

Immunotherapeutic strategies targeting natural killer T cell responses in cancer

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Received: 22 April 2016 / Accepted: 22 June 2016 / Published online: 8 July 2016
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Abstract Natural killer T (NKT) cells are a unique subset of lymphocytes that bridge the innate and adaptive immune system. NKT cells possess a classic $\alpha\beta$ T cell receptor (TCR) that is able to recognize self and foreign glycolipid antigens presented by the nonclassical class I major histocompatibility complex (MHC) molecule, CD1d. Type I NKT cells (referred to as invariant NKT cells) express a semi-invariant V α 14J α 18 TCR in mice and V α 24J α 18 TCR in humans. Type II NKT cells are CD1d-restricted T cells that express a more diverse set of TCR α chains. The two types of NKT cells often exert opposing effects especially in tumor immunity, where type II cells generally suppress tumor immunity while type I NKT cells can enhance anti-tumor immune responses. In this review, we focus on the role of NKT cells in cancer. We discuss their effector and suppressive functions, as well as describe preclinical and clinical studies utilizing therapeutic strategies focused on harnessing their potent anti-tumor effector functions, and conclude with a discussion on potential next steps for the utilization of NKT cell-targeted therapies for the treatment of cancer.

Keywords NKT cells · CD1d · Cancer immunotherapy · α -Galactosylceramide

Introduction

The initial discovery of conserved V α 14 chains across several hybridoma lines was quite unusual (Imai et al. 1986) because T cell receptors (TCRs) are highly diverse, with 70–80 α chain genes giving an enormous diversity to TCR α . Then a population of thymocytes possessing a single V β chain (V β) was described and posited to “represent a distinct lineage” (Fowlkes et al. 1987). It was this discovery that prompted the search for a subtype characterized by an invariant V α 14 chain with V β chains of limited diversity. Koseki et al. discovered that the frequency of the V α 14⁺ cells was much higher than expected, with V α 14⁺ cells comprising as much as 10–20 % of T cells in the livers of mice (Koseki et al. 1990). Almost a decade after the discovery of this remarkable population of V α 14⁺ cells, Adachi et al. noted a significant reduction in β 2-microglobulin-deficient mice (Adachi et al. 1995). Later, Lantz and Bendelac ascertained that the V α 14⁺ cells were CD1d restricted and it became clear that this was a distinct population of T cells (Lantz and Bendelac 1994).

NKT cell subsets and cytokine profiles

NKT cells have been characterized according to their expression of CD4 or lack thereof, particularly the distinct Th1 and Th2 cytokine profiles of these subsets. In humans, CD4⁺ NKT cells produce Th1 and Th2 cytokines, whereas the CD4⁻ subset, which includes both CD8⁺ and double negative (DN), primarily produces Th1 cytokines (Godfrey et al. 2010). In addition, NKT cells are phenotypically similar to effector T

This article is published in the Special Issue *CD1, MRI, NKT, and MAIT: Evolution and Origins of Non-peptidic Antigen Recognition by T lymphocytes* with Guest Editor Dr. Dirk Zajonc

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cells because they express non-lymphoid tissue homing chemokine receptors such as CCR2, CCR5, and CXCR3.

Mouse NKT cells also include CD4⁺ and CD4⁻ subsets, with different subsets possessing diverse functional profiles. There is heterogeneity within CD4⁺ NKT cell subset in regard to the expression of the IL-17 receptor B (IL-17RB), a receptor for IL-25. CD4⁺ IL-17RB⁺ NKT cells are reported to produce high levels of IL-13 and IL-4, but little IFN- γ in response to IL-25 stimulation. Another subset of NKT cells within the NK1.1⁻ CD4⁻ subset is the retinoic acid receptor-related orphan receptor (ROR) γ ⁺ NKT cells, which produces IL-17A and IL-22 (Michel et al. 2007; Terashima et al. 2008; Watarai et al. 2012).

