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Type II NKT cells: a distinct CD1d-restricted immune regulatory NKT cell subset

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Abstract

Type II natural killer T cells (NKT) are a subset of the innate-like CD1d-restricted lymphocytes that are reactive to lipid antigens. Unlike the type I NKT cells, which express a semi-invariant TCR, type II NKT cells express a broader TCR repertoire. Additionally, other features, such as their predominance over type I cells in humans versus mice, the nature of their ligands, CD1d/lipid/TCR binding, and modulation of immune responses, distinguish type II NKT cells from type I NKT cells. Interestingly, it is the self-lipid-reactivity of type II NKT cells that has helped define their physiological role in health and in disease. The discovery of sulfatide as one of the major antigens for CD1d-restricted type II NKT cells in mice has been instrumental in the characterization of these cells, including the TCR repertoire, the crystal structure of the CD1d/lipid/TCR complex, and their function. Subsequently, several other glycolipids and phospholipids from both endogenous and microbial sources have been shown to activate type II NKT cells. The activation of a specific subset of type II NKT cells following administration with sulfatide or lysophosphatidylcholine (LPC) leads to engagement of a dominant immunoregulatory pathway associated with the inactivation of type I NKT cells, conventional dendritic cells, and inhibition of the proinflammatory Th1/Th17 cells. Thus, type II NKT cells have been shown to be immunosuppressive in autoimmune diseases, inflammatory liver diseases, and in cancer. Knowing their relatively higher prevalence in human than type I NKT cells, understanding their biology is imperative for health and disease.

Keywords

NKT cells; CD1d; Antigen presenting cells; Lipids; Sulfatide; LPC

Introduction

Conventional or major histocompatibility complex (MHC)-restricted T cells are an integral part of adaptive immunity, which is a highly evolved and delayed response system present in higher vertebrates like mammals. In recent years, the discovery of innate-like T cells called natural killer T cells (NKT cells) has revealed a more rapid response system. The NKT cells, in contrast to conventional T cells, generally respond to lipid antigens presented on a MHCI-like molecule called CD1d by antigen-presenting cells (APCs). Broadly, there are two types

of NKT cells (Godfrey et al. 2015; Kumar and Delovitch 2014). One type expresses a semi-invariant T cell receptor (TCR) (V α 14-J α 18 in mice and V α 24-J α 15 in humans) and is called the iNKT or type I NKT cells. Type I NKT cells have been characterized primarily on the basis of their reactivity to the marine-sponge derived glycolipid α -galactosyl ceramide (α GalCer), which forms a stable α GalCer/CD1d tetrameric reagent that can be used to track them (Benlagha et al. 2000; Matsuda et al. 2000). In contrast, the other type of NKT cells, referred to as type II, use relatively diverse TCR α and β chains and are not reactive to α GalCer but recognize different lipid antigens. In this review, we will discuss some of the key distinguishing features of type II NKT cells and their role in immune modulation.

The first direct demonstration of a major subset of self-glycolipid reactive type II NKT cells was carried out in the context of an autoimmune disease when Jahng et al. discovered their reactivity to the myelin-derived glycolipid sulfatide presented in the context of CD1d, the only isoform of the CD1 family present in mice (Jahng et al. 2004). The sulfatide-reactive type II NKT cell subset was demonstrated to be distinct from type I NKT cells as well as from other non- α GalCer-reactive CD1d-restricted NKT cell hybridomas, including V α 3/V α 8+ hybridomas (Jahng et al. 2004). In addition to type II NKT cell activation, sulfatide has been shown to be presented by CD1a, CD1b, and CD1c molecules to T cell clones derived from human PBMC (Shamshiev et al. 2002). Earlier studies had indirectly demonstrated the presence of autoreactive CD1d-restricted V α 14J α 18-NKT cells expressing diverse TCR repertoire in class II MHC-deficient mice (Cardell et al. 1995). Additionally, another study also revealed broader diversity of TCR expressed by CD1d reactive murine T cell clones (Behar et al. 1999). Thus, reactivity to sulfatide was the first demonstration that a self-glycolipid is recognized by a subset of CD1d-restricted type II NKT cells in mouse and paved the way for further characterization of this important cell type.

Antigens recognized by type II NKT cells

Generally, lipids derived from mammalian or microbial sources are presented by the CD1d molecule. Classifying them on the basis of chemical nature, CD1d ligands for type II NKT cells can be broadly divided into two major subclasses: sphingolipids and glycerolipids or phospholipids (see Table 1 and Fig. 1). Historically, the first CD1d ligand to be studied in depth was the type I NKT cell activator α GalCer, originally derived from a marine sponge. Thus, synthetic α GalCer or α GlcCer (α Glucosylceramide) were able to activate V α 14+ NKT cells (Kawano et al. 1997). More recently, among self lipids, iGb3 and β -glucosyl ceramide, which are abundantly produced in mammalian cells, have been reported to be endogenous ligands for type I NKT cells (Brennan et al. 2011; Zhou et al. 2004b). However, later evidence has contested this finding by demonstrating that α GalCer is produced in miniscule proportions in mammalian cells and is in fact the active endogenous ligand for type I NKT cells (Kain et al. 2014). How this endogenous ligand, present in very low quantities, shapes the physiology and pathology of type I NKT cells remains to be investigated and will be discussed by others in this issue.

Self-glycosphingolipid antigens.

