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Anti-tumor Potential of Type-I NKT cells against CD1d-positive and CD1d-negative Tumors in Humans

Leonid S. Metelitsa¹

Leonid S. Metelitsa: lsmeteli@txccc.org

¹Texas Children's Cancer Center, Center for Cell & Gene Therapy, Departments of Pediatrics and Immunology, Baylor College of Medicine, 1102 Bates Ave, Suite 1770, Houston, TX 77030, Tel: (832) 824-4395, Fax: 832-825-1206

Abstract

V α 24-invariant Natural Killer T cells (NKTs) are strictly CD1d-restricted, and CD1d expression has been found in several types of leukemia and lymphoma as well as in brain tumors suggesting that these malignancies could be targeted for direct NKT-cell cytotoxicity. Several studies have revealed strong positive associations between the numbers of tumor-infiltrating or circulating NKTs with improved disease outcome in patients with diverse types of CD1d-negative solid tumors. The mechanism by which NKTs mediate anti-tumor activity against CD1d-negative tumors has long remained enigmatic. Recent evidence indicates that NKTs can suppress tumor growth indirectly by targeting CD1d-positive elements of tumor-supportive stroma such as tumor-associated macrophages. This review summarizes the current knowledge about the mechanisms that regulate NKT-cell localization to the tumor site and their interaction with the tumor microenvironment. The discussed strategies for pharmacologic modulation and genetic engineering of NKTs may lead to development of effective and broadly applicable immunotherapies of cancer.

1. Introduction

V α 24-invariant or type-I Natural Killer T cells (NKTs) are an evolutionary conserved sub-lineage of T cells that are characterized by reactivity to self- and microbial-derived glycolipids presented by monomorphic HLA classI-like molecule CD1d. They express an invariant TCR α -chain, V α 24-J α 18 which is preferentially paired with V β 11 [1–5]. The first discovered ligands for NKTs were agelasphins and their synthetic analogue, α -Galactosylceramide (α GalCer, KRN7000), glycolipids with an α -anomeric linked galactose moiety that were derived from the marine sponge, *Agelas mauritianus* or the sponge-colonizing *Sphingomonas* bacteria and shown to have potent anti-tumor activity in mice [6]. Several recent studies indicate that NKTs could have been selected in evolution primarily for their role in antimicrobial defense [7–9]. It has been demonstrated that NKTs are required for host protection from some forms of Gram-negative bacteria such as *Sphingomonas* [8] or *Borrelia* [10] that do not contain LPS in their bacterial walls and, therefore, are unable to activate dendritic cells (DCs) via TLR-4. Instead of LPS, these microbes contain activating ligands for NKTs. NKTs can also be activated by as yet poorly defined endogenous antigens such as isoglobotrihexosylceramide (iGb3) [11;12] that are presented by DCs upon stress response to infection [13;14]. The elusive nature of the endogenous NKT ligands could in part be explained by recently reported findings indicating that these glycolipids constantly degrade and only accumulate in response to TLR-mediated inhibition of α -galactosidase A enzymatic activity in DCs [15]. Therefore, NKTs can sense both microbial and self-derived glycolipid antigen and therefore participate in a broad spectrum of immune responses ranging from protection from infection to autoimmunity [16–19].

NKTs are long-lived lymphocytes that develop in thymus and are present even in neonates as functional cells with effector-memory phenotype [20;21]. Unlike conventional T cells which are positively selected by thymic epithelial cells, NKTs are selected by CD1d-expressing CD4/CD8-double-positive thymocytes [22]. Recent studies revealed that expression of PLZF transcription factor immediately after positive selection enables intrathymic expansion and effector/memory differentiation of NKTs [23;24]. A number of other transcription factors are selectively involved in NKT-cell development [21], indicating that these cells have a unique differentiation program that reflects their distinct functional properties. NKTs may be viewed as innate memory T cells. Even in the resting state, they carry pre-formed mRNA for both type-1 and type-2 cytokines and produce large amounts of these cytokines within hours after activation [25]. Therefore, NKTs serve as an early source of cytokines that provide initial signals for other cells of the immune system to initiate innate and adaptive responses and protect host from infection and tumor growth.

