Type I natural killer T cells: naturally born for fighting

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Type I natural killer T cells (NKT cells), a subset of CD1d-restricted T cells with invariant Vαβ TCR, are characterized by prompt production of large amounts of Th1 and/or Th2 cytokines upon primary stimulation through the TCR complex. The rapid release of cytokines implies that type I NKT cells may play a critical role in modulating the upcoming immune responses, such as anti-tumor response, protection against infection, and autoimmunity. As a bridge between innate and adaptive immunity, type I NKT cells differentiate and mature upon stimulations to achieve and maintain a homeostasis. Orchestrating with other arms of adaptive immunity, type I NKT cells show strong cytotoxic effects in response to various tumors in a direct and/or indirect manner(s). This review will focus primarily on type I NKT cell development, homeostasis, and effector functions, especially in anti-tumor immunity, and followed by their potential applications in treatment of cancers.

Keywords: NKT cells; tumor immunity; cell development and homeostasis; Th1/Th2 cytokines

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Introduction
Type I natural killer T cells, referring to as invariant NKT cells (thereafter abbreviated as NKT cells), are the most extensively studied due to its unique Vα14-Jα18 TCR in mouse (Vα24-Jα18 in humans)[5–7], while type II NKT cells with a diverse TCR repertoire are less well studied. Unlike conventional T cells, which respond to foreign peptides presented by MHC class I or II molecules through interaction with their αβ TCR, NKT cells recognize antigenic glycolipids presented by the MHC class I-like molecule CD1d[5, 4]. Mice with deletion of CD1d gene eradicates NKT cells including both type I and type II. Because the positive selection of these two NKT cell subtypes is strictly dependent on CD1d during thymic ontogeny. Exogenous glycolipid α-galactosylceramide (α-GalCer) derived from marine sponge Agelas mauritianus or symbiotic microorganism has commonly been used to identify and activate NKT cells in vitro. Recently, several endogenous mammalian self lipids have been defined as CD1d ligands recognized by NKT cells, including isoglobotrihexosylceramide (Gb3) and other phosphatidyl inositol compounds, but these are still controversial and need to be further confirmed[5–6]. However, we found that the infection of Epstein-Barr virus (EBV), but not human T-cell leukemia virus type I (HTLV-1), can profoundly promote EBV-associated CD8` NKT cell development in humans and human-thymus/liver-SCID (hu-thym/liv-SCID) chimeras, suggesting whether other types of antigen are involved in differentiation and maturation of NKT cells is still unknown[7–9].

Unlike the T cells, unconventional glycolipid-reactive NKT cells that bridge innate and adaptive immunity set the keynote and the tone for the subsequent adaptive immune responses through expression of Th1/Th2 cytokines in response to glycolipid antigens. After activation, NKT cells rapidly produce Th1, Th2 cytokines and various chemokines, as well as up-regulate co-stimulatory molecules to respond to the infections, tumors and autoimmune disorders[7, 10–12]. Upon expression of CD4 and CD8 molecules, the NKT cells are divided into two main subpopulations including CD4` and CD4` NKT cells, and the subpopulation of CD4` NKT cells is further subdivided into CD4`CD8` double negative (DN) and CD8` single positive (SP) NKT cells, which is limited in human beings. It is widely believed that CD8 is expressed on a minor proportion of human NKT cells, but it is usually acquired after egression from the thymus[13]. The finding of limited correlation between human thymic CD4` NKT cells and peripheral CD4` NKT cells, including DN and CD8` SP, has raised a requisition on the direct evidence for origin of DN and CD8` NKT cells. Many papers from independent groups have described that CD4` NKT cells usually produce both Th1 and Th2 cytokines, whereas CD4` NKT cells including human CD8` NKT cells are skewed more toward Th1 cytokines[14]. Once it occurs, the initial production of Th1/Th2 cytokines may leads to a corresponding adaptive immunity towards Th1 or Th2 response,

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CD4 is lost in parts of these cells, which results in generation of total NKT cells during initial stages of development, while the surface CD8 molecule is constantly down-regulated in (stage3), and ultimately develop into mature NKT cells [1, 19].

Recently published data that EBV-induced CD8⁺ NKT cells display negative; SP, single positive.

It is clear that the NKT cells originate from a common precursor pool of CD4⁺CD8⁻ double positive (DP) thymocytes, which have undergone random TCR gene rearrangement and expression[17]. Unlike conventional T cells depend on thymic epithelial cells during positive selection, NKT cell precursors are positively selected by CD1d⁺ DP thymocytes in the thymic cortex[31]. Once the interaction between TCRs expressed on DP thymocytes and self glycolipids presented by CD1d-bearing DP thymocytes occurs, NKT cell precursors undergo consecutive differentiation and maturation stages, including CD24⁺CD44⁻NK1.1⁻ (stage 0), CD24⁺CD44⁺NK1.1⁺ (stage 1), CD24⁻CD44⁻NK1.1⁻ (stage 2), and CD24⁻CD44⁺NK1.1⁺ (stage3), and ultimately develop into mature NKT cells[1, 19]. The surface CD8 molecule is constantly down-regulated in total NKT cells during initial stages of development, while CD4 is lost in parts of these cells, which results in generation of CD4⁺ SP and DN NKT cells, but the actual existence of murine CD8⁺ SP NKT cells is still unknown. However, a fraction of NKT cells expressing CD8 markers, predominantly CD8αα, are present in normal persons and healthy people with latent EBV infection[9].