As mentioned above, the very first antigen defined for a subset of murine type II NKT cells was sulfatide, a sulfated glycolipid enriched in the membranes of various tissues, e.g., myelin of the central nervous system (CNS), pancreas, kidney, and liver. Human CD1d-reactive type II NKT cells also have been shown to be reactive to sulfatide and lysosulfatide, leading to apoptosis of renal tubular cells and cytotoxicity of epithelial cells in vitro, respectively (Fuss et al. 2014; Yang et al. 2011). Interestingly, sulfatide is not a limiting factor in the e type II NKT cells, as these cells have been found in *CST*^{-/-} and *CGT*^{-/-} mice, which are genetically deficient in the cerebroside sulfotransferase (CST) and UDP-galactose ceramide galactosyltransferase (CGT), respectively, key enzymes in the generation of the sulfatides (Arrenberg et al. 2010; Jahng et al. 2004). Similarly, a type II NKT cell hybridoma cell line was found to be highly autoreactive to splenocytes from *CST*^{-/-} mice (Blomqvist et al. 2009). These data suggest that there may be considerable cross-reactivity in self-antigens involved in the thymic positive selection of type II NKT cells. Although type II NKT cells generally express TCR α - and β -chains, recently, sulfatide-reactive CD1d-restricted T cells in peripheral human blood have been shown to also express $\gamma\delta$ TCR (Bai et al. 2012; Luoma et al. 2013).

Additionally, β GlcCer and β GalCer have been identified as self-lipid ligands that are able to activate type II NKT cells (Rhost et al. 2012; Roy et al. 2008). Interestingly, the lysoforms of glycolipids which lack the fatty acid chain were the most potent activators of the type II NKT cells. In addition, isoforms with a longer fatty acid chain (C24) were found to be better stimulators than those with C16 in these assays. A recent demonstration by Nair and coworkers has shown that two major sphingolipids accumulating in Gaucher disease (GD), β -glucosyl ceramide 22:0 and glucosyl sphingosine, are recognized by human type II NKT cells (Nair et al. 2015). In an experimental model of GD, the recognition of these sphingolipids by murine CD1d-restricted type II NKT cells also has been confirmed.

Although several mammalian glycosphingolipids have been shown to activate type II NKT cells, this has not yet been reported for microbial glycosphingolipids. Whether the microbial glycosphingolipids completely evade recognition by type II NKT cells by molecular mimicry, or small structural modifications do not allow effective formation of the lipid/CD1d-TCR complex, or the semi-invariant TCR of type I NKT cells are better suited for microbial glycosphingolipids are possibilities that remain to be investigated.

Self-glycerolipid/phospholipid antigens.

Mammalian phospholipids form another group of self-lipid antigens for type II NKT cells. They differ in structure from their microbial counterparts especially in the fatty acyl chains. These structural differences have been shown to alter recognition by the type I NKT cells (Kinjo et al. 2011; Macho-Fernandez and Brigl 2015). Similar to the glycolipids, in the case of phospholipids, the lysoform was also found to be a relatively more potent stimulator of type II NKT cells. Lysophosphatidylcholine (LPC) has been shown to be recognized by both human and murine type II NKT cells (Chang et al. 2008a; Macho-Fernandez and Brigl 2015; Maricic et al. 2014a). Notably, while LPC can be recognized by a few human type I NKT cell clones, it is not recognized by murine type I NKT cells (Fox et al. 2009; Gapin et al.

2013; Gumperz et al. 2002; Pei et al. 2011). Interestingly, the TCR repertoire of LPC-reactive type II NKT cells appears to at least partially overlap with sulfatide-reactive type II NKT cells as a sulfatide reactive type II hybridoma can also be activated by LPC (Maricic et al. 2014a). Lysophospholipids are generated following phospholipid hydrolysis and are found in high concentration at sites of inflammation (Knowlden and Georas 2014; Sevastou et al. 2013). Thus, even in the absence of external ligands, as would be present in infection, these modified ligands can potentially play a role in the regulation of inflammation-induced pathology or autoimmunity.

Non-self-lipid antigens.

One major source of natural ligands for NKT cells is provided by microbial species, which resides within the mammalian host in a steady state or during infection. Analogs for bacterial-derived glycolipids from *Sphingomonas* species were shown to stimulate both murine and human type I NKT cells (Wu et al. 2005). Subsequently, several microbial antigens from *Mycobacterium tuberculosis* or *Corynebacterium glutamicum* were identified to be ligands for type I NKT cells (Tatituri et al. 2013). Phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylinositol bound to CD1d molecules stimulate type II NKT cell hybridomas irrespective of their microbial or mammalian origins suggesting that type II NKT cell activation plays a role during infection. Recently, a similar strategy was employed to identify a microbial type II NKT cell ligand, phosphatidylglycerol, from *Listeria monocytogenes* (Wolf et al. 2015). However, in this case, the bacterial antigen was found to be more potent in activating type II NKT cells than the mammalian counterpart. The bacterial but not mammalian antigen-CD1 tetramer complex could also stain type II NKT cells. Thus, identifying novel ligands for type II NKT cells opens up doors for characterizing the role of this population in pathological settings. The availability of lipid antigens for type II NKT cells from exogenous and endogenous sources begs the question whether this represents degeneracy, molecular mimicry, or promiscuity of the TCR recognition.

