregulatory function of type II NKT cells in murine disease models.

The glycosphingolipid (GSL) sulfatide has previously been shown to be a stimulatory self-ligand for type II NKT cells. Sulfatide exists naturally as a mixture of different isoforms and is abundant in organs such as the central nervous system, gastrointestinal tract, kidneys and the pancreas where it has important functions. We demonstrate that naturally existing isoforms, including C24:1 sulfatide and lyso-sulfatide, activate type II NKT cells. Organ specific isoforms in particular, but not non-physiological isoforms, of sulfatide induced efficient activation of type II NKT cells. Despite the potent activation of NKT cells by natural sulfatide isoforms, the autoreactivity of the type II NKT cells to CD1d-expressing cells was not dependent on sulfatide production by the stimulatory cells, demonstrating that other self-lipids were causing autoreactivity. In a search for such lipids, isolated from stimulatory cells, we identified two novel NKT cell activating self-GSLs, β -glucosylceramide and β -galactosylceramide and defined their stimulatory isoforms. However, by using antigen presenting cells deficient in all GSLs we could demonstrate that the autoreactivity of the type II NKT cells did not require GSLs. In summary, we demonstrate that natural isoforms of sulfatide, β -glucosylceramide and β -galactosylceramide are ligands for type II NKT cells, suggesting that they may play a role to activate type II NKT cells upon increased exposure in autoimmunity or tumor immunity. We also find that the CD1d-dependent natural autoreactivity of the type II NKT cells depends on lipids other than GSLs.

Sulfatide is present in pancreatic β -cells that are targets for autoimmune destruction in type I diabetes (T1D). We demonstrate immune reactivity to sulfatide in non-obese diabetic mice that spontaneously develop T1D. However, treatment of these mice with sulfatide, to activate immunomodulatory type II NKT cells, did not confer protection from T1D. In contrast, we found that sulfatide treatment significantly improved the survival rate of mice with *Staphylococcus aureus* sepsis. The protective effects mediated by sulfatide required CD1d but not type I NKT cells, suggesting that activated type II NKT cells ameliorated sepsis development. Protection was associated with reduced serum levels of pro-inflammatory cytokines and improved platelet counts.

In conclusion, our results provide novel information on the activation of type II NKT cells, and expands our understanding of their immunomodulatory capacity to improve disease outcome.

Keywords: NKT cells, GSL, Activation, T1D, *S. aureus* sepsis

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunförsvarets viktiga uppgift är att skydda oss mot invaderande mikroorganismer. Immunförsvaret är uppbyggt av immunceller vars roll är att urskilja det som är främmande, samtidigt som de skall vara toleranta mot kroppens egna vävnader. Detta innefattar ett enormt komplext system som i stora drag består av två delar, det medfödda immunsystemet och det adaptiva immunförsvaret. Det medfödda immunförsvaret har till uppgift att snabbt skydda oss mot hotande mikroorganismer, vilket resulterar i ett mindre energikrävande system som saknar specificitet och immunologiskt minne. Däremot, aktivering av det adaptiva immunförsvaret medför nästintill oändlig specificitet och skapar dessutom ett minne som skyddar mot senare infektioner av samma sort. För att särskilja mellan egna och främmande substanser så finns det regulatoriska celler. Dessa celler kan reglera immunförsvaret så att vi inte skapar ett immunförsvaret mot kroppsegna eller ofarliga främmande substanser. Det finns dessutom celler som fungerar som en länk mellan det medfödda och det adaptiva immunförsvaret. Dessa celler aktiveras snabbt och medverkar i att forma det adaptiva immunförsvaret. Utformningen av det adaptiva immunförsvaret medför en risk att utveckla celler som känner igen och attackerar kroppsegna substanser. Detta kan leda till utveckling av autoimmuna sjukdomar, så som typ I diabetes eller multiple scleros (MS). Naturliga mördar T celler är exempel på celler som fungerar som en länk mellan det medfödda och det adaptiva immunförsvaret. Dessa celler genomgår en unik mognadsprocess i brässen som innebär att de selekteras av kroppsegna lipider och blir autoreaktiva. Det medför att de som mogna celler snabbt kan aktiveras och därmed kan de medverka till att styra det adaptiva immunförsvaret. Studier har visat att de naturliga mördar T cellerna medverkar i regleringen av flera autoimmuna sjukdomar, och kan stärka immunsvaret mot infektioner och cancer. Exakt hur dessa celler aktiveras är idag okänt.

Här visar vi att en typ av naturliga mördar T celler (typ II) aktiveras av olika former av den kroppsegna lipiden sulfatid. Sulfatid tillhör en grupp av lipider kallade glykosfingolipider och finns i organ såsom centrala nervsystemet och bukspottkörteln, där sulfatid underlättar vid nervsignalering, respektive medverkar vid frigörandet av insulin. Vi visar att naturliga mördar T celler aktiveras av organspecifika isoformer av sulfatid men att den naturliga autoreaktiviteten inte beror på sulfatid. För att undersöka vilka lipider som medverkar i autoreaktiviteten så analyserade vi lipider från celler som ger upphov till stark naturlig aktivering av naturliga mördar T celler. Från den

studien hittade vi ytterligare två glykosfingolipider som gav upphov till naturliga mördar T cellers aktivering, nämligen galaktosylceramid och glucosylceramid. Vidare fann vi att lipider från glykosfingolipid gruppen inte var nödvändiga för uppkomst av autoreaktivitet av naturliga mördar T celler, vilket betyder att kroppsegna lipider involverade i autoreaktiviteten av typ II naturliga mördar T celler måste vara av en annan sort. Vi visar även i en djurmodell att behandling med glykosfingolipiden sulfatid leder till aktivering av typ II naturliga mördar T celler som dämpar immunförsvaret vid akut blodförgiftning vid allvarlig bakterie infektion, så kallad septisk chock, utlöst av systemisk närvaro av *Staphylococcus Aureus* bakterien. Sulfatid behandling medförde en minde akut inflammationsprocess och till följd av det så förbättrades blodflödet något, och även koagulationsprocessen. I en djurmodell för typ I diabetes så medförde behandling med sulfatid inga effekter på utvecklingen av sjukdomen.

Sammanfattningsvis så har vi medverkat i att brädda kunskapen om hur naturliga mördar T celler aktiveras och hur man kan manipulera immunsystemet och motverka sepsis vid akut systemisk bakterieinfektion, genom att aktivera just naturliga mördar T celler.



LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals (I-IV).

- I. Maria Blomqvist*, Sara Rhost*, Susann Teneberg, Linda Löfbom, Thomas Osterbye, JanEric Månsson and Susanna Cardell. **Multiple tissue-specific isoforms of sulfatide activate CD1d-restricted type II NK T cells.**
Eur. J. Immunol. 2009, 39, 1726-1735

- II. Sara Rhost, Linda Löfbom, Britt-Marie Rynmark, Bo Pei, Jan-Eric Månsson, SusannTeneberg, Maria Blomqvist and Susanna L. Cardell. **Identification of novel glycolipid ligands activating sulfatide specific type II natural killer T (NKT) lymphocytes.**
Eur. J. Immunol. 2012, 42, 2851-60

- III. Sara Rhost, Linda Löfbom, Jan-Eric Månsson, Maria Blomqvist and Susanna L. Cardell. **Sulfatide treatment to ameliorate type 1 diabetes in non-obese diabetic mice.**
Manuscript

- IV. Jakub Kwiecinski*, Sara Rhost*, Linda Löfbom, Jan-Eric Månsson, Maria Blomqvist, Susanna L. Cardell and Tao Jin (2013). **Sulfatide attenuates Staphylococcus aureus sepsis through a CD1d-dependent pathway.**
Infection and Immunity, 2013, 81, 1114-20

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ABBREVIATIONS

NKT	Natural killer T
MZ	Marginal zone
TCR	T cell receptor
BCR	B cell receptor
NK	Natural killer
MHC	Major histocompatibility complex
DP	Double positive
Th	T helper
β_2m	β_2 microglobulin
GSL	Glycosphingolipid
APC	Antigen presenting cell
DC	Dendritic cell
CNS	Central nervous system
ER	Endoplasmic reticulum
GalCer	Galactosylceramide
DN	Double negative
IL	Interleukin
iGb3	Isoglobotrihexosylceramide
GlcCer	Glucosylceramide
LN	Lymph node
BM	Bone marrow
LPS	Lipopolysaccharide
PAMPs	Pathogen-associated molecular patterns
TLR	Toll like receptor
EAE	Experimental autoimmune encephalomyelitis
MS	Multiple sclerosis
LSD	Lysosomal storage disease
TNF	Tumor necrosis factor
T1D	Type I diabetes
NOD	Non obese diabetic
WT	Wild type
S.aureus	Staphylococcus aureus



INTRODUCTION

General introduction

We are constantly exposed to infectious agents, but in spite of that, most of the time our immune system is able to fight off these infections. The first line of defense against pathogens occurs through mechanical barriers such as the epithelial layer, as well as through different chemical substances. A break through this barrier by harmful pathogens leads to the activation of the innate immune system, resulting in inflammation. Following activation of the innate immune system and subsequent inflammation, activation of the adaptive immunity takes place [1]. The adaptive immunity involves populations of cells with tremendous diversity in antigen recognition due to somatic rearrangement of genes that generates highly specific receptors [2]. This is in contrast to innate immune cells that are activated by pathogen associated molecular patterns on pathogens, so called PAMPs, through recognition by non-rearranged receptors [3]. Activation of the adaptive immunity will develop into memory against the specific pathogen, resulting in protection against infection upon re-exposure to the same pathogen. In contrast, the innate immune system does not form any specific memory. Although these two systems have distinct functions, interplay between them is important to establish an efficient protection against harmful pathogens as well as to create a good immunological memory.

To facilitate the link between the innate and the adaptive immune response, there are cells that possess features of both innate and adaptive immunity [4-7]. These innate-like cells rapidly exert their effector functions and communicate with cells of both innate and adaptive immunity. Notably, innate-like lymphocytes are situated in tissues such as skin, intestine, lung

and liver, rather than in blood or secondary lymphoid structures such as lymph nodes and spleen, which are the main site for adaptive lymphocytes. This results in an effective first line of defense against pathogens and a complement to innate immunity for further activation of immune cells in the adaptive immunity. The activated memory phenotype of innate-like lymphocytes is crucial for their immediate response and it indicates prior exposure to self antigen [4]. The innate-like lymphocytes include natural killer T (NKT) cells, $\gamma\delta$ T cells, CD8 $\alpha\alpha$ T cells, marginal zone B cells (MZB) and B1-B cells. In this thesis, the activation and immunoregulatory ability of NKT cells have been studied.

Hybrids of innate and adaptive immune cells – “innate-like lymphocytes”

A hallmark of the innate-like lymphocytes is the recurrent expression of receptors with similar specificity. This is found for both B cell receptors (BCR) and T cell receptors (TCR) that recognize common molecular structures from pathogens as well as self antigens. In contrast to conventional lymphocytes, which are part of the adaptive immune response, activation of innate-like lymphocytes leads to rapid expression of effector functions. In terms of NKT cells, they are a major source of a range of different cytokines of both T helper (Th) 1 and Th2 type. Many innate-like T lymphocytes are restricted by non-classical major histocompatibility complex (MHC) class I molecules. The MHC class I-like molecule CD1d represents one of these unconventional MHC class I complexes. Development of functional NKT cells requires CD1d expression in the thymus [5, 6].

CD1 molecules

CD1d and other isoforms of CD1

MHC class I like CD1 molecules are a lineage of antigen presenting proteins that have evolved to present lipid antigens to T cells [7]. The CD1 genes encode non-polymorphic proteins that associate with β_2 -microglobulin (β_2m) [8]. Five CD1 molecules have been identified in humans, CD1a-e [9-12]. They are divided into three groups according to their sequence similarities in $\alpha 1$ and $\alpha 2$ domains, where CD1a-c make up group 1, CD1d group 2 and CD1e is an intermediate and separated in group 3. In humans, group 1 CD1 molecules present lipid antigens to clonally diverse T cells that mediate immunity to microbial lipid antigens. By contrast, CD1d molecules present lipid antigens to NKT cells (reviewed in [13]). In mice, CD1d is the only CD1 molecule expressed. Similar to MHCI, the α -chain of CD1d folds into three domains, $\alpha 1$, $\alpha 2$ and $\alpha 3$, which associate with β_2m [14]. The antigen binding groove of CD1d consists of two large hydrophobic pockets, A' and F'. The binding groove is closed at both ends but is accessible at the top of the molecule through a narrow opening [15]. CD1d binds lipids of various structures, such as glycosphingolipids (GSLs) [16, 17] and phospholipids [18]. Further, a binding capacity of non-lipid molecules has also been documented [19].

CD1d expressing cells

In mice, CD1d is mainly found on professional antigen presenting cells (APC) such as dendritic cells (DCs), macrophages and B cells [20-23]. Among those APCs, DCs are the most potent APCs in stimulating NKT cells by ligand [24]. Localization of CD1d to the endocytic system in these monocyte derived DCs was observed, which suggests a possible mechanism for achieving efficient antigen loading onto CD1d. Interestingly, splenic

marginal zone (MZ) B cells display the highest CD1d expression among B cells in mice [21]. MZ B cells belong to the innate-like lymphocytes and localize in the MZ of the spleen. The MZ is positioned at the interface between the non-lymphoid red pulp and the lymphoid white pulp. In MZ, specialized APCs capture foreign antigens circulating in blood for further activation of the immune system. NKT cells interact productively with B cells. Leadbetter and colleagues show that when using haptenated lipid antigen for type I NKT cells, type I NKT cells localized to the B cell area and provided cognate help for class-switched antibody responses [25, 26]. Further, consistent with their higher level of CD1d, MZ B cells induced more proliferation of type I NKT cells than follicular B cells, suggesting that MZ B cells are efficient at activating type I NKT cells. Further, in liver, sinusoid-lining endothelial cells express high levels of CD1d, while Kupffer and DC have somewhat lower levels. This suggests that in liver, where NKT cells are highly abundant, they are constantly surrounded by CD1d expressing cells [27]. During chronic viral hepatitis infection, CD1d expression on hepatic APCs increases in parallel with the progression of inflammation and subsequent tissue damage [28], suggesting an increased interaction between hepatic APCs and NKT cells. In the central nervous system (CNS), CD1d is expressed on microglia cells, and during inflammation, their expression of CD1d increases significantly [29]. Accumulation of NKT cells has been observed in CNS during an ongoing inflammatory response [30], indicating that NKT cells may interact with CD1d on microglia. Importantly, CD1d is also expressed on CD4⁺CD8⁺, double positive (DP) thymocytes [31], which is indispensable for NKT cell development. The role of CD1d expressing DP thymocytes will be discussed later in this thesis. The outcome of NKT cell stimulation will depend on which of these APCs that interacts with the NKT cell, and moreover, the activation state of the APC will influence its capacity

to activate NKT cells, through modulation of CD1d expression and through other mechanisms.