Selective decrease of NKT cell number and/or their functional activity have been reported in patients with diverse types of cancer [26–28], suggesting that NKTs may play an important role in the anti-tumor immune responses and, conversely, an escape from NKTs may contribute to tumor progression. Our group has originally demonstrated that NKTs infiltrate primary human tumors in a subset of children with neuroblastoma (NB) and that NKT-cell infiltration is associated with an improved long-term disease-free survival [29], a finding that has been since extended to other malignancies [30;31]. NKT-cell infiltration in primary tumors served as an independent factor of favorable outcome in patients with colorectal cancers [30]. Low levels of circulating NKTs predicted a poor clinical outcome in patients with head and neck squamous cell carcinoma [31]. The anti-tumor potential of the type-I NKTs has been demonstrated in numerous tumor models in mice as summarized in the recent reviews [32–34]. There are other as yet poorly characterized CD1d-reactive T cells that have non-V α 24(V α 14) invariant or variable TCR α (type-II NKT) as well as non-CD1d-reactive T cells that express NK markers [4]. Several reports have demonstrated that type-II NKTs may play a regulatory role in tumor immunity and counteract type-I NKTs [35]. The present review focuses on the anti-tumor properties of human type-I NKTs and their potential application for the immunotherapy of both CD1d-positive and CD1d-negative malignancies.

2. Direct NKT cell cytotoxicity against CD1d-positive tumors

CD1d is preferentially expressed in hematopoietic cells, especially those of myelomonocytic and B-cell lineages and malignancies originating from the corresponding tissues often express CD1d. We originally demonstrated that CD1d is expressed on the majority of leukemia cells in patients with acute myeloid leukemia: AML, M4 and M5 as well as Juvenile myelomonocytic leukemia (JMML) [36]. Moreover, after pulsing with α GalCer, primary leukemia cells from these patients were sensitive to NKT-cell killing. CD1d expression has also been demonstrated on tumor cells in patients with B-cell malignancies such as B-precursor acute lymphoblastic leukemia with (MLL)/AF4 gene rearrangement [37] and B cell chronic lymphocytic leukemia (B-CLL) [38]. Many murine leukemia and lymphoma cell lines express CD1d, and a recent report demonstrated that NKTs play a protective role in A20 murine B-cell lymphoma model [39]. This protection correlated with the level of CD1d expression on lymphoma cells and was lost in NKT-deficient mice. The most recent study of CD1d expression in human lymphomas found that CD1d is expressed on the surface of the Hodgkin and Reed-Sternberg (HRS) cells in half of the classical Hodgkin lymphoma (cHL) cases whereas in non-Hodgkin lymphoma (NHL) only 30% tumors stained positive [40]. Interestingly, NKTs were present at high frequencies in primary cHL tumors and reactive lymph nodes regardless of CD1d expression on tumor cells. However, the functional role of tumor-infiltrating NKTs in cHL biology and disease

outcome is yet to be determined. We recently demonstrated that NKTs may co-localize with CD1d-positive tumor-associated monocytes/macrophages (TAMs) in the microenvironment of CD1d-negative tumors [41]. Similar to our findings in NB, a recent report demonstrated that an increased number of TAMs was strongly associated with shortened survival in patients with cHL [42]. Therefore, targeting both HRS cells and TAMs for immunotherapy with NKTs and/or their ligands may represent an attractive therapeutic strategy for patients with recurrent/resistant cHL. CD1d is also expressed on the tumor cells of another hematologic malignancy, multiple myeloma [43]. However, patients with multiple myeloma have functionally defective NKTs incapable of IFN γ production upon antigenic stimulation that may relate to the mechanism of the tumor progression [28;43;44]. The functional deficiency of NKTs in myeloma patients is reversible after *ex vivo* stimulation with α -GalCer-pulsed DCs, suggesting that ex-vivo expanded NKTs or appropriately stimulated endogenous NKTs could be used for immunotherapy of this disease [28;44].

