



Type II NKT Cells: An Elusive Population With Immunoregulatory Properties

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Natural killer T (NKT) cells are unique unconventional T cells that are reactive to lipid antigens presented on the non-polymorphic major histocompatibility class (MHC) I-like molecule CD1d. They have characteristics of both innate and adaptive immune cells, and have potent immunoregulatory roles in tumor immunity, autoimmunity, and infectious diseases. Based on their T cell receptor (TCR) expression, NKT cells are divided into two subsets, type I NKT cells with an invariant TCR α -chain (V α 24 in humans, V α 14 in mice) and type II NKT cells with diverse TCRs. While type I NKT cells are well-studied, knowledge about type II NKT cells is still limited, and it is to date only possible to identify subsets of this population. However, recent advances have shown that both type I and type II NKT cells play important roles in many inflammatory situations, and can sometimes regulate the functions of each other. Type II NKT cells can be both protective and pathogenic. Here, we review current knowledge on type II NKT cells and their functions in different disease settings and how these cells can influence immunological outcomes.

Keywords: type II NKT cells, CD1d, sulfatide, T cell receptor, health and disease

INTRODUCTION

Although conventional MHC-restricted T cells have been the main attraction for immunologists, unconventional T cells are continuously gaining increased attention. Despite of their low frequencies compared to conventional T cells, the subsets of unconventional T cells play an important role in various autoimmune diseases, cancers and infections, and are present both in human and mice. These cells include CD1d-restricted NKT cells, $\gamma\delta$ TCR expressing T cells, and MR1-restricted mucosal associated invariant T cells (1). NKT cells are reactive to lipid antigens presented on the non-polymorphic MHC class I-like molecule CD1d (2). These cells are activated early in immune responses and can express immunoregulatory activities that determine different immune outcomes.

In contrast to conventional T cells that express highly diverse TCR, NKT cells appear comparatively limited in its TCR repertoire and ligand reactivities. Based on the nature of their TCR expression, NKT cells comprise two main categories, invariant or type I NKT cells, and diverse or type II NKT cells. Type I NKT cells, the most extensively studied subgroup, express a semi-invariant V α 14-J α 18 TCR in mice, and V α 24-J α 18 in humans paired with a limited repertoire of V β -chains (V β 8.2, V β 7, and V β 2 in mice and V β 11 in humans) (1, 3), while the less explored type II NKT cells utilize a more diverse TCR repertoire (4–7). In mice, type I NKT cells outnumber type II NKT cells,

while in humans the type II NKT cells are more frequent. Type I NKT cells are reactive to the marine sponge-derived glycolipid α -galactosylceramide (α -GalCer) (8), which forms a stable α -GalCer/CD1d tetramer reagent that can be used for detection of type I NKT cells (9, 10). Availability of α -GalCer/CD1d tetramers opened up new avenues of NKT cell research and laid the foundation for important findings in the field of immunoregulation. In contrast, type II NKT cells are not reactive to α -GalCer (11, 12), instead they are thought to make up an oligoclonal population that recognizes a diverse repertoire of lipid antigens (5, 7, 13). As ligand/CD1d tetramer reagents only recognize a fraction of type II NKT cells, and due to the lack of specific surface markers, it is currently not possible to identify the entire type II NKT cell population using single or combined reagents. Instead, type II NKT cells are often defined by indirect approaches. One way of identifying this population is by their CD1d-reactivity and absence of the V α 14 (mouse) or V α 24 (human) TCR α -chains. In a few cases, ligands recognized by type II NKT cells can be loaded on CD1d-tetramers and used to identify subsets of the population (14). Type II NKT cells are abundant in liver of both mice (15) and human (16), although the frequency of the entire type II NKT cell population in different organs is still unknown. However, recent advances in the field have highlighted the importance of type II NKT cells in different diseases. In this review, we discuss recent progress in the study of type II NKT cells and their critical role in different disease settings.