The earliest NKT precursors may also be susceptible to negative selection in the presence of strong agonist ligands. A dose- and time-dependent deletion of NKT cells is induced by the injection of high dose of the glycolipid agonist α-GalCer to fetal thymic organ culture (FTOC) or persistent injection

**Basic pathway of NKT cell development**

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Moreover, expression of co-receptors on NKT cell precursors, which might be more favorable targets, facilitate the selection of NKT cells by invasive pathogens. Thymus appears to be important for generation of intermediate NK1.1⁻ NKT cells in mice, but the majority of NKT cells emigrating from thymus to peripheral organs are NK1.1⁺, suggesting maturation of NKT cells occurs mainly in the periphery, although the acquisition of the NK receptors NK1.1 (CD161 in humans) or Ly49 is not required for thymic emigration[20].

Once committed to the NKT cell lineage, numerous transcription factors, cytokines, chemokines, and costimulatory molecules have been documented that are uniquely required for maturation and homeostasis of NKT cells, but dispensable for conventional T cell development. At least two key control points exist in NKT cell development, which govern NKT cell selection to give rise to immature NKT cells branched from the conventional T cell development pathway and NKT cell maturation with a series of phenotypic and functional changes, respectively[5, 21]. The basic pathway of NKT cell development is illustrated in Figure 1.

The earliest NKT precursors may also be susceptible to negative selection in the presence of strong agonist ligands. A dose- and time-dependent deletion of NKT cells is induced by the injection of high dose of the glycolipid agonist α-GalCer to fetal thymic organ culture (FTOC) or persistent injection
to young adult mice\[22\]. Similarly, the intrathymic NKT cell development is specifically blocked by the TCR-mediated selective signals, which strongly suggests NKT cells are subject to negative selection if they are highly reactive to self-glycolipid ligands during their development\[23\]. However, whether negative selection of NKT cells really exists in vivo during inherent cell development is still unclear, because α-GalCer is not considered to be a natural mammalian product. Furthermore, over-expression of CD1d resulted in a thymic deletion of high-affinity CD1d-restricted NKT cells in transgenic mice, as well drastically reduced frequency of NKT cells in FTOC, suggesting negative selection of NKT cells may be influenced by ligand expressing cells in the thymus through altering ligand density. Particularly, CD1d expressing thymic stromal cells, mainly DCs, rather than DP thymocytes mediated negative selection of NKT cells, and CD4\(^+\) NKT cells formed the majority of these deleted NKT cells\[22\]. Rarity in humans, even deficiency in mice, of CD8\(^+\) SP NKT cells suggests a possible mechanism of CD8-mediated negative selection during CD8\(^+\) SP NKT cell development. However, Engel and colleagues believe that the exclusion of CD8\(^+\) SP NKT cells from murine NKT cells is a by-product of enhanced expression of CD4 molecules on NKT cells, which is regulated by the transcription factor Th, Poxviruses and Zinc-finger (POZ), and Krüppel family (Th-POK)\[30\].

**Controls of NKT cell development and homeostasis**

Complete absence of NKT cells in mice deficient in recombinase subunits RAG-1 and RAG-2 or the TCR Ja18 segment demonstrated that successful rearrangement of TCRα gene segments Va14 to Ja18 is absolutely necessary to the subsequent selection of DP thymocytes\[25, 26\]. The signaling and transcription factors mentioned below act as master regulators on the NKT cell lineage, but distinct from conventional T cells\[49\]. Retinoic acid-related orphan receptor-γt (ROR-γt), a master regulator of NKT cell lineage, but distinct from conventional T cells, is located in alymphoplasia (aly/aly), leads to a unique reduction in NKT cell number due to close link between these genes and IL-15/IL-15R signaling pathway. A majority of NKT cells assembles in the liver may be attributable to Id2, which regulates the expression of chemokine receptor CXCR6 and anti-apoptotic molecules to determine the maturation and localization of NKT cells\[37\].