Antigen processing and presentation to type II NKT cells

Mechanisms involved in the processing and presentation of lipid antigens to NKT cells can impact the outcome of an immune response. In the case of type II NKT cells, lysosulfatide has been used to investigate mechanisms for antigen processing/presentation of self-lipids (Roy et al. 2008). Similar antigen presentation mechanisms for LPC have also been shown to activate human type I NKT cells (Fox et al. 2009). The cellular compartments like endosomes and lysosomes are characterized by a reduction in pH or acidification that allows for efficient antigen processing and loading of antigen on to CD1d molecules. Roy et al. demonstrated that on one hand, acidification (pH 5 or 6) enhances type II NKT activation and on the other hand, increasing endosomal pH in the presence of monensin or concanamycin inhibits antigen presentation. However, prosaposin and saposins that help in the loading of glycolipids on to CD1d (Zhou et al. 2004a) do not have a significant role in the presentation of lysosulfatide to type II NKT cells (Roy et al. 2008). In the presence of brefeldin A, a known inhibitor of anterograde antigen transport from endoplasmic reticulum (ER) to Golgi, lysosulfatide presentation is unperturbed, thus negating a major role of CD1d

recycling. However, the role of recycling at the endosomal level is critical since a weak base called primaquine, which blocks trafficking of proteins through endosomes, inhibits lysosulfatide presentation. The relevance of protein synthetic machinery in lysosulfatide presentation to type II NKT cells was further emphasized by the fact that the inhibition of an ER-resident protein, which helps in the loading of CD1d, the microsomal triglyceride transfer protein (MTP), inhibits lysosulfatide presentation. The role of a major intracellular signaling molecule, PI3 kinase, was also established by employing the well-known inhibitor Wortmanin. In another study, the accumulation of CD1d in the lysosomal compartment owing to the lack of a triple arginine motif results in the inhibition of lipid presentation to both NKT cells without effecting cell surface CD1d expression (Shin et al. 2012). In contrast, a truncated CD1d tail inhibits antigen presentation to type I but not type II NKT cells suggesting differential modes of cellular signaling for two subsets (Chiu et al. 1999; Chiu et al. 2002; Jayawardena-Wolf et al. 2001). Since type I and type II NKT antigens bind to CD1d molecules, is there a competition for presentation to either of the two subsets? Though the binding affinity for α GalCer is highest among all the known NKT cell ligands, competitive inhibition by type II antigen for CD1d cannot be ruled out. Indeed, it has been demonstrated that sulfatide can inhibit loading of α GalCer onto CD1d in dendritic cells in vitro and in vivo (Kanamori et al. 2012). However, the physiological and pathological relevance of this inhibition remains to be ascertained.

Recognition of the lipid/CD1d complex by a type II NKT TCR

The CD1d binding groove consists of two binding pockets, the A' and the F', in which lipid antigens dock. The first demonstration of how the ceramide and the fatty acid chain in sulfatide molecules bind to CD1d was made possible by studying the crystal structure of the cis-tetracosenoyl sulfatide/CD1d complex at 1.9 Å resolution (Zajonc et al. 2005). The fatty acid chain was found to occupy the large A' pocket while the sphingosine chain was docked in the smaller F' pocket. Interestingly and in contrast to the α -linked glycosyl head group in α GalCer, the β -linked head group in sulfatide was found to be projected away from the binding groove on CD1d, allowing it to interact with the TCR. Apart from this distinction conferred by the β -linkage of the head group, another striking difference was observed by two independent groups in terms of antigen recognition by the TCRs of a type I versus type II NKT cell. For both sulfatide-CD1d and lysosulfatide-CD1d binding to a type II NKT TCR, the TCR molecule was found to be docked over the A' pocket of CD1d. However, in contrast to the type I NKT TCR, the type II NKT TCR in both cases contacted the respective lipids using the CDR3 β loop while the CDR3 α loops associated with the CD1d (Girardi et al. 2012; Girardi and Zajonc 2012; Patel et al. 2012; Rossjohn et al. 2012).

Also interesting is the fact that in spite of the lysosulfatide lacking the fatty acyl chain, it binds to the same A' pocket of CD1d as occupied by the cis-tetracosenoyl sulfatide. Since both these studies used the same TCR from a type II NKT hybridoma XV19/Hy19.3 (Jahng et al. 2004), it is not yet known whether all other type II NKT TCRs will dock onto CD1d in a similar fashion. These structural elucidations highlight the key difference between the TCR-antigen recognition of type I NKT cells and that of type II NKT cells. They also suggest a relative structural closeness between MHC-restricted and CD1d-restricted antigen recognition by conventional T cells and type II NKT cells, respectively. Thus, the type II

NKT-TCR, unlike the type I NKT-TCR, docks above the A' pocket of CD1d in an antiparallel fashion resembling the situation with conventional T cells. These structural studies have important applications for the development of NKT cell-targeted interventions in autoimmune and other inflammatory diseases.

Characteristics of type II NKT cells

Transcription factors required for their development.