Although the majority of non-hematopoietic solid tumors are CD1d negative, CD1d expression by tumor cells has been reported in malignant glioma [45]. In that work, K. Dhodapkar et al. demonstrated that some tumor cells and the angiogenic vessels in primary high-grade gliomas expressed CD1d and that unlike in other solid tumors in adults, the number and functional status of peripheral blood NKTs were preserved. Moreover, authors demonstrated that ex-vivo expanded NKTs from glioma patients could kill ligand-pulsed CD1d-positive human glioma cells in a CD1d-dependent manner. However, NKT-cell localization to the tumor site was not analyzed and it's yet to be determined whether NKTs can control growth of CD1d+ gliomas *in vivo*. An ongoing study in our lab has revealed CD1d expression in 9 of 20 primary tumors from patients with medulloblastoma, the most common pediatric brain tumor. After treatment with α GalCer, CD1d-positive medulloblastoma cell lines were highly sensitive to direct NKT-cell cytotoxicity *in vitro*, and intracranial injection of human NKTs in NOD/SCID mice with established orthotopic human medulloblastoma xenografts significantly delayed tumor growth (D. Liu et al., manuscript in preparation). Further therapeutic development for CD1d-positive brain tumors will require understanding of the mechanism of NKT-cell trafficking to CNS and to CNS tumors.

3. NKT-cell trafficking and localization to the tumor site

Several studies examined expression of chemokine receptors by freshly isolated peripheral blood human NKTs and evaluated their functional responses to a number of chemokine ligands [29;46–49]. Consistent with their effector-memory phenotype, the chemokine receptor profile of NKTs resembles that of effector-memory T cells, since the majority of NKTs express tissue-homing chemokine receptors: CCR2, CCR5, CCR6, CXCR3, and CXCR6. Of interest, some chemokine receptors were differentially expressed by CD4⁺ (CCR4) and CD4^{neg} (CXCR6) NKT subsets which were known to have distinct cytokine profiles *in vivo* [50;51]. This may enable selective targeting of functionally distinct NKT subsets to the tissues and differentially affect the microenvironment. Alternatively, NKT cell subsets may acquire their different cytokine profiles because of their localization to tissues with distinct microenvironment. Observations in mouse models of autoimmune and infectious disease also suggest that the dual immunoregulatory role of NKTs may depend on their trafficking pattern. For example, mouse NKTs that were recruited by MIP-2 to the spleen induced peripheral tolerance in a model of anterior chamber-associated immune deviation [52]. By contrast, NKTs that were recruited by CCL2 to the lungs mediated delayed-type hypersensitivity in a model of pulmonary infection with *Cryptococcus neoformans* [53]. Selective genetic deletion of NKTs in J α 18^{-/-} mice suppressed Th1 responses and prevented clearance of the infection. CCL2 production preceded NKT cell-mediated inflammatory responses in wild type mice, whereas the lack of lung infiltration

with NKTs in CCL2^{-/-} mice (NKTs remained functional) was associated with the failure to develop protective immunity against the fungus. Therefore, NKT-cell localization to the site of disease is essential for the development of protective immunity.

Recent studies of NKT-cell trafficking in cancer patients have demonstrated that NKTs localize to primary and metastatic tumors in subsets of patients with common types of cancer, both in children and adults. These included NB, myeloma, lung adenocarcinomas, colon cancer, HNSCC, hepatocellular carcinoma, and liver metastases of melanoma [28–30;54;55]. All studies that examined clinical outcome found that greater numbers of either circulating or tumor-infiltrating NKTs were associated with better outcome in patients [29–31]. However, only a few studies have examined the mechanism of NKT-cell localization to the tumor site. We found that NKTs migrate toward NB cells in a CCL2-dependent manner and infiltrate tumors that express high levels of CCL2 [29]. The decreased NKT-cell localization to tumors with high-level MYCN oncogene expression was due to MYCN-mediated transcriptional repression of the CCL2 promoter [49]. It has also been demonstrated that transcriptional repression of CCL2 and other chemokines for NKT and effector-memory CD8 T cells was associated with a general immune escape mechanism associated with STAT3-mediated oncogenic signaling in tumor cells [56] (Fig. 1). Therefore, the avoidance of tumor infiltration by immune effector cells, including NKTs is coupled with oncogenic transformation and needs to be considered in cancer immunotherapy.