Coquet et al. demonstrated that mouse NKT cell populations exhibit diversity in their ability to produce cytokines, depending on the organ and subsets. NKT cell subpopulations from thymus, spleen, and liver produced 19 cytokines with differences of 10- to 100-fold in response to anti-CD3/CD28 stimulation, depending on the organ (Coquet et al. 2008). Liver-derived NKT cells produced equal or higher amounts of IFN- γ , as compared to thymic NKT cells in response to CD3/CD28 stimulation (Coquet et al. 2008). The majority of NKT cells found in the periphery express both IL-4 and IFN- γ mRNA (Stetson et al. 2003). Thus, NKT cells are primed to exert their effector functions could thus provide immediate protection at sites of pathogen entry, while conventional T cells are undergoing antigen-specific expansion (Stetson et al. 2003). Moreover, production of these cytokines may facilitate conventional T cell differentiation, with NKT cells serving as primary initiators of antigen-specific responses.

Depending on the lipid presented to NKT cells in the context of CD1d, differential cytokine profiles can be induced (Zajonc and Girardi 2015). The prototypical NKT cell agonist is α -galactosylceramide (α -GalCer), a glycolipid originally isolated from a marine sponge, *Agelas mauritanus* (Kawano et al. 1997). α -GalCer induces rapid cytokine production and proliferation and has been extensively studied as an adjuvant in cancer. For example, α -GalCer induces IL-4, IL-13, and IFN- γ , but β -GalCer is a poor inducer of IFN- γ , TNF- α , GM-CSF, and IL-4 gene expression (Ortaldo et al. 2004).

IL-12p70 and IL-23 are members of a small family of heterodimeric cytokines predominantly produced by dendritic cells (DCs) and macrophages. IL-12p70 is involved in the induction and amplification of the Th1 response, while IL-23 mediates inflammatory responses, through induction of expansion of Th17 cells (Ortaldo et al. 2004). Uemura et al. demonstrated that when NKT cells are co-cultured with DCs, NKT cells enhance the IL-12p70 production while downregulating IL-23 production by DCs (Uemura et al. 2009).

Effects of cytokines produced by NKT cells

NKT cells can mediate anti-tumor activity via multiple mechanisms (Fig. 1). First, they can directly kill tumor cells. Second, they can induce maturation of dendritic cells in a CD40-CD40L-dependent manner (Fujii et al. 2007), thus initiating adaptive anti-tumor immunity. Finally, they activate NK cells and T cells by producing pro-inflammatory cytokines, such as IFN- γ and TNF- α . Using mouse tumor models of FBL-3 erythroleukemia and B16 melanoma, it was shown that in the absence of NKT cells, NK and T cells could not mediate tumor rejection (Cui et al. 1997).

In vivo administration of α -GalCer rapidly activates NKT cells to release Th1 and Th2 cytokines, which contribute to the activation of NK cells, dendritic cells, and T lymphocytes. Immature DCs can present antigens to NKT cells, which induce DC maturation, which in turn provides the necessary co-stimulation for NKT cell activation (Zaini et al. 2007).

Co-stimulatory requirements

NKT cells constitutively express cytokine mRNA and can synthesize cytokines in the absence of CD28 signaling, unlike conventional T cells, which require CD28 for cytokine gene transcription (Wang et al. 2009). Cytokine production by NKT cells is independent of CD28/CD40 co-stimulatory pathways. While CD28^{-/-} mice have NKT cells, CD28 and CD40 signaling has been shown to be required for expansion of the

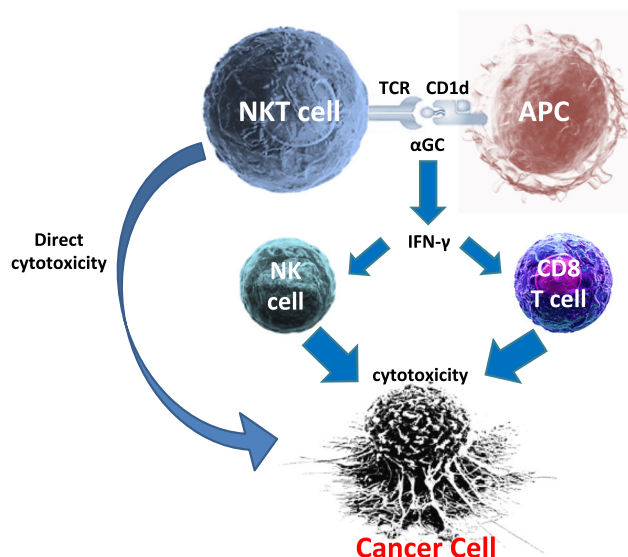


Fig. 1 NKT cells bridge innate and adaptive immune response. NKT cells have been shown to augment anti-tumor responses due, in part, to their capacity for rapid production of large amounts of IFN- γ , which acts on NK cells to target MHC negative tumors, and also, to target CD8 cytotoxic T cells to promote killing of MHC-positive tumors

NKT cells *in vivo*. Thus, NKT cells can be activated without CD28 co-stimulation, while the production of downstream factors such as IL-12 by DCs may be necessary to mount a full NKT cell response (Uldrich et al. 2005).