Pathways of antigen processing and presentation on CD1d

The CD1d molecule is similar in structure to that of MHC class I, with a transmembrane heavy chain with three α -domains, non-covalently attached to β_2m [13, 32]. Upon synthesis, the heavy chain of CD1d is translocated into the endoplasmic reticulum (ER) where N-linked glycans are attached and association with β_2m and self-lipids takes place [8]. Following assembly in ER, CD1d molecules are rapidly transported via Golgi to the plasma membrane through the secretory route. Further, CD1d is recycled from the membrane. Directed by the cytoplasmic tail of CD1d, it internalizes from the plasma membrane via clathrin coated pits and moves through early and late endosomes to the lysosome [33]. The transport of CD1d from the plasma membrane to endosomes requires the adaptor protein (AP) complex AP-2, after which an AP-3 dependent transport of CD1d from endosomes to the lysosome takes place (figure 1). Studies have shown that CD1d binds endogenous lipids, including glycosylphosphatidylinositols (GPIs) in ER [34-36] that may be exchanged for other lipids when CD1d is recycled through endosomal and lysosomal compartments. As for today, GPIs have not been shown to be antigenic for NKT cells, which suggests that GPIs might function as chaperons, facilitating the assembly of CD1d molecule in ER.

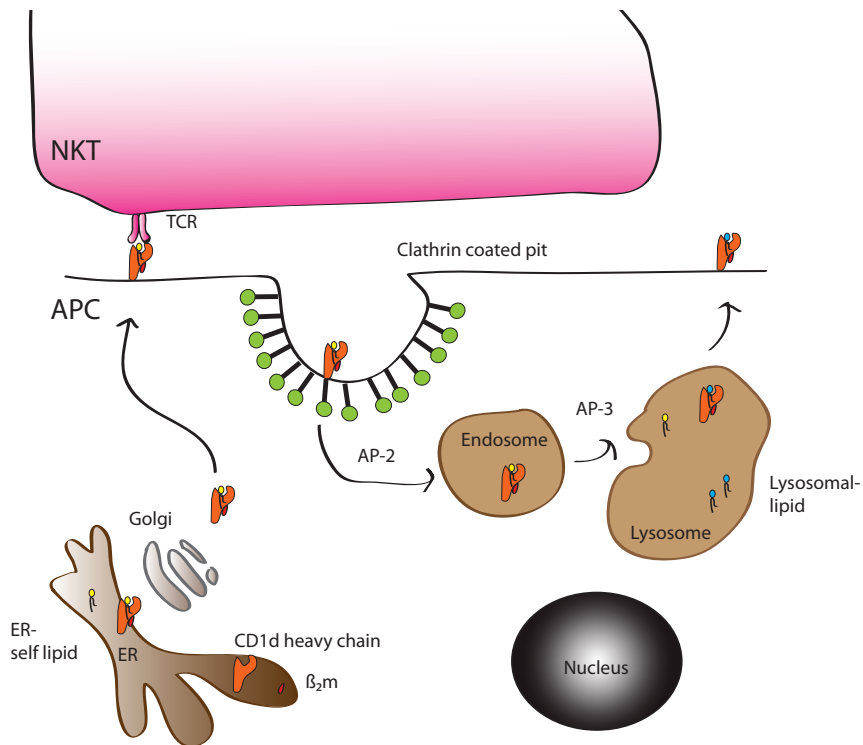


Figure 1. Mouse CD1d trafficking. CD1d heavy chain assembles with β_2 -microglobulin (β_2m) in endoplasmic reticulum (ER) where it binds ER derived self-lipids and further transport via the Golgi complex to the plasma membrane takes place. From the plasma membrane, CD1d is internalized through clathrin coated pits and is directed to endosomes by adaptor protein complex (AP)-2 and further to the lysosome by AP-3. In endosomes and lysosome, ER derived self-lipids on CD1d can be replaced with other self or foreign lipids, followed by transport back to the plasma membrane.

NKT cells

The history of NKT cells

In 1986, Taniguchi and colleagues described a $V\alpha 14$ – $J\alpha 18$ TCR α chain cloned from a suppressor T cell hybridoma [37]. A few years later, a population of TCR $\alpha\beta$ cells expressing the NK marker NK1.1 was discovered in C57BL/6 mice and named NKT cells [38, 39]. Further investigations

demonstrated that these V α 14–J α 18 invariant TCR α chain expressing cells were CD1d restricted [40], autoreactive, and differed from conventional T cells in that they expressed intermediate levels of TCR, had a bias toward V β 8 expression and notably, produced high levels of immunoregulatory cytokines such as IFN- γ and IL-4 (reviewed in [39, 40]). In 1995, Cardell and colleagues found a population of CD1d restricted TCR $\alpha\beta$ cells [41]. Instead of the invariant V α 14–J α 18 TCR α chain, these cells expressed a diverse set of TCR α - and β -chains [43]. A couple of years later, Kawano et al discovered that the V α 14–J α 18 expressing NKT cells recognized the GSL α -galactosylceramide (α -GalCer) derived from a marine sponge [44]. Subsequently, α -GalCer loaded on CD1d tetramers could be used as a tool to study V α 14–J α 18-expressing NKT cells [45, 46] which significantly helped to move the NKT cell research field forward. Following the discovery of α -GalCer, investigations on V α 14–J α 18 TCR expressing NKT cells increased extensively, while the lack of specific reagents has limited the studies of the non-V α 14–J α 18 expressing NKT cells. However, in 2004 there was a breakthrough in the study of these NKT cells. The GSL sulfatide was demonstrated as a CD1d-restricted ligand for a subset of non- V α 14–J α 18 NKT cells, which allowed further studies of this novel CD1d-restricted NKT cell population [30].

Definition of NKT cells

The consensus in the field is that NKT cells are defined as CD1d restricted TCR $\alpha\beta$ cells, and this is the definition that will be used throughout this thesis. Two main subsets of NKT cells have been described. Type I NKT cells (or invariant NKT) bearing the V α 14–J α 18 (mouse) TCR α -chain paired with V β 8.2, V β 7 or V β 2, or the corresponding segments V α 24– J α 18 in humans, paired with V β 11. Type II NKT cells (or diverse NKT), in contrast, carry diverse TCRs [42, 43]. In addition to TCRs or BCRs, NKT

cells also express receptors that do not require gene recombination, and are generally not expressed by conventional lymphocytes. These receptors sense cellular stress, such as during infections and are divided into activating receptors, which include NKG2D and NK1.1, and inhibitory NK receptors such as NKG2A [44].

NKT cell subsets

Dividing the NKT cell subsets

The subdivision of NKT cells according to their expression of TCRs is one way of distinguishing different NKT cells. In addition, NKT cells can be divided by their expression of CD4 and CD8 [45]. In mice, type I NKT cells are either CD4⁺ or CD4⁻CD8⁻ (double negative, DN). However, in humans, CD4⁻CD8⁺ NKT cells also exist. In humans, a difference in function of CD4⁺ and DN type I NKT cells has been shown [42, 46, 47]. CD4⁺ NKT cells were shown to produce cytokines of both Th1 and Th2 type, including both IFN- γ and IL-4, whereas the major cytokine production by DN NKT cells was of Th1 type, suggesting that the expression profile of CD4 and CD8 divides NKT cell cells into functionally different subsets. Further, DN type I NKT cells seem to express more NK receptors, such as NK.1.1, 2B4, NKG2A and NKG2D [46-48], suggesting that they are more similar to NK cells in comparison to CD4⁺ NKT cells. Further, it was shown that DN type I NKT cells more efficiently induced protection against methylcholanthrene-induced sarcomas in mice [49] and when comparing cells from spleen, liver and thymus it was shown that liver derived DN type I NKT cells were required for rejection of sarcomas. This suggests that not only the expression of CD4 or CD8 contributes to distinguish functional differences of NKT cells, in addition different tissue locations seems to provide NKT cells of diverse functions. Whether the expression profile of CD4 and CD8 goes together with functional differences in type II NKT is not well explored. However,

Kadri et al demonstrated that TCR transgenic DN type II NKT cells express higher levels of NK markers such as CD49b, Ly49G2, and CD122 in comparison to the CD4⁺ population, and only CD4⁺ cells could prevent type 1 diabetes induction [43], suggesting that also type II NKT cells may be divided into functionally different subsets according to CD4 expression.

Type I NKT cells

Through the use of α -GalCer loaded CD1d tetramers, type I NKT cells have been extensively studied. In mouse thymus, type I NKT cells represent ~0,5% of all thymocytes [48] and in the liver where they are most abundant, they represent as much as ~30% of the T cell population. In spleen, they represent ~ 2,5% of all T cells and in peripheral lymph nodes and blood, they represent a population of ~0,5% of T cells. In liver, type I NKT cells seem to patrol the sinusoids and during steady state condition, they stay in the liver by their expression of CXCR6, the receptor for CXCL16, expressed by endothelial cells lining the sinusoids [27]. In addition, high expression of CD1d on liver resident Kupffer cells seems to be important for retaining the NKT cells in the liver [27]. Further, the expression of lymphocyte function associated antigen 1(LFA-1) and the interaction with intracellular adhesion molecule 1 (ICAM-1) expressed on NKT cells as well as production of interleukin (IL) -15 by hepatic stellate cells (Ito) appear crucial for the maintenance of NKT cells in liver [50]. Recently, a subset of type I NKT cells that lack NK1.1 and produces high amounts of IL-17 and low amounts of IFN- γ and IL-4 has been identified [57]. It was demonstrated that these IL-17 producing NKT cells were highly abundant in lungs. During airway neutrophilia induced by endotoxin exposure, they significantly increased the inflammation. Notably, in humans, the frequencies of type I NKT cells appear to be lower, approximately ten times less than the population observed in mice [51].

Type II NKT cells

In contrast to the semi-invariant TCR expressed by type I NKT cells, type II NKT cells express diverse TCRs and are non responsive towards α -GalCer. Analysis of autoreactive, CD1d restricted, non-V α 14 T hybridoma cells derived from short term TCR β activated CD4⁺, NK1.1 positive or negative splenocytes from MHC class II-deficient mice demonstrated a bias towards V α 3.2-J α 9, together with V β 8-chains [52]. This suggests that the type II NKT cell population might include subpopulations that are invariant and may be activated by the same lipid antigens. Interestingly, a population of non-V α 14, α -GalCer reactive cells has been identified [53]. This NKT cell population expresses V α 10-J α 50 paired with V β 8 and is named “V α 10 NKT cells”. Similar to type I NKT cells, V α 10 NKT cells recognize α -GalCer and isoglobotrihexosylceramide (iGb3), however showed a preference for α -glucosylceramide (α -GlcCer). In addition, V α 10 NKT cells are strongly activated by the microbial lipid ligand α -glucuronosyl diacylglycerol (α -GlcA-DAG) derived from *Mycobacterium smegmatis*. V α 10 NKT cell produced 10- to 100-fold more IL-4, IL-13 and IL-17A than did type I NKT cells in response to α -GlcA-DAG, suggesting that they are functionally distinct. Further, sulfatide reactive cells belong to the type II NKT cell subset. When using sulfatide loaded CD1d tetramers, Kumar and colleagues could demonstrate a population of ~ 5% of T cells in liver and ~ 0,2% of T cells in spleen that stained positive. This represents approximately 20% the size of the type I NKT cell population in these organs. Further, human type II NKT cells have been shown to recognize lyso-phosphatidylcholine (LPC) isolated from plasma derived from multiple myeloma patients. These cells produced high levels of IL-13 and were found at increased frequencies in patients with multiple myeloma compared to healthy individuals [54].

These data strengthen the concept that there are, indeed, subpopulations of type II NKT cells expressing invariant TCR and/or having shared lipid antigen specificity. As a consequence of their diverse TCR, no universal lipid antigen can be expected to identify the entire population of type II NKT cells. As a result of the inability to identify the entire type II NKT cell population, the only certain way to investigate the type II NKT cells is through their CD1d restriction and the expression of non-V α 14 TCR α chains.

Development of T lymphocytes in the thymus

Thymus provides the microenvironment essential for the development of T cells from hematopoietic stem cells. From early thymic progenitors, DN cells develop through four stages (DN1-DN4) distinguished by differential expression of CD25, CD44, and CD117. At the DN2 stage, the rearrangement at the TCR γ , TCR δ , and TCR β gene loci is initiated which is completed at the DN3 stage [55, 56], where $\alpha\beta$ versus $\gamma\delta$ T cell fate is specified [57]. The $\gamma\delta$ rearrangements at the DN2 stage can give rise to $\gamma\delta$ T cells already at this stage. Further, the maturation of $\alpha\beta$ -thymocytes involves expression of the pre-TCR that induces the expression of CD4 and CD8, and transition to the DP stage and initiation of TCR α recombination. The TCR α is randomly rearranged and for type I NKT cells, the formation of V α 14-J α 18 allows the recognition of selecting self-lipids presented on CD1d after pairing with an appropriate TCR β chain. Thus, at the DP stage, positive selection of $\alpha\beta$ -thymocytes takes place and NKT cell development diverges from that of conventional T cells.

NKT cell development

The development of NKT cells is distinct from that of conventional T cells even though they originate from the same DP precursor. The study of NKT cell development has been possible by the use of α -GalCer tetramers specific for the type I NKT cells. Due to the lack of unique reagents for type II NKT cells, most information available describes the development of type I NKT cells. At the DP stage, DP cells to become NKT cells are positively selected by other DP thymocytes expressing self-lipid CD1d complexes (figure 2). This is in contrast to the selection of conventional T cells, which have been selected by cortical thymic epithelial cells, bearing MHC molecules. The different stages of thymic NKT cell development, and important factors for each stage, is depicted in figure 2. The selection of NKT cells by CD1d expressing self-lipids induces an activated memory phenotype already at “stage 0” in development, which is distinct from the naïve phenotype of mature conventional single positive (SP) thymocytes. NKT cells are only selected when CD1d is expressed on DP thymocytes [31, 58-62], indicating that DP thymocytes possess crucial signals that are significant for positive selection of NKT cells, and/or that DP thymocytes have a unique capacity of presenting lipids required for selection of NKT cells. CD1d^{-/-} mice, or mice with defects in CD1d processing and presentation, lack mature NKT cells [63]. The positive selection event by CD1d-lipid complex with TCR requires ligation of both TCR and a costimulatory molecule, signaling lymphocytic activation molecule (SLAM) that signals via SLAM-associated protein (SAP) and the downstream Src kinase (FynT) [64-66]. In comparison to conventional T cells that depend on the Ras-MAP kinase pathway (RAS-Mek1), this pathway seems dispensable for NKT cells. In contrast, NKT cells are deficient in mice lacking FynT, demonstrating the importance of SLAM-SAP-FynT signaling pathway in the developmental program of NKT cells

[67, 68]. Also, at this point, induction of promyelocytic leukemia zinc finger (PLZF) and runt related transcription factor 1 (Runx1), and subsequently myelocytomatosis oncogene (c-Myc) transcription factor and early growth response (Egr) transcription factor 2 appears to be important. PLZF is expressed during the complete development of type I NKT cells, starting after the positive selection of DP thymocytes to the terminally differentiated stage of NKT cell development in peripheral tissues [69]. With exception of two other innate-like lymphocyte, the MR1-restricted, mucosal-associated invariant T (MAIT) cells and $\gamma\delta$ T cells [70], PLZF is only detected at high levels in NKT cells. Following successful CD1d-TCR ligation, NKT cells enter “stage 0” indicated by expression of CD24, CD4 and CD69 (CD24^{high}, CD4⁺, CD8^{low} and CD69^{high}). Further maturation occurs through downregulation of CD24 and CD8 to reach the mature CD4⁺ NKT cell stage with low expression of CD44 in “stage 1”. These “stage 1” NKT cells remain in thymus where they continue their developmental program into “stage 2” by upregulating CD44 and IL-15 β receptor (CD122) that mediate the induction of low basal transcription of Th2 followed by Th1 cytokines. Upon leaving the thymus, NKT cells starts to express NK lineage receptors such as NK1.1 and enter “stage 3”. The transcription factor T-bet is essential for the transition from “stage 2” to “stage 3”. Some NKT cells in “stage 3” reside in the thymus (figure 2), however, with as yet unknown functions. Upon leaving the thymus, NKT cells preferentially migrate to the liver, however they are also present in spleen, bone marrow, lung and gut. The frequencies in lymph nodes (LN) are relatively low due to the lack of expression of homing receptors such as CD62L and CCR7 on NKT cells (reviewed in [38, 63, 71]). Notably, an IL-17 producing subset of type I NKT cells, mentioned above, is abundant in the lung, inguinal LN, and mesenteric LN, but hardly detectable in the liver and bone marrow (BM) of both C57BL/6 and BALB/c mice. This distinct NKT cell population expresses CCR7, in contrast to other type I

NKT cells. This suggests that there might be functionally distinct populations of NKT cells that locate specifically to these locations [72] [73].

