Published in final edited form as:

Cancer Immunol Immunother. 2014 March ; 63(3): 199–213. doi:10.1007/s00262-013-1509-4.

The immunoregulatory role of type I and type II NKT cells in cancer and other diseases

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Abstract

NKT cells are CD1d-restricted T cells that recognize lipid antigens. They also have been shown to play critical roles in the regulation of immune responses. In the immune responses against tumors, two subsets of NKT cells, type I and type II, play opposing roles and cross-regulate each other. As members of both the innate and adaptive immune systems, which form a network of multiple components, they also interact with other immune components. Here we discuss the function of NKT cells in tumor immunity and their interaction with other regulatory cells, especially CD4⁺CD25⁺Foxp3⁺ regulatory T cells.

Keywords

Tumor immunity; NKT cells; regulatory T cells (Treg); interaction among regulatory cells

Immunotherapy of cancer is no longer science fiction after the FDA approval of sipuleucel-T (Provenge) and ipilimumab (Yervoy), milestone achievements of the field of tumor immunology [1, 2]. However, there is still a long way to go for this new modality of cancer treatment to achieve true success to become a conventional therapy. Since immune therapy deals with the immune system of patients, a better understanding of the system is critical to improve treatment strategies. One kind of factor limiting the success of immunotherapy is negative feedback mechanisms that have developed to prevent deleterious self-reactive immune responses. Thus, targeting this aspect of the immune system is a reasonable approach to develop immunotherapy. In fact, based on the success of Ipilimumab, which targets CTLA-4, a receptor that transmits negative signals, expressed on activated T cells and regulatory T cells, more efforts are now being made to develop new drugs targeting molecules involved in suppression of immune activation. It is clear that multiple types of cells such as myeloid derived suppressor cells (MDSC) [3], CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells [4], NKT cells [5], M2 macrophages [6], tumor associated macrophages (TAM) [7] as well as molecular pathways that together constitute a network to control immune responses by negative feedback. Consequently there is increasing interest in the interaction among those cell types [6, 8, 9]. For example, NKT cells have been shown to interact with MDSC (see reviews [9, 10]). Although it is of great interest to understand the

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Conflict of interest

Authors declare they have no conflict of interest.

interaction of NKT cells with all these regulatory cells, in this article due to space limitations, we are going to mainly discuss the several roles of NKT cells in the regulation of tumor immunity and their interaction with Treg cells.

NKT cells

NKT cells are T cells that recognize antigens presented by a class Ib MHC molecule, CD1d. Distinct from conventional T cells that recognize protein (peptide) antigens presented by MHC molecules, NKT cells recognize lipid antigens (mainly glycolipids and glycerol lipids) [11, 12]. Although they were originally described as a T cell population constitutively expressing an NK cell marker, NK1.1, the expression of NK receptors is no longer part of the definition of NKT cells, as some CD1d-restricted NKT cells do not express NK cell markers and activated conventional T cells also express many NK cell markers [13]. There are at least two distinct types of NKT cells, type I and type II NKT cells (Table 1). Type I or “invariant” NKT cells (sometimes called iNKT cells) express a semi-invariant TCR α chain with a V α 14J α 18 gene segment (V α 24J α 18 in humans) paired with a very limited TCR β repertoire (V β 8, 7, 2 in mice and V β 11 in humans). They recognize a prototypic glycolipid antigen, α -galactosylceramide (α -GalCer or KRN7000). Type I NKT cells have two major subsets based on the expression of CD4 and CD8. In mice, the majority are CD4⁺ (~90%) type I NKT cells, and the rest are CD4⁻CD8⁻ double negative (DN) NKT cells. In humans, there is a subset expressing CD8 $\alpha\alpha$ as well, although their biological function is not well studied yet. The other type is called type II or non-invariant NKT cells. They do not express the V α 14J α 18 TCR α chain and are also either CD4⁺ or CD4⁻CD8⁻. They also do not recognize α -GalCer, but rather other lipid antigens. Recently it was reported that some $\gamma\delta$ T cells also recognize antigens presented by CD1d. Those $\gamma\delta$ T cells can be considered a component of type II NKT cells [14]. Unlike conventional CD4⁺ T cell functional subsets, subsets of NKT cells cannot inter-convert. Also unlike conventional T cells, among which different subsets with the same antigen specificity can be induced or found, the two subsets of NKT cells recognize different sets of antigens. This unique feature of NKT cells makes it relatively easy to study the function of NKT cells *in vivo* since administration of an NKT cell antigen can specifically activate one subset of NKT cells. Another difference between NKT cells and conventional T cells is the breadth in the specificity of the TCR. It is known that a TCR of conventional T cells has limited flexibility in terms of the structure of antigen that it recognizes. However, the TCR of NKT cells (at least for type I NKT cells) seems to be able to recognize antigens of fairly diverse structures. It is also worth noting that although the proportion of NKT cells (approximately 1 % for type I in mouse spleens) sounds very small compared to conventional T cells (approximately 30 % and 10 % for CD4⁺ T cells and CD8⁺ T cells, respectively, in mouse spleens), one can consider that having almost 1% of spleen cells with the same antigen specificity in the naïve repertoire is an extremely high precursor frequency. From this point of view, it is not a total surprise to find critical roles for NKT cells in regulating immune responses in various settings.