The development of NKT cells is dependent on the antigen presentation molecule CD1d expressed on the cells of both non-hematopoietic and hematopoietic origin (Chun et al. 2003; Schumann et al. 2005). Similar to MHC-restricted conventional T cells, different subsets of type I NKT cells express unique combinations of transcription factors. However, unlike in conventional T cells, in all NKT cells, the transcriptional program is driven by promyelocytic leukemia zinc finger (PLZF) and Gata-3 (Brennan et al. 2013). The Th1-like type I NKT cells, associated primarily with liver and spleen, also express T-bet, while the Th17-like type I NKT cells associated with lymph nodes, the lungs, and skin express ROR γ t instead. The Th2-like type I NKT cells seem to operate in a Th2-like manner in the lungs and intestine because of a lack of coexpression of transcription factors T-bet or ROR γ t. Whether plasticity operates in type I NKT cells or whether type II NKT cells can also be subclassified in a similar fashion needs to be investigated. An investigation in the *Ja18*^{-/-} IL-4 GFP reporter mice has indicated employment of similar transcription factors in the development of type II NKT cells (Zhao et al. 2014). PLZF and an adaptor molecule called signaling lymphocyte activation-molecule associated protein (SAP) is crucial in the development of the type II NKT cells similar to type I NKT cells. It is interesting to note that in these reporter mice, TCR β + GFP⁺ cells respond to β GlcCer but not to sulfatide or phospholipids. It is not known whether this is due to the lack of TCR repertoire-reactive to sulfatide or other self-lipids in these reporter mice.

The diversity of TCR repertoire.

Type II NKT cells express a relatively diverse TCR repertoire in comparison to type I NKT cells. However, sulfatide-CD1d tetramer + cells from naïve mice predominantly use V α 3/V α 1-J α 7/J α 9 and V β 8.1/V β 3.1-J β 2.7 TCR gene segments (Arrenberg et al. 2010). The oligoclonal TCR repertoire of sulfatide/CD1d-tetramer + cells is reminiscent of a protein antigen-reactive MHC-restricted conventional T cells and is in sharp contrast to a type I NKT cell. Another contrasting TCR repertoire feature from type I NKT cells is that the CDR1 α and CDR3 α regions in sulfatide-reactive type II NKT cells were found to show variability. However, in the CDR2 β region, the potential CD1d binding tyrosine residues were conserved among members of the two NKT cell subsets and confirmed later by crystal structure of the trimolecular complex (Girardi et al. 2012). Thus, type II NKT cells possess features in the TCR repertoire of both conventional and innate-like T cells. It will be interesting to investigate whether other specific lipid antigen-reactive type II NKT cells are also oligoclonal and possess similar features for lipid recognition. Of special interest in this regard is the demonstration of type II NKT cells with oligoclonal TCR repertoire reactive to sphingolipids accumulating in Gaucher disease (Nair et al. 2015).

Responsiveness to cytokines and other environmental stimuli.

The influence of the cytokine milieu has been suggested to be critically important in the activation of NKT cells in general. However, how the two subsets respond to the cytokine environment might be different. Indeed, during infection with HBV, it was demonstrated that activation of type II NKT responding to lysophospholipids was independent of the proinflammatory cytokine IL-12, in contrast to type I NKT cells (Zeissig et al. 2012). This supports the observation that IL-12 β 1 gene expression was found to be threefold higher in type I NKT cells when compared to that in type II NKT cells in 24 $\alpha\beta$ TCR transgenic mice (Rolf et al. 2008). In this study, receptors for another proinflammatory cytokine IL-18 were expressed at similar levels in both the NKT cell subsets suggesting their similar behavior to such a stimulus. Interestingly, expression of IL-2 β (CD122) was similar but IL-2 α (CD25) expression was several fold higher in type I NKT cells. Receptors for other major proinflammatory cytokines IL-1, IFN- γ , and IL-6 were expressed at low levels on both the NKT cell subsets. However, it is likely that the levels of gene expression in the steady state may be different from that in inflammatory conditions. Type I and II NKT cells also differ in the expression of retinoic acid receptor γ (RAR γ), which was found to be expressed several fold higher in type I than in type II NKT cells (Maricic et al. 2015). Accordingly, type I NKT cells were preferentially inhibited following stimulation with a RAR γ agonist both in vitro and in vivo.

It has been shown that type I NKT cells can be activated either directly through TCR stimulation or indirectly by cytokines (IL-12, IL-18, or type I IFN) produced through Toll-like receptor (TLR)-mediated signaling in dendritic cells (DCs) (Brigl et al. 2011; Kinjo et al. 2013). Thus, type I NKT cells can be activated in the absence of TCR ligation. In contrast, TCR stimulation appears to be the main pathway for type II NKT cell activation. Using type II NKT hybridomas, TCR engagement by CD1d-presented lipid, but not TLR signaling, has been shown to be necessary for their activation (Roy et al. 2008; Tatituri et al. 2013). In support of the dependence of type II NKT activation relying on TCR engagement and not merely on TLR signaling is the fact that in many experimental conditions in which type I NKT cells are activated by TLR signaling in APCs, type II NKT cells remain unactivated (Marrero et al. 2015).

In addition to their responsiveness to cytokines, an important question which still remains to be addressed is whether there exist different subsets of type II NKT cells similar to that in the case of type I NKT cells or T helper cells like Th1, Th2, and Th17 (Kim et al. 2015). Along with distinct transcription factors, cytokine receptors are also unique in these subsets. Thus, surface expression of IL-12 α , IL-25 α , and IL-23 α is associated with Th1-like, Th2-like, and Th17-like type I NKT cells, respectively (Brennan et al. 2013). Similar to type I NKT cells, it is likely that the variants of the lipid ligands or the antigen-presenting cells can also change the cytokine secretion pattern of type II NKT cells (Kumar and Delovitch 2014). Furthermore, suppressor of cytokine signaling -1 (SOCS-1) molecule was shown to orchestrate the balance between type I and type II NKT cells in the periphery (Hashimoto et al. 2011). Peripheral NKT cells from SOCS-1-deficient mice did not respond to the type I NKT ligand α GalCer, had a marginal labeling with α GalCer-CD1d tetramers, and exhibited

significantly reduced expression of the invariant TCR while responding to the ligand sulfatide thereby confirming their phenotype to be type II NKT.