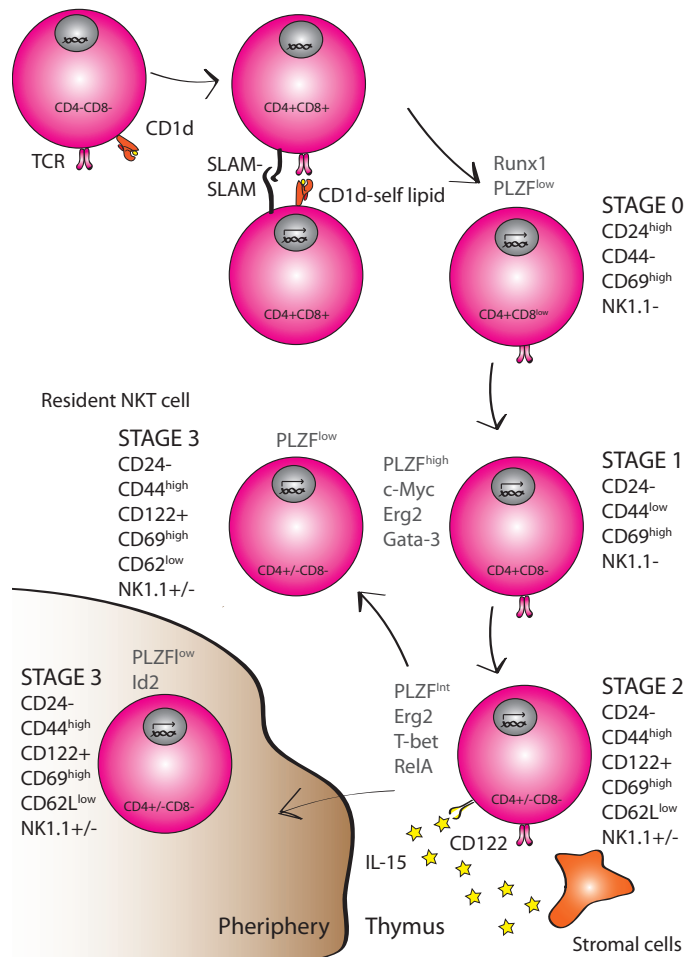


Figure 2. Type 1 NKT cell development. NKT cells diverge from conventional T cell development when the TCR interact with CD1d expressing self-lipids. The positive selection event by CD1d-lipid complex with TCR requires ligation of both TCR and a costimulatory molecule, signaling lymphocytic activation molecule (SLAM) that signals via SLAM-associated protein (SAP) and the downstream Src kinase (FynT). Further development of NKT cells occurs in a stepwise manner, “stage 0” to “stage 3”, where expression of different transcription factors as well as cell surface proteins differs. At “stage 2”, NKT cells leave the thymus and complete the development peripherally. Some NKT cells stay in thymus as “stage 3”.

NKT cell activation

NKT cells have various functions in the immune system. They have the capacity of rapidly and robustly producing a range of different cytokines as well as the capacity of killing other cells [74]. Type I NKT cells express high levels of granzyme B, perforin and FasL consistent with their cytotoxic capacity. The dual production of IFN- γ and IL-4 has been a hallmark in terms of NKT cell activation. Indeed, the secretion of IFN- γ and IL-4 by type I NKT cells has been shown as early as 2 hours after *in vivo* exposure to α -GalCer [75]. High levels of IFN- γ and IL-4 mRNAs in resting NKT cells is provided as an explanation for this rapid response and subsequent cytokine release [4, 76]. Consequently they have an “activated-yet memory” phenotype. The fact that NKT cells can produce both inflammatory and anti-inflammatory cytokines (figure 3A) is key to their importance in different diseases, such as autoimmunity, infection, cancer and allergy (reviewed in [38]). The role of NKT cells in some of these diseases will be brought up later in this thesis.

Interestingly, CD1d is a non-polymorphic molecule, in contrast to MHC molecules, which are highly polymorphic. This suggests that CD1d carries out conserved antigen presenting functions [77]. The activation of NKT cells can occur in different ways, and involve distinct activation systems. Since NKT express receptors of both NK cells and T cells, they are capable of being activated by TCR-CD1d-lipid interactions (figure 3B, D-E), through non-rearranged innate receptors such as NK receptors (figure 3F) [78], as well as by cytokines (figure 3C). During the course of infection, NKT cells can be activated by a direct presentation of bacterially derived lipids [79-83] (figure 3D). Notably, some bacterial lipid antigens stimulating NKT cells via CD1d are derived from lipopolysaccharide (LPS) deficient gram-negative bacteria, and it has been suggested that bacterially derived lipids may

function as substitutes to the lack of PAMPs such as LPS to activate NKT cells (reviewed in [38]). Further, bacterial infections can modulate GSL synthesis in APC by either increasing biosynthesis or inhibiting degradation enzymes, which leads to accumulation of GSLs known to stimulate NKT cells [84, 85]. The recognition of PAMPs by toll like receptors (TLRs) on APCs has been shown to induce presentation of stimulatory self-lipids to NKT cells (figure 3B) [84, 85], demonstrating that lipid synthesis and presentation of self-lipids are altered during TLR engagement. In addition, during infections, NKT cells can be activated by TCR independent mechanisms including cytokines released by APCs. These cytokines include type I IFN, IL-12 and IL-18 [86, 87]. Further, theoretically, NKT cells could also be activated by lipid ligands derived from damaged tissues (figure 3E). For instance, sulfatide present in β -cells of pancreatic islets or CNS [88] might be taken up by APCs and be presented to type II NKT cells.

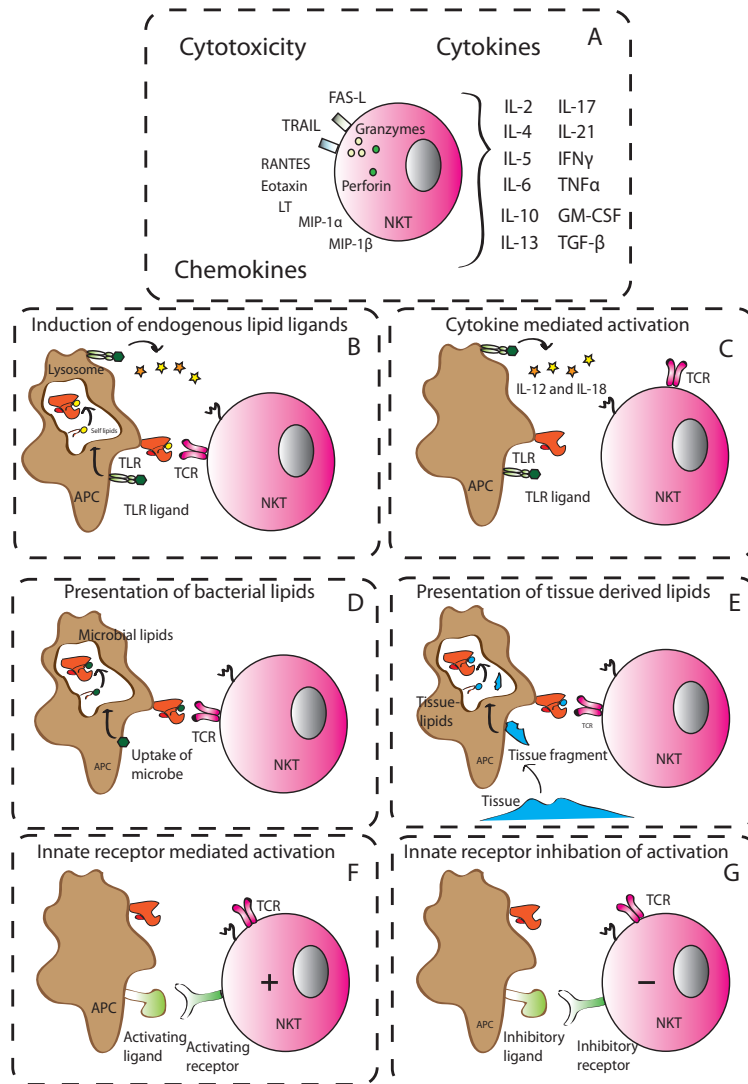


Figure 3. Different modes of NKT cell activation. (A) NKT cells have different functions in the immune system; they possess killing activity, and they produce an array of different cytokines and chemokines upon activation through (B) the induction of stimulatory endogenous self-lipids in response to pathogenic microbes, or (C) they can be activated by the release of cytokines from activated APCs that have encountered bacteria, or (D) a direct presentation of bacterially derived lipid antigens. (E) Hypothetically NKT cells can also be activated by tissue derived self-lipids from destructed tissues. (F-G) Further, NK cell receptors can mediate activation or inhibition of activation of NKT cells. Modified from [74].

NKT cell ligands

The diversity of recognized lipid antigens

The interaction between lipid-CD1d complex and the NKT cell TCR represents a central event in NKT cell activation. As for today, a great diversity of lipids presented on CD1d and stimulating NKT cells has been demonstrated. One major difference between bacterial lipids and mammalian lipids is the linkage of the sugar to the lipid backbone. Bacterial lipids have α -linkage, whereas mammalian lipids are β -linked. Interestingly, both types of lipids can bind to CD1d and activate NKT cells. Thus, despite the conserved structure of CD1d, it can bind to an array of different lipid-based antigens [38, 39, 74, 89-98].

Bacterial ligands

In 1997, Kawano et al discovered that the marine sponge derived GSL α -GalCer efficiently stimulates type I NKT cells [99]. It is thought that α -GalCer is a bacterially derived lipid produced by a putative bacterium that is symbiotic with the sponge. Since the discovery of α -GalCer, several microbial ligands have been demonstrated to activate type I NKT cells [53, 79-81, 83, 100-104]. Using soluble murine CD1d bound to plastic, Fischer et al. identified phosphatidylinositol mannoside (PIM) with four mannose residues and two saturated palmitate (C16) acyl chains (PIM₄) derived from *Mycobacterium tuberculosis* as an activating mycobacterial lipid ligand for type I NKT cells [103]. PIM₄ induced CD1d dependent IFN- γ production by splenic type I NKT cells, isolated from V α 14-J α 18 transgenic mice. In contrast to stimulation with α -GalCer, no IL-4 was produced in the PIM₄ stimulated cultures. Staining with PIM₄ loaded CD1d tetramers demonstrated that 0.26% of liver NKT cells (CD3⁺NK1.1⁺) and 0.6% of splenic NKT cells (CD3⁺NK1.1⁺) stained positive. The low frequencies of PIM₄ positive, type I

NKT cells indicate that the β -chain might influence the interaction with PIM₄ to a greater extent than with α -GalCer, resulting in a subpopulation of type I NKT cells recognizing PIM₄.

Ever since α -GalCer was demonstrated as a ligand for type I NKT cells, GSLs have been a focus in the search for lipid antigens activating NKT cells. The cell wall of *Sphingomonas paucimobilis* contains α -glucuronosylceramide (α -GlcUCer) that was demonstrated as a ligand for type I NKT cells [82, 83]. The use of α -GlcUCer loaded CD1d dimers demonstrated that 0.5% of liver α -GalCer positive NKT cells stained positive, again pointing to a decisive role for the TCR β -chain of the type I NKT TCR in the recognition of certain bacterial lipids. Further, the highly pathogenic gram-positive bacterium *Borrelia burgdorferi* that causes Lyme disease contains stimulatory lipid ligands of the family α -galactosyldiacylglycerols (α GalDAGs) for type I NKT cells [81]. α GalDAGs loaded tetramers stained a population in liver that was 23% of the α -GalCer positive cells. Another highly pathogenic gram-positive bacterium, *Streptococcus pneumoniae* as well as the gram-positive pathogen, group B Streptococcus have been demonstrated to contain activating lipid ligands such as α -glucosyldiacylglycerols (α GlcDAGs) for type I NKT cells [102].

Endogenous ligands

Autoreactivity

The TCR selection of DP thymocytes by CD1d expressed on DP cells, initiates the developmental program that provides NKT cells with their unique characteristics such as autoreactivity. Hence NKT cells have been termed “autoreactive by design” [105]. This natural autoreactivity is easily demonstrated in *in vitro* assays using NKT cell hybridomas. Several NKT cell hybridomas derived from both type I and type II NKT cells are

autoreactive towards different CD1d expressing APCs without the addition of exogenous lipid antigens [40, 41, 106, 107]. In addition, several studies show that the autoreactivity of NKT cells can be increased by altered presentation of endogenous ligands during infections or APC activation. Based on current knowledge, figure 4 depicts different circumstances of CD1d-dependent NKT cell autoreactivity, where we call the latter situation "Induced peripheral autoreactivity". We suggest that the CD1d-dependent autoreactivity that is demonstrated by NKT hybridomas to APC *in vitro* may represent a low degree of autoreactivity of NKT TCR *in vivo* to resting APC, and have termed this "Steady state peripheral autoreactivity" (as in figure 4), or natural autoreactivity. Whether the lipids required for TCR selection of NKT cells in thymus (the first mode of autoreactivity in figure 4) are playing a role in the peripheral steady state or induced autoreactivity of NKT cells is not known, nor if peripheral steady state and induced autoreactivity depend on the same or overlapping ligands. Below, we will use these terms to describe different modes of NKT cell autoreactivity.