Self antigens for NKT cells

Although tumor cells are derived from “self”, it is now widely accepted that they frequently express antigens that are not in normal cells, often at low levels. These tumor-associated

antigens are recognized by the immune system and shown to be good target candidates for the immunotherapy of cancers. In this context, it is important to identify endogenous lipid antigens recognized by NKT cells as well as to understand the functions of NKT cells under physiological conditions without involvement of exogenous foreign antigens. Despite the identification of many pathogen-derived lipid antigens during the last decade [15–17], limited information is available about endogenous lipid antigens for NKT cells, although it seems that a variety of cell lipids can bind to CD1d [18, 19] (Fig 1). There are studies strongly suggesting that tumors also produce antigens that can activate NKT cells. While a glycosphingolipid α -GalCer (KRN7000), a synthetic form of glycosphingolipid isolated originally from a marine sponge, has been extensively used to study type I NKT cells because of its strong agonistic activity, humans and mice cannot make glycosphingolipids with an alpha-linked sugar moiety and there is a recent study suggesting that endogenous lipids involved in autoreactivity of NKT cells may not be glycosphingolipids [20].

Phosphatidylinositol, phosphatidylethanolamine, and phosphatidylglycerol were reported to be recognized by a mouse type I NKT cell hybridoma, which has a weak reactivity to α -GalCer [21]. Although these phospholipids are among the first endogenous antigens demonstrated to be recognized by the TCR of mouse NKT cells, their stimulatory capacity seems weak for human NKT cells [22]. Interestingly, the type I NKT cell hybridoma with strong activity to α -GalCer does not react with these phospholipids. Isoglobotrihexosyl ceramide (iGb3) was the first endogenous glycosphingolipid shown to activate both mouse and human type I NKT cells [23]. However, it subsequently was reported that it may not exist in humans [24] and the importance for NKT cells was questioned [25, 26].

Recently it was reported that β -glucosylceramide (β -GlcCer) with a C24:1 acyl chain in the ceramide moiety can activate both mouse and human type I NKT cells [27]. The synthesis of β -GlcCer was up-regulated in LPS-stimulated DCs. Inhibition of β -GlcCer synthesis in APCs (BM-DCs) decreased LPS-induced activation of type I NKT cells, which requires both DC-derived IL-12 and recognition of self antigen presented by CD1d through TCRs. This set of data strongly suggests that β -GlcCer is a physiologically relevant self antigen for type I NKT cells. It is interesting that β -GlcCer variants with different lengths of acyl chains can be recognized by two type II NKT cell hybridomas [27, 28] suggesting that even though the acyl chain is not exposed to TCRs because it is buried in the binding groove of CD1d [17], it makes a significant contribution to determine which type of NKT cell can respond. It was also reported that phosphatidylglycerol and diphosphatidylglycerol (or cardiolipin) can be recognized by a type II NKT cell hybridoma [29]. Cardiolipin presented by CD1d was also reported to be recognized by $\gamma\delta$ T cells in mice [30] and type II NKT cells in humans [14]. GD3, a disialoganglioside relatively enriched in certain types of tumors, is one of a few lipids derived from tumors to be able to stimulate NKT cells. It is also suggested that this lipid antigen can be cross-presented by APCs to activate type I NKT cells [31]. A recent study suggested that GD3 has high affinity for CD1d molecules, and can suppress type I NKT cell-activation by α -GalCer in vivo by competing for the binding to the CD1d [32].

Sulfatide ($\text{SO}_3\text{-3Gal}\beta\text{1Cer}$) with a C24:1 acyl chain is the first endogenous antigen demonstrated to be recognized by type II NKT cells [33]. Sulfatide-loaded CD1d-tetramer-reactive type II NKT cells are a non-redundant population distinct from α -GalCer-loaded

CD1d-tetramer reactive type I NKT cells. Although this form of sulfatide is enriched in myelin sheaths of the central nervous system, other isoforms are highly expressed in other organs (e.g., pancreatic β -cells, kidney) and cancers [34]. A study with a sulfatide-reactive type II NKT cell hybridoma demonstrated that sulfatide C24:1 is the most potent to activate the hybridoma compared to shortened (C16:1) and saturated (C24:0) isoforms and that sulfatide lacking an acyl chain, lysosulfatide, has much higher potency to activate the hybridoma [35]. Lysophosphatidylcholine (LPC) was identified as a $V\alpha 24^-V\beta 11^-$ type II NKT cell-agonist antigen enriched in myeloma patients' plasma samples [36]. LPC-reactive type II NKT cells showed a more Th2 (IL-13) skewed cytokine profile, suggesting that these are immunosuppressive. Recently Fox et al., reported that LPC is also recognized by the majority of human type I NKT cell clones tested expressing a semi-invariant receptor [22]. In this study, the authors examined the stimulatory activity of multiple kinds of lipid antigens previously identified as CD1d binding antigens [18], and observed that except for lysosphingomyelin, which has the same head group, choline, linked by a phosphate ester to a hydrocarbon tail, none of them (phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, tetra-acylated cardiolipin, lyso-phosphatidylethanolamine, lyso-phosphatidylcholine, lyso-phosphatidylglycerol, lysophosphatidic acid, sphingomyelin, ganglioside GM3) had strong stimulatory activity. It is speculated that LPC induces activation of type I NKT cells at inflammatory sites [37]. Concordant with the notion that signaling from TLRs increases the capacity of DCs to stimulate autoreactivity of type I NKT cells by changing the composition of lipid antigens presented by CD1d [27, 38, 39], it is possible to speculate that tumors in an environment of chronic inflammation have a distinct lipid composition from normal cells. Further studies are required to better understand lipid antigens that modulate NKT cell activities.