Functional role of type II NKT cells

Broadly, type II NKT cells have been demonstrated to be the innate arm of immunoregulatory T cells: the majority of their function being attributed so far to their role in inhibiting the proinflammatory functions of type I NKT cells, conventional T cells, and dendritic cells. We have described their functional role in detail in inflammation and autoimmune disease in two recent reviews (Bandyopadhyay et al. 2016; Marrero et al. 2015). Recent studies in the context of gut immunity have suggested that just like other T cells, they may not be committed to a predetermined function, but their role may be guided by the immediate tissue environment (Liao et al. 2012). Additionally, type II NKT cells can also have an important influence on the B cells: although the lack of type II NKT cells did not change the mature phenotype of B cells, alum-induced adjuvant effect on T cell-dependent antibody production was compromised (Shah et al. 2012). Here, we will briefly describe some of the key functions of type II NKT cells (Fig. 2).

Influence of APCs on function of type II NKT cells.

The function of NKT cells is influenced by CD1d-bearing APCs in different tissues, such as sialoadhesin (CD169) + macrophages (Barral et al. 2010), CD8 α + DCs in spleen and lymph nodes (Arora et al. 2014; Ko et al. 2007; Ushida et al. 2015), B cells, tumor cells, and non-hematopoietic cells like epithelial cells (Fais et al. 2004; Lang et al. 2008; Olszak et al. 2014; Rakhshandehroo et al. 2014; Schrumph et al. 2015; Stein-Streilein 2002; Yang et al. 2007). In addition, the level of CD1d expression on APC subsets and factors governing such expression like TGF- β and interferon have a profound effect on NKT cells (Raghuraman et al. 2006; Ronger-Savle et al. 2005). Apart from the CD1d mode of antigen presentation, APCs can also play crucial roles in controlling the effect of NKT cells by modulating the cytokine environment. The clear demonstration of APCs influencing type II NKT cell activation and function was validated by our previous study (Halder et al. 2007) wherein the administration of sulfatide induced a selective activation of CD11c^{int}B220⁺/PDCA-1⁺ plasmacytoid DCs (pDCs) but not of myeloid DCs (CD11c^{high}CD11b⁺). Furthermore, type I NKT cells subsequently accumulated in the liver in an IL-12 and MIP-2-dependent manner but were anergized. Additionally, anergy induction in type I NKT cells in the recipients can be adoptively transferred by CD11c + hepatic DCs (mostly cDCs) from sulfatide-treated wild type but not from animals deficient in IL-12. Studies are underway to address whether pDCs and cDCs cross-talk or whether both are required for anergy induction in the type I NKT cells. Recent literature indicates that the B220 + CD11c^{int} population also contains other cells including B cells. Toward this, our recent data also indicate that B cells are required for sulfatide-mediated activation of type II NKT cells (Halder et al., manuscript in preparation).

It has also been shown that chronic administration of α GalCer leads to anergy induction in type I NKT cells (Parekh et al. 2005). However, this anergy induction is different than that mediated following sulfatide or microbial infection. Thus, α GalCer—but not sulfatide—

microbial-mediated anergy in type I NKT cells require programmed death 1 (PD1)/PD ligand (PDL) 1 signaling (Chang et al. 2008b; Parekh et al. 2009). These interactions between type I and type II NKT cells have important implications in vaccine design where adjuvant properties of type I NKT cells are crucial.

Regulatory role of type II NKT cells in disease

Autoimmune diseases.

The first demonstration of type II NKT function was described in a murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). Jhang and coworkers employed sulfatide-CD1d tetramers for the first time to identify type II NKT cells during EAE and found them to accumulate (Jahng et al. 2004) in CNS tissue. In contrast, α GalCer-tetramer reactive type I NKT cells though also present in the CNS did not accumulate, implying a distinct function for the type II NKT cells. Indeed, treatment with sulfatide protected mice from EAE in a CD1d-dependent manner (Jahng et al. 2004). In a subsequent study, it was observed that sulfatide-mediated activation of immune regulatory pathway also inactivated the microglia population in the CNS (Maricic et al. 2014b). The mechanism of sulfatide reactive type II NKT cells in EAE involved the tolerization of conventional DCs and microglia and the inhibition of the effector function of the encephalitogenic myelin protein reactive CD4 T cells (Jahng et al. 2004; Maricic et al. 2014b).

In the case of type II NKT cell-mediated protection in spontaneously diabetic non-obese diabetic (NOD) mice, two mechanisms were demonstrated. In the first, type II NKT cells were found to be protective by the secretion of IFN- γ and involving costimulators inducible costimulator (ICOS) and PD-1 (Duarte et al. 2004; Kadri et al. 2012). In another demonstration, the type II NKT ligand sulfatide, especially the C24 isoform, was found to be protective by inducing the anti-inflammatory cytokine IL-10 from DCs (Subramanian et al. 2012). Notably, an enrichment of sulfatide-tetramer + type II NKT cells was also observed in the pancreatic nodes of the NOD mice during active diabetes, similar to that of their accumulation in the CNS during EAE (Jahng et al. 2004). Since commensal microbiota has a major impact on NKT cells, it is likely that this may influence the activity of type II NKT cell-mediated protection of spontaneous diabetes (Rhost et al. 2014).