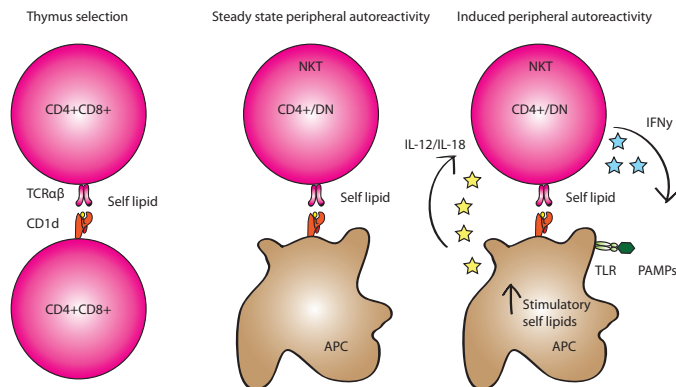


Figure 4. *Self-lipids are involved in the positive selection of NKT cells in the thymus, as well as in "steady state" and "induced" autoreactivity in the periphery.* As for today, whether the lipids presented on CD1d in these three situations are the same, overlapping or completely different is not known.

Type I NKT cell ligands

Due to potent regulatory properties of NKT cells, extensive studies have focused on identifying how they can be activated and especially the ligands underlying their CD1d-dependent autoreactivity. In 2000, Gumperz et al. demonstrated that both type I and type II NKT cell hybridomas were activated by phospholipids derived from a tumor cell line [108] when presented on plate-bound CD1d. Phospholipids derived from the tumor cells included phospholipids such as phosphatidylinositol, phosphatidylethanolamine and phosphatidylglycerol. Further, Stanic et al demonstrated that β -GlcCer deficient APCs (APCs deficient in glucosylceramide synthase (GCS), see figure 5) fail to stimulate type I NKT cell autoreactivity [109]. Further, they found that β -GlcCer itself did not stimulate type I NKT cells indicating that lipids downstream of β -GlcCer, dependent on β -GlcCer synthesis, could be responsible for the autoreactivity of type I NKT cells. The following year, Zhou et al identified iGb3 as an activating ligand for type I NKT cells [110]. Mice deficient in the lysosomal glycosphingolipid degrading enzyme β -hexosaminidase b subunit ($\text{Hexb}^{-/-}$, see figure 5) were shown to exhibit a significant decrease in type I NKT cells. Cells staining positive with α -GalCer tetramers were reduced by 95%. This suggested that iGb3 is involved in the thymic positive selection of NKT cells. A report from another group seemed to confirm the role of iGb3 as a ligand for type I NKT cells, demonstrating that deficiency in α GalA (as in α GalA^{-/-} mice and human Fabry disease. See figure 5), resulting in accumulation of iGb3 as well as Gb3, caused overstimulation and deletion of type I NKT cells [85]. In contrast, a subsequent study demonstrated that mice deficient in iGb3 synthase (iGb3S) have normal development of type I NKT cells with no functional abnormalities, suggesting that iGb3 is unlikely to be involved in thymic development of type I NKT cells [111]. It also remains controversial whether iGb3 is involved in thymic selection of type I NKT cells in humans

[112]. Moreover, in a recent study using $\alpha\text{GalA}^{-/-}/\text{iGb3S}^{-/-}$ (αGalA and iGb3S , see figure 5) double knockout mice, unable to degrade Gb3 and iGb3 and deficient in iGb3 , the number of type I NKT cells decreased to the same extent as in $\alpha\text{GalA}^{-/-}$ deficient mice, unable to degrade Gb3 and iGb3 . The conclusion from this last study was therefore that the accumulation of Gb3 and not iGb3 was sufficient to decrease the number of type I NKT in the $\alpha\text{GalA}^{-/-}/\text{iGb3S}^{-/-}$ double deficient mice. Consequently, they proposed that it was accumulation of Gb3 rather than iGb3 that caused type I NKT cell deficiency also in the $\alpha\text{GalA}^{-/-}$ single deficient mice [113].

The demonstration that several self-GSLs stimulated type I NKT cells and influenced their development, suggested that GSLs might be required for the autoreactivity of type I NKT cells. However, in 2011, Pei et al demonstrated that the autoreactivity of type I NKT cells did not require GSLs [114], by using APCs deficient in sphingosine long-chain base subunit 1 (LCB1, see figure 5) and subsequently have a dramatic deficiency in GSLs and sphingomyelin. The independence of GSLs in the autoreactivity of type I NKT cells was demonstrated with both NKT hybridoma cells and primary TCR transgenic type I NKT cells. Interestingly, this suggested that GSLs are not required for natural autoreactivity of type I NKT cells, yet there are GSLs that can activate type I NKT cells. The following year, it was demonstrated that $\beta\text{-GlcCer}$ accumulates in APC after LPS stimulation and contributes to type I NKT cell activation during *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae* or *Listeria monocytogenes* bacterial infection [115]. This indicates that the GSL $\beta\text{-GlcCer}$ functions as a self-lipid ligand, responsible for induced autoreactivity of type I NKT cells during infections. Even though $\beta\text{-GlcCer}$ plays a role as a self-lipid ligand in peripheral autoreactivity of type I NKT cells, its function in thymic development is not clear. In 2012, Facciotti et al demonstrated that

the ether-bonded plasmalogen C16-lyso-phosphatidylethanolamine (pLPE), derived from mouse thymocytes activates type I NKT cells [116]. Mice lacking glyceronephosphate O-acyltransferase, and thereby deficient in ether-lipid synthesis and plasmalogens, have reduced numbers of type I NKT cells and impaired ability to activate and promote full maturation of type I NKT thymocytes. This suggests that pLPE is involved in the positive selection of type I NKT cells. Further, tumor associated disialoganglioside (GD3) has been demonstrated to activate type I NKT cells [117]. In mice immunized with a human melanoma cell line expressing GD3, 0.29 % of CD3⁺ T cells in spleen stained positive for CD1d-tetramer loaded with GD3, while these cells were not detected in non-immunized mice. In addition, lyso-phosphatidylcholine (LPC) and lyso-sphingomyelin (LSM) activate a fraction of human type I NKT cells [118].

Type II NKT cell ligands

The first lipid ligand described to activate type II NKT cells was sulfatide [30]. Jahng and colleagues demonstrated that sulfatide is a ligand for type II NKT cells and that sulfatide administration protected mice from developing experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS). Further, sulfatide has been shown to be recognized by CD1a-, CD1b-, and CD1c- restricted human T cells, indicating that the same self-lipid antigen can, in some cases, be presented by different CD1 isoforms [119]. The activation of mouse type II NKT cells by CD1d presentation of sulfatide will be discussed in detail later in this thesis.

LPC derived from plasma of myeloma patients has been demonstrated to activate human type II NKT cells [54], and LPC specific T cells increase in myeloma patients, compared to healthy individuals. Further, a recent publication demonstrated that during hepatitis B infection, lyso-phospholipids are produced in infected hepatocytes by the action of secretory

phospholipases and subsequently presented on CD1d resulting in the activation of type II NKT cells [120]. Among lipids induced by the secretory phospholipase A₂ (sPLA₂), the activating lipid ligands lyso-phosphatidylethanolamine (LPE) with different sphingosine bases, as well as C18:1 lyso-LPC were identified. Further, it was demonstrated that the CD1d-dependent activation of type II NKT cells by these lyso-phospholipids induced downstream activation of type I NKT cells by an indirect, TCR independent mechanism. Further, in 2005, Agea et al. demonstrated that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) derived from cypress pollen grains were able to stimulate the proliferation of CD1d restricted non-V α 24 NKT cells from cypress sensitive individuals [121]. The major type of both PC and PE had 18:2/18:2 fatty acids.

Non-lipid ligands

Early reports have shown a peptide binding capacity of CD1d demonstrating that antigens presented on CD1d may not be limited to lipidic molecules [15, 122-124]. Further, human type II NKT cells have been described to recognize a small non-lipidic molecule derived from a mixture of acetylated synthetic lipopeptides [19]. This non-lipidic molecule was identified as 9-fluorenyl methyloxycarbonyl-N α -2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl-L-arginine (PPBF) and was shown to induce activation of non-V α 24 NKT cells. Further, CD1d has been demonstrated to bind non-polar hydrophobic fluorescent probes, named anilinonaphthalene sulfonic acid (ANS) [125]. These probes become fluorescent when bound to hydrophobic regions of proteins and thereby it was possible to measure their binding to CD1d. In addition, a recent report illustrates that the type II collagen peptide 707-721 (CII₇₀₇₋₇₂₁) was presented on CD1d, and that CII₇₀₇₋₇₂₁-specific CD1d-restricted NKT cells produce IL-4, IFN- γ , IL-17A, TNF- α , and TGF- β upon peptide stimulation [15, 122-124].

Glycosphingolipids

Biosynthesis of GSL

The term glycosphingolipid applies to compounds that contain a ceramide base connected to a monosaccharide or more complex saccharide structures [126]. GSLs can be subdivided into neutral or acidic groups where neutral GSLs involve the globo-, lacto-, neoacto- and ganglio series, while the acidic GSLs are the sialic acid carrying gangliosides and sulfated GSLs such as sulfatide (figure 5). GSLs are believed to play important roles in a variety of cellular processes, such as cell recognition, growth, development and differentiation. The biosynthesis and degradation of GSLs involves numerous enzymes that act at various subcellular locations.

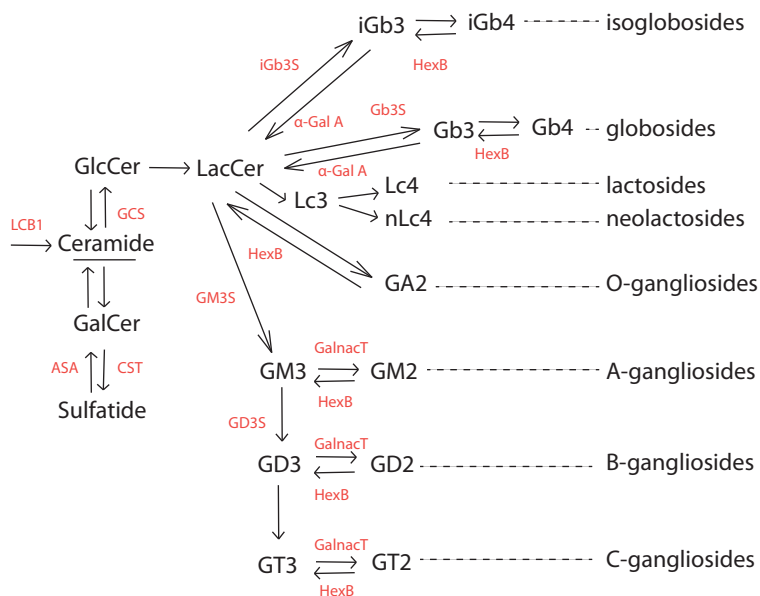


Figure 5. GSL metabolism. GSLs are subdivided in neutral or acidic groups, where neutral GSL are the globo-, lacto- and neolacto, and ganglio-series, and the acidic GSL are the gangliosides and sulfated GSLs. Enzymes involved in synthesis and degradation are shown in red. Modified from [113]

The biosynthesis of GSLs occurs in a stepwise manner, where the first step is the addition of a single carbohydrate moiety to an acceptor lipid substrate, ceramide [127]. Initially, the biosynthesis of GSLs occurs in the membranes of ER, where ceramide is synthesized (figure 6). Thereafter, ceramide is translocated from the cytoplasmic face of ER to the luminal side of Golgi apparatus by the transport protein CERT. On the luminal side, sphingomyelin synthase 1 converts ceramide into sphingomyelin (SM) by transfer of a phosphorylcholine headgroup from phosphoglycerolipids. In addition, through the action of glucosylceramide synthase (GCS, see figure 5), synthesis of GlcCer occurs at the cytoplasmic face of ER as well as in early Golgi (cis) and subsequently flips into the Golgi lumen, where synthesis of more complex GSLs take place by a series of glucosyltransferases (the GSL series are shown in figure 5) (reviewed in [127]). These complex GSLs consist of α and β -linked glucose, galactose, *N*-acetylglucosamine, and/or *N*-acetylgalactosamine. Many of these complex GSLs have one or more sialic acid residues in their glycan structure and are named gangliosides [128]. In comparison to GlcCer, GalCer synthesis occurs on the luminal face of the ER and then traffics through the Golgi, where it may be sulfated to form sulfatide or more complex sulfated GSLs. During synthesis and passage through the ER/Golgi compartments, GSL may be available for loading on newly synthesized CD1d (see figure 1 and 6), however, isolation of lipids loaded on CD1d in these compartments have revealed a dominance of glucosylphosphatidylinositol [34].

From Golgi, synthesized GSLs are transported via vesicles to the plasma membrane where the GSLs become part of the membrane, facing the extracellular milieu. Through invaginated vesicles, GSLs and other components of the plasma membrane are internalized into endosomes. This results in the glycan structure of GSLs facing the endosomal lumen. Fusion

of endosomes with lysosomes makes the GSLs available for cleavage into smaller structures, by lysosomal hydrolases with the help from activator proteins such as saposins. Recycling of CD1d from the cell surface results in the localization of CD1d to these endosomal/lysosomal compartments (figure 1) where the lipids loaded on CD1d can be exchanged, and GSL loaded onto CD1d. Finally, the GSLs are broken down to their individual components and are available to be reprocessed. The degradation products of GSLs can be recycled to ER-Golgi for reuse. Defects in GSL catabolism can have large impact and influence several biological functions, which is clearly seen in a number of inherited metabolic disorders caused by deficiency in GSL degrading enzymes within lysosomes. These diseases are called sphingolipidoses and fall into the group of lysosomal storage diseases (LSD). NKT cells are often severely affected in these situations, which may be explained by a direct effect resulting from the lack or accumulation of specific GSL, or an indirect effect caused by a general major disturbance in GSL homeostasis. The most common LSD is Gaucher disease [129] caused by deficiency in glucosylceramidase activity resulting in accumulation of GlcCer. Patients suffering from this disease can develop neurological damage, however, the majority of patients have a less severe outcome where accumulation of GlcCer is restricted to tissue macrophages, which cause hepatosplenomegaly, pancytopenia, and skeletal deterioration.

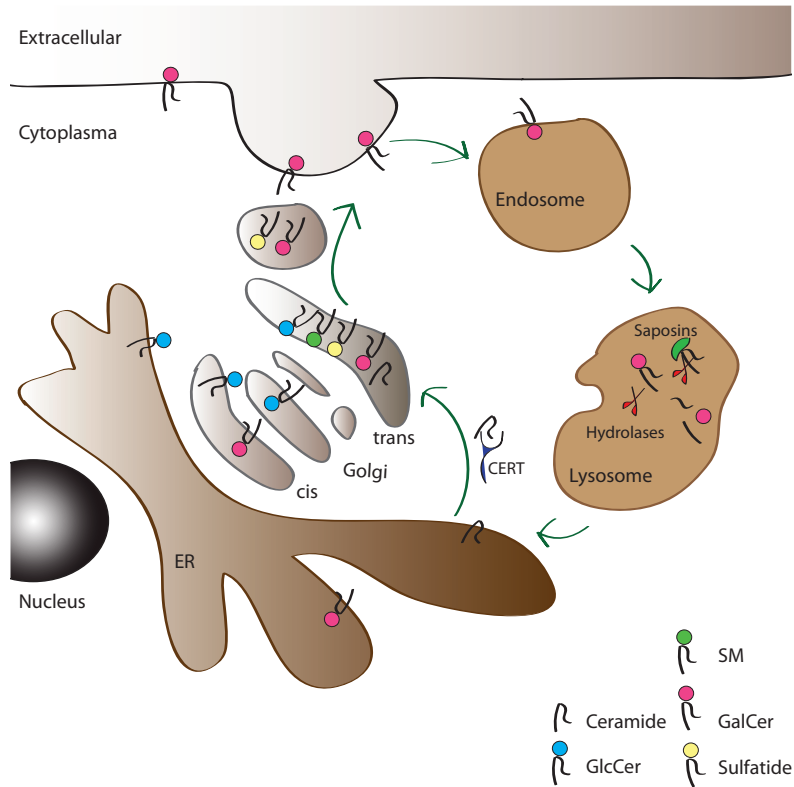


Figure 6. Intracellular pathways of GSL biosynthesis. Biosynthesis of GSL starts in ER and Golgi where different carbohydrates are added to ceramide by different GSL synthases. Thereafter, the GSLs are transported via vesicles to the plasma membrane. Through invaginated vesicles, GSLs internalize into endosomes where after fusion with lysosomes occur. In lysosomes, GSLs are cleaved into smaller structures and are available to be reprocessed.