ANTIGENS FOR TYPE II NKT CELLS

MHC-II-deficient mice facilitated the discovery of type II NKT cells, when it was noted that while conventional CD4⁺ cells were absent, there was still a significant population of peripheral CD4⁺ cells (4). Among CD4⁺ T cell hybridomas developed from these mice, several were CD1d-autoreactive and they expressed different TCR. These and other type II NKT cell hybridomas were instrumental for the identification of different lipid antigens (5, 11, 15, 17–19). With the identification of more CD1d-restricted ligands for type II NKT cells, the ligands could be categorized into different subtypes - sphingolipids and glycerolipids or phospholipids (7, 20). As α -GalCer is not recognized by type II NKT cells, taken together, this suggests that type I NKT cells and type II NKT cells react to different sets of CD1d-presented antigens, consistent with their distinct TCR, although there are some ligands that are recognized by both NKT subsets.

Consistent with the CD1d-autoreactivity of many type II NKT cell hybridomas, several self lipids have been defined as ligands. Sulfatide, a well studied ligand for type II NKT cells, was first identified as a ligand for the type II NKT cell hybridoma XV19 expressing a V α 1/V β 16 TCR (4, 15, 19). Subsequently, type II NKT cell reactivity to sulfatide has been shown by independently derived hybridomas (15, 19, 21). Structural analysis of the XV19 TCR in complex with CD1d and sulfatide revealed similarities to both TCR-MHC interactions and type I NKT TCR-CD1d (22, 23). A significant population of sulfatide/CD1d tetramer-reactive cells, around 0.2–4% in different organs, were identified

in naive mice (15). Sulfatide reactive cells displayed diverse TCR with oligoclonal expansions, and preferentially used 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 segments (24). While type I NKT cells possess germline-encoded CDR3 region of the TCR α chain, type II NKT cells demonstrated predominantly non-germline but also germline encoded CDR3 of TCR α and TCR β chains (24). The substantial fraction of sulfatide/CD1d-reactive cells, and the different TCR used for CD1-restricted sulfatide reactivity emphasized the diverse but oligoclonal nature of the TCR of these cells. Using XV19 hybridoma reactivity, we demonstrated that lysosulfatide, which lacks the fatty acid chain, was the most stimulatory sulfatide isoform, followed by C24:1 and C24:0 (19). Surprisingly, mice genetically deficient in cerebroside sulfotransferase (CST^{-/-}) or UDP-galactose ceramide galactosyltransferase (CGT^{-/-}), key enzymes required in the synthesis of sulfatides, showed the presence of sulfatide reactive type II NKT cells (15, 24). These findings suggest that sulfatide is dispensable for the development of sulfatide-reactive type II NKT cells. Further, the sulfatide reactive XV19 hybridoma showed high autoreactivity to splenocytes from CST^{-/-} mice (19). These findings may be explained by reactivity to several ligands by one TCR, shown by the fact that the self-lipids β -glucosylceramide and β -galactosylceramide as well as self-phospholipids can activate the sulfatide reactive XV19 type II NKT cells (17, 25).

Using lipid loaded tetramers, significant populations of human and mouse type II NKT cells were identified reactive to β -glucosylceramide and glucosylsphingosine, lipids that accumulate in Gaucher's disease (26). Sequencing of TCR β -chains of the human tetramer positive cells revealed diverse TCR. Lysophosphatidylcholine (LPC) is another ligand recognized by both murine and human type II NKT cells (25, 27, 28). LPC is also recognized by a few human type I NKT cells, but not by murine type I NKT cells (29–31).

Type II NKT cells have also been shown to react to ligands of microbial origin. Phosphatidylglycerol, diphosphatidylglycerol (or cardiolipin), and phosphatidylinositol from *Corynebacterium glutamicum* or *Mycobacterium tuberculosis* can activate various type II NKT, but not type I NKT, hybridomas, in a CD1d-dependent manner. The specificities of these hybridomas for different lipid antigens were distinct, albeit partially overlapping (18). In a subsequent study, a more potent phosphatidylglycerol antigen from *Listeria monocytogenes* was identified that had a structure distinct from previously identified mammalian or microbial variants, as it contains short, fully saturated anteiso fatty acid lipid tails (32). Type II NKT cells may not only recognize lipid antigens but a recent study suggests that they also can recognize hydrophobic peptides presented on CD1d (33, 34). A report by Nishioka et al. found that a rat type II NKT cell clone, reactive to vascular endothelial cells, could recognize a CD1d-presented hydrophobic peptide derived from sterol carrier protein 2, a protein implicated in intracellular lipid transfer.