Monocytes and other immature myelomonocytic precursors of TAMs can localize to the tumor site in response to CCL2 [57;58], the same chemokine that attracts NKTs. Monocytes, however, are also known to respond to multiple other tumor-derived chemotactic signals that are not recognized by NKT or other T cells [59]. The majority of these factors (VEGF, endothelin, angiopoietin-2) are produced in hypoxic conditions and drive TAM migration to the hypoxic areas [59–61]. However, we recently demonstrated that NKTs co-localize and are often in direct contact with TAMs in primary NB [41]. This raises the question of how NKTs migrate into TAM-rich hypoxic areas inside the tumor. To investigate NKT-cell *in vivo* trafficking and antitumor potential, we developed a NB/TAM model [41] (Fig. 2A–B) and examined kinetics of NKT-cell homing to the xenografts of tumor cells alone (NB) or with monocytes (NB/TAM). Fig. 2C demonstrates that maximum tumor infiltration occurs 48 h after NKT-cell transfer and the frequency of NKTs in NB/TAMs is 8 fold higher than in NB sites ($P<0.001$, t-test). We also found CD1d blocking had no effect on NKT-cell homing either to NB or NB/TAM sites (data not shown), indicating that the enhanced NKT-cell localization to NB/TAM cannot be explained by an antigen-specific retention. Based on this observation, we hypothesized that TAMs produce (a) chemokine(s) to attract NKTs. In hypoxia, human monocytes [62;63] and macrophages [64] have been shown to selectively up-regulate CCL20, a selective chemoattractant for iDCs, effector-memory T cells, and NKTs [48;65]. Of interest, NF- κ B is required for CCL20 transcription, and hypoxia-inducible transcription factor-1 (HIF-1 α) increases NF- κ B binding to its site in CCL20 promoter [64]. NF- κ B has also been shown to serve as a link between the innate immunity and the hypoxic response through transcriptional regulation of HIF-1 α [66]. However, the exact mechanism of the cellular innate response to tumor hypoxia and its relationship with the adaptive immunity remains largely unknown. In our ongoing studies, CCL20 has emerged as a strong candidate for a chemokine that mediates NKT-cell migration towards TAMs in NB (data not shown), suggesting that NKTs may co-localize with TAMs as a part of a novel innate response to tumor-induced hypoxia.

4. Indirect control of tumor growth by NKTs via regulation of CD1d-positive TAMs

The majority of examined solid tumors are CD1d-negative so that tumor cells cannot be a direct target for NKT-cell cytotoxicity [33;67]. Consistent with observations in mice, human NKTs could activate cytotoxicity of NK cells against NB cells [67]. However, NK cells poorly localize to established tumors in humans [68] and are at extremely low levels in primary NBs [69]. This suggests that NK cells may cooperate with NKTs to control the tumor initiation or the metastatic process, but are unlikely themselves to mediate NKT-cell function in established tumors. An alternative target for NKTs in established tumors could be CD1d-positive cells in the tumor stroma. We demonstrated that monocytes were the only CD1d-positive subset of human leukocytes that co-localized with NKTs to NB xenografts in NOD/SCID mice [49], suggesting that NKTs may interact with monocytes and their progeny in the tumor microenvironment. The accumulating evidence indicates that TAMs contribute to tumor progression in various types of cancer [60;70;71]. They are the dominant subset of CD45+ leukocytes that accumulate in human tumors [72] and suppress immune cell function via multiple mechanisms [59;61;72;73]. In a NB model, they directly support tumor growth, where in response to Galectin-3-binding protein secreted by NB cells, TAMs produce large amounts of IL-6, which stimulates proliferation of NB cells in a STAT3-dependent manner [41]. In a recent report, we demonstrated that TAMs can cross-present NB-derived glycolipids and be killed by NKTs in a CD1d-restricted manner that suppresses tumor growth in a humanized NB/TAM model in NOD/SCID mice [41]. Therefore, NKTs may mediate anti-tumor activity via removal of TAM-mediated growth support for tumor cells.