Roles of type I NKT cells in tumor immune regulation in mice and humans

The majority of studies on the role of NKT cells in the regulation of tumor immunity have used α -GalCer. When type I NKT cells are stimulated with α -GalCer, they produce IFN- γ . Simultaneously, they activate antigen-presenting cells (APCs) through CD40-CD40L interaction, especially inducing DCs to mature and up-regulate co-stimulatory receptors such as CD80 and CD86. DCs also produce IL-12 upon the interaction with type I NKT cells. IL-12 induces more IFN- γ production by other T cells and plays a critical role together with IFN- γ in the activation of downstream effectors such as NK cells, CD8⁺ T cells and $\gamma\delta$ T cells [40]. In this pathway, IFN- γ has been shown to be necessary, as α -GalCer does not induce protective immune responses in IFN- γ deficient mice. The interaction of type I NKT cells with APCs licenses APCs to render them able to cross-prime to CD8⁺ T cells through the induction of CD70 and CCL17 [41–43]. Due to this ability of type I NKT cells, type I NKT cell agonists have been shown to have adjuvant activity when used with a vaccine [44–46]. The great success of α -GalCer in mouse tumor models led to clinical trials of this compound in cancer patients (reviewed in [47, 48]). Recent reports of trials suggest that use of α -GalCer as an adjuvant for DC-based vaccines is a promising approach [49–51].

We recently found a new class of type I NKT cell agonist, β -ManCer [52]. This compound has an identical ceramide structure to that of α -GalCer (KRN7000), which contributes to the binding with CD1d, with a beta-linked mannose instead of alpha-linked galactose. It has

been believed in the field that the alpha-linked sugar moiety is a critical feature of α -GalCer to elicit tumor immunity. Therefore, the discovery of relatively strong anti-tumor activity of β -ManCer was unexpected. While the protection induced by β -ManCer was type I NKT cell-dependent, the protection was independent of IFN- γ but dependent on TNF- α and nitric oxide synthase (NOS). Furthermore, consistent with the distinct mechanism of protection, α -GalCer and β -ManCer synergized to induce tumor immunity when suboptimal doses were used. In addition, β -ManCer has much weaker ability to induce long-term anergy in type I NKT cells than α -GalCer [53]. Similar to α -GalCer, β -ManCer can enhance the effect of a tumor vaccine [54]. Thus, type I NKT cells can use multiple pathways/mechanisms dependent on the antigens that they recognize. It is still unclear how antigens recognized by the same TCR induce distinct downstream pathways for anti-tumor immune responses. Nevertheless, the ability of β -ManCer to induce tumor protection by a novel mechanism, synergize with α -GalCer, avoid anergy induction, avoid neutralization by natural anti-alpha-Gal antibodies found in humans and other primates, and to stimulate human type I NKT cells [52] all motivate the translation of β -ManCer to human clinical trials in cancer.

The role of type I NKT cells in immunosurveillance was reported in multiple spontaneous tumor models including a methylcholanthrene (MCA)-induced tumor model, a p53 deficiency model and a TRAMP model [55–57]. In these studies, tumors grew faster in $J\alpha 18^{-/-}$ mice, which lack type I NKT cells, and/or $CD1d^{-/-}$ mice, which lack both type I and type II NKT cells, either treated with carcinogen or crossed with oncogene transgenic mice compared with NKT cell-intact mice, suggesting that type I NKT cells play a critical role in natural immunosurveillance. In a model with MCA-induced cell lines, reconstitution of $J\alpha 18^{-/-}$ mice with liver NKT cells restored the resistance against tumors even when the NKT cells were from perforin deficient mice, suggesting that the lytic activity is not critical for the type I NKT cell's ability to control tumor growth [58], even though NKT cells can lyse $CD1d$ -expressing tumor cells [59]. It is also important to point out that when sorted NKT cell subpopulations were adoptively transferred, the liver $CD4^{-}CD8^{-}$ population was the only subset of type I NKT cells that reconstituted immunosurveillance against lung metastasis of the B16 melanoma in $J\alpha 18^{-/-}$ mice [60].

The hypothesis that type I NKT cells contribute to tumor immunosurveillance is also supported by some clinical observations suggesting that the number of type I NKT cells can be an independent predictor of prognosis. There was a significant reduction in the number of circulating NKT cells in patients with solid tumors compared to healthy donors [61]. It was also shown that the low number of circulating type I NKT cells is an independent predictor of poor overall survival and disease-free survival in patients with head and neck cancer [62] and is associated with poor overall survival in acute myeloid leukemia [63]. A high number of tumor infiltrating type I NKT cells is reported to be an independent prognostic factor for overall survival and disease-free survival in primary colorectal cancer patients [64].

In contrast, there are some reports suggesting an immunosuppressive role of type I NKT cells. NKT cell-deficient $J\alpha 18^{-/-}$ and $CD1d^{-/-}$ mice were reported to develop fewer liver metastases of B16LS9 melanoma from primary tumors in either eyes or spleens than wild-type mice [65]. In this model, Type I NKT cells seem to suppress NK cell-mediated tumor immunosurveillance by an IL-10-dependent mechanism. In a Burkitt's-like B cell lymphoma

model with a Triple-Transgenic B cell Lymphoma from E-mu-MYC/BCR^{HEL}/sHEL transgenic mice, type I NKT cell-deficient J α 18^{-/-} mice had significantly lower tumor burden compared to wild-type mice and CD1d^{-/-} mice, which lack both types of NKT cells [66]. The tumor burden was correlated with the frequency of tumor antigen specific CD8⁺ T cells. These data paradoxically suggest that type I NKT cells suppress CD8⁺ T cell-mediated tumor immunosurveillance while type II NKT cells facilitate it. As mentioned below, this is a surprising finding since multiple other studies suggest that type I NKT cells enhance and type II NKT cells suppress tumor immunity. While many solid tumor cell lines tested were negative for CD1d expression [67], when tumor cells express CD1d and present endogenous antigens and/or α -GalCer to NKT cells, the NKT cells are prevented from full activation to facilitate immunosurveillance [68–70]. The outcome of the interaction between type I NKT cells and CD1d-expressing tumor cells may also be affected by the expression level of CD1d on the tumors, as high expression levels of CD1d on tumor cells facilitated NKT cell-mediated tumor immunosurveillance while low levels of CD1d expression promoted metastasis [70].