Considering the relatively large populations of primary type II NKT cells that recognize identified lipid ligands, one may speculate that the number of antigens recognized by type II NKT cells is limited. On the other hand, a single type II NKT cell TCR can bind several different antigens. Further, the type II

NKT cells that bind a given lipid/CD1d-tetramer have diverse but oligoclonal TCR. Therefore, type II NKT TCR show degeneracy for antigen recognition. Importantly, the TCR repertoire of type II NKT cells appears to largely overlap between mice and human.

MODES OF TYPE II NKT CELL ACTIVATION

Having characteristics of both NK and T cells, NKT cells can respond to either innate (TCR-independent) or adaptive (TCR-dependent) stimulation. Type I NKT cells not only respond very rapidly to stimulation through TCR by secreting diverse cytokines but also to cytokines (IL-12, IL-18, and type I IFN) alone or produced by Toll-like receptors (TLR)-activated DCs, in the absence of TCR-engagement (35). However, information regarding the activation of type II NKT cells is still scarce. In contrast to type I NKT cells, lysophospholipid-reactive type II NKT cell activation was independent of IL-12 during hepatitis B virus (HBV) infection (28). A subset of type II NKT cells were shown to respond with partially CD1d-independent IFN- γ production when co-cultured with CpG stimulated DC (36). This implies TCR-independent activation of type II NKT cells on the one hand, but also suggests that TLR activation of DC may have upregulated type II NKT cell ligands on CD1d. It therefore seems feasible that like type I NKT cells, type II NKT cells are not limited to activation by TCR-engagement but can also be activated independently of TCR in a proinflammatory cytokine milieu. However, this needs to be directly addressed. Thus, type II NKT cells can be activated through the TCR by exogenous antigens, such as microbial lipids, or self-lipids that may be upregulated on CD1d in activated DC. Moreover, they can likely be activated indirectly by pathogen derived or endogenous TLR-ligands acting on DC, or by inflammatory cytokines independently of the TCR. It is likely that under most circumstances, both TCR-engagement and TCR-independent stimulatory signals contribute to type II NKT cell activation.

FEATURES OF TYPE II NKT CELLS

Type I and type II NKT cells share several features that makes them different from conventional T cells, but the two subsets often have distinct functions in specific immune reactions (5, 7, 13). Transcription factors play an important role in the development of MHC-restricted conventional T cells, and their combinations guide the functional profile of these cells. The transcription factor promyelocytic leukemia zinc finger (PLZF), induced by TCR signaling by agonist self-ligands after positive selection, is crucial for the development of type I NKT cells (37, 38). Type I NKT cells can be sub-grouped into distinct functional subsets based on the combinations of transcription factors such as PLZF, T-bet and ROR γ t: NKT1 (PLZF^{low}T-bet⁺), NKT2 (PLZF^{hi}T-bet⁻) and NKT17 (ROR γ t⁺) cells that secrete TH1-, TH2-, and TH17-like cytokine patterns, respectively, upon activation (39, 40). Whether type II NKT cells follow similar developmental pathways and can be sub-grouped in similar functional subsets is not yet clearly understood. Studies have found that at least a subset of type II NKT cells have a constitutive