Sulfatide

The GSL sulfatide is formed in Golgi by the action of cerebroside sulfotransferase (CST, see figure 5 and 6) catalyzes the addition of a sulfate group in 3-position of GalCer to form sulfatide. The GSL sulfatide is a naturally existing GSL in several mammalian tissues such as CNS, gastrointestinal tract, kidneys and pancreas. In these organs, the fatty acid chain of sulfatide varies in length and degree of saturation. In CNS, sulfatide consists mainly of isoforms with long unsaturated fatty acids such as C24:1,

while isoforms in pancreas are dominated by shorter saturated fatty acids such as C16:0 (see structures in figure 7A-B) [130, 131]. This indicates that different isoforms of sulfatide might have different functions in distinct tissues. Studies have shown that sulfatide co-localizes with insulin within pancreatic β -cells and are believed to stabilize the insulin crystals within the granules of the beta cells and furthermore facilitate the release of insulin [132]. In CNS, sulfatide has important functions in the myelin sheath such as in the development of paranodal junctions [133]. In metachromatic leukodystrophy the enzyme arylsulfatase A (ASA, see figure 5), which degrades sulfatide, is deficient and leading to the accumulation of sulfatide and lyso-sulfatide in lysosomes of various tissues. This leads to demyelination and neurological deterioration (reviewed in [134]). The lyso-form of sulfatide lacking the fatty acid chain, lyso-sulfatide, (figure 7C) also exists naturally but at low levels, however lyso-sulfatide, as well as other lyso-GSLs are increased in lysosomal storage diseases [135-140].

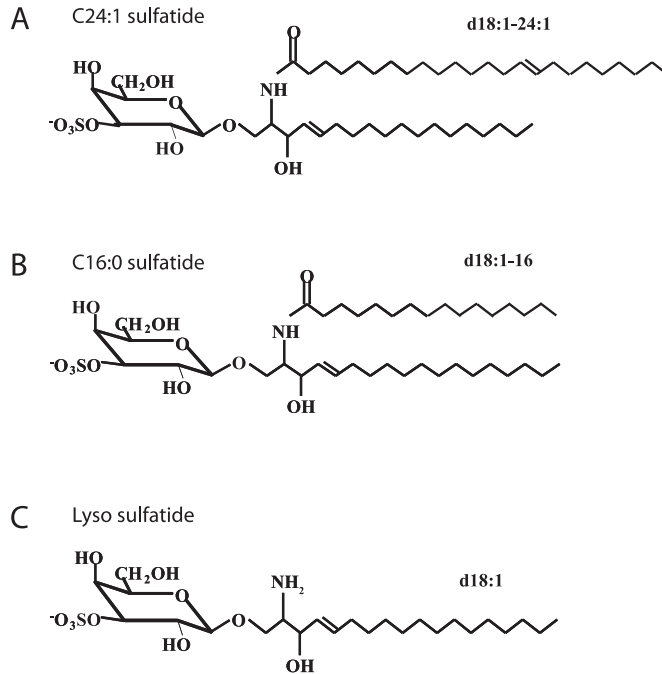


Figure 7. Naturally existing sulfatide isoforms. Semi-synthetic sulfatide isoforms with d18:1 sphingosine (1,3-dihydroxy-2-aminooctadecene) and fatty acid chain of (A) C24:1, (B) C16:0 and (C) without fatty acid chain (lyso), synthesized from native sulfatide.

NKT cell functions

The unique functions of NKT cells

NKT cells are functionally different from conventional MHC restricted T cells in terms of their activation state. NKT cells have an activated memory phenotype with high expression of CD44 and the IL-2 receptor β (CD122) in contrast to the naïve phenotype of conventional T cells. During an immune response NKT cells are rapidly activated, indicated by a fast production of high amounts of cytokines, including IFN- γ , IL-4, IL-10, IL-13, IL-17, IL-21 and tumor necrosis factor (TNF) [38, 46, 72, 141-143]. The type of cytokine

profile produced depends on signals, such as IL-12, obtained during activation.

When activated, NKT cells can promote or suppress the immune system (reviewed in [74, 144]). By comparing wild type mice with mice deficient in type I NKT cells (J281^{-/-}), or mice deficient in all NKT cells (CD1d^{-/-}), it has been possible to study the natural role of type I and type II NKT cells in several immune settings. Collectively, these studies have demonstrated that type I and type II NKT cells seem to have similar functions in some settings, however in other situations they can counteract as well as regulate each other [145, 146]. This demonstrates that the two subpopulations of NKT cells can have very different functions in immunity. Further, *in vivo* targeting of NKT cells by administration of activating lipid ligands such as α -GalCer has demonstrated the potent regulatory ability of activated NKT cells [91]. Structural variants of α -GalCer have been developed that activate type I NKT cells and bias the immune response in either a Th1 or Th2 direction depending on the modification. This demonstrates the importance of studying the role of different lipid isoforms, as small changes in structure, such as the length and saturation of the fatty acid chain, can have a great impact on the outcome of the immune response [94, 98].

NKT cells in autoimmunity

NKT cells have been linked to several autoimmune diseases, however the role of NKT cells in autoimmune diseases has not always been associated with beneficial effects. In some autoimmune setting, they may also have a pathogenic role. In patients with rheumatoid arthritis, numbers of type I NKT cells were decreased in peripheral blood and synovium [147-149] and it was suggested that low numbers of NKT cells contribute to the development of rheumatoid arthritis. A pathogenic role for NKT cells was established using a

mouse model for rheumatoid arthritis, in which the disease is induced by immunization with heterologous type-II collagen. Mice lacking type I NKT cells have less severe rheumatoid arthritis compared to control mice [150], and the lack of type I NKT cells resulted in a Th2 bias, which was beneficial for protection [151]. In MS, decreased numbers of type I NKT cells in peripheral blood of patients has been demonstrated. Interestingly, this reduction seems to correlate with relapse of the disease [152-154], while numbers of type I NKT cells were increased during the remission phase [155]. Further, an induced activation of either type I or type II NKT cells by the administration of activating lipid ligands has been shown to influence the development of the mouse model of MS [156-158]. Sulfatide administration at the induction of EAE protected the mice from developing the disease in a CD1d dependent manner. The protection was associated with lower levels of IFN- γ and IL-4 produced by autoantigen reactive T cells. In systemic lupus erythematosus (SLE), where an impaired clearance of apoptotic cells gives rise to development of autoantibodies, NKT cells have been suggested to have an important protective role. Mice that spontaneously develop autoantibodies and nephritis (NZB/NZW F1 mice) and were deficient in NKT cells have more severe nephritis [159]. Further, old $J\alpha 18^{-/-}$ mice, deficient in type I NKT cells, spontaneously develop a SLE like syndrome with production of antibodies against double stranded DNA and proteinuria [160], indicating that NKT cells can regulate the production of autoantibodies towards apoptotic cells. In 2010, Wermeling et al demonstrated that type I NKT cells have a suppressive role in the development of autoantibodies against an increased load of apoptotic cells in the circulation. The protective effect seen by type I NKT cells was through a cognate interaction between NKT cells and CD1d expressing B cells [161].

Type II NKT cells in experimental autoimmune encephalomyelitis

In MS and its mouse model EAE, the myelin sheath in CNS is the target for an autoimmune inflammatory process [162], which consequently leads to degradation of the myelin sheath accompanied by neurological problems and paralysis. Sulfatide is highly abundant in CNS; approximately 20% of all galactolipids in CNS are sulfatide, which indicates its importance in this organ. Interestingly, circulating T cells reactive towards GSL are more frequent in MS patients than control donors [163]. Among those GSLs are sulfatide, which suggests that glycolipids including sulfatide are released during the destruction of the myelin sheath and induce the activation of immune cells. It was demonstrated that these GSL reactive T cells were producers of proinflammatory cytokines such as TNF- α and IFN- γ , and it was suggested that they might promote, rather than control the autoinflammatory process in the CNS. Later, Jahng et al demonstrated that CD1d restricted type II NKT cells were activated by sulfatide *in vitro*, and that sulfatide reactive cells accumulate in CNS during the process of demyelination in EAE. These CNS infiltrating sulfatide reactive type II NKT cells produced IFN- γ but not IL-4. Interestingly, sulfatide administration completely protected mice from developing EAE in a CD1d-dependent manner, suggesting that the protective effect of sulfatide was mediated through CD1d-restricted NKT cells. Injection of sulfatide reduced the production of IFN- γ and IL-4 by autoantigen reactive T cells indicating a dampened activation of autoaggressive cells. Subsequent studies investigating the role of IFN- γ in EAE have shown that IL-17, rather than IFN- γ plays a role in the pathogenesis. Notably, IFN- γ counteracts IL-17 production. This might explain how IFN- γ producing type II NKT cells could have a protective role in EAE [164].

The immunomodulatory role of sulfatide *in vivo*

Since the discovery that sulfatide activates immunomodulatory type II NKT cells in EAE, sulfatide administration has been used in several immune settings in an attempt to modify immunity. In addition to its effects in EAE, sulfatide has been demonstrated to suppress tumor immunity resulting in increased tumor growth. In contrast, in the same tumor model, administration of α -GalCer was protective. When type I and type II NKT cells were simultaneously activated by α -GalCer and sulfatide, type II NKT cells suppressed the protective effect by type I NKT cells [145], suggesting that type II NKT cells could down modulate both tumor immunity and type I NKT cells when activated by ligand. In concanavalin A induced hepatitis, where type I NKT cells play an important role in mediating the disease, sulfatide administration mediated protection by the induction of anergy in type I NKT cells [146]. ConA induced hepatitis results in the infiltration of neutrophils and a cytokine burst in the liver, but after sulfatide treatment this did not take place due to the anergy of type I NKT cells. In hepatic ischemic reperfusion injury, the protective effect induced by sulfatide was associated with reduced IFN- γ production by pathogenic type I NKT cells and reduced hepatic recruitment of myeloid subsets, which normally occur during hepatic ischemic reperfusion injury [165]. Further, it has also been shown that sulfatide inhibits human immunodeficiency virus (HIV) replication in mice [166]. Virus replication was lowered significantly up to 4-8 weeks post infection. Altogether, this suggests that sulfatide, through its activation of sulfatide reactive type II NKT cells has strong immunomodulatory capacity.

Type 1 diabetes

Autoimmune type 1 diabetes (T1D) is caused by a T cell mediated destruction of insulin producing β -cells in the pancreatic islets of Langerhans. Animal models, such as the non-obese diabetic (NOD) mouse [167], that

spontaneously develops T1D similar to the human disease have provided successful tools to study the mechanisms involved in the disease pathogenesis. In NOD mice, diabetes develops in two stages which involves an initial phase where cells infiltrate the pancreas without destruction of β -cells, followed by a second phase when the infiltrating cells start to destroy β -cells (reviewed in [168]). In the NOD model, it is well established that different types of regulatory cells control the onset and progression of T1D. This was illustrated by crossing mice with a transgenic TCR (BDC2.5 NOD [169, 170]) specific for a pancreatic peptide, to mice lacking the ability of rearrange T and B cell receptors. TCR transgenic mice that lacked endogenous T and B cell receptors developed an early and very rapidly progressing disease, caused by the absence of immunoregulation [170]. In addition, NKT cells were suggested to have a protective role in T1D when it was shown that NOD mice have impaired numbers and functions of type I NKT cells [171, 172]. Further, transfer of DN thymocytes, most likely containing a large fraction of NKT cells, to NOD mice prevented the onset of T1D [172]. Also type II NKT cells have been implied in T1D protection; over expression of a type II NKT cell TCR ($V\alpha 3.2^+V\beta 9^+$) in NOD mice almost completely prevented the onset of disease [173]. These cells produced high levels of IFN- γ and low levels of IL-4. In 2012, Kadri et al. demonstrated that $CD4^+$, but not DN, TCR transgenic $V\alpha 3.2^+V\beta 9^+$ type II NKT cells efficiently down regulated T1D induced by BDC2.5 NOD T cells in adoptive transfer experiments, through a mechanism that was dependent on the inducible T-cell costimulator (ICOS) and programmed cell death 1 (PD-1) pathways [174].

Moreover, activation of type I NKT cells by α -GalCer protects NOD mice from developing T1D [175-178]. The most efficient protection was observed with repeated α -GalCer administrations for several weeks. When

administering an analog of α -GalCer (OCH), with a truncated sphingosine chain, which gives rise to reduced levels of IFN- γ and high levels of IL-4 after stimulation of type I NKT cells, a more effective protection against T1D in NOD mice was observed compared to α -GalCer treatment [179]. This confirms that minor changes in structures of lipid antigens may cause great differences in terms of the activation and induced effector functions of NKT cells [180].

It is interesting to note that the type II NKT cell ligand sulfatide is present in β -cells of the pancreatic islets. Since T1D is an autoimmune disease where a specific destruction of pancreatic β -cells occurs, it seems possible that upon destruction, β -cell associated GSLs such as sulfatide may be released and induce an immune response, similar to what has been shown in MS and EAE [163]. Notably, it is known that human patients with T1D, but not healthy individuals, have autoantibodies against sulfatide in serum [88], supporting this concept. These findings prompted our studies of the possible role of sulfatide reactive NKT cells in T1D.