Human CD4\(^+\) NKT cells predominantly respond to IL-15, whereas CD4\(^-\) NKT cells are more responsive to IL-7 by coupling with up-regulated receptors, which is consistent with cytokine signaling pathways initiated by IL-15/IL-15R and IL-7/IL-7R. These pathways play a central role in proliferation and survival of NKT cells, but not conventional T cells\[13, 38\]. Mice with a deficiency in several genes, including IL-2Rβ (CD122), interferon-regulatory factor 1 (IRF-1), Fyn, Ets, lymphotixin (LT) and the hypomorphic allele of the NIK located in alymphoplasia (aly/aly), leads to a unique reduction in NKT cell number due to close link between these genes and IL-15/IL-15R signaling pathway. NK-like phenotype is acquired by human NKT cells in thymus and does not require peripheral expansion, which suggests that final maturation of NKT cells is completed mainly in thymus, although it does not exclude that CD161\(^-\) NKT thymic emigrants may acquire CD161 in periphery as well\[33\]. In our study, mouse fetal and neonatal NKT cells homogenously express IL-7Ra (CD127) and respond vigorously to IL-7 in vitro by proliferating and differentiating into mature CD161\(^+\) effector cells. Similarly, the maturation of human CD8\(^+\) SP NKT cells is also affected, which suggests that IL-7/IL-7R signaling is responsible for the expansion and maturation of NKT cells during fetal and postnatal lifecycles\[9, 39\]. Mutation of signaling lymphocytic activation molecule-associated protein (SAP), which is responsible for recruitment and activation of the Src kinase Fyn, leads to a deficiency of NKT cell development and results in X-linked lymphoproliferative (XLP) syndrome in humans\[40\]. Small microRNAs (miRNAs) regulate basic functions such as cellular proliferation, apoptosis, lineage commitment, and differentiation in the immune system as regulatory elements\[41\]. So the deletion of dicer in DP thymocytes resulted in a dramatic NKT
cell-autonomous defect in both thymic and peripheral compartments, supporting that dicer-dependent miRNAs play a critical role in differentiation and homeostasis of NKT cells[42]. Although the influence of costimulatory signaling, including CD28/B7 and CD40/CD40L, on NKT cell stimulation is controversial, reduced frequency of NKT cells in liver and spleen resulted from the blockage of CD28/B7 signaling pathway in vivo through transgenic CTLA-4Ig expression. This may point to a possibility that these signaling pathways are critical in periphery expansion of NKT cells, though, this remains to be formally investigated[43].

**Polarization of Th1 and Th2 cytokines in NKT cells**

The predominant characteristic of NKT cells is rapid production of copious amounts of Th1 and/or Th2 cytokines following stimulation[15]. Human cord blood exhibits a stronger Th2 response after polyclonal stimulation compared to adult NKT cells. However, neonatal NKT cells could be shifted towards a Th1 response under more polarizing conditions, while adult NKT cells are more resistant to shift in cytokine production[44]. Liver-derived DN NKT cells seem more protective than the ones from spleen and thymus. This suggests that different effects of NKT cells on tumor growth also depend on their tissue of origin[45]. This shift or switch between Th1 and Th2 cytokines produced by NKT cells is possibly a requirement of development, or even a byproduct of maturation. Yuling et al have reported that EBV-induced CD8⁺ NKT cells are prone to express more IFN-γ under the stimulation of α-GalCer, whereas CD4⁺ NKT cells bias towards secretion of IL-4 and IL-10, which is in accord with the finding that CD4⁺ NKT cells have the superior capacity to produce Th2 cytokines over CD4⁺ NKT cells including CD8⁺ NKT cells in humans, which preferentially induce Th1 cytokines production[7, 8]. This provides an explanation for separate immunoregulatory responses of individual subset of NKT cells, which could significantly affect the direction of an immune reaction. The detailed information on differences of three distinct functional subpopulations of NKT cells is listed in Table 1.

Invariant NKT cells could be induced under different conditions to mediate opposite effects by altering the balance of cytokine profiles[4]. Activation of NKT cells after α-GalCer treatment can alter the balance of secreted cytokines from a Th2-bias to a Th1-bias[46]. The activation/up-regulation of T-bet and GATA-3 is responsible for the induction of chromatin remodeling Th1 and Th2 cytokine genes locus and expression of Th1 and Th2 cytokines, respectively[31, 47]. IFN-γ