Besides TAMs, NKTs may systemically interact with CD1d+ myeloid-derived suppressor cells (MDSCs), which often accumulate during tumor growth and contribute to immune escape and tumor progression [74]. A recent report has demonstrated that CD11b+GR1+ MDSCs regulate immune response in a model of influenza A viral infection and that these cells are in turn controlled by NKTs in a CD1d-dependent manner [75]. The authors demonstrated that MDSC-mediated suppression of T-cell proliferation was mediated by ARG1 and NOS activity and that interaction between NKTs and MDSCs resulted in CD40-dependent inhibition of both enzymes' activity and enhanced secretion of IL-12. Importantly, both CD1d^{-/-} (lack CD1d and all NKTs) and J α 18^{-/-} (lack only type-I NKTs) mice had increased numbers of MDSCs, and adoptive transfer of NKTs to J α 18^{-/-} but not to CD1d^{-/-} mice returned MDSCs numbers to wt levels, and restored immunocompetence. These results indicate that NKT-MDSC interaction is CD1d-restricted and antigen-specific. The role of NKTs in the control of MDSCs has not been investigated in the context of anti-tumor immunity. There was, however, a report that MDSCs suppressed NKT-cell activity in tumor-bearing mice via nitric oxide-production *in vitro* [76]. This study demonstrated that administration of retinoic acid (RA), which is known to induce terminal differentiation of MDSCs [77], restored NKT-cell responses and the anti-metastatic activity of α GalCer. Therefore, as yet poorly understood complex reciprocal interaction likely exist between NKTs and MDSCs in the tumor-bearing host, and the sum effect of these interactions may influence the outcome of the anti-tumor immune response (Fig. 3). Our preliminary results demonstrate that MDSC numbers are significantly increased in CD1d^{-/-} compared to wild type tumor-bearing mice. A systematic evaluation of the reciprocal NKT-MDSCs interactions will be important for the development of NKT-cell based cancer immunotherapy.

The tumor microenvironment may affect NKT-cell viability and function, suggesting that an effective immunotherapeutic strategy with NKTs should consider the effect of tumor on NKTs and their homeostatic requirements. To examine the effect of tumor cells and tumor-induced hypoxia on the functional activity of NKTs, we activated NKTs by TCR stimulation

with OKT3 mAb and examined their proliferation in CFSE dilution assay under normoxia or hypoxia and with or without contact with NB cells. Fig. 4 demonstrates that hypoxia inhibits NKT-cell proliferation and the inhibition is strongly enhanced in the presence of NB cells. Studies in mice have demonstrated that NKT-cell development and homeostatic maintenance largely depend on IL-15 [78]. Our group reported that IL-15 also stimulates proliferation and enhances survival of human NKTs [20]. Unlike in the mouse, however, human CD4-negative (mostly CD8/CD4-double negative, DN) NKTs express much higher levels of IL-2R β than CD4+ subset so that IL-15 preferentially expands DN NKTs [20]. Importantly, DN NKTs are more cytotoxic than CD4+ NKTs, and a recent report demonstrated that only DN NKTs are required for anti-tumor responses *in vivo* [79]. This suggests that expression of IL-15 in NKTs for therapeutic purposes would support expansion, persistence, and anti-tumor activity of DN NKTs in cancer patients. We indeed found that transduction of NKTs with IL-15 cDNA protects them from the inhibitory effects of NB cells and hypoxia (Fig. 4), suggesting that IL-15 or NKTs engineered to express IL-15 (and a suicide gene) could be used therapeutically to enhance NKT-cell activity at the tumor site.