NKT cells in infections

Due to the rapid production of high amounts of inflammatory cytokines, NKT cells possess a powerful capacity to enhance immunity against several infections. In murine models, NKT cells have been described to induce immunity to several pathogens such as viruses, gram-positive bacteria, gram-negative bacteria, fungi, parasites and helminths [181]. In 2000, Kumar et al demonstrated that *Borrelia burgdorferi* (Bb) infected CD1d^{-/-} mice showed increased Bb specific IgG antibodies and developed infection induced arthritis, suggesting that NKT cells play a role in the protection from Bb infection in mice [182]. NKT cells can polarize conventional CD4⁺ T cells into either Th1 or Th2 and thereby modulate the immune system to several

microbial infections, including bacteria and helminths. NKT cells might also have negative effects such as causing increased liver injury during *Salmonella* infection [183]. In *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* lung infections, type I NKT cells have been shown to induce protection in mice. Protection was associated with recruitment of type I NKT cells to the site of infection that subsequently promoted the recruitment of neutrophils. Moreover, activation of type I NKT cells by administration of α -GalCer inhibited hepatitis B virus (HBV) replication in the liver of HBV transgenic mice. The inhibition induced by α -GalCer was associated with induction of IFN- γ and IFN- α/β in the liver [184]. In addition, type II NKT cells have also been shown to play a role in the defence to HBV infection, using the same transgenic model [185]. The first insight concerning a role for type II NKT cells during infections was obtained from studies of mice infected with diabetogenic encephalomyocarditis virus (EMCV-D). A protective effect was observed both by type I and type II NKT cells [186]. Using $J\alpha 18^{-/-}$, $CD1d^{-/-}$ and C57BL/6 (wildtype (WT)) mice, it was demonstrated that during infection with the parasite *Trypanosoma cruzi*, type II NKT cells increased the anti-parasite response while type I NKT cells limited the response. Type I NKT cell deficient $J\alpha 18^{-/-}$ mice developed increased weight loss and mortality as well as an augmented splenomegaly compared to $CD1d^{-/-}$ and WT mice [187]. This suggests that type I and type II NKT cells may perform different immunological functions in immunity against infections. A divergent function of type I and II NKT cells was also observed during helminth infection by *Schistosoma mansoni* where type I NKT cells promoted a Th1 cell differentiation whereas type II NKT cells provided help for a Th2 cellular response. Recently, HBV infection has been demonstrated to induce the activation of type II NKT cells by a $CD1d$ -dependent recognition of HBV induced lyso-phospholipids derived from ER

[120]. Subsequently to activation of type II NKT cells, an IL-12-mediated indirect activation of type I NKT cells occurred. The absence of NKT cells or ER-associated transfer of lipids onto CD1d diminished the HBV infection, suggesting that NKT participate in the viral control by recognition of lysophospholipids.

NKT cells in sepsis

The occurrence of systemic bacterial infection is the foremost health problem leading to deaths in hospital care units worldwide [188]. One major bacterium causing sepsis is the gram-positive *Staphylococcus aureus* (*S. aureus*) [189]. The hallmarks of sepsis consist of an initial hyperreactive phase with excessive inflammation, followed by a anti-inflammatory/immunosuppressed, latent phase [190]. During the initial phase, after systemic exposure to bacteria, there is a massive release of pro-inflammatory cytokines such as TNF, IL-1, IL-6 and IFN- γ from circulating activated monocytes, macrophages and other immune cells. In a mouse model for sepsis, injection of the endotoxin LPS into mice causes a lethal chock syndrome that is mediated by IL-12 and subsequent IFN- γ production, followed by the production of pro-inflammatory cytokines such as TNF- α and IL-1 and other inflammatory mediators [191-193]. APC derived IL-12 induces the activation of NKT cells, which rapidly produce high amounts of IFN- γ [194], as well as increase their cytotoxicity in the liver [195]. In the absence of NKT cells, the mortality after a lethal dose of LPS was significantly reduced [196]. Mice deficient in type I NKT cells ($J\alpha 18^{-/-}$) had significant survival advantages with reduced serum concentrations of IFN- γ and TNF- α compared to wild type controls, suggesting that type I NKT cells play a role in the initiation of septic shock induced by LPS. However, administration of α -GalCer before or shortly after LPS challenge reduced sepsis in these mice [197]. Interestingly, protected mice showed decreased

frequencies of NKT cells expressing IFN- γ and higher frequencies expressing IL-10 suggesting that ligand mediated modulation of NKT cells in this situation resulted in a shift towards a protective response. Even though type I NKT cells have pathogenic effects in the LPS model of severe sepsis, they may have beneficial functions in less severe sepsis-like situations, such as endotoxemia and microbial infections, by contributing to increased clearance of pathogens [198]. To achieve a balance between a proper and robust inflammatory response resulting in an efficient clearance of bacteria without overstimulation and subsequent septic shock is of major concern in the treatment of sepsis.

NKT cells in tumor immunity

α -GalCer was first discovered as a potent antitumor agent, and the role of NKT cells in tumor immunity was revealed when α -GalCer was found to activate type I NKT cells, a requirement for the antitumor activity [99]. Further, IL-12 was known to possess antitumor properties [199] but the downstream effects of IL-12 was not described. Through the use of $J\alpha 18^{-/-}$ mice, Cui et al were able to demonstrate the significant effect of type I NKT cells in antitumor immunity driven by IL-12 [200], dependent on a direct contact between type I NKT cells and tumor cells. Additionally, activating type I NKT cells by α -GalCer resulted in type I NKT cell mediated killing of tumor cells through a CD1d-independent, NK-like mechanism [201]. Moreover, an antitumor function of type I NKT cells in the absence of exogenously administered stimulators such as IL-12 or α -GalCer has been described [144] using the chemical carcinogen methylcholanthrene (MCA) that induces tumor formation. Here, the protective activity was depending on IFN- γ production by NKT cells and subsequently the activation of NK cells, while perforin expression on NKT cells was not required. Furthermore, it was

demonstrated that DN and not CD4⁺ NKT cells derived from liver, but not spleen or thymus, mediated the protective activity.

While type I NKT cells have been described to possess a protective role in tumor immunity, type II NKT cells can give rise to opposite effects [202] [145]. In different tumor models, *J α 18^{-/-}* mice have been demonstrated to have an accelerated tumor growth compared to *CD1d^{-/-}* mice, suggesting that type II NKT cells reduce tumor immunity while type I NKT cells promote it. A series of studies demonstrated that CD4⁺ type II NKT cells suppressed antitumor CD8⁺ T cells by the production of IL-13, that in turn induced secretion of TGF- β by myeloid cells, resulting in increased tumor growth [145]. In addition, it was shown in two different tumor models, in line with previous data, that α -GalCer administration enhanced tumor immunity and protected mice, while sulfatide administration activated type II NKT cells that suppressed tumor immunosurveillance resulting in increased tumor growth [145].

AIM

Innate-like lymphocytes, such as NKT cells have unique characteristics and regulatory functions in the immune system. They are autoreactive cells that have an activated memory phenotype, which makes them respond rapidly and vigorously to activation with the expression of effector functions. The overall aims of this thesis were to expand the knowledge on how to activate type II NKT cells as well as to evaluate their immunoregulatory function in autoimmunity and infection.

Specific aims

1. To evaluate the activation of type II NKT cells by the glycosphingolipid sulfatide and to determine the role of sulfatide in the autoreactivity of type II NKT cells
2. Identify self-lipids that are involved in the autoreactivity of type II NKT cells
3. To investigate whether sulfatide treatment could prevent type 1 diabetes development through the induction of immunomodulatory type II NKT cells
4. To investigate whether sulfatide treatment could ameliorate *Staphylococcus aureus* induced sepsis through the induction of immunomodulatory type II NKT cells



KEY METHODOLOGY

Cells

For analyzing sulfatide reactivity and autoreactivity of type II NKT cells, the type II NKT hybridoma XV19 was used [41]. These cells were generated from CD4⁺ T cells from MHCII^{-/-} mice and were initially identified as CD1d-autoreactive, and later demonstrated to be sulfatide reactive CD1d restricted type II NKT cells [30]. T cell hybridomas are generated by fusion of antigen-specific T cells with a tumor cell, usually a thymoma. Properties of the tumor fusion partner are inherited, which means that T cell hybridomas constantly divide, however they do not retain functions of the original primary T cells. Of importance, T cell hybridomas are generally less dependent than primary cells to co-stimulation for activation, and are commonly used for detailed studies of antigen reactivity [203]. When activated by antigens, presented on APCs, T cell hybridomas produce IL-2. The IL-2 production produced is a direct correlation of the activation of the T cell hybridoma. As APCs for presentation of GSLs we have used the DC line, JawsII cells [204], unless otherwise stated. We found that JawsII cells are optimal APCs for our studies due to the fact that they naturally express high levels of CD1d, and efficiently present lipids on CD1d while they induce low levels of autoreactivity of XV19 cells. Further, to obtain physiologically relevant subsets of DCs, we cultured bone marrow (BM) cells in the presence of FLT3L, which is a growth factor that gives rise to the majority of mouse DC populations found *in vivo* [205, 206].

Glycosphingolipids

Native sulfatide at a purity of >95% (determined by thin layer chromatography and mass spectrometry) had been isolated from pig brain

[207]. Lyso and sulfatide isoforms containing the fatty acids of caprylic acid (C8:0), lauric acid (C12:0), palmitic acid (C16:0), oleic acid (C18:1), lignoceric acid (C24:0) and nervonic acid (C24:1) were produced from pig brain-derived native sulfatide [208]. The solubility in culture medium at final concentration of the various sulfatide isoforms was verified by thin layer chromatography in selected experiments, and was found to be >80%. Native GlcCer and GalCer, and production of their semi-synthetic isoforms have been described before [209]. The synthesized isoforms were free of detectable contaminations in the analysis by thin layer chromatography and mass spectrometry, revealing a purity of >99%. Semi-synthetic lyso-lactosylceramide, lyso-sphingomyelin, lyso-GM1 and lyso-globotriaosylceramide were purchased from Matreya (PA, USA).

Cellular lipid extracts and fractionation

A20CD1d cells [107] give rise to very high autoreactivity of XV19 cells, and therefore we hypothesized that A20CD1d cells present potent stimulatory self lipids for XV19 cells. To identify stimulatory lipid ligands presented by A20CD1d cells we isolated cellular lipids and fractionated them into different lipid fractions, according to their solubility. It should be noted that when we started the project, GSLs were the main class of lipids known to stimulate NKT cells. The GSLs α -GalCer and iGb3 [99, 110] had been identified as stimulatory ligands for type I NKT cells and the GSL sulfatide for type II NKT cells [30]. Therefore we isolated lipids from A20CD1d according to protocols for purification of GSLs.

T cell hybridoma assays

We have used two different methods to evaluate the activation of XV19 cells by GSLs; APC-based assay and APC-free assay, where CD1d is bound to

plastic plates (plate-bound CD1d). These two methods are considerably different. Lipid loading onto CD1d in the APC assay can take place on the surface of the APC, but also through uptake and intracellular loading, therefore, several parameters in addition to the TCR-ligand-CD1d interaction will determine the ability of a certain lipid to stimulate an NKT cell. When using plate-bound CD1d, a direct loading of the lipid onto CD1d has to take place.

IL-2 analysis

CTLL-2 cells [210] or IL-2 ELISA were used as readouts for activation of XV19 cells, and subsequently produced IL-2. CTLL-2 cells are dependent on IL-2 for survival, thereby they can be used for analysis of IL-2 in supernatant from XV19 hybridoma assays. When using CTLL-2 cells as readout for IL-2 production, XV19 hybridoma stimulation is measured by ³H-thymidine incorporation and therefore expressed as cpm determined in the CTLL assay. A titration of IL-2 was always included as reference to ensure that the responses were in the linear response range. During the course of the study, IL-2 ELISA became more frequently used to determine the activation of the T cell hybridomas. Because IL-2 ELISA provides a quantitative measurement of the amounts of IL-2 produced, we are now routinely using this method as readout for IL-2 production.

Mice

NOD mice are a commonly used mouse model to study T1D. NOD mice spontaneously develop T1D similar to the human disease, caused by the destruction of β -cells in the Langerhans islets of the pancreas [167]. In NOD mice, the T1D process starts as early as 2 weeks after birth when β -cells within pancreas experiences a wave of β -cell death, followed by presentation

of pancreas derived antigens in the draining pancreatic lymph nodes leading to the activation of autoaggressive T cells. After 3-4 weeks, insulinitis is initiated and an accumulation of immune cells take place in the pancreas. When approximately 80% of the islets have been destroyed and insulin production is too low to regulate blood glucose levels, clinical signs of diabetes occur, starting from around 12 weeks of age. At 25–30 weeks of age, around 80% of female NOD mice have become diabetic, but only 20–30% of the male mice become diabetic. The diabetes incidence may fluctuate to some degree due to different animal houses, depending how clean the environment is as well as other factors.

Staphylococcal sepsis induction

To evaluate the role of NKT cells in sepsis we have used *S. aureus*. Specifically, we have used the TSST-1 producing *S. aureus* LS-1 strain. The LS-1 strain was isolated from a spontaneously arthritic NZB/W mouse [211], and is a well established strain in for studies of staphylococcal sepsis and arthritis in mice [212, 213].

RESULTS AND DISCUSSION

The activation of type II NKT cells by naturally existing GSLs

Physiological isoforms of sulfatide stimulate type II NKT cells (Paper I)

After the discovery of type II NKT cells in 1995 [41], the activation and functional ability of the cells were not well known for several years. Almost a decade later, the self-lipid sulfatide was identified as a stimulatory lipid ligand for type II NKT cells [30]. Notably, sulfatide is highly abundant in pancreas and CNS [130, 131], where sulfatide has important functions. In CNS, sulfatide is critical for the maintenance of the myelin sheath and axon structure [214]. Mice deficient in CST (see figure 5) and thereby lacking sulfatide develop neurological abnormalities as well as defects in spermatogenesis, which results in paralysis and sterility [133]. In pancreas, sulfatide is believed to be involved in the release of insulin by stabilizing insulin crystals within the granules of the β -cells [132]. Further, in these organs, sulfatide exists in different isoforms. The main isoforms of sulfatide in CNS have long unsaturated, such as C24:1, and saturated, such as C24:0, fatty acids [132]. In pancreas, sulfatide exists in isoforms with saturated fatty acids. The short fatty acid (C16:0) isoform comprises one and the longer 24 carbon atom (C24:0) the other major species in islets [132, 215]. Notably, in MS or T1D, myelin proteins in CNS or pancreatic β -cells are targets for destruction by autoaggressive cells [162], suggesting that during the tissue destruction phase of these autoimmune diseases, sulfatide may be released from these tissues and subsequently activate type II NKT cells (figure 3E). Interestingly, T cells reactive to myelin GSLs are increased in peripheral blood of patients with MS [163]. Further, native sulfatide extracted from

bovine brain, administered simultaneously with EAE induction completely protects mice from developing EAE in a CD1d dependent manner [30], indicating an induced protective role of sulfatide reactive type II NKT cells in EAE. Altogether this suggests that a fraction of type II NKT cells are activated by sulfatide in these diseases and that these sulfatide reactive cells may possess immunoregulatory properties. Due to the fact that sulfatide exists naturally in different isoforms in certain organs, we sought to determine whether sulfatide reactive type II NKT cells are differently activated by these tissue specific isoforms.