| Table 1. Comparison of distinct functional subpopulations of NKT cells |
|---------------------------------------------------------------|
| **Feature**       | **CD4⁺ NKT**                                      | **CD4⁻ CD8⁻ NKT**                      | **CD8⁺ NKT**                                      | **References** |
| Frequency         | High frequency (normal), or high frequency (specific stimulation) | Low frequency                           | Low frequency (normal), or high frequency (specific stimulation) | 7–9, 13, 45, 59, 61, 69, 70, 76, 80 |
| Mouse             | High frequency                                     | Low frequency                           | Undetectable                                      | 16, 20, 24, 53, 71,72 |
| Distribution      | 70%–80% of hepatic NKT cells; 55%–75% of splenetic and thymic NKT cells; ~60% of myeloid and lymphatic NKT cells (individual variations) | ~25% of hepatic NKT cells; 25%–35% of splenetic and thymic NKT cells; 35%–45% of myeloid NKT cells (individual variations) | Very few in Thymus, Liver, Spleen, bone marrow, Lymph nodes (normal); ~25% of thymic NKT cells and hepatic NKT cells (EBV challenge) | 7–9, 11–14, 18, 20, 44, 45, 61, 70-72 |
| Development       | Thymus-dependent origin, mature in thymus or in periphery, thymic proliferation and output | Thymus-dependent origin, mature in thymus or in periphery, thymic proliferation and output | Thymus-dependent origin, mature in thymus and thymic output, peripheral expansion | 7–9, 13, 17, 18, 20, 22–24, 30 |
| Cytokines         | High levels, but depending on conditions            | High levels                             | High levels                                        | 7–9, 11, 13, 14, 44, 45, 59, 61, 80 |
| Th1               | High levels, but depending on conditions            | Very low levels                         | Undetectable level                                 | 11–14, 44, 45, 53, 59, 61, 80 |
| Th2               | High levels, but depending on conditions            | Perforin/granzyme, TRAIL (depending on conditions), FasL (expressed on very few or no cells) | Perforin/granzyme                                  | 7–9, 12, 45, 69 |
| Cytotoxic molecules | FasL(CD95L), TRAIL, Perforin/granzyme (depending on conditions) | Perforin/granzyme, TRAIL (depending on conditions), FasL (expressed on very few or no cells) | Perforin/granzyme                                  | 7–9, 12, 45, 69 |
| Associated diseases | Autoimmunity diseases (type I diabetes, allergic asthma, SLE, infections, transplantation associated diseases (GVHD and allograft tolerance), some tumor types | Some tumors, transplantation associated diseases (GVHD and allograft tolerance), infections | Some tumors (EBV-associated malignancies), infections | 7–9, 11–14, 16, 45, 53, 59, 61, 80 |
deficient mice exhibited an increased incidence of carcinoma, suggesting that IFN-γ-dependent immune responses might be effective in the promotion phase of carcinogenesis[48]. Reconstitution of NKT cells from ICOS KO mice to NKT-deficient mice failed to develop airway hyperreactivity, because the ICOS/ICOSL interaction contributes to peripheral homeostasis of CD4+ NKT cells and production of Th2 cytokines such as IL-4 and IL-13[49]. The Th1 and Th2 effects of periphery NKT cells were abolished in CD28-deficient mice under the stimulation of α-GalCer, while Th1-like function was suppressed in CD40-deficient mice but with rather enhanced Th2 effect, which suggests that CD28- and CD40-mediated costimulatory signals differentially contribute to the regulation of Th1 and Th2 functions of NKT cells[50]. Polarization of NKT cells is based on intrinsic DC-mediated modulation of the Th1/Th2 balance, which is mainly dependent on IL-12 or IL-4 produced by mature DCs in vivo. To achieve the Th1/Th2 cytokine balance, a negative feedback loop selectively inhibits prolonged Th1 or Th2 responses of NKT cells through counter-regulation of Th2 or Th1 cytokines by mature DCs in vivo[51, 52]. In turn, immature DCs up-regulate MHC class II and costimulatory molecules in response to the stimulation of CD40 expressed on NKT cells, and activate NKT cells by autocrine IL-12 to express more IFN-γ.

It is clear that NKT cells that are protective against tumors are skewed toward Th1 cytokines, while type II NKT cells suppress tumor immunosurveillance based on their production of Th2 cytokines[53]. The potentially opposite effect of these two subsets of NKT cells was also determined in CD1d KO mice lacking both subtypes of NKT cells with decreased production of Th1 and Th2 cytokines but Jα18 KO mice lacking only NKT cells with reduced IFN-γ during murine schistosomiasis[54]. Jα18 KO mice are more susceptible to tumorigenesis and lack anti-tumor CTLs-mediated responses at the early time point, which demonstrates that NKT cells contribute to the natural anti-tumor immunosurveillance through Th1 cytokines during early tumor growth. However, the stimulation of type II NKT cells suppresses anti-tumor immunosurveillance by Th2 cytokines secretion[55]. Type I and type II NKT cells define a new immunoregulatory axis of opposing forces analogous to Th1 and Th2 cells since they not only have opposing functions but also counter-regulate each other, and any alterations of this axis may result in infectious diseases, autoimmune diseases and tumors. However, the detailed mechanism of suppressing anti-tumor immunosurveillance of type II NKT cells is not possible to define completely. Because it lacks specific markers to determine the selective development of type II NKT cells alone. Importantly, this immunoregulatory axis between type I and type II NKT cells could be a major determinant in the subsequent immune response to any skewing.

**NKT cells in tumor immunity**

Understanding of cross talk between various arms, cells and molecules of the immune system is instrumental in achieving novel immunotherapy protocols for cancers. Type I NKT cells mediate both protective and regulatory immune functions, including anti-tumor responses, protection against pathogens, maintenance of transplant tolerance and inhibition of autoimmunity, whereas type II NKT cells suppress anti-tumor surveillance in certain model systems[56]. A large mount of studies demonstrated the anti-tumor activity of NKT cells stimulated by α-GalCer or by IL-12 in vivo[57, 58]. Invariant NKT cells induce the so-called adjuvant effect on anti-tumor immunity by activating other anti-tumor cytolytic cells mainly through the Th1 cytokine cascades, although NKT cells have their own lytic activity and cause direct lysis of various tumor cells[59, 60]. The numerical and functional alteration of circulating Va24Vβ11+ NKT cells has arisen in patients with different types of tumor. The number of circulating Va24 NKT cells significantly decreased in patients with colon cancer, head and neck cancer, breast cancer, renal cell cancer, and melanoma. IFN-γ producing ability of single NKT cell was preserved, whereas the responsiveness of Va24Vβ11+ human NKT cells was still low in patients with lung cancer, advanced prostate cancer or other undefined advanced cancers except glcoma, even after stimulation with α-GalCer or granulocyte-macrophage colony stimulating factor (G-CSF)[61-64]. Significantly enriched NKT cells in surgically resected specimens of lung tumor and colorectal tumor may explain the decrease of circulating NKT cells in quantity[65, 66]. Some lipid components derived from tumor cell membranes have been demonstrated to bond with CD1d and stimulate NKT hybridoma cells, so the dysfunction of NKT cells is possibly attributable to tumor-derived ligands[67].