5. The use of NKT ligands as adjuvants for cancer vaccines

Because of the ability of NKTs to serve as an alternative to TLR danger-sensing system that provides an initial innate signal to activate adaptive immune responses, NKT-cell ligands have been extensively tested as adjuvants for vaccines against microbial pathogens and tumors. This area of NKT-cell research has recently been reviewed elsewhere [80;81]. Since then our group has demonstrated a potent adjuvant effect of NKT ligands, α -Glucuronosylceramide (GSL1) and α GalCer for a cancer vaccine carried by a live attenuated *Salmonella* vector [82]. The rationale of combining NKT ligands with *Salmonella*-based vectors came from the studies of NKT-cell-mediated response early in the course of *Salmonella* infection. Despite the fact that *Salmonella* does not have glycolipid antigens, which could directly activate NKTs, TLR-mediated signaling in DCs in response to *Salmonella* invasion leads to IL-12 and IL-18 production [13;14] and generation or accumulation [11;15] of NKT-cell endogenous ligands in DCs that in turn activate NKT-cell-mediated help to DCs, creating a positive amplification loop that plays an important role in the generation of protective immunity against *Salmonella*. Consistent with the described mechanism of NKT-cell help to DCs in *Salmonella* infection, we have demonstrated that NKT ligands strongly enhance IL-12 production induced by an attenuated *Salmonella* vector in human DCs and that *in vivo* co-administration of a NKT ligand with *Salmonella*-based survivin vaccine enhances effector-memory T cell responses. Furthermore, a combined use of survivin vaccine with GSL1 produced potent anti-tumor activity in mouse models of CT26 colon carcinoma and orthotopic DBT glioblastoma [82]. From the clinical application prospective, *Salmonella*-based vectors might have an advantage over other types of live bacterial vectors for recombinant vaccines because of excellent safety record of an FDA-approved oral *Salmonella* vaccine (*S. typhi* strain Ty21a, Vivotif[®]) in children and in adults [83;84] and potent immunogenicity with low toxicity in humans of more recently developed attenuated *S. typhi* strains, CVD 908 and CVD 909 [85].

6. Pending questions and future directions

Despite the progress in the understanding of NKT-cell biology and their role in tumor immunity, many important questions remain unanswered that impedes translation of NKT-cell anti-tumor potential into effective immunotherapies in cancer patients. In order to determine whether a certain tumor can be targeted for direct NKT-cell cytotoxicity, primary tumors need to be screened for CD1d expression on the surface of tumor cells. So far only a few types of solid tumors in humans have been characterized for CD1d expression. The

rapid progress in tissue microarray technology should facilitate this task in the near future [86]. However, there could be a heterogeneity of CD1d expression within the same type of tumor like we observed in medulloblastoma so that only a subset of patients may have CD1d-positive tumors. The functional status of CD1d expression (e.g. the ability to present α GalCer to NKTs) on the tumor cells need to be tested using established cell lines of the same tumor type or short-term cultures of primary tumor cells when it is possible. Those types of cancer that express functional CD1d could be targeted for direct NKT-cell cytotoxicity using either synthetic NKT ligands or/and *ex-vivo* expanded NKTs.

The localization of NKTs to the tumor site is another critical issue that may determine the efficacy of NKT-cell therapy. NKT-cell trafficking may depend on the tumor type and its chemokine profile [87]. Even within the same tumor, NKTs may use different signals to traffic from one tumor compartment to another. For example, in NB they may be directed by CCL2 and CCL20 in oxygenated and hypoxic tumor areas, respectively. Furthermore, the expression of NKT-cell chemoattractive chemokines can be controlled by oncogenes like MYCN in NB [49] or STAT3 in other tumors [88], suggesting that drugs that target oncogenic signals could synergize with NKT-cell immunotherapy via enhancement of effector cell localization to the tumor site. For example, sunitinib that induces tumor cell apoptosis via inhibition of STAT3 and AKT signaling pathways in several types of cancer including CD1d+ medulloblastoma [89;90] could be tested in combination with NKT ligands. Sunitinib may also enhance NKT-cell localization and anti-tumor activity against CD1d-negative tumors since the drug is also a potent inhibitor of MDSCs [89;91], which regulate NKT cell activity.

The discovery of the NKT-TAM axis in the mechanism of NKT-cell indirect anti-tumor activity against CD1d-negative tumors [41] may guide a rational design of NKT-cell immunotherapy alone or in combination with the current therapies. One such combination could include RA. It is known that CD1d expression in monocytes/macrophages is strongly up-regulated by RA via direct binding of RAR α to RARE site in CD1d promoter [92;93]. RA is also known to induce terminal differentiation of MDSCs [77] leading to restoration of NKT-cell functional activity and anti-tumor potential [76]. Since RA is currently a standard of therapy in high-risk NB [94], it is important to explore whether CD1d/NKT system contributes to RA anti-tumor efficacy and whether RA can sensitize tumor microenvironment for therapy with NKT ligands. Next, it will be important to determine the nature of the endogenous ligand(s), which are presented by TAMs to NKTs. We recently demonstrated that monocytic cells can cross-present NB-derived glycosphingolipids to NKTs [41]. Our preliminary data indicate that this cross-presentation requires lipid antigen processing in monocytic cells. The mechanism of intracellular trafficking and loading of glycolipid antigens to CD1d in monocytes and DCs has been well described in murine systems [5;95;96]. Exogenous lipids (e.g. from tumor cells) enter endosomes as lipoprotein complexes, from which lipids are extracted and loaded into recycling CD1d by a group of lipid transfer proteins, saposins, especially saposin B [95] while a fraction of exogenous lipids can be sorted to the ER and loaded into CD1d by the microsomal triglyceride lipid transfer protein (MTP) [97]. Studies with human CD1d indicate that access to lysosomal lipid transfer proteins is important for presentation of exogenous antigens [98–100]. However, the signals that control the lysosomal trafficking of human CD1d molecules differ from those in mice and remain poorly understood [100]. Additional studies are required to define the mechanism of CD1d-mediated cross-presentation of tumor-derived glycolipids to NKTs in humans.