production of IL-4 like type I NKT cells, and these type II NKT cells could be identified as IL-4-reporter⁺ cells that did not bind the α -GalCer/CD1d-tetramer, or were present in mice lacking type I NKT cells (28, 36). These type II NKT cells exhibited similar developmental requirements as type I NKT cells - PLZF along with signaling lymphocyte activation molecule-associated protein (SAP) played a crucial role (36). The cells were PLZF^{hi} and had an activated phenotype (CD44⁺, CD62L⁻, CD69^{hi}) comparable to type I NKT cells. In other studies, mouse and human type II NKT cells were described as PLZF^{int} and having a more resting phenotype (24, 26, 41, 42). In addition, several reports suggest that type I and type II NKT cells have different cytokine-producing capacities, and interestingly, that IL-13 may be more produced by type II NKT cells (15, 26, 27, 36, 41, 42). Taken together, studies using TCR transgenic mice, lipid/CD1d-tetramers, and IL-4-reporter mice show that type II NKT cells are diverse also in their phenotype, cytokine secretion and activation state. While they frequently express NKT cell characteristic markers such as PLZF, NK receptors and CD122, some type II NKT cells seem more similar to type I NKT cells while others have a resting phenotype or different functional characteristics. This is in line with the diverse activities that have been associated with type II NKT cells.

TYPE II NKT CELLS IN DIFFERENT DISEASES

Multiple Sclerosis (MS) and Experimental Autoimmune Encephalomyelitis (EAE)

MS is a demyelinating autoimmune disease of the central nervous system (CNS) in which myelin-derived protein/lipid antigens are targets for autoreactive T cells. The myelin sheath is a rich source of sulfatide. Sulfatide-reactive T cells are more frequent in peripheral blood of MS patients than in healthy individuals (43), and CD1d-sulfatide reactive type II NKT cells, but not type I NKT cells, accumulate in the CNS in the experimental autoimmune encephalomyelitis (EAE) mouse model for MS (15). Thus, sulfatide-reactive type II NKT cells are thought to play a role in EAE, and *in vivo* administration of sulfatide at the time of EAE induction prevented the disease in a CD1d-dependent manner (15). Sulfatide-reactive type II NKT cells induced anergy of type I NKT cells, tolerized DCs and CNS microglia and inhibited the effector function of the pathogenic autoantigen-reactive CD4 T cells (15, 44).

Type 1 Diabetes (T1D)

T1D is an autoimmune disease in which autoreactive T cells target pancreatic β -cells of the Langerhans' islets. We have shown that type II NKT cells from 24 α B transgenic mice can protect NOD mice from disease, dependent on costimulators inducible costimulator (ICOS) and PD1 interactions (45, 46). Besides being a major lipid in the myelin sheath, different species of sulfatide are also present in pancreatic β -cells and could serve as ligands for type II NKT cells during T1D immunopathogenesis (47). Sulfatide/CD1d-tetramer positive cells were indeed enriched in pancreas-draining lymph nodes in non-obese diabetic (NOD)

mice. Sulfatide administration to NOD mice reduced T1D incidence and islet-specific T cell responses by inducing secretion of the anti-inflammatory cytokine IL-10 from DCs (48, 49), however, protection from T1D in NOD mice by sulfatide was not found in a second study (50).

Tumor Immunity

A role for NKT cells in tumor immunity is well established; particularly for type I NKT cells (51). In many human cancers, low levels of circulating type I NKT cells correlate with a poor prognosis; therefore, these cells have been targeted in a series of clinical trials (52). In some cancer forms, type I and type II NKT cells play opposing roles; while type I NKT cells promote, type II NKT cells suppress tumor immunity. Immunosuppression by type II NKT cells was shown to be mediated by IL-13 production resulting in the activation of TGF- β -secreting Gr1⁺CD11b⁺ myeloid derived suppressor cells (MDSCs), which in turn suppressed tumor-specific CD8⁺ T cells or type I NKT cells (51). A similar scenario may be present in multiple myeloma patients, in which IL-13 secreting LPC/CD1d-tetramer positive type II NKT cells were several fold increased in peripheral blood (27). It may therefore be a way forward to target tumor immunosuppression by type II NKT cells in cancer treatment, as discussed in a recent review (53). However, the roles of NKT cells in tumor immunity are more complex, as both type I and II NKT cells can promote tumor immunity, and there are other cancers in which both type I or type II NKT cells can suppress tumor immunity, as recently discussed in more detail (53, 54). The factors that determine whether NKT cells promote or suppress tumor immunity are not well understood, but it is possible that NKT cell activation by CD1d-expressing tumor cells will favor immunosuppressive functions.