Type II NKT cells regulate immune responses

Type II NKT cells are much less well characterized than type I NKT cells. In contrast to type I NKT cells, in which the whole population can be stimulated with the single antigen α -GalCer, there is no known antigen that can uniformly stimulate all type II NKT cells. Based on studies with NKT cell hybridomas, which inherit the antigen specificity of their TCRs from the NKT cells used for the fusion, there is significant diversity in the antigen specificity among clones. In view of the fact that there is no widely available tool to identify type II NKT cells, currently three approaches have been used to study the biological function, especially *in vivo* functions, of type II NKT cells in mice. The first is to use a TCR transgenic mouse that overexpresses the 24 $\alpha\beta$ TCR from the type II NKT cell clone VIII24 [71]. The second is to use sulfatide to specifically stimulate a fraction of type II NKT cells [33]. The third is to compare the immune responses in two NKT cell deficient mice, J α 18^{-/-} mice lacking type I NKT cells but retaining type II NKT cells, and CD1d^{-/-} mice deficient in all NKT cells.

Studies with transgenic mice

Studies with 24 $\alpha\beta$ TCR transgenic mice and sulfatide have demonstrated that type II NKT cells have immunosuppressive function. They also utilize multiple different mechanisms to suppress immune responses.

NOD mice, that have a genetic susceptibility to develop type I diabetes, do not develop diabetes when made transgenic for the 24 $\alpha\beta$ TCR. Likewise, adoptive transfer of the transgenic T cells also delays the disease onset in an NOD $scid$ model with transferred diabetogenic CD4⁺ T cells [72]. Recently the involvement of ICOS and PD-1, but not Foxp3⁺ Treg cells or immunoregulatory cytokines (IL-4, IL-10, IL-13 and TGF- β), was implicated in the immunoregulation by 24 $\alpha\beta$ T cells [73]. It is also important to point out that 24 $\alpha\beta$ T cells do not react with sulfatide.

Studies using sulfatide

Sulfatide reactive type II NKT cells have also been shown to play a role in autoimmune diseases. Because one of the organs enriched for sulfatide isoforms is the pancreas, it was speculated that sulfatide-reactive type II NKT cells might be involved in the immune regulation of type I diabetes. In fact, sulfatide-reactive type II NKT cells are enriched in draining lymph nodes of the pancreas during the disease development [74]. Moreover, *in vivo* treatment with sulfatide significantly reduced the incidence of the disease in NOD mice. These studies clearly suggest that type II NKT cells with different antigen specificity play a critical role in the regulation of type I diabetes.

Experimental autoimmune encephalomyelitis (EAE) is a model of multiple sclerosis, an autoimmune disease with autoreactive T cells targeting myelin, which is enriched in sulfatide and its analogues. During disease progression, the number of sulfatide reactive type II NKT cells increases in CNS tissues. *In vivo* sulfatide treatment prevents antigen-induced disease, correlated with suppression of cytokine production by myelin oligodendrocyte glycoprotein (MOG)-reactive CD4⁺ T cells [33].

Sulfatide reactive type II NKT cells can also prevent Concanavalin A (ConA)-induced hepatitis, in which type I NKT cells are necessary for the disease onset. In this model, *in vivo* treatment with sulfatide prior to ConA treatment prevented the disease. The sulfatide treatment results in accumulation of sulfatide-reactive type II NKT cells in the liver. The type II NKT cells together with pDCs induce IL-12 and MIP-2 production, which recruit type I NKT cells to the liver and anergize them [75]. In contrast, in HBV transgenic mice, type II NKT cells seemed to exacerbate the inflammation, although the antigen specificity of those type II NKT cells was not defined [76]. The immunosuppressive role of sulfatide-reactive type II NKT cells was also reported in models of hepatic ischemic reperfusion injury, sepsis and airway inflammation [77–79]. It is also worth noting that sulfatide-reactive type II NKT cells were reported to interact with renal tubular cells to attenuate apoptosis when activated with sulfatide in renal ischemia-reperfusion injury [80].

Studies using $\text{J}\alpha 18^{-/-}$ and $\text{CD1d}^{-/-}$ mice

Although both type II NKT cells with the 24 $\alpha\beta$ TCR or those specific for sulfatide have generally been found to be immunosuppressive, studies using the third approach comparing immune responses in wild-type, $\text{J}\alpha 18^{-/-}$ and $\text{CD1d}^{-/-}$ suggest that type II NKT cells can have diverse faces in the modulation of immune responses. In infection with the protozoan intracellular parasite *Trypanozoma cruzi*, $\text{CD1d}^{-/-}$ mice behaved similarly to wild-type mice while $\text{J}\alpha 18^{-/-}$ mice showed significantly higher susceptibility to the infection, accompanied by production of pro-inflammatory cytokines. Thus, type II NKT cells can promote inflammatory responses in this infection but counteract protective immunity [81]. The two types of NKT cells play reverse roles in infection with the extracellular parasite *Schistosoma mansoni*. $\text{J}\alpha 18^{-/-}$ mice showed a more Th2 skewed immune response whereas $\text{CD1d}^{-/-}$ mice showed a more Th1 skewed response, suggesting that type II NKT cells facilitate Th2 responses and implicating opposing roles of two types of NKT cells [82]. A contribution of type II NKT cells to the control of EAE was also shown in a therapeutic setting with tolerogenic DCs without using sulfatide [83]. In this study, it was shown that B7-H1-

deficient DCs could induce better protection accompanied by increased Th2 cytokine production and by reduced IL-17 and IFN- γ production. The increased effect of the treatment was due to activation of type II NKT cells by endogenous antigens presented by the DCs. Type II NKT cells in J α 18^{-/-} mice can also suppress GVHD [84], but the suppression is lost in either J α 18^{-/-}IL-4^{-/-} or J α 18^{-/-}IFN- γ ^{-/-} mice, suggesting that both IL-4 and IFN- γ are necessary for the suppression.