As the first line responder, NKT cells promptly secrete large amounts of inflammatory cytokines to promote cytotoxic effects in response to antigens in a direct and/or indirect manner. Activated NK cells have been well characterized in their ability to directly mediate anti-tumor responses through the delivery of cytotoxic effector molecules such as granzyme and perforin[68]. In our study, EBV-induced CD8+ NKT cells produced, remarkably, more perforin than their counterpart CD4+ NKT cells, which suggests a direct tumor cell-killing ability of NKT cells in vivo[69]. NKT cells directly eradicate target cells through cell death inducing ligands FasL and TNF-related apoptotic induced ligand (TRAIL)[69]. The recruitment of leukocytes into tissues is dependent on a series of adhesive and activation steps mediated by adhesion molecules and interaction of chemokine and chemokine receptors, which restrict their tissue and microenvironmental distribution[70-72]. In this regard, NKT cells resemble effector T cells because CD4+, CD8+, and DN subsets uniformly express non-lymphoid-tissue-homing chemokine receptors, such as CCR2, CCR5, and CXCR3[70]. The production of RANTES by NKT cells is important for the recruitment of APCs and the induction of regulatory T cells. It suggests that NKT cells can influence immune responses by the regulation of chemokines expression, which is likely to shape the ensuing immune responses[73, 74]. In addition, NKT cells can enhance antigen specific B cell response to secrete elevated levels of IgG, which leads to antibody-dependent cellular cytotoxicity (ADCC) to lyse cancer cells mediated by NK cells[75]. Moreover, NKT cells facilitate proper
DCs maturation and also improve migration of more mature DCs towards tumor sites from lymph nodes[76, 77]. Interaction of chemokines and its receptors facilitate licensed DCs for cross-priming naïve CTLs in a helper T cell-dependent way[78]. Activated NKT cells also induce DCs to up-regulate CCL17 acting with its receptor CCR4 expressed on CTLs, which is distinct from classical interaction of CCR5 and its three ligands, and might permit an additional level of selectivity in CTL attraction for NKT cell-licensed cross-priming[79, 80]. A semi-permeable transwell membrane co-culture system prevented the interaction between syngeneic spleen CD3+ T cells and NKT cells, which strongly suggests the synergy of NKT cells is direct cell-cell contact dependent[11]. In turn, murine hepatic NKT cells show negative regulatory activities on recruitment, activation and effector functions of intrahepatic γδT cells dependent on TLR3 signaling pathway[81].

It is important to note that potency of anti-tumor mediated by NKT cells may also be influenced by the type, complexity, and composition of tumor microenvironment in which they interact with neoplastic cells and other immune cells, although NKT cells may not infiltrate all tumors. Inflammatory mediators and effector molecules possibly released by tumor-infiltrating immunocytes are a contributing factor in suppression or promotion of angiogenesis and tumor growth. Expansion and activation of other immune effector cells are associated with activation of NKT cells in vivo. Immature DCs are activated by NKT cells to secrete IL-12 and IL-15, which are responsible for the activation of NK cells along with IFN-γ production. Moreover, activated DCs induced by NKT cells present tumor-derived antigens to activate conventional CD4+ Tregs and CD8+ CTLs, which enriches the adjuvant effect of NKT cells[82]. Th1 cytokines from activated NKT cells responded to α-GalCer promote cell-mediated cytotoxicity and maturation of DCs, which are involved in the development of cognate T cell responses[83]. The EBV-induced human CD8+ NKT cells cooperated with syngenic CD3+ T cells to kill or suppress EBV-associated tumors upon induction of Th1-bias, and the synergy is enhanced by addition of CD4+ NKT cells, which secrete high levels of IL-2 to maintain the transferred cells[7, 8]. Thymic CD8+ NKT cells induced by EBV could significantly drive CD3+ T cells to produce high levels of IFN-γ combined with autocrine Th1 cytokines to resist tumors, which is consistent with IFN-γ-mediated anti-tumor activity of NKT cells on α-GalCer stimulation[84, 85]. Additionally, the synergistic effect of EBV-exposed thymic CD4+ and CD8+ NKT cells also significantly suppressed EBV-associated malignancies and prolonged animal survival[5].