Ulcerative Colitis (UC)

UC is a one of two forms of inflammatory bowel disease (IBD) characterized by Th-2-driven mucosal inflammation and tissue destruction in the colon. In UC, sulfatide-reactive type II NKT cells producing IL-13 were increased and suggested to have a colitogenic role (55, 56). Interestingly, in a mouse model for UC, a similar function was attributed to IL-13 producing type I NKT cells that were required to induce the disease (57). However, in different mouse models for IBD, type I NKT cells were either pathogenic or protective (56). Microbial components are abundant in the intestine, which are likely to activate the NKT cells during IBD and compromised intestinal barrier function. 24 $\alpha\beta$ TCR transgenic mice with elevated CD1d expression spontaneously developed colitis but here IFN- γ and IL-17 were the main players, not IL-13 (58). Moreover, a recent study has suggested the involvement of type II NKT cells in dextran sulfate sodium-induced colitis in mice provided choline-deficient diet (59).

Obesity

Obesity is a disease of low-grade adipose-tissue inflammation with a potential cancer risk (60). Both protective and pathogenic roles of type I NKT cells in obesity has been reported by different studies as these cells can either produce anti-inflammatory

cytokines IL-4 and IL-10 or pro-inflammatory cytokines IFN- γ , in adipose tissues (61–65). However, type II NKT cells exacerbated diet-induced obesity, as deduced from mice that lack type I NKT cells compared with CD1d^{-/-} mice (lacking both cell types) (66). Similarly, another recent study indicated that type II NKT cells in *ldlr*^{-/-} mice promote spontaneous obesity, as *ldlr*^{-/-} or *ldlr*^{-/-}CD1d^{-/-} mice are less obese and have less adipose tissue inflammation than *ldlr*^{-/-}J α 18^{-/-} mice (67). By contrast, it was recently shown that sulfatide-induced type II NKT cells prevented high fat diet-induced obesity in mice by regulating adipose tissue inflammation, and their transfer into obese mice resulted in improved weight loss and glucose tolerance (68).

Liver Inflammation and Hepatitis

Type I and type II NKT cells can play opposing roles in liver inflammation (69). Type II NKT cells are more frequent in human liver than type I NKT cells (16). In liver ischemic-reperfusion injury or a conanavalin A-induced hepatitis model, type I NKT cells were rapidly activated and elicited liver inflammation. This was inhibited by sulfatide-activated type II NKT cells through the activation of plasmacytoid DCs and the production of IL-12 and MIP-2 that induced anergy in type I NKT cells (70, 71). In another study, type II NKT cells inhibited alcohol-induced liver disease in a similar manner (72). Interestingly, sulfatide-reactive type II NKT cells that express IL-13R α 2 were detected in human liver and suggested to play a role in the protection from liver fibrosis (73). Thus, sulfatide activated type II NKT cells regulate pro-inflammatory type I NKT cells in liver inflammation.

Infectious Diseases

A role for NKT cells in infectious diseases is well established (74). In fact, as described above, bacteria and viruses can stimulate type I NKT cells without TCR engagement in an innate-like manner. Data suggest that also type II NKT cells can be activated by infections in an innate-like manner, without TCR stimulation. The two NKT subsets exert an opposing role in response to certain infectious agents. In *Trypanosoma cruzi* infected mice, contrary to type I NKT cells, type II NKT cells showed a proinflammatory effect by reducing the titers of pathogen-specific antibodies (75). By contrast, an opposing role was shown in *Schistosoma mansoni* infection where type II NKT cells skewed the profile to Th2 cytokine secretion with decreased IFN- γ , here type I NKT cells supported IFN- γ secretion (76). We have demonstrated a protective role of sulfatide-activated type II NKT cells in a murine model for *Staphylococcus aureus* sepsis accompanied with decreased TNF- α and IL-6 (77). Further, as mentioned earlier, type II NKT cells could be activated by the glycolipid components from *Corynebacterium glutamicum* or *Mycobacterium tuberculosis* (18) and phosphatidylglycerol from *Listeria monocytogenes* (32). Studies have also established a role for NKT cells in antiviral immune responses. Earlier reports have shown that CD1d- and NKG2D-mediated activation of type II NKT cells in HBV infection caused liver damage in mice (78, 79). However, it was more recently found that modified self-lipids such as phosphatidylethanolamine and lysophosphatidylethanolamine, produced during HBV infection,