NKT cells express CD28 and CD28-CD80/CD86 co-stimulation is necessary for IL-4 production, which is drastically inhibited in presence of anti-CD80 and anti-CD86 antibodies (Hayakawa et al. 2001). Moreover, blockade of CD80 and CD86 resulted in a marked but partial inhibition of IFN- γ production. CD28- and CD40-deficient B6 mice have impaired IFN- γ production, but IL-4 production is impaired in CD28-deficient mice only. In addition, NKT cells from CD28 or CD40-deficient mice have impaired cytotoxic activity. Thus, CD154-CD40 blockade polarizes NKT cells towards a Th2 phenotype, with Th2 cytokine production being dependent on CD28 co-stimulation. On the other hand, IFN- γ production is dependent on IL-12 production by DCs and requires CD154-CD40 interaction, while CD28 is able to regulate IFN- γ production independently of IL-12, suggesting that IFN- γ production is dependent on both CD28 and CD40 co-stimulation in C57BL/6 mice (Hayakawa et al. 2001).

α -GalCer has been shown to promote DC maturation and subsequent antigen-specific T cell responses, with CD40-CD40L necessary for optimal DC-NKT cell interaction (Croudace et al. 2008). Moreover, TNF- α -induced maturation of DCs led to OX40L upregulation, which, through engagement of OX-40 on NKT cells, activated NKT cells (Zaini et al. 2007). IL-12 produced by DCs is critical for IFN- γ production by NKT cells and requires CD154-CD40 interaction. Due to their ability to rapidly produce a wide variety of cytokines, NKT cells can polarize the immune response towards a Th1 profile; while in other cases, NKT cells drive Th2 responses. In addition, NKT cells play an immunoregulatory or immunosuppressive role in some systems, usually through their production of the Th2-type cytokines; while in other systems, they appear to promote enhanced cell-mediated immunity via production of Th1-type cytokines. Work focused on delineating the factors that drive cytokine polarity of the NKT cell response and influence NKT cell responses in the context of initiating or regulating the host's innate and adaptive immune system are ongoing.

Suppression of NKT cell activation

As a pillar of innate immunology and initiation of the adaptive immune response, methods of NKT cell suppression are of great interest in disease pathology. Decreased suppression results in inflammatory diseases, while increased suppression or dysfunction of the immune system is often a characteristic of exacerbated chronic infections, autoimmunity, and cancer.

Cancers are the masters of immunosuppression. Some types of tumors can suppress NKT cells via shedding of inhibitory glycolipids (Sriram et al. 2002; Webb et al. 2012).

There have been three primary focuses of cellular suppression of NKT cell activation in anti-tumor immunity: regulatory T cells, immature myeloid cells, and NKT cells. Regulatory T cells (Tregs) are a well-known immunoregulatory component, and thus, the Treg:NKT interaction was one of the first to be examined closely. *In vitro* studies testing V α 24⁺ NKT proliferation, cytokine production, and direct cytotoxicity indicated that CD4⁺CD25⁺ Tregs can inhibit NKT responses in a dose-dependent, contact-dependent manner (Azuma et al. 2003). Yanagisawa et al. discovered that NKT cell responses are suppressed by the nitric oxide production of myeloid-derived suppressor cells (MDSCs) and that this suppression can be subverted by forced maturation of the MDSCs using all-*trans*-retinoic acid (Yanagisawa et al. 2006). In addition, it was shown that CCL20-producing tumor-associated macrophages (TAMs) can act as a hypoxic trap for tumor-infiltrating NKT cells, resulting in a loss of NKT cell function within the tumor microenvironment (Liu et al. 2012). Lastly, NKT cells can inhibit anti-tumor immunity (Osada et al. 2005). This phenomenon initially seemed very contradictory, but is explained by two concepts. First, the initial studies looked at bulk NKT cells. Type II NKT cells can be suppressive in the context of anti-tumor immunity by producing IL-13 that induces MDSCs (Ambrosino et al. 2008; Marrero et al. 2015; Terabe et al. 2005). Secondly, type I NKT cells are heterogeneous and the context of activation may affect their anti-tumor capacity through Th2 skewing (Renukaradhya et al. 2006). As shown by Izhak et al., there is an intricate network of suppression in the tumor microenvironment with multiple levels of suppression acting at once (Izhak et al. 2013). Collectively, these studies demonstrate that understanding NKT cell suppression within the tumor microenvironment mediated by tumors, Tregs, MDSCs, and type II NKT cells is extremely important in the context of developing effective cancer immunotherapy.