In clinical trials, therapeutic strategies mainly focus on the reconstitution of an adequate number of functional NKT cells either by active immunization or adoptive transfer of NKT cells activated in vitro[86]. With identification of glycolipids with high specificity to NKT cells and following high responsiveness to tumors, α-GalCer (KRN700) and its variants have been continuously identified and tested as powerful anti-tumor immunotherapeutic agents[87]. Exogenous glycolipid α-GalCer specially promotes the peripheral expansion of human CD8+ NKT cells from EBV infected human thymus transplanted SCID chimeric mouse, whereas the moderate cell proliferation for CD4+ NKT cells is noted[5]. Administration of α-GalCer-pulsed DCs activate murine NKT cells and eradicate established metastatic tumor foci in models of the mouse liver and lung metastasis. They may exert a greater anti-tumor activity than α-GalCer alone[87]. It possibly ascribes this difference to dysfunction of APCs in patients with cancer along with numerical and functional deficits of NKT cells, or free α-GalCer anergizes NKT cells after initial activation[88]. Autologous in vitro expanded and activated NKT cells were adoptively transferred to patients with cancers as a potential immunotherapeutic strategy. Endogenous murine NKT cells were also activated and expanded to inhibit tumor metastasis through intravenous injection of α-GalCer-pulsed DCs at intervals. Faster and more robust secondary immune activation points to the existence of NKT cells activation and/or memory induction[89]. Moreover, in vitro expansion and polarization methods of circulating NKT cells facilitate the production of activated NKT cells and improve therapies based on NKT cells[90]. Some clinical trials with α-GalCer-pulsed DCs as a phase I study show obvious tumor regression without severe adverse immunological and clinical responses[91]. Although adoptive transfer of in vitro activated circulating Va24/Vδ11 human NKT cells with α-GalCer seems to makes up for the deficiency of number and/or function of endogenous target NKT cells, refusion of expanded and activated NKT cells in vivo is accompanied with potential problems including in vivo premature clearance of activated NKT cells and dysfunction of APCs[92]. In contrast to therapeutic efficiency in animals, few clinical trials to date has succeeded in achieving significant efficacy against human tumors in vivo, which reflects a species-related difference in NKT cell activity or a planning difference to treat advanced cancer patients and adoptively tumor-transplanted animals. Thus immunotherapy based on NKT cells to bolster anti-tumor immunity might be a potent new tool for modulating immune responses against malignancies. Overall, these events that occur in tumor-immunity by NKT cells are illustrated in Figure 2. Since a limited space, detailed description of other important functions of NKT cells, such as protection against infection, maintenance of transplant tolerance, and inhibition of autoimmunity were not included in this review.

**Conclusion remarks**

Since NKT cells were discovered nearly three decades ago, there have been great advances in the understanding of NKT cell development, homeostasis, effector function, and therapeutic applications. However, there are still some perplexities that have not yet been completely resolved in spite of the progress made. Why is there so much individual variation between individuals, even congenic mouse strains and identical twins? In contrast to the mouse, very few CD8+ NKT cells reside in normal person and a significantly increased number of CD8+ NKT cells is found in healthy persons with latent EBV infection. This raises the question what the CD8+ NKT cell
origin and development pathways might be. How could the homeostasis of NKT cells and their production of Th1/Th2 cytokines be properly regulated or controlled in vivo? Another challenge in the field is to properly manipulate this balance of functional distinctive type I and type II NKT cells in the treatment of pathogenic states, particularly, in tumor therapy. Furthermore, understanding how to maintain this balance within the normal immune system is vitally important. Immuno-therapeutic approaches targeting NKT cells should break tolerance and prove to be useful in the induction of more effective immune responses for the improved treatment of cancers, infections and autoimmune diseases. A major paradox is the ability of NKT cells to synchronously promote and suppress immune responses since functionally distinct subpopulations of NKT cells and the communication with other immune arms exist. In order to completely understand the role of NKT cells, significant markers should be identified to differential diagnosis type II NKT cells and more work needs to be done to determine their functions other than the suppressive activity.

In conclusion, an important point is that various clinical applications based on NKT cells to treat malignancies, even combined with other effectors and tools, possibly become potential immunotherapy for cancers.

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References

1. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. Annu Rev Immunol 2007; 25: 297–336.
2. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. Annu Rev Immunol 2005; 23: 877–900.
3. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. Nat Immunol 2010; 11: 197–206.
4. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-restricted NKT cells. J Clin Invest 2004; 114: 1379–88.
5. Gapin L. iNKT cell autoreactivity: what is 'self' and how is it recognized? Nat Rev Immunol 2010; 10: 272–7.
6. Godfrey DI, Pellicci DG, Patel O, Kjer-Nielsen L, McCluskey J, Rossjohn J. Antigen recognition by CD1d-restricted NKT cell receptors. Semin Immunol 2010; 22: 61–7.