induced activation of liver type II NKT cells that enhanced anti-viral immune responses, demonstrating the dual role of type II NKT cells in this infection (28). Evidence for a protective role of type II NKT cells has also been shown in human immunodeficient virus-1 (HIV-1) infection, where sulfatide-induced type II NKT cells reduced viral replication in humanized severe combined immunodeficiency (SCID-Hu) mice (80), and induced type I NKT cell energy (81).

Graft vs. Host Disease (GVHD)

GVHD is a serious complication that can follow bone marrow transplantation as a treatment for hematological malignancies. Type II NKT cells have been implicated in the regulation of GVHD. Using a mouse GVHD model, it was shown that type II NKT cells in donor bone marrow protected recipient mice from GVHD by producing IFN- γ , which induced apoptosis of donor T cells, and IL-4, that deviated the immune response toward a protective Th2 type (82). In contrast, in this model, type I NKT cells did not have an effect. In this context it is interesting to note that type II NKT cells are more frequent than type I NKT cells in human bone marrow. Human bone marrow derived type II NKT cells displayed a TH2 cytokine profile and suppressed mixed lymphocyte reactions (83).

Gaucher's Disease (GD)

GD is an inherited metabolic disease caused by a deficiency of lysosomal glucocerebrosidase, characterized by progressive lysosomal storage of β -glucosylceramide and glucosylsphingosine (84) with an increased cancer risk (85). A functionally unique subset of type II NKT cells, reactive to β -glucosylceramide and glucosylsphingosine, was identified in wild type mice and healthy humans (26). These cells constitutively expressed a T-follicular helper phenotype and provided efficient B cell help. Strikingly, these cells were activated and expanded in human GD and its murine disease model, and it was speculated that they might contribute to the increased risk of B cell malignancy observed in GD (26).

CONCLUSIONS AND FUTURE PERSPECTIVES

Studies so far have well documented the diverse roles of type II NKT cells in different immunological contexts. The

picture that emerges is of a population with diverse functions and phenotypes, substantially more heterogeneous than type I NKT cells. Recent publications have further established that type II NKT cells are activated and potentially involved in several human diseases, while their targeting in mouse disease models have provided promising results. Type II NKT cells are activated by a range of lipid antigens, and although different type II NKT TCR recognize the same lipids, the TCR repertoire is oligoclonal in nature. One of the greatest challenges is to further define the entire population of type II NKT cells. The identification of additional ligands that can be used to identify type II NKT cells with CD1d-tetramers will be instrumental in this quest. Considering the recognition of several lipids by the same type II NKT cell TCR, it will be of importance to elucidate the role of ligand specificity vs. a more promiscuous CD1d-reactivity for type II NKT cell development and activation. The relative role of TCR mediated/adaptive activation and innate, TCR-independent/cytokine mediated stimulation will also be critical to determine. So far type I NKT cells have been targeted in several clinical trials. Considering that type II NKT cells are more abundant in humans, and have been shown to play an immunoregulatory role in several diseases, the type II NKT cells have a largely unexplored immunotherapeutic potential. We have great expectations that the coming years will see rapid progress in this field.

AUTHOR CONTRIBUTIONS

AKS, PT, and SC made intellectual contribution to the work. AKS and SC wrote the text. PT critically read the manuscript.

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