Type II NKT cells in tumor immunity

In multiple tumor models, tumors grow in both wild-type and J α 18^{-/-} mice while tumor burden is much less in CD1 KO mice, suggesting that type II NKT cells play a critical role in suppression of natural tumor surveillance [67, 85], in contrast to type I NKT cells that are known to have anti-tumor activity as described above. In a subcutaneous 15-12RM fibrosarcoma and in lung metastases of the CT26 colon carcinoma, type II NKT cells, especially the CD4⁺ subset, produced IL-13, which induced TGF- β production by CD11b⁺Gr-1⁺ myeloid cells [86, 87]. TGF- β inhibits activation of tumor specific CD8⁺ CTLs, effector cells that kill tumor cells. The immunosuppressive type II NKT cells in these models may be sulfatide-reactive type II NKT cells since treating mice with sulfatide increases tumor burden [88]. In the same tumor models, activation of sulfatide-reactive type II NKT cells can partially or completely abrogate the protective effect of type I NKT cell activation (proliferation and cytokine production) by α -GalCer, defining an immunoregulatory axis between the two types of NKT cells [88–90]. The interaction between type I and type II NKT cells may also affect the number of NKT cells, since J α 18^{-/-} mice tend to have more type II NKT cells in their livers [91]. Suppression of type I NKT cell functions by sulfatide-activated type II NKT cells is not limited to tumors, but was also reported in an airway inflammation model [78] where type I NKT cells have been reported to play a critical role in the pathogenesis [92, 93]. The immunosuppressive role of type II NKT cells, probably through IL-13, TGF- β and CD11b⁺Gr-1⁺ myeloid cells, was reported in a model with a B cell lymphoma transfected with CD1d but not without CD1d [85]. In human multiple myeloma patients, type II NKT cells (V α 24⁻ CD1d-restricted T cells) were reported to increase in peripheral blood compared with normal healthy donors [36]. Those type II NKT cells are specific for lysophosphatidylcholine species isolated from patients' plasma. These type II NKT cells have a Th2-skewed cytokine profile with high expression levels of IL-13.

Function of NKT cells within the network of immunoregulatory cells: Type I NKT cells support Treg cells

As described above, NKT cells have immunoregulatory functions. Another suppressive T cell population known to be involved in the regulation of immune responses including tumor immunity is the CD4⁺CD25⁺Foxp3⁺ regulatory T cell (Treg cell). There are two types of Treg cells, natural Treg (nTreg) cells and induced Treg (iTreg) cells. nTreg cells develop in the thymus and iTreg cells develop from conventional naive CD4⁺ T cells in the periphery under the influence of IL-2 and TGF- β . Treg cells, especially nTreg cells, were discovered as CD4⁺ T cells involved in self-tolerance [94]. Soon after the discovery of Treg cells, they were reported to suppress tumor immunity [95]. Now both types of Treg cells are thought to

be involved in the suppression of tumor immunity. The mechanisms used by Treg cells to suppress immune responses include cell-to-cell contact and secretion of IL-10 and TGF- β . Treg cells are frequently enriched in tumor masses, and the balance between effector T cells and Tregs in a tumor mass is believed to determine prognosis [96, 97].

Interplay between NKT cells and Treg cells has been reported in many different settings. It seems that interactions between Treg cells and NKT cells, especially type I NKT cells, play an important role in induction of tolerance. In a model of autoimmune myasthenia gravis, in which the disease is induced in mice by immunization with the self-antigen, acetylcholine receptor (AChR), repeated treatment with α -GalCer inhibits the development of the disease by inducing proliferation and suppressive activity of Treg cells [98]. IL-2, necessary for the induction of Treg cells, is highly expressed by type I NKT cells. The support of Treg cell proliferation by type I NKT cells through the production of IL-2 is also observed in humans [99]. Interestingly, in this model, without stimulation with α -GalCer, there is no apparent role of type I NKT cells, because the disease develops similarly in wild-type, $\text{J}\alpha 18^{-/-}$ and $\text{CD1d}^{-/-}$ mice upon the immunization with AChR [100].

A more drastic difference in the face of type I NKT cells with or without being stimulated with α -GalCer has been reported in ileitis induced by *Toxoplasma gondii* infection, accompanied by overexpression of IFN- γ [101]. Without involvement of α -GalCer, type I NKT cells exacerbate ileitis, since $\text{J}\alpha 18^{-/-}$ mice are more resistant than wild-type mice. In contrast, α -GalCer administration prior to the infection decreases inflammation by increasing the number of IL-10-producing Treg cells. The effect of α -GalCer-induced activation of type I NKT cells on Treg cells may be partly due to induction of anergy of NKT cells and skewing of the cytokine profile of type I NKT cells toward Th2 [102, 103]. To collaborate with Treg cells, type I NKT cells can also recruit Treg cells to the organ where type I NKT cells are located. When type I NKT cells were activated with α -Gal-C18-Cer, an analogue of α -GalCer which does not induce liver injury but still induces IFN- γ production by type I NKT cells, IFN- γ from type I NKT cells induced chemokine CXCL10/IP-10 expression in hepatocytes [104] (Fig 2A). Treg cells expressing CXCR3 (a receptor for CXCL10) were recruited to the liver where activated type I NKT cells were located. Although CXCL10 has been implicated to recruit pro-inflammatory cells that express CXCR3, in this case neutralization of CXCL10 reduced the number of Treg cells and increased the number of IFN- γ -expressing NK cells.