Utilization of NKT cells in vaccines for cancer treatment

Cancer therapies that incorporate NKT cell agonists have shown promise in improving vaccination strategies. Dendritic cell vaccines have been on the cusp of incorporation into standard cancer immunotherapy since their inception over two decades ago, but their complexity has slowed their progress. In an attempt to control all the variables, the first iterations of DC vaccines involved *ex vivo* antigen pulsing and maturation of monocyte-derived DCs (MoDCs) and suffered from low efficacy. *In vivo* maturation of DCs produces much better results, but the process must be carefully controlled to

produce the desired Th1, CTL skewed anti-tumor immune response. DC maturation signals can include microbial products that trigger Toll-like receptors (TLRs) and co-stimulation provided by conventional T cells or NKT cells which occurs at a much higher frequency than antigen-specific conventional T cells at the start of an adaptive immune response (Vincent et al. 2002). The activated, memory phenotype of NKT cells makes them a natural choice for *in vivo* DC maturation. Upon reinfusion, DCs present α -GalCer to NKT cells via CD1d and the NKT cells in turn supply maturation signals to the DC (Toura et al. 1999).

The complexities of traditional DC-based vaccines have encouraged research into simpler methods such as using NKT cell activation as a type of adjuvant. NKT cells activated by α -GalCer stimulate anti-tumor immunity via IFN- γ that enhances the innate response through NK cell activity and the adaptive response via DC production of IL-12 and encouragement of a Th1, CTL response (Nakagawa et al. 2001). In mice, the ability of NK cells to be activated by IFN- γ from NKT cells and IL-12 from APCs has been well established (Eberl and MacDonald 2000). While the experiments to determine the ability of NKT cells to transactivate NK cells with the help of DCs have not been performed in humans, the interplay between NKT cells and the innate and adaptive immune systems is very important. It has been demonstrated in human cancer patients that NKT cell stimulation by α -GalCer-loaded mature DCs leads to increased IL-12 serum levels and a robust T cell response with the ability to produce memory CD8 T cells (Chang et al. 2005).

Protein antigen co-delivered with *i.v.* α -GalCer results in rapid DC maturation and induction of an effective adaptive immune response to challenge with tumors expressing that antigen (Fujii et al. 2003b; Hermans et al. 2003). This response is dependent on co-stimulatory molecules such as CD40 and CD80/86. DCs cross-present necrotic cell antigen more effectively than soluble antigen, therefore irradiated tumor cells administered α -GalCer (*i.v.*) as an adjuvant resulted in even more potent immune response (Liu et al. 2005). This concept was demonstrated in another study by induction of anti-tumor immunity to poorly immunogenic cancers such as plasmacytoma and myelomonocytic leukemia after *i.v.* administration of irradiated tumor cells loaded with α -GalCer (Shimizu et al. 2007). Other effective cell options for adjuvant loading have included antigen-loaded MDSCs and allogeneic, antigen mRNA-transfected fibroblasts (Fujii et al. 2009; Ko et al. 2009a).

Although α -GalCer has been shown to be relatively safe even at high doses, there has been a significant amount of research into alternative NKT-activating ligands for use as adjuvants. Tumors such as human melanoma can express NKT cell-activating ligands that enhance specific targeting (Wu et al. 2003). Because NKT cells can produce Th1, Th2, and immunosuppressive cytokines upon activation, the most