During investigations in systemic lupus erythematosus (SLE), the role of type II NKT cell has been reported as either pathogenic (secreting IFN γ and of splenic origin) or protective (secreting IL-4 and from bone marrow) (Zeng et al. 1998). Our unpublished data on the administration of the type II NKT ligand sulfatide supports a protective role of these cells in SLE-like disease in (*NZB X NZW*) F1 mice (Halder R et al. unpublished observation).

Inflammation-induced pathology.

The liver constitutes a large depot for both NKT cell subsets where they play crucial and opposing roles in non-microbe-mediated hepatic inflammatory diseases (Bandyopadhyay et al. 2016; Kumar 2013). This cross-regulation between type I vs. type II NKT cells was first demonstrated by our group in the Concanavalin A-induced murine hepatitis model (Halder

et al. 2007). The sulfatide-mediated anergy induction in type I NKT cells in liver results in a significant decrease in the cytokine burst and inflammatory cascade following challenge with ConA. Using the same liver disease model, we further demonstrated this cross-regulatory mechanism using another type II NKT cell ligand, LPC (Maricic et al. 2014a). Further, we investigated whether this mechanism is also employed during sterile hepatic inflammation and injury. During liver transplant or hepatic surgeries, ischemic reperfusion injury is a major complication and represents a model for sterile inflammation. We demonstrated that in *Ja18*^{-/-} mice, lacking type I but not type II NKT cells, ischemic reperfusion injury was significantly diminished compared to wild type (Arrenberg et al. 2011). Moreover, a similar reduction in disease in *Ja18*^{-/-} mice was observed in the alcohol-induced liver disease (Maricic et al. 2015). Sulfatide-mediated activation of type II NKT cells in both models decreased the accumulation of proinflammatory myeloid cells and neutrophils and consequently inhibited inflammation and liver injury. Additionally, direct inhibition of type I NKT cells by administration of all-trans retinoic acid or a RAR- γ agonist resulted in protection from alcoholic liver disease (ALD) as well as from CCL4-induced liver fibrosis (Maricic et al. 2015). Sulfatide-mediated protection was also demonstrated in murine models of ischemic-reperfusion injury in kidney and in asthma (Yang et al. 2011; Zhang et al. 2011).

It is important to mention that unlike the type I NKT antigen α GalCer, the route of administration plays an important role in activating the sulfatide-reactive type II NKT cells and the subsequent anergy induction in type I NKT cells. Sulfatide administration only by the intraperitoneal route, not intravenous or subcutaneous routes, results in inactivation of type II NKT cell (Maricic et al. 2014b). This raises the question of whether activation of type II NKT cells by sulfatide requires a specific cell subset present in the peritoneal cavity. For example, it is not known whether water-insoluble lipids require specific lipid-binding proteins, antibodies, or other specialized cells, including B1 cells enriched in the peritoneal cavity that helps in its transport or presentation to type II NKT cells.

In addition to antigen-mediated activation of a specific subset of type II NKT cell population, several studies have used bulk population from *Ja18*^{-/-} mice to indirectly examine their role in different experimental conditions. For example, in a murine model of graft versus host disease (GVHD), both IFN- γ and IL-4-producing type II NKT cells provide protection using different mechanisms (Kim et al. 2007). Thus, IFN- γ -producing type II NKT cells induced apoptosis of donor cells, while IL-4-secreting type II NKT cells skewed the response toward a Th2-phenotype. Interestingly, in another study, it was found that IL-25 treatment had a beneficial effect in high-fat diet-induced obesity and caused infiltration of innate cells into adipose tissue including type II NKT cells. Furthermore, the adoptive transfer of type II NKT cells in obese mice improved weight loss and stabilized glucose homeostasis in recipients (Hams et al. 2013).

In Gaucher disease in humans, a novel type II ligand-specific NKT cell was also suggested to contribute to the B cell-mediated pathological response (Nair et al. 2015). Similarly, in gut tissue, type II NKT cells may have a colitogenic role. Transgenic mice overexpressing an autoreactive TCR from a CD1d-reactive type II NKT cell hybridoma spontaneously developed colitis (Liao et al. 2012).

Adoptive transfer of these cells also resulted in the development of colitis in the recipients. Consistently, in ulcerative colitis (UC) in humans, one of the two major forms of inflammatory bowel disease (IBD), lysosulfatide/CD1d-tetramer + and IL-13-secreting cells from the lamina propria are thought to have a proinflammatory or colitogenic role (Fuss et al. 2014).

Immune surveillance of cancer.

The initial indirect evidence indicating an important role for type II NKT cells in tumor immunoregulation came when Terabe et al. demonstrated that CD1d-mediated presentation and not CD4 + CD25 + Treg lead to suppression of antitumor responses in the murine models of colon carcinomas, fibrosarcoma, and mammary carcinoma. In *Ja18*^{-/-} mice, such suppression was maintained thereby leading to the conclusion that type II NKT cells were involved in immunoregulation (Terabe et al. 2005). In a subsequent study, using specific induction of type II NKT cells with sulfatide, a confirmation of their role in a more deterministic way was obtained (Ambrosino et al. 2007). The latter work also demonstrated the mutual antagonism of type I and type II NKT cells in affecting the control of antitumor responses. Additional evidence of a suppressive role for type II NKT cells against immunosurveillance in B cell lymphoma was found to be mediated by induction of myeloid-derived suppressor cells (CD11b + Gr1+) (Renukaradhya et al. 2008) leading to protection (Teng et al. 2009). A role for type II NKT cells as well as CD25+ Tregs in immunosuppression was also found in models of colorectal and renal cancer (Izhak et al. 2013). This suggests a collaborative mechanism in between the two NKT cell subsets and the conventional Tregs.