When using the sulfatide reactive, CD1d restricted type II NKT cell hybridoma XV19 [41] to study the stimulatory capacity of sulfatide isoforms we found that sulfatide without fatty acid chain (lyso) has the greatest capacity of activating XV19 cells, followed by C24:1>C24:0>C12>C16:0>C18:1 isoforms (figure 8). The activation of XV19 cells by C24:1, C24:0 and C16:0 sulfatide isoforms indicates that indeed the type II NKT cells are activated by sulfatide isoforms known to exist in different mammalian tissues such as CNS and pancreas, during normal conditions. Interestingly, semi-synthetic isoforms with short fatty acid chains (C8 and C12) and C18:1, that are not known to exist naturally, only weakly or moderately stimulated XV19 cells. Also, in addition to XV19 cells, two additional type II NKT cell hybridomas are activated by sulfatide, 14S.15.5D [108] (figure 4, paper I) and IC8.DC1 [6], which suggest that the recognition of sulfatide may be a common reactivity of type II NKT cells. Interestingly, the TCR gene segments used by XV19 [216] and 14S.15.5D [108] cells are different, suggesting that even though type II NKT cells express diverse TCR, some type II NKT cells can recognize the same lipid antigen [217]. Altogether, this suggests that type II NKT cells are efficiently activated by sulfatide isoforms known to exist in different organs, suggesting

that sulfatide reactive type II NKT cells may have a natural role in autoimmune diseases related to tissue destruction of these sulfatide containing organs, such as CNS and pancreas.

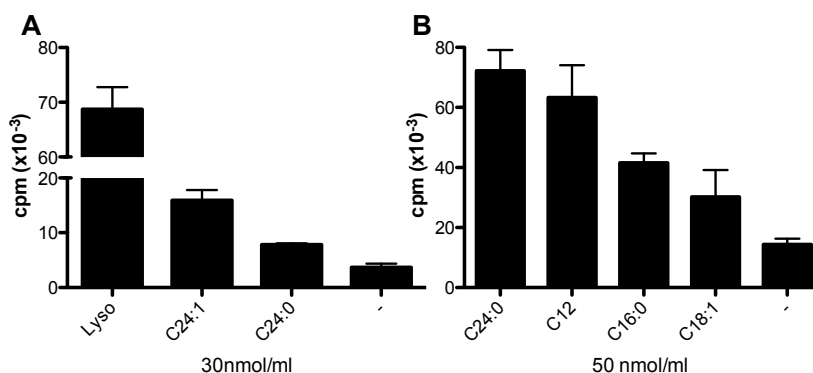


Figure 8. Sulfatide isoforms activate XV19 cells. (A) 30 nmol/ml or (B) 50 nmol/ml of indicated sulfatide isoforms were added together with JawsII cells as APCs for 3-4 h before addition of XV19 cells, followed by incubation over night. IL-2 produced by XV19 cells was analyzed by CTLL-2 assay. (-) Indicate without lipid in culture

In 2012, Patel and colleagues presented the crystal structure of CD1d bound to C24:1 sulfatide in association with the XV19 TCR [216]. The TCR of XV19 cells was cloned and sequenced and identified as V α 1-J α 26/V β 16. In contrast to the parallel docking mode over the F' pocket of type I NKT cell the V α 14-J α 18 TCR, the TCR of XV19 cells binds orthogonally above the A' pocket of CD1d. Further, the XV19 TCR contact with CD1d:C24:1 sulfatide was dominated by non germline encoded residues, where the CDR3 α loop makes contacts with CD1d and the CDR3 β loop shapes the specificity for sulfatide. This is in contrast to type I NKT cell TCR recognition of CD1d: α -GalCer, where interactions are dominated by germline encoded segments within the invariant V α -J α chain and the V β chain. Notably, some degree of conservation of the CDR3 β loop of type II

NKT cells TCR has been observed among sulfatide reactive type II NKT cell TCRs, indicating common interactions for type II NKT cells with sulfatide and possibly other β -linked lipid antigens [217].

Novel lipid ligands identified for XV19 type II NKT cells (Paper II)

The fact that different APCs give rise to different degree of autoreactivity of XV19 cells indicates that this might be due to presentation of more or less stimulatory lipid ligands. Therefore we fractionated APCs that induce significantly high autoreactivity of XV19 cells (figure 1B, paper II) and indeed, we could identify stimulatory lipid fractions that stimulate XV19 cells. Mass spectrometry demonstrated that the neutral GSL, β -GlcCer (GlcCer) with fatty acid chain lengths of C16:0 and C24:0 were dominating lipid species within the active fraction (figure 2E, paper II). When using semi-synthetic variants of C24:0 and C16:0 GlcCer we were able to demonstrate a CD1d dependent activation of XV19 cells by these isoforms (figure 3, paper II). In addition to GlcCer, we could demonstrate that the structurally similar β -GalCer (GalCer), which is the precursor to sulfatide, also stimulates XV19 cells (figure 4, paper II). The relative activation pattern by different isoforms of GlcCer and GalCer were similar to sulfatide (see structures in figure 9), where lyso-forms give rise to superior activation, followed by saturated or unsaturated C24 length of the fatty acid chain, however, the stimulation of XV19 cells is greater when using sulfated GalCer (sulfatide) compared to GlcCer and GalCer. This indicates that differences in positioning of the OH-group of the hexose has no importance for activation of XV19 cells, however sulfated galactose gives rise to increased activation, suggesting that the sulfate group enhances the interaction between CD1d-lipid-TCR. The crystal structure of XV19 TCR to CD1d:C24:1 sulfatide reveal that the sulfate moiety form contact with TCR, however it did not form

any intricate hydrogen-bonding network [216]. From this, it was suggested that the sulfate moiety does not enhance the interaction between CD1d-lipid-TCR, however further analysis are required. Further, the absence of stimulation of XV19 cells by lyso-sphingomyelin, lyso-monosialotetrahexosylganglioside and lyso-lactosylceramide demonstrates that the activation by lyso-forms was not a general feature of GSL lyso-forms, but appeared specific for sulfatide, GlcCer and GalCer (figure 4A, paper II). Interestingly, lyso-sulfatide is normally not present in high amounts in mammalian tissues, however it has been shown that patients suffering from the lysosomal storage disease metachromatic leukodystrophy have higher levels of lyso-sulfatide in the brain [218]. Further, lyso-sulfatide, along with other lyso-GSLs bound to high density lipoproteins (HDL) have been associated with reduced inflammation in the development of arteriosclerosis [219]. Lyso-GSLs:HDL binding to its receptor induce cellular signaling and restriction of inflammation such as inhibition of NF_κB. Interestingly, several other studies also demonstrate that lyso-compounds are highly stimulatory for NKT cells (see [220]), indicating that NKT cells can be activated in situations where you have accumulation of lyso-GSLs, including sulfatide. However, whether this does occur needs to be investigated.

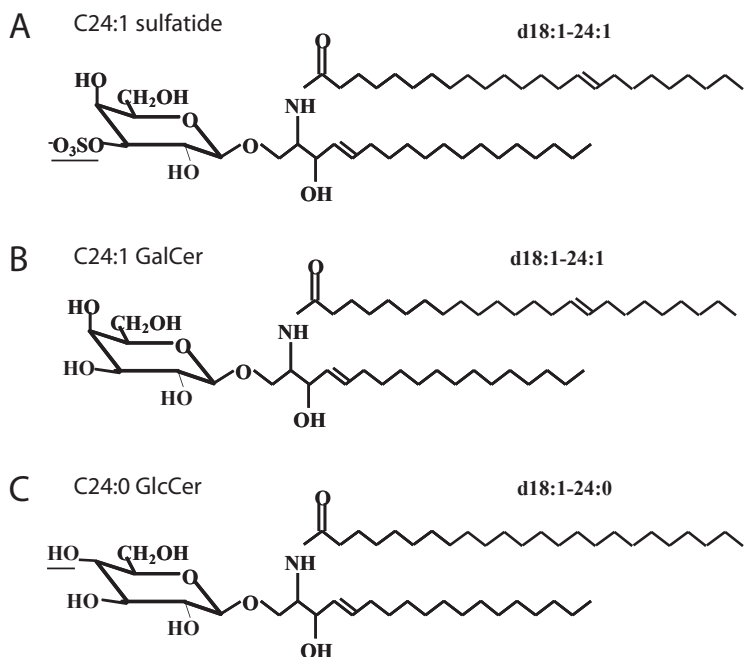


Figure 9. Structure of stimulatory GSLs for type II NKT cells. GSL isoforms with d18:1 sphingosine (1,3-dihydroxy-2-aminooctadecene) with saturated or unsaturated fatty acid chains of C24. **(A)** C24:1 sulfatide, **(B)** C24:1 GalCer and **(C)** C24:0 GlcCer.

Further, it is possible that increased stimulation of XV19 cells with lyso-GSLs compared to C24 isoforms in the *in vitro* assays might be due to that lyso-compounds are less hydrophobic, as they lack the fatty acid chain. This increases the polarity and solubility, which may lead to increased accessibility of lyso-GSLs for loading on CD1d molecules and thereby enhance the activation of XV19 cells. However, this scenario may not be valid *in vivo*, where CD1d-mediated presentation of lyso-GSLs will be affected by additional factors. Notably, transport of lipids by lipid binding proteins in serum, and binding of these proteins to cellular receptors and uptake is suggested to significantly affect the presentation of lipid ligands on CD1d [221]. Apolipoprotein E (apoE) associated with very-low-density

lipoprotein (VLDL) has been demonstrated to target di-GalCer for uptake via the LDL receptor, which results in delivery to lysosomes and subsequent processing and loading onto CD1d for presentation. Interestingly, this pathway seems dispensable for presentation of α -GalCer [222], indicating that different lipids require distinct transport mechanisms in serum to achieve optimal uptake by APCs followed by presentation on CD1d *in vivo*.

Notably, in addition to the docking mode of the XV19 TCR to CD1d loaded with C24:1 sulfatide, the molecular interaction of the XV19 TCR with CD1d and lyso-sulfatide was also recently solved. A comparison demonstrates remarkable similarities in XV19 TCR interaction with CD1d and exposed ligand, even though these two isoforms of sulfatide are structurally significantly different [216] [223]. Also, the affinity of XV19 TCR for CD1d:C24:1 was higher than for CD1d:lyso-sulfatide. This suggests that differences in the molecular recognition and affinity of this type II NKT cell TCR to CD1d:lyso-sulfatide does not explain the increased activation of type II NKT cells by lyso-sulfatide in comparison to C24:1 sulfatide. Further, when using an APC-free assay where CD1d is coated on plastic plates, we observed strong activation of XV19 cells by the lyso-forms and C24 length of the acyl chain of sulfatide, GlcCer and GalCer (figure 5, paper II), demonstrating that the increased activation of XV19 cells in the APC assay was not due to more efficient uptake and loading on CD1d by the lyso-GSLs. In addition, this also establishes that stimulation with lyso-GSL was not due to induction of co-stimulation or other non-specific factors in the APC. Whether there are biological consequences, such as biased cytokine production, which has been described with the use of different α -GalCer analogues [224-226], induced specifically by lyso-sulf, GlcCer and GalCer and other stimulatory lyso-compounds needs to be addressed.

The role of GSLs in the autoreactivity of type II NKT cells (Paper I and II)

The autoreactivity is a hallmark for NKT cells and the role and identity of CD1d-presented ligands in this interaction have been extensively studied lately. Not only in the context of how NKT cells are selected by CD1d-ligands, but also for the fact that NKT cell autoreactivity seems to be altered during immune responses such as to infections. Importantly, the selection of NKT cells in the thymus requires CD1d presentation of self-lipids [63, 227]. As for today, the self-lipids involved in the thymic positive selection of NKT cells are not fully known. Further, the identity of the self-lipids involved in "steady state" and "induced" autoreactivity to CD1d, and whether they are the same lipids, are also unresolved questions (figure 4).

The GSL iGb3 was first suggested to be involved in the autoreactivity of type I NKT cells [110]. However, recent studies have shown conflicting results and whether the autoreactivity of type I NKT cells involves recognition of iGb3 is not clarified [113]. Despite this, GSLs seem to possess efficient stimulatory capacity for NKT cells. Since we found that physiological isoforms of sulfatide, produced during normal conditions in cells, activate a set of type II NKT cells, we hypothesized that the natural autoreactivity of the sulfatide reactive type II NKT cells was dependent on sulfatide. We found that when using splenocytes from mice lacking CST (see figure 5), the enzyme required to catalyze the addition of a sulfate group of GalCer to form sulfatide, we observed no alteration in the autoreactivity of XV19 cells (figure 6A-B, paper I). This demonstrated that the natural autoreactivity of NKT cells (called "steady state" autoreactivity in figure 4) is not dependent on sulfatide.

Knowing that the autoreactivity of XV19 cells does not require sulfatide we speculated that the novel stimulatory self-lipids that we identified for XV19 cells, GlcCer and GalCer, may instead be involved. When using APCs deficient in glycosyltransferase (GCS, see figure 5), and thereby lacking GlcCer and all downstream GSLs, we found that GlcCer synthesis was not required for the autoreactivity of XV19 cells, excluding GlcCer and a range of different complex GSLs in the induction of natural autoreactivity of the XV19 cells (figure 6A, paper II). From our experiments we could therefore exclude sulfatide and the majority of other GSLs, however, GalCer may still be responsible for autoreactivity of XV19 cells. We therefore used APCs deficient in sphingosine long-chain base subunit 1 (LCB1, see figure 5), having a dramatic deficiency in sphingomyelin and all GSLs, but we detected no alteration in autoreactivity of XV19 cells when we compared mutant and wild type cells (figure 6B, paper II). This suggests that neither GalCer, nor any other GSL were necessary for the natural autoreactivity of XV19 NKT cells. As mentioned earlier, similar findings have been published regarding the natural autoreactivity of type I NKT cells.

However, studies have shown that GSL synthesis is altered during bacterial infections [228], opening the possibility that GSLs could be involved in the increased activation of NKT cells by activated DCs. CpG oligonucleotides stimulating TLR9 on DCs induced production of charged GSLs that mediated type I NKT cell stimulation in the presence of type I interferons [229]. Further, TLR ligand activation of human APCs has been shown to modulate lipid biosynthesis resulting in increased activation of type I NKT cells to secrete IFN- γ [84]. The activation was dependent on both CD1d and soluble factors such as IL-12. Another study demonstrates that infections with microbes inhibit the action of α -GalA (see figure 5), which is the rate limiting enzyme for iGb3 turnover [85], leading to accumulation of self-

lipids such as iGb3 in lysosomes of APCs, which further induces vigorous activation of type I NKT cells. Also, it was proposed that during steady state, iGb3 is constantly degraded by that action of α -GalA. Further, this demonstrates that the pool of GSLs in DCs is altered after TLR triggering, which leads to increased activation of type I NKT cells. Whether the altered representation of GSLs in activated APCs influences the autoreactivity of type II NKT cells is not known, however, we have observed increased autoreactivity of XV19 cells to LPS-stimulated DCs (figure 10), supporting this concept. Thus, it is possible that GSLs, including sulfatide, GlcCer and GalCer, may be involved in induced autoreactivity of type II NKT cells represented by XV19 cells.

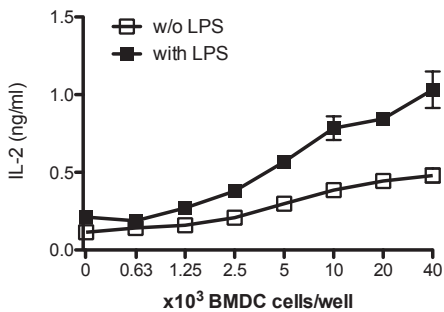


Figure 10. Increased autoreactivity of XV19 cells to LPS activated APCs. FLT3L derived bone marrow dendritic cells (BMDC) were cultured with or without (w/o) 1 μ g/ml LPS over night, before co-culture with XV19 cells over night. IL-2 produced by XV19 cells was analyzed by ELISA.