The interplay between NKT cells and Treg cells does not always require activation by α -GalCer. In an oral tolerance induction model with nickel, Treg cells and tolerogenic APCs were generated in mice orally treated with nickel [105]. These cells can transfer the tolerance to a naïve mouse. In $\text{J}\alpha 18^{-/-}$ mice, the tolerance is not induced, suggesting the necessity of type I NKT cells in the tolerance induction. In this system, the induction of tolerogenic APCs requires CD4^{+} type I NKT cells that produce IL-4 and IL-10 (Fig 2B). The tolerogenic APCs then induce Treg cells. Once Treg cells are established, they can transfer the tolerance even in $\text{J}\alpha 18^{-/-}$ mice. Thus, in this case, type I NKT cells (especially the CD4^{+} subset) help Treg cell induction through the induction of tolerogenic APCs, but type I NKT cells are not required for the effector function of Treg cells.

Indirect induction of Treg cells by type I NKT cells was also reported in control of type I diabetes by LCMV infection in two diabetes models, a transgenic mouse expressing LCMV nucleoprotein in pancreatic β cells and proinsulin 2^{-/-} mice [106]. In these models, LCMV infection induced TGF- β -producing pDCs through type I NKT cells in pancreatic draining lymph nodes. IL-10 and PD-1-PD-L1 pathways were required for the recruitment of TGF- β -producing pDCs by type I NKT cells (Fig 2B). Then, TGF- β -producing pDCs converted naïve CD4⁺ T cells into TGF- β -producing Treg cells, which suppressed CD8⁺ T cells that caused the diabetes.

In influenza virus A (H3N1) infection in suckling mice but not in adult (8 week-old) mice, CD4⁺CD8⁻ type I NKT cells producing IFN- γ expanded in a T-bet- and TLR7-dependent manner [107]. The infected suckling mice became resistant to allergen-induced airway hyperreactivity (AHR), which is a key feature of asthma. Adoptive transfer of these type I NKT cells could transfer the protection, and Treg cells were necessary for the NKT cells to transfer the protection, suggesting that Treg cells are downstream of and regulated by type I NKT cells.

The collaboration between NKT cells and Treg cells has been well studied in bone marrow transplantation to treat leukemia and lymphoma [108]. It is critical to control graft vs host disease (GVHD), but retain the graft vs tumor (GVT) effect to control the disease. A key to the success of the bone marrow transplant is conditioning patients prior to the transplantation, and the combination of total lymphocyte irradiation (TLI) and anti-thymocyte serum (ATS) has been shown to prevent GVHD. After this conditioning, a great enrichment of type I NKT cells (which became >90% of all TCR $\alpha\beta$ ⁺ cells) in the host was observed. IL-4 from these type I NKT cells facilitated donor Treg cell division, accumulation, and IL-10 production as well as PD-1 expression. These Treg cells from the donor preferentially suppressed GVHD but not GVT. IL-4 also up-regulated PD-1 expression and reduced IFN- γ production of conventional CD4⁺ T cells. Both PD-1 and Tim-3 on CD8⁺ T cells were induced by IL-4 as well [109]. PD-1⁺Tim-3⁺ CD8⁺ T cells are typical exhausted T cells. The enrichment of type I NKT cells in the host T cell population after TLI/ATS preconditioning observed in mouse studies was confirmed in patients with lymphoid malignant disease or acute leukemia who underwent the preconditioning [110, 111]. Also in a study with patients of hematological malignancy treated with hematopoietic stem cell transplantation conditioned by busulfan and Cyclophosphamide, together with cyclosporine and methotrexate GVHD prophylaxis, the patients that received a high number ($>4 \times 10^6$ /kg) of donor Foxp3⁺ Treg cells in the transplant had better disease-free survival [112]. However, patients who received fewer ($<0.6 \times 10^6$ /kg) type I NKT cells also had better disease-free survival. It may be possible that a certain type of preconditioning of recipients selectively enriches type I NKT cells with immunosuppressive functions that are not abundant in normal healthy donors.

Although many studies suggest that type I NKT cells support Treg cells, there are some studies showing that type I NKT cells can also suppress Treg cells. V α 14 TCR transgenic mice have a 5- to 10- fold increase in the number of type I NKT cells with an unusually high proportion of CD4⁺CD8⁻ cells (approximately 50% compared to approximately 10 % in wild-type mice) [107]. The V α 14 TCR Tg mice have reduced nTreg cells [113]. Also type I

NKT cells, through production of IFN- γ , suppressed induction of iTreg cells in a culture of naïve CD4⁺ T cells stimulated with anti-CD3 plus IL-2 and TGF- β

Type I NKT cells, which produce IL-4 and IL-13, are implicated for the pathogenesis of allergic asthma in both mouse and human studies, although some studies suggested a limited role of type I NKT cells. In allergic asthma patients, it was reported that CD4⁺ type I NKT cells expressed increased levels of NKp30 and NKp46 although there was no increase in NKG2D and CD226 [114]. They also had increased expression of granzyme B and perforin. Those CD4⁺ type I NKT cells showed increased cytotoxicity against autologous Treg cells, and the cytotoxic potential correlated with disease duration. However, these observations were not made with non-allergic asthma patients. This study suggests that type I NKT cells not only produce effector cytokines but also actively eliminate regulatory cells that control pathogenic immune responses. Thus this study raises the possibility that type I NKT cells not only contribute to pathogenesis of AHR by making Th2 cytokines, IL-4 and IL-13 [93], but also actively counteract disease-controlling Treg cells in patients.