Infectious diseases.

A dual role for type II NKT cell participation in the anti-infectious immune response and infection-induced inflammation has been demonstrated in certain viral, bacterial, and parasitic infections (Table 2).

A pathogenic role for type II NKT cells in hepatitis B virus (HBV) infection was observed more than a decade ago when NK1.1-positive NKT cells were found to accumulate in the liver of HBV-transgenic mice with acute hepatitis (Baron et al. 2002). These NKT cells were found to be non-reactive to α GalCer and did not express V α 14 and mediated hepatitis when adoptively transferred. In contrast, using mice deficient in both type I NKT and type II NKT cells (*CD1d*^{-/-}) and mice deficient in only type I NKT cells (*Ja18*^{-/-}), a protective function was demonstrated in infection with diabetogenic encephalomyocarditis virus (EMCV-D) and in HBV infection (Exley et al. 2001; Zeissig et al. 2012). In the case of EMCV-D, a higher severity of disease was observed in *CD1d*^{-/-} when compared to wild-type (WT) or *Ja18*^{-/-} mice. While there was a gross deficiency in the activation of both CD4+ and CD8+ T cell immunity in *CD1d*^{-/-} mice infected with HBV, in *Ja18*^{-/-} animals, only the CD4 + T cell compartment was affected. The lack of CD8 + T cell activation resulted in higher pathology strongly suggesting that type II NKT cells play a protective role in this instance. Further evidence of a protective role of type II NKT cells was obtained in human immunodeficient virus-1 (HIV-1) infection, when specific activation with sulfatide reduced viral replication in humanized severe combined immunodeficiency (SCID) mice

(Sundell et al. 2010). In humans, CD1d was found to be upregulated in hepatitis C-infected liver while type II NKT cells produced IFN- γ and small amounts of IL-13 (Durante-Mangoni et al. 2004; Exley et al. 2002). Type II NKT cells have also been shown to complement antiparasite protective immune responses in schistosomiasis and *Trypanosoma cruzi* infection (Macho-Fernandez and Brigl 2015).

Although several bacterial antigens have been shown to be ligands of type II NKT cells as described earlier, evidence for bacterial infection relying on type II NKT cells is still minimal. Both *Staphylococcus aureus* and *Salmonella typhimurium* infection have been shown to be independent of NKT cells (Berntman et al. 2005; Kwiecinski et al. 2013). However, treatment with sulfatide in *S. aureus* infection conferred protection in a CD1d-dependent and type I NKT cell-independent way supporting a protective role for the activation of type II NKT cell by its ligand sulfatide (Kwiecinski et al. 2013). Whether activation of type II NKT cells with specific ligands can cause modulation of *Salmonella typhimurium* infection remains to be seen. Of particular interest is the fact that *Salmonella typhimurium* is a well-known enteric pathogen which causes colonic inflammation in mice and gastroenteritis in humans. As mentioned earlier, bulk type II NKT cells have been demonstrated to play a pathogenic role in spontaneous colitis (Liao et al. 2012). Whether a role for sulfatide reactive type II NKT cell is present in *Salmonella typhimurium*-mediated colitis remains to be determined. Interestingly, sulfatide-treated *Salmonella typhimurium* infection was recently shown to exhibit increased human neutrophil apoptosis in vitro suggesting a role for sulfatide in the *Salmonella typhimurium* immune interaction which needs to be further investigated in vivo (Grishina et al. 2015).

Conclusion and future directions

Type II NKT cells form a unique niche in the cellular repertoire of the immune regulatory response. They express diverse TCRs and as a result, their study has been limited by this very nature of this diversity. There is no single unique molecule or a set of molecules, which define the phenotype or function of this cell subset as a whole. Thus, tracking type II NKT cells experimentally or in humans remains quite challenging. Utilization of sulfatide-tetramers has helped greatly in studying antigen-specific type II NKT cell subsets in different conditions. However, unlike the α GalCer/CD1d-tetramers, sulfatide/CD1d-tetramers are highly unstable and hence difficult to use. Future technology in type II NKT cell investigation may rely largely on identifying relatively specific cell surface markers and ligands enabling activation of a specific subset of type II NKT cells.

The diverse nature of the type II NKT TCR repertoire as well as how the TCR docks onto CD1d may not be unique as it has recently been shown also to mirror even in an atypical type I NKT cell (Le Nours et al. 2016). If commensal flora could provide ligands for type II NKT cells, it is likely that they may behave differently in gut tissues. Thus, while being anti-inflammatory in the liver, type II NKT cells in the gut may play a pathogenic role. Thus, how local milieu in different tissues may condition their activity differently still need to be studied.

The fact that type II NKT cells are more numerous than type I NKT cells in humans makes them attractive candidates for investigating their role in human physiology and pathology. It will be important to develop specific reliable reagents to identify and characterize type II NKT cells in humans and also to use lipid/CD1d-tetramers to distinguish their activation in peripheral blood and in other tissues. Recent investigations leading to the identification of a novel ligand in Gaucher disease is a strong case in point and should serve as a prototype for future studies. Finally, immune regulatory properties of type II NKT cells using stable analogs with better bioavailability of sulfatide or LPC should be exploited for potential therapeutic interventions in various inflammatory and autoimmune disorders.