An alternative model to explain autoreactivity of type I NKT cells was put forward by Gapin and coworkers, suggesting that it can arise from the direct contact between CD1d and the type I NKT TCR [230]. They demonstrated that the presence of unique sequences within the hypervariable CDR3 β loop can greatly influence type I NKT TCR affinity for CD1d presenting self antigens by direct interaction with CD1d, [230-233]. This would result in that a broad range of CD1d associated self antigens would allow autoreactivity as long as they are not interrupting the germline encoded recognition of the CD1d by type I NKT TCR. However, for sulfatide reactive type II NKT cells, non-germline

sequences seem to dominate the interaction with CD1d presenting self antigens, suggesting that this hypothesis might not be generally applicable for type II NKT cells.

Immunomodulation with sulfatide to ameliorate disease development

The effect of sulfatide treatment on type I diabetes development (Paper III)

Sulfatide autoantibodies in type I diabetes prone non-obese diabetic (NOD) mice

The increase of sulfatide reactive cells in MS and EAE [30, 163] suggests that sulfatide is released during the destruction of the myelin sheath in CNS and evokes an immune response. Sulfatide is also present in pancreatic β -cells, which is the target for destruction during T1D, suggesting that sulfatide may be presented to immune cells in a stimulatory manner also in this autoimmune disease. In support of this, it has been shown that human patients with T1D, but not healthy individuals, have autoantibodies against sulfatide in serum [234]. In paper III, our aim was to evaluate whether sulfatide induces an immune response in the NOD mouse model for T1D, such as the production of autoantibodies and stimulation of sulfatide reactive type II NKT cells. We also sought to determine whether the administration of sulfatide to NOD mice would suppress the development of T1D. The results show that the majority of NOD mice, like non diabetic C57BL/6 mice, only have low or background levels of sulfatide autoantibodies, (figure 1A, paper III). However, we observed that approximately 25% of NOD mice demonstrated increased serum reactivity to sulfatide indicating that autoantibodies against sulfatide may arise during T1D pathogenesis, as found in humans. However, comparing NOD mice newly diagnosed with T1D and healthy NOD mice we did not observe a significant difference, suggesting

that the increased levels of autoantibodies in a few individual NOD mice were independent of clinical manifestations of T1D (Figure 1B, paper III), unlike the human situation.

Previously, it has been shown that old $J\alpha 18^{-/-}$ mice, lacking type I NKT cells, on the non autoimmune C57BL/6 genetic background, have increased concentrations of autoantibodies in serum [160], similar to the situation in systemic lupus erythematosus (SLE). Further, the production of autoantibodies towards an increased load of apoptotic cells in the circulation was regulated by type I NKT cells in a CD1d dependent manner [161]. This suggests that NKT cells are able to regulate the production of autoantibodies in mice. Therefore we investigated whether a lack of NKT cells would reveal an increased propensity for production of sulfatide autoantibodies in NOD mice, however, CD1d-deficient NOD mice lacking all NKT cells did not show elevated levels of antibody reactivity to sulfatide (figure 1C, paper III).

The production of autoantibodies against self-structures such as self-lipids expressed by apoptotic cells can be induced even in non-autoimmune mouse strains by repeated injection of apoptotic cells [161, 235]. However, injection of native sulfatide according to the protocol for induction of autoantibodies towards apoptotic cells [161], did not evoke antibody reactivity towards sulfatide in autoimmune prone NOD mice nor in healthy C57BL/6 mice (figure 2, paper III).

The immunomodulatory effect of sulfatide treatment in T1D prone NOD mice

We have previously shown that lyso-sulfatide, as well as C24:1 sulfatide, was more stimulatory for the type II NKT cell hybridoma XV19 than native sulfatide (figure 3, paper I). Therefore we speculated that C24:1 sulfatide may be a good candidate for activation of type II NKT cells *in vivo*.

However, even though lyso-sulfatide induced high stimulation of XV19 cells, low stimulation of primary cells has been observed (unpublished data), suggesting that lyso-sulfatide may not be the optimal choice for CD1d-dependent immunomodulation to ameliorate disease in NOD mice. When testing spleen cells derived from C57BL/6 mice and non diabetic NOD mice we found that C24:1 sulfatide gave rise to somewhat increased stimulation compared to native sulfatide (figure 3A, paper III). In addition, an increased proliferative response was observed by NOD cells compared to cells from C57BL/6 mice, suggesting that C24:1 sulfatide is a good candidate for sulfatide treatment of NOD mice.

Several studies using the type I NKT cell ligand α -GalCer have demonstrated a protective effect on T1D progression in NOD mice, when administered at different time points [236]. Further, native sulfatide has previously been shown to modulate autoimmune diseases, infections and tumor immunity [30, 165, 237, 238], suggesting that sulfatide administration is able to regulate immunity in a range of different immune settings. We selected a treatment protocol, which was used successfully with α -GalCer and its analogs in NOD mice [236]. Hence we injected native sulfatide i.p. twice a week during three weeks starting with five or eight week old NOD mice. At the age of five weeks, infiltrates of autoaggressive T cells start to appear in the pancreas [239], indicating T1D initiation, albeit without clinical manifestation. Injecting sulfatide from this early time point did not influence the progression of disease (figure 4A, paper III). In addition, when administering sulfatide to eight week old mice, a time point when NOD mice normally have considerable infiltration in the pancreas also failed to influence the disease progression (figure 4B, paper III). Thus, we did not observe reduced T1D development in NOD mice after sulfatide administration at different time points using the present protocol. However, a comprehensive review has

demonstrated the complexity of modulating T1D in NOD mice [240]. The timing of distinct treatments to achieve optimal protection of T1D varies extensively with the particular treatment. Therefore it is possible that the lack of effect by sulfatide in our hands may be due to a sub-optimal administration protocol, including an inappropriate age of treated mice. At this time, Subramanian and colleagues published that native sulfatide, administered i. p. once a week for a three week period to twelve week old NOD mice significantly reduced spontaneous development of T1D in NOD mice [241], suggesting a role for sulfatide reactive type II NKT cells in modulating T1D progression in NOD mice. However, when we used the same treatment protocol, native as well as the highly stimulatory sulfatide isoform C24:1 failed to reduce the T1D incidence (figure 11). Therefore, at this time, it is unclear to what extent sulfatide treatment is able to reproducibly modulate T1D in NOD mice, and further investigations are required to resolve this issue.

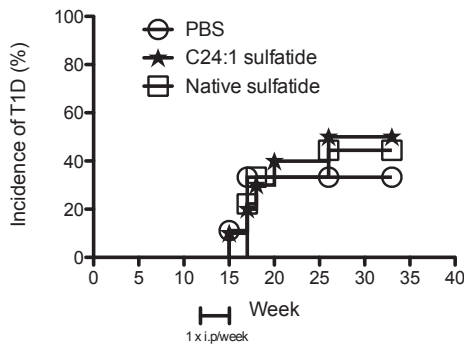


Figure 11. Sulfatide did not reduce T1D incidence when administered to 12 week old NOD mice. 25 nmol C24:1 or native sulfatide were administered i. p. to NOD mice once a week for a three week period starting at twelve weeks of age.

The effect of sulfatide treatment in *S. aureus* infection (Paper IV)

NKT cells did not influence the course of *S. aureus* induced sepsis

Due to the fact that NKT cells rapidly and robustly produce a range of different cytokines [74], such as IFN- γ and other pro-inflammatory cytokines, we speculate that NKT cells can play a role in the dysregulated immune response during *S. aureus* induced sepsis. In the generalized Shwartzman reaction, an experimental model for sepsis in which a lethal shock syndrome is induced by two consecutive injections of LPS [191-193], type I NKT cells have a pathogenic role. It was demonstrated that mice lacking type I NKT cells had significantly increased survival and lower levels of serum IFN- γ and TNF- α , suggesting that type I NKT cells promote an excessive pro-inflammatory response with increased lethality in this endotoxic shock model [242]. In paper IV, we investigated whether NKT cells play a role in *S. aureus* induced sepsis in a mouse model developed to more closely represent the human life threatening sepsis condition caused by systemic infection by *S. aureus* [212] [213].

We first investigated whether NKT cells (see figure 12A-B) were activated during *S. aureus* sepsis.

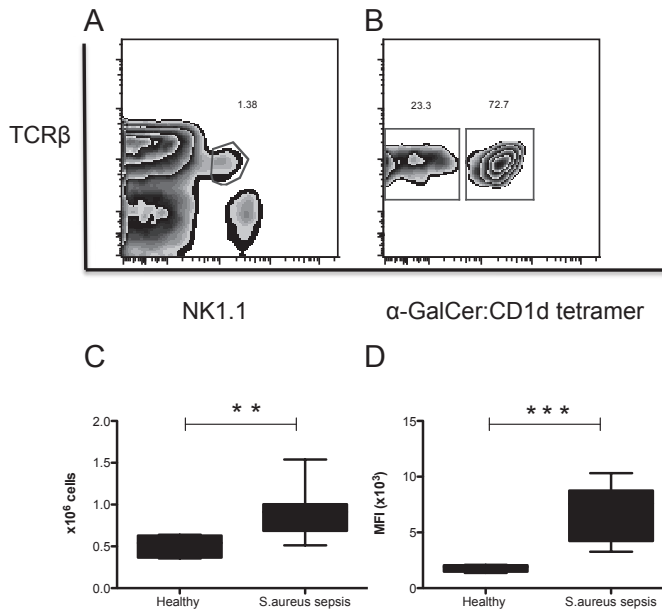


Figure 12. Activated type I NKT cells accumulate in spleen, three days post *S. aureus* infection. (A) Live, B220⁻TCR β ⁺NK1.1⁺ were gated for further analysis of (B) α -GalCer (PBS57) tetramer negative and positive cells. (C) Absolute numbers of type I NKT cells in spleen (D) Mean fluorescence intensity of CD69 on splenic type I NKT cells.

We could demonstrate that the numbers of type I NKT cells (figure 12C) as well as the α -GalCer-negative, NK1.1⁺TCR β ⁺ cells, most likely containing a large fraction of type II NKT cells, were increased in spleen and liver three days post infection. Further, these cells demonstrated an increased expression of the activation marker CD69, suggesting that NKT cells become activated and expand in spleen and liver during the course of *S. aureus* infection (figure 12D). However, we observed no effect on the severity of sepsis in mice lacking type I NKT cells (J α 18^{-/-}), nor in mice deficient in all NKT cells (CD1d^{-/-}) (figure 2A-B, paper IV), suggesting that even though NKT cells are activated by the infection, they do not influence the survival of *S. aureus* infected mice.

Sulfatide attenuates experimental *S. aureus* sepsis through type II NKT cells

In the Shwartzman reaction, activation of type I NKT cells by administration of α -GalCer could in fact replace the priming dose of LPS demonstrating the early pathological effect induced by type I NKT cells [243]. In terms of sulfatide, it has been shown to inhibit HIV replication in mice [166], suggesting that sulfatide, through its activation of sulfatide reactive type II NKT cells can modulate the immune response to infections. Therefore, we investigated the immunomodulatory role of sulfatide in *S. aureus* induced sepsis and were able to show that sulfatide, administered one hour before and three days post inoculation of *S. aureus*, significantly improved the survival rate of infected mice (figure 3A-B, paper IV). The protective effect induced by sulfatide was observed both in moderate and severe sepsis, demonstrating the effectiveness of sulfatide treatment. One dose of sulfatide on day three post infection did not mediate protection (figure 3C, paper IV), demonstrating lack of therapeutic effect of this single dose of sulfatide. However, the positive effect of two administrations of sulfatide encourages further studies to optimize the timing of the primary and secondary sulfatide dose to achieve a therapeutic effect by sulfatide in *S. aureus* sepsis. Further, the amelioration by sulfatide treatment of *S. aureus* sepsis required CD1d, but was lost in $J\alpha 18^{-/-}$ mice (figure 4, paper IV). This suggests that significant protection was mediated by type II NKT cells, and independent of type I NKT cells, in this model.

Mice inoculated with *S. aureus* and treated with sulfatide demonstrated similar bacterial counts in blood, liver and kidneys three days post infection (figure 5A, paper IV). However, the pro-inflammatory cytokine burst induced by *S. aureus* infection was decreased after sulfatide administration. Already at day one, serum IL-6 levels were significantly reduced. The reduction of

IL-6 was more pronounced day three post infection, when a decrease in TNF- α was also observed (figure 5B-C, paper IV). This demonstrates an anti-inflammatory effect of sulfatide at early time points. In addition, sulfatide significantly increased platelet counts, suggesting that sulfatide might dampen the disseminated intravascular coagulation in *S. aureus* sepsis (figure 6A, paper IV). Notably, the pro-inflammatory burst was reduced by sulfatide treatment, yet the bacterial count was similar in treated and untreated mice, indicating that sulfatide treatment limited adverse effects of inflammation, albeit without diminishing the control of bacterial growth, which is promising for future development of putative clinical applications.

CONCLUDING REMARKS

The naturally activated steady state phenotype is a hallmark of NKT cells and a consequence of their unique developmental program in the thymus, which involves selection by self-lipids. As a result, NKT cells rapidly exert their effector functions upon activation and are able to regulate the immune system in several immune settings, such as autoimmunity and infections. Knowing how NKT cells are activated is necessary for understanding their immunoregulatory ability. As for today, most information concerns the type I NKT cells, due to the fact that efficient reagents identifying type I NKT cells are available for these cells but not type II NKT cells. Importantly, studies have shown that type I and type II NKT cells are phenotypically and functionally different from each other, which emphasizes the importance of gaining more information of the type II NKT cells. Notably, data so far suggest that humans, in contrast to mice, have an increased size of the diverse (type II), CD1d restricted T cells compared to invariant type I NKT cells.

In this thesis we demonstrate that type II NKT cells are activated by the GSLs sulfatide, GlcCer and GalCer, which are all abundant GSLs in mammals. However, the autoreactivity of the type II NKT cell hybridoma XV19 was not dependent on GSLs, suggesting that self-lipids mediating steady state autoreactivity of type II NKT cells are non-GSLs. Despite the fact that the autoreactivity of type II NKT cells is independent of GSLs, one can speculate that these stimulatory GSLs might be involved in induced peripheral activation. Also, the fact that sulfatide is abundant in tissues such as pancreas and CNS, which is the target for self destruction during autoimmune type I diabetes and MS, indicates that sulfatide may activate type II NKT cells during the destruction phase of these autoimmune diseases, as has been shown in the murine EAE model. Further, the GSL sulfatide

significantly protects mice from developing severe sepsis, induced by *S. aureus* infection. The protective effect induced by sulfatide in *S. aureus* sepsis was mediated by type II NKT cells. This suggests that induced activation of type II NKT cells by administration of the GSL sulfatide provided potent immunomodulatory effects by these cells.

Altogether this thesis has expanded the knowledge on the activation of type II NKT cells and their immunomodulatory ability. The finding that the GSLs, sulfatide, GlcCer and GalCer induce efficient activation of type II NKT cells suggests a potential for effectively activate this potent immunomodulating type II NKT cell subset in different immune settings.

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