pressing issue for this research is ensuring a Th1 phenotype of NKT cell activation. Indeed, many NKT cell antigens elicit differing cytokine and activation profiles (Wu et al. 2005). α -C-GalCer is a promising synthetic C-glycoside analog of α -GalCer because it shows much higher specificity for Th1 skewing *in vivo* when tested in anti-tumor models (Schmiege et al. 2003). While alpha-C-GalCer has been extremely effective in mice, it has shown reduced capacity in humans, leading to investigation of C-glycoside analogs (Li et al. 2009). Synthetic glycolipids that have shown potential for the strong Th1 skewing needed in cancer immunity include PyrC- α GalCer, 7DW8-5, NU- α GalCer, and DB06-1 and highlight the need to characterize how different glycolipid structures affect binding to CD1d and interaction with the NKT TCR (Aspeshlagh et al. 2011; Aspeshlagh et al. 2013; Birkholz et al. 2015; Li et al. 2010). Similarly, the NKT antigen β -mannosylceramide represents a complementary adjuvant to α -GalCer by inducing anti-tumor immunity that is dependent on TNF- α and NOS instead of IFN- γ (O'Konek et al. 2011). The phenotype of the DC interacting with the NKT cell can also impact its anti-tumor capacity. For instance, cooperative stimulation of TLR4 or TLR9 on DCs and NKT cells enhances the resulting adaptive immune response (Hermans et al. 2007; Paget et al. 2007). Administering anti-tumor antibodies with TLR agonists and NKT-activating ligands enhances NK-mediated ADCC (Moreno et al. 2008). CD1d fusion proteins allow a more physiologic stimulation of NKT cells that eliminates the risk of anergy. α -GalCer-loaded CD1d-anti-HER2 scFV has been shown to localize activated NKT cells to the tumor site and induce anti-tumor adaptive immunity (Corgnac et al. 2012; Stirnemann et al. 2008). In allergic airway inflammation models, α -GalCer-antigen conjugates have been used to induce tolerance to allergens (Anderson et al. 2014). The cumulative work of the past decade has made consideration of NKT cells in vaccine design second nature.

NKT cells in preclinical studies

One of the earliest preclinical studies that determined the significance of NKT cell-mediated anti-tumor responses was demonstrated by the IL-12-mediated rejection of tumors (Cui et al. 1997). It was initially hypothesized that IL-12-induced anti-tumor effects were mediated mainly by NK cells or CD8⁺ T cells (Tahara 1995). To elucidate the specific mechanism of IL-12, $J\alpha 18^{-/-}$ mice (lacking the invariant TCR) were generated and injected with melanoma cell lines, which preferentially metastasize to the liver. Liver metastases were greater in $J\alpha 18^{-/-}$ mice than WT and were similar to RAG^{-/-} mice. This study confirmed that NKT cells are primarily responsible for IL-12-mediated tumor rejection and shed light on the functions of the various subsets of lymphocytes *in vivo*.

Since this initial study, many advances have been made in our understanding of NKT cells, in general, and their anti-tumor properties, as reviewed in (McEwen-Smith et al. 2015; Robertson et al. 2014). To date, NKT cell responses to a variety of solid tumors and hematopoietic malignancies have shown preclinical efficacy.

Hematological malignancies

NKT cells are known to exert their effector functions by recognition of CD1d on target cells. Thus, expression of CD1d is important for tumor recognition and subsequent elimination (Haraguchi et al. 2006). Many hematological malignancies, including myelomonocytic leukemia (Metelitsa et al. 2003), chronic and acute lymphoblastic leukemia (CLL and ALL; Fais et al. 2005; Anastasiadis et al. 2014), and lymphoid neoplasms (Guo et al. 2014), express CD1d molecules and the costimulatory proteins needed to induce an anti-tumor immune response by NKT cells. Despite this, most of these cancers are poorly immunogenic, and CD1d expression correlates with a poor prognosis (Fais et al. 2005). However, these malignancies are primed for NKT cell-based immunotherapeutic interventions, and numerous preclinical studies demonstrate their potential. Studies by Dhodapker's group have identified CD1d-binding ligands that are inflammation-associated lysophospholipids isolated from myeloma patients (Chang et al. 2008).

Our lab has assessed the NKT cell responses in spontaneous model of B cell lymphoma. It was found that treatment of these tumor-bearing mice with a single intravenous dose of α -GalCer resulted in delayed disease progression and prolonged survival compared to vehicle treated controls (Li et al. 2014a). Additional treatment methods involve α -GalCer incorporated into vaccination strategies. Consistent with our work, a single vaccination with α -GalCer-loaded A20 lymphoma cells elicited effective anti-tumor immunity against tumor challenge and tumor regression (Chung et al. 