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Fig. 1.

Examples of lipid antigens known to stimulate type II NKT cells. Chemical structures of some of the mammalian self-lipids recognized by the CD1d-restricted type II NKT cells are shown. Lysosulfatide and cis-tetracosenoyl sulfatides have been used to study the crystal structure of lipid/CD1d-TCR complex

Immunoregulatory mechanism(s)

- cDC tolerization
- Type I NKT anergy or inhibition
- Neutrophil inhibition
- Inhibition of Th1/Th17
- Inhibition of hepatic stellate cell activation
- pDC activation
- B cell interactions
- Co-stimulator ICOS/PD-1-mediated regulation
- Microglial Inactivation
- Cytokine (IL-4/IFN- γ /IL-25)-mediated regulation

- Autoreactive TCR transgenic and IL-13/IL-13R+ cells in Ulcerative colitis

Fig. 2.

Immunoregulatory properties of type II NKT cells in various autoimmune and inflammatory conditions. Broadly, self-lipid-mediated activation of type II NKT cells induces a dominant immunosuppressive mechanism maintaining tolerance in several tissues including liver and confers protection against autoimmune and inflammatory diseases. Some of the disease conditions are mentioned in parenthesis along with each affected tissue. Liver: concanavalin A-induced hepatitis, alcoholic liver disease (*ALD*), ischemic reperfusion injury (*IRI*); central nervous system (*CNS*): experimental autoimmune encephalomyelitis (*EAE*), a murine model of multiple sclerosis; airway: allergic inflammation; pancreas: type I diabetes in non-obese diabetic (*NOD*) mice; bone marrow in a murine model of graft versus host disease (*GVHD*); kidney: glomerulonephritis in mice and *IRI*; adipose tissue: high-fat diet-induced obesity; colon: spontaneous colitis in transgenic mice and a correlation of IL-13R expression in ulcerative colitis (*UC*) patients; various models of cancer. In some cases like colitis, type II NKT cells may have a proinflammatory role. **Bold blue arrows** depict anti-inflammatory, and **red-dashed arrow** suggests a potential proinflammatory mechanism

Table 1

Lipid antigens recognized by type II NKT cells

Source of lipid	Chemical nature	Example
Self	Sphingolipids	<ul style="list-style-type: none"> • Sulfatides and lysosulfatide • βGlcCer • Glucosylsphingosine • βGalCer
	Glycerolipids/phospholipids	Major component of the cell membrane <ul style="list-style-type: none"> • Phosphatidylinositol • Phosphatidylethanolamine • Phosphatidylglycerol • Lysophospholipids like LPC, LPE, lysosphingomyelin, lysoplatelet-activating-factor
Non-self	Sphingolipids	Not known
	Glycerolipids/phospholipids	<ul style="list-style-type: none"> • Phosphatidylglycerol (<i>Mycobacterium tuberculosis</i>, Mtb); • Phosphatidylglycerol (PG), di-phosphatidylglycerol (DPG), phosphatidylinositol (<i>Corynebacterium glutamicum</i>, Cg); • Unidentified apolar lipids from Mtb and Cg • PG and DPG from <i>Listeria monocytogenes</i>
Non-self and non-microbial	Phospholipids	• Pollen-derived lipids for gamma delta TCR

Table 2

Involvement of type II NKT cells in microbial infections

Types of infection	Example of infectious species	Influence on disease outcome	references
Viral	<ul style="list-style-type: none"> • EMCV-D • HIV-1 • HBV 	<ul style="list-style-type: none"> • Enhanced disease in <i>CD1d</i>^{-/-} mice in comparison with WT or <i>Ja 18</i>^{-/-} mice • Sulfatide administration reduced viral burden in humanized SCID mice. • a. Induced acute hepatitis • b. Reduced T cell immunity and increased pathology in <i>CD1d</i>^{-/-} compared to <i>Ja 18</i>^{-/-} mice 	<ul style="list-style-type: none"> • Exley et al. (2001) • Sundell et al. (2010) • a. Baron et al. (2002) • b. Zeissig et al. (2012)
Bacterial	<ul style="list-style-type: none"> • <i>Staphylococcus aureus</i> • <i>Salmonella</i> • Typhimurium 	<ul style="list-style-type: none"> • Sulfatide induces protection from sepsis, but no change in lethality was not altered in <i>CD1d</i>^{-/-} or <i>Ja 18</i>^{-/-} mice. • Lethality was not altered in <i>CD1d</i>^{-/-} mice. 	<ul style="list-style-type: none"> • Berntman et al. (2005) • Kwiecinski et al. (2013)
Parasitic	<ul style="list-style-type: none"> • <i>Schistosoma</i> • <i>Trypanozoma cruzi</i> 	<ul style="list-style-type: none"> • Promotes a Th2 response and this response is decreased in <i>CD1d</i>^{-/-} mice. • Increased sensitivity and pathology in <i>Ja 18</i>^{-/-} mice in comparison with WT or <i>CD1d</i>^{-/-} mice 	<ul style="list-style-type: none"> • Faveeuw et al. (2002); • Magalhaes et al. (2010); • Mallevaey et al. (2007) • Duthie et al. (2005)