7. Yuling H, Ruijing X, Xiang J, Li L, Lang C, Jie X, et al. EBV-induced human CD8+ NKT cells suppress tumorigenesis by EBV-associated malignancies. Cancer Res 2009; 69: 7935–44.
8. Xiao W, Li L, Zhou R, Xiao R, Wang Y, Ji X, et al. EBV-induced human CD8+ NKT cells synergise CD4+ NKT cells suppressing EBV-associated tumours upon induction of Th1-bias. Cell Mol Immunol 2009; 6: 367–79.
9. Yuling H, Ruijing X, Xiang J, Li L, Lang C, Jie X, et al. EBV promotes human CD8+ NKT cell development. PLoS Pathog 2010; 6: e1000915.
10. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, 3rd, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature 2005; 434: 525–9.
11. Sen Y, Yongbl Y, Yuling H, Luokun X, Li H, Jie X, et al. V alpha 24-invariant NKT cells from patients with allergic asthma express CCR9 at high frequency and induce Th2 bias of CD3+ T cells upon CD226 engagement. J Immunol 2005; 175: 4914–26.
12. Tao D, Shangwu L, Qun W, Yan L, Wei J, Junyan L, et al. CD226 expression deficiency causes high sensitivity to apoptosis in NK T cells from patients with systemic lupus erythematosus. J Immunol 2005; 174: 1281–90.
13. Baev DV, Peng XH, Song L, Barnhart JR, Crooks GM, Weinberg KI, et al. Distinct homeostatic requirements of CD4+ and CD4- subsets of Valpha24-invariant natural killer T cells in humans. Blood 2004; 104: 4150–6.
14. Lee PT, Benlaga K, Teyton L, Bendelac A. Distinct functional lineages of human Valpha24+ natural killer T cells. J Exp Med 2002; 195: 637–41.
15. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Xu H, Nguyen H, et al. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. J Exp Med 2003; 197: 907–18.
16. Pellicci DG, Ulrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(-)CD4(+)+CD1d-dependent precursor stage. J Exp Med 2002; 195: 835–44.
lymphocyte homing and proliferation. Immunity 1998; 9: 669–76.

40 Pasquier B, Yin L, Fendaneche MC, Relouzat F, Bloch-Queyrat C, Lambert N, et al. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J Exp Med 2005; 202: 695–701.

41 Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. Cell 2009; 136: 26–36.

42 Fedeli M, Napolitano A, Wong MP, Marciais A, de Lalla C, Colucci F, et al. Dicer-dependent microRNA pathway controls invariant NKT cell development. J Immunol 2009; 183: 2506–12.

43 Ulrich AP, Crowe NY, Kyparissoudis K, Pellicci DG, Zhan Y, Lew AM, et al. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. J Immunol 2005; 175: 3092–101.

44 Kadowaki N, Antonenko S, Ho S, Rissoan MC, Soumelis V, Porcelli SA, et al. Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. J Exp Med 2001; 193: 1221–6.

45 Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. J Exp Med 2002; 202: 1279–88.

46 Ru W, Peijie C. Modulation of NKT cells and Th1/Th2 imbalance after alpha-GaCer treatment in progressive load-trained rats. Int J Biol Sci 2009; 5: 338–43.

47 Kim PJ, Pai SY, Brigi M, Besra GS, Gumperz J, Ho IC. GATA-3 regulates the development and function of invariant NKT cells. J Immunol 2006; 177: 6650–9.

48 Wakita D, Chamoto K, Ohkuri T, Narita Y, Ashino S, Sumida K, et al. Fedeli M, Napolitano A, Wong MP, Marciais A, de Lalla C, Colucci F, et al. Dicer-dependent microRNA pathway controls invariant NKT cell development. J Immunol 2009; 183: 2506–12.

49 Akbari O, Stock P, Meyer EH, Freeman GJ, Sharpe AH, Umetsu DT, et al. ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. J Immunol 2006; 177: 6650–9.

50 Williams JA, Lumsden JM, Yu X, Feigenbaum L, Zhang J, Steinberg SM, et al. Regulation of thymic NKT cell development by the B7-CD28 costimulatory pathway. J Immunol 2008; 181: 907–17.

51 Minami K, Yanagisawa Y, Iwabuchi K, Shinohara N, Harabayashi T, et al. Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance via dendritic cell and natural killer T cell interactions. Blood 2005; 106: 1685–93.

52 Onoe K, Yanagawa Y, Minami K, Iijima N, Iwabuchi K. Th1 or Th2 balance regulated by interaction between dendritic cells and NKT cells. Immunol Res 2007; 38: 319–32.

53 Park JM, Terabe M, van den Broeke LT, Donaldson DD, Berzofsky JA. Unmasking immunosurveillance against a syngeneic colon cancer by elimination of CD4+ NKT regulatory cells and IL-13. Int J Cancer 2005; 114: 80–7.

54 Mallevaey T, Fontaine J, Breuilh L, Paget C, Castro-Keller A, Vende- ville C, et al. Invariant and noninvariant natural killer T cells exert opposite regulatory functions on the immune response during murine schistosomiasis. Infect Immun 2007; 75: 2171–80.

55 Ambrosino E, Terabe M, Halder RC, Peng J, Takaku S, Miyake S, et al. Cross-regulation between type I and type II NKT cells in regulating tumor immunity: a new immunoregulatory axis. J Immunol 2007; 179: 5126–36.

56 Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, et al. A nonclassical non-Valpha14/Alphabeta18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. J Exp Med 2005; 202: 1627–33.

57 Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. J Immunol 1999; 163: 2387–91.

58 Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 1997; 278: 1623–6.

59 Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B, et al. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. J Immunol 2001; 167: 3114–22.

60 Terabe M, Berzofsky JA. The role of NKT cells in tumor immunity. Adv Cancer Res 2008; 101: 277–348.

61 Molling JW, Koijen W, van der Vliet HJ, Boomsma MF, Kruizenga H, Smorenburg CH, et al. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. J Int Cancer 2005; 116: 87–93.