The strategy of isolating and expanding antigen-specific T cells as a therapeutic intervention has been validated in anti-viral and anti-tumor immunity [101;102]. The genetic modification of T cells with chimeric antigen receptor (CARs) for re-directed antigen

specificity is one strategy to generate effector cells for adoptive therapy that does not rely on pre-existing anti-tumor T-cell immunity and can overcome immune tolerance [103;104]. A recent phase I trial demonstrated that autologous Epstein Barr Virus (EBV)-specific T cells genetically modified to express CAR specific for GD2 on NB cells can induce remissions [105]. Accumulated evidence indicates that the therapeutic effect of the virus-specific T cells depends on their ability to persist for long-term after infusion, which is due to their reactivity to latent EBV antigens [106]. NKTs could serve as an alternative platform for generating CAR-modified effector cells for cancer immunotherapy because their long-term persistence and effector functions are supported by the intrinsic factors and do not depend on infection. Moreover, NKTs have innate anti-tumor properties discussed in this review that could be combined with tumor-specific CAR expression. Another approach is to redirect NKT-cell cytotoxicity against tumor cells using bispecific fusion proteins as it has recently been demonstrated with CD1d fusion to HER2-specific scFv in a B16 melanoma model [107]. Therefore, NKT-based immunotherapies could be designed to target both tumor cells and tumor-supportive microenvironment.

There is a growing evidence that co-stimulation plays a critical role in the activation, expansion, and survival of NKTs. Murine and human NKTs constitutively express CD28 and rapidly upregulate CD40L on activation [108;109]. It also has been shown that blocking antibodies to B7 or CD28 inhibit the proliferation of murine NKTs induced by α GalCer [110]. Several reports demonstrated that B7:CD28, CD40:CD40L, and OX40:OX40L pathways are important for the expansion and subsequent systemic cytokine production by NKTs [25;111]. NKTs also express OX40 and intratumoral administration of DCs modified to express OX40L induced OX40-dependent NKT-cell accumulation and IFN- γ production at the tumor site that resulted in a potent suppression of tumor growth [112]. Another major co-stimulatory molecule 4-1BB (CD137) is important for NKT cell homeostasis and function [113]. CD137 is not expressed on quiescent NKTs but was rapidly induced upon TCR engagement, and CD137 stimulation by an agonistic anti-4-1BB mAb promoted NKT cell activation resulting in enhanced cytokine production of NKTs in response to α GalCer [114]. Further studies of the NKT-cell co-stimulatory requirement may provide a rationale for combined use of NKT ligands with therapeutic antibodies targeting co-stimulatory molecules and for the design of tumor-specific CAR expression with appropriate co-stimulatory endodomains for optimal NKT-cell expansion, *in vivo* persistence, and anti-tumor activity.

In summary, several studies have revealed strong positive associations between the numbers of tumor-infiltrating or circulating NKTs with improved disease outcome in diverse types of cancer. Functional CD1d expression has been found in several types of leukemia and lymphoma as well as in brain tumors in children and adults, suggesting that these malignancies could be targeted for direct NKT-cell cytotoxicity using synthetic NKT ligands or/and adoptive transfer of *ex-vivo* expanded NKTs. In CD1d-negative tumors, NKTs have been shown to suppress tumor growth indirectly by targeting CD1d-positive elements of tumor-supportive stroma such as TAMs. Regardless of the mode of NKT-cell anti-tumor activity, NKTs must localize to the tumor site and remain functional in the tumor microenvironment. Recent findings indicate that tumors have evolved various escape mechanisms that result in the inhibition of NKT-cell trafficking and effector functions. The enabling NKT-cell tumor localization and functional activity at the tumor site via pharmacological modulation of oncogenic signaling in tumor cells or/and genetic engineering of NKTs as discussed in this article will lead to development of effective and broadly applicable immunotherapies of cancer.