Treg cells suppress type I NKT cells

Even though type I NKT cells provide help to Treg cells, paradoxically, Treg cells suppress type I NKT cells as they suppress conventional T cells. *Streptococcus pneumoniae* infection can prevent asthma mediated by type I NKT cells [115]. In this model, *S. pneumoniae* components type-3-polysaccharide (T3P) and pneumolysin (Ply) suppress the development of symptoms of asthma. The mechanism of the suppression is that exposure to both T3P and Ply induced Treg cells that suppressed pathogenic type I NKT cells. Suppression of type I NKT cells by Treg cells was also seen in a tumor setting. In a model with 3-methylcholanthrene (MCA) induced tumor cell lines, immunization with antigens defined by “serological identification of antigens by recombinant expression cloning” (SEREX) induced Treg cells, which accelerated tumor growth [116]. In this model, the tumor antigen-specific Treg cells reduced the number of type I NKT cells. The adoptive transfer of type I NKT cells in Ja18^{-/-} mice reduced tumor burden, indicating that type I NKT cells promote tumor immunosurveillance. However, when the adoptive transfer of type I NKT cells was followed by immunization with the SEREX-defined antigens, the protective effect of type I NKT cell transfer was abrogated, suggesting that Treg cells suppressed protective type I NKT cells. Interestingly Treg cells preferentially reduced the number only of type I NKT cells but not of conventional T cells.

Similarly, Treg cells suppress type I NKT cells in humans. Treg cells suppressed both proliferation and cytokine production (both Th1 and Th2 cytokines) of type I NKT cells stimulated with α -GalCer-pulsed allogeneic DCs, which also activated Treg cells through the TCR signal [117]. Treg cells also suppress cytotoxic activity of type I NKT cells against tumor cell lines. All three subpopulations of type I NKT cells, CD4⁺, CD4⁺CD8⁻ and CD8⁺, could be suppressed through a cell-to-cell contact mechanism. Recently Venken et al., reported that the susceptibility of type I NKT cells to suppression by Treg cells may depend on the strength of the TCR signal to type I NKT cells [118]. In this study, autologous DCs were used to stimulate both type I NKT cells and Treg cells in culture. Treg cells could suppress type I NKT cell proliferation when type I NKT cells were stimulated with weak

agonists, OCH (α -GalCer analog with truncated sphingosine chain), C9:0 (α -GalCer analog with truncated acyl chain), BbGLIc (monogalactosyl diacylglycerol lipid originally extracted from *Borrelia burgdorferi*) and SPN-Glc-DAG (glycosphingolipid containing glucuronic acid originally extracted from *Sphingomonas* spp), but not when type I NKT cells were stimulated with strong agonists, α -GalCer and naphthylurea- α -GalCer. Interestingly Treg cells also preferentially suppress IL-4 but not IFN- γ production by NKT cells. Treg cells suppressed both conventional CD4⁺ T cells and type I NKT cells. While the suppression of conventional T cells by Tregs was mediated by cell-to-cell contact, the suppression of type I NKT cells required both cell-to-cell contact and IL-10. Treg cells co-cultured with type I NKT cells stimulated with bacterial-derived weak agonists expressed higher levels of Foxp3 and IL-10. This study suggests that under physiological conditions, in which type I NKT cells recognize weak agonists, they facilitate the suppressive activities of Treg cells and can be suppressed by Treg cells.

This hypothesis is further supported by studies showing that depletion or blockade of Treg cells facilitated the adjuvant effect of α -GalCer on tumor vaccines [119, 120]. In a subcutaneous B16F10 melanoma model, it was shown that the therapeutic effect of an α -GalCer-loaded tumor cell vaccine was enhanced by short-term elimination of Foxp3⁺ Treg cells at the time of vaccination in Depletion of REGulatory T cells (DEREG) mice. The combination treatment induced protection mediated by both NK cells and CD8⁺ T cells through IFN- γ . In a subcutaneous B16.OVA model, the effect of a DC vaccine pulsed with both tumor-derived antigen and α -GalCer to induce anti-tumor immunity mediated by CD8⁺ T cells was further enhanced by blockade of Treg cells by anti-CD25.

Three-way interplay among Tregs, type I NKT cells and type II NKT cells

So far we discussed cases in which immunosuppressive Treg cells suppressed functions of type I NKT cells, including their anti-tumor function. Treg cells have been extensively studied in the context of tumors and it is widely believed that they are the major T cell subset to suppress host immune response. However, there has been limited success in Treg cell-targeted therapy [121], suggesting that other suppressors can substitute. Studies with mouse tumor models clearly suggest that Treg cells are not always necessary for the suppression of tumor immunity. Even in the first paper reporting the immunosuppressive role of Treg cells in tumor immunity, it was shown that anti-CD25 to block/deplete Treg cells (mostly nTreg cells) was not always effective to reduce tumor burden [95]. On the other hand, as described above, type II NKT cells also have immunosuppressive functions. Studies with mouse tumor models show that it was tumor model-dependent whether Treg cells or type II NKT cells played a dominant role in the suppression of tumor immunity.