62 Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, et al. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. J Immunol 2001; 167: 4046–50.

63 Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. J Immunol 2002; 168: 6494–9.

64 Dhodapkar KM, Cirignano B, Chamian F, Zagzag D, Miller DC, Finlay JL, et al. Invariant natural killer T cells are preserved in patients with glialoma and exhibit antitumor lytic activity following dendritic cell-mediated expansion. Int J Cancer 2004; 109: 893–9.

65 Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, et al. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. Clin Cancer Res 2005; 11: 7322–7.

66 Song L, Asgharzadeh S, Salo J, Engell K, Wu HW, Sposto R, et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. J Clin Invest 2009; 119: 1524–36.

67 Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. J Exp Med 2003; 197: 1667–76.

68 Fehninger TA, Cai SF, Cao X, Bredemeyer AJ, Presti RM, French AR, et al. Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. Immunity 2007; 26: 798–811.

69 Nieda M, Nicol A, Koezuka Y, Kikuchi A, Lapteva N, Tanaka Y, et al. TRAIL expression by activated human CD4(+)V alpha 24(+)NKT cells induces in vitro and in vivo apoptosis of human acute myeloid leukemia cells. Blood 2001; 97: 2067–74.

70 Kim CH, Johnston B, Butter EC. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)/V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. Blood 2002; 100: 11–6.

71 Cullen R, Germanov E, Shimaoka T, Johnston B. Enhanced tumor metastasis in response to blockade of the chemokine receptor CXCR6 is overcome by NKT cell activation. J Immunol 2009; 183: 5807–15.

72 Germanov E, Veinotte L, Cullen R, Chamberlain E, Butter EC, Johnston B. Critical role for the chemokine receptor CXCR6 in homeo-stasis and activation of CD1d-restricted NKT cells. J Immunol 2008; 181: 81–91.

73 Faunce DE, Stein-Streilein J. NKT cell-derived RANTES recruits APCs and CD8+ T cells to the spleen during the generation of regulatory T
cells in tolerance. J Immunol 2002; 169: 31–8.
74 Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T[H(1)]1 priming. Nat Immunol 2004; 5: 1260–5.
75 Galli G, Pittoni P, Tonti E, Malzone C, Uematsu Y, Tortoli M, et al. Invariant NKT cells sustain specific B cell responses and memory. Proc Natl Acad Sci U S A 2007; 104: 3984–9.
76 Montoya CJ, Jie HB, Al-Harthi L, Mulder C, Patino PJ, Rugeles MT, et al. Activation of plasmacytoid dendritic cells with TLR9 agonists initiates invariant NKT cell-mediated cross-talk with myeloid dendritic cells. J Immunol 2006; 177: 1028–39.
77 Vuylsteke RJ, Molenkamp BG, van Leeuwen PA, Meijer S, Wijnands PG, Haanen JB, et al. Tumor-specific CD8+ T cell reactivity in the sentinel lymph node of GM-CSF-treated stage I melanoma patients is associated with high myeloid dendritic cell content. Clin Cancer Res 2006; 12: 2826–33.
78 Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature 2006; 440: 890–5.
79 Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, et al. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. Nat Immunol 2010; 11: 313–20.
80 Moreno M, Molling JW, von Mensdorff-Pouilly S, Verheijen RH, Hooijberg E, Kramer D, et al. IFN-gamma-producing human invariant NKT cells promote tumor-specific antigen-specific cytotoxic T cell responses. J Immunol 2008; 181: 2446–54.
81 Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. Nat Immunol 2002; 3: 867–74.
82 Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, et al. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. Blood 2004; 103: 383–9.
83 Liu K, Ido yaga J, Charalambous A, Fujii S, Bonito A, Mordoh J, et al. Innate NKT lymphocytes confer superior adaptive immunity via tumor-capturing dendritic cells. J Exp Med 2005; 202: 1507–16.
84 Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med 2003; 198: 267–79.
85 Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antitumor effect of alpha-galactosylceramide. Blood 2002; 99: 1259–66.
86 Crowe NY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. J Exp Med 2002; 196: 119–27.
87 Yu KO, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, et al. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. Proc Natl Acad Sci USA 2005; 102: 3383–8.
88 Fujii S, Shimizu K, Tazbirkova A, Lin H, Yamaura A, Ide K, et al. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. Blood 2004; 103: 383–9.
89 van der Vliet HJ, Molling JW, Nishi N, Masterson AJ, Kolgen W, Porcelli SA, et al. Polarization of Valpha24+Vbeta11+ natural killer T cells of healthy volunteers and cancer patients using alpha-galactosylceramide-loaded and environmentally instructed dendritic cells. Cancer Res 2003; 63: 4101–6.
90 Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosylceramide loaded mature dendritic cells in cancer patients. J Exp Med 2005; 201: 1503–17.
91 Bagnara D, Ibarici A, Corselli M, Sessarego N, Tenca C, De Santanna A, et al. Adoptive immunotherapy mediated by ex vivo expanded natural killer T cells against CD1d-expressing lymphoid neoplasms. Haematologica 2009; 94: 967–74.