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8. Abbreviations

αGalCer	α -galactosylceramide
GSL1	α -Glucuronosylceramide
(i)DCs	(immature) dendritic cells
G3BP	galectin-3-binding protein
EFS	event-free survival
MDSCs	myeloid-derived suppressor cells
NB	neuroblastoma
NKTs	V α 24-invariant NKTs
RA	retinoic acid
TAMs	tumor-associated monocytes/macrophages.

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Figure 1. Summary of the prior work on NKT-cell migration and function in NB

Tumor cells secrete CCL2, which attracts NKTs. Monocytes also localize in response to CCL2 and other factors and differentiate to TAMs. TAMs secrete IL-6 that supports NB cell survival and proliferation in a STAT3-dependent manner. NKTs do not recognize CD1d-negative tumor cells, but can recognize and kill or inhibit TAMs, which cross-present tumor-derived glycolipids via CD1d. This provides an indirect mechanism of NKT-cell anti-tumor activity via removal of stromal support to tumor cells. MYCN overexpression in a subset of NBs represses CCL2 expression, leading to the absence of NKTs in these tumors despite the presence of TAMs (which can respond to lower amounts of CCL2 or other chemokines). This mechanism could contribute to immune escape by MYCN-amplified NBs.

Figure 2. NKT-cell activity in NB/TAM model

(A) CHLA-255/luc cells were s/c injected alone (NB) or with monocytes (NB/TAM) in the right or left flanks of the same mice ("Control"). Other groups in addition received NKTs in both flanks ("NKT") alone or with anti-CD1d 51.1 mAb ("NKT+anti-hCD1d"). Shown are bioluminescence (BL) images at day 21. (B) NKTs (2×10^7 cells/mouse) were i/v injected to mice with 21-day established s/c tumor+monocytes xenografts and 48 hr later tumor tissues were analyzed for presence of NKTs (6B11, red) and TAMs (CD68, green). (C) Experimental set-up as in B with or w/o anti-CD1d. NB and NB/TAM xenografts were analyzed at indicated time points by FACS for NKT-cell frequency as percent of human CD45+ cells. Data are from one of at least 2 experiments.

Figure 3. Hypothetical role of NKTs in the immune response to CD1d-negative solid tumors
Tumor (e.g. NB) cells secrete chemokines (e.g. CCL2), which attract monocytes and NKTs. Monocytes differentiate to TAMs and migrate to hypoxic areas in response to VEGF and other factors. TAMs produce IL-6 and stimulate tumor growth as well as STAT3-mediated immune suppression. Under the influence of hypoxia and tumor-derived factors, TAMs produce CCL20 that attracts iDCs and NKTs to TAM-rich areas. TAMs and iDCs express CD1d and present tumor-derived or endogenous glycolipids to NKTs. Activated NKTs inhibit TAMs and promote iDC maturation followed by effective antigen presentation to CD8 T cells, leading to anti-tumor CTL generation. NKTs also systemically control and are controlled by MDSCs, and the result of this reciprocal regulation affects the efficacy of anti-tumor response.

Figure 4. NKTs are inhibited by hypoxic tumor environment and can be rescued by IL-15 expression

In vitro expanded NKTs from a healthy individual were transduced with a retroviral vector encoding IL-15 and the suicide gene iCaspase-9 with a selectable marker based on a truncated form of CD34 (iCasp9.ΔCD34/IL-15v) [115]. CFSE-labeled parental or IL-15-expressing NKTs were stimulated with OKT3 mAb and cultured for 4 days under normoxia (21% O₂) or hypoxia (1% O₂) with or without addition of SKN-BE2 NB cells (1:1). Shown are plots from one of two experiments with similar results.