What determines which suppressive T cell population plays a dominant role to suppress tumor immunity? What is the relationship between these two regulatory T cells? A recent study with a subcutaneous CT26 tumor suggests that type I NKT cells play a key role to determine the relationship [91]. It has been shown that anti-CD25 treatment can induce tumor rejection of subcutaneous CT26 tumors in wild-type mice [67, 122]. Surprisingly, this treatment had no effect on tumor growth in J α 18^{-/-} mice, while it was still effective in CD1d^{-/-} mice [91]. However, simultaneous treatment with anti-CD1d, which blocks

antigen presentation by CD1d, and anti-CD25, reduced tumor burden in $J\alpha 18^{-/-}$ mice, suggesting that concurrent suppression of tumor immunity by type II NKT cells in $J\alpha 18^{-/-}$ mice accounted for the failure of Treg blockade alone to protect. The protective effect of anti-CD25 treatment in $J\alpha 18^{-/-}$ mice was also restored when the mice were reconstituted by type I NKT cells. These results suggested that when type II NKT cells are counteracted by type I NKT cells, Treg cells are dominant suppressors (Fig 3A). However, when type II NKT cells are not counteracted by type I NKT cells, both Treg cells and type II NKT cells need to be eliminated to remove immune suppression (Fig 3B). Thus, a third T cell, the type I NKT cell, regulates the regulators. Given the fact that type I NKT cells in cancer patients are dysfunctional and/or are reduced in number [97], it is possible that the immunological status of cancer patients mimics that of $J\alpha 18^{-/-}$ mice. This may provide an explanation for the limited success of Treg cell targeted therapy in patients, and suggests that blockade of both Treg and type II NKT cells may be necessary to unmask immunosurveillance in many human cancers.

Conclusions

We have seen that type I and type II NKT cells play key roles in the regulation of tumor immunity. Most often, type I NKT cells contribute to protection against cancer, usually through an IFN- γ -mediated mechanism, although exceptions exist, such as with the novel type of NKT agonist represented by β -ManCer. Also, most often, type II NKT cells suppress tumor immunity. However, in both cases, there are exceptional role reversals that complicate the picture. Moreover, type I and II NKT cells cross-regulate each other, forming an immunoregulatory axis that may influence the balance of other immune responses. Indeed, the NKT cell regulatory system does not exist in a vacuum, but rather serves as an important part of a large interactive network of regulatory cells, including $CD4^+CD25^+Foxp3^+$ Treg cells, MDSCs, M2 macrophages, regulatory DCs and others. One key player is the $CD4^+CD25^+Foxp3^+$ Treg cell. Type I NKT cells can promote induction of Treg cells, but conversely, Treg cells can suppress type I NKT cells. Type II NKT cells can suppress tumor immunity in parallel with Treg cells although the interaction between them still remains to be elucidated. Which one dominates depends on a third T cell, the type I NKT cell, that regulates the balance between the regulators. As humans with cancer often have deficient type I NKT cell numbers or function, it may be necessary to block both type II NKT cells and Treg cells in order to effectively promote tumor immunity. NKT cells may also be exploited in tumor immunity by mediating the effect of some vaccine adjuvants targeting these cells. Thus, overall, the important role of immune regulation by NKT cells must be taken into account in designing effective immunotherapy for cancer.

Acknowledgments

This research was supported by the intramural research program of the National Cancer Institute, NIH and the Gui Foundation.

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Figure 1.

Structures of α -GalCer and self lipid antigens that can activate NKT cells.

Figure 2. Type I NKT cells provide help to Treg cells

A. When type I NKT cells are activated by a strong agonist (e.g. α -GalCer, α -Gal-18-Cer), they produce a large amount of cytokines including IL-2 and IFN- γ . IL-2 from type I NKT cells activates Treg cells. In some cases, IFN- γ from type I NKT cells induces production of a chemokine, CXCL10 (IP-10), by hepatocytes to attract CXCR3-expressing Treg cells to the liver.

B. In another scenario in which type I NKT cells are activated by a weak agonist such as bacterial lipids or endogenous lipids, the activated type I NKT cells up-regulate PD-1 on their surface and produce IL-4 and IL-10 and possibly other cytokines. These cytokines and interaction through PD-1-PD-L1 instruct DCs or pDCs to produce TGF- β . TGF- β induces iTreg cells. IL-4 also can induce production of IL-10 and up-regulate PD-1 in Treg cells that can also suppress type I NKT cells.

Fig 3A

Normal state

Fig 3B

cancer patients

Figure 3. Relationship among type I NKT cells, type II NKT cells and Treg cells

A. Type I NKT cells enhance and type II NKT cells suppress tumor immunity. They also cross-regulate each other. This balance may depend on lipid antigens presented by CD1d in cancer patients, and this balance may determine the clinical effect of Treg blockade. As shown in Fig 2, type I NKT cells also support Treg cells although Treg cells suppress type I NKT cells. Treg cells also suppress tumor immunity.

B. In cancer patients, who have suppressed type I NKT cell function, type II NKT cells are not adequately counter-regulated by type I NKT cells. Under this condition, even if Treg cells are blocked, there is no clinical effect since type II NKT cells still suppress tumor immunity, and both Treg cells and type II NKT cells must be blocked to improve tumor immunity.

Table 1

Characteristics of two types of NKT cells

	Type I	Type II
TCR α	V α 14Ja18 (mice) V α 24Ja18 (humans)	diverse: some V α 3.2Ja9, V α 3Ja7, V α 1Ja7 (mice)
TCR β	V β 2, 7, 8 (mice) V β 11 (humans)	diverse: some V β 8, V β 3 Some $\gamma\delta$ T cells are also CD1d restricted
surface markers	CD4, double negative CD8 (humans) CD44, CD69	CD4, double negative
antigens recognized	α -GalCer	PPBF (phenyl 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonate)
endogenous antigens	GD3, iGb3, β -GlcCer	sulfatide, β -GlcCer, PG, lyso-PC, cardiolipin
role in cancer (with exceptions)	enhance immunity	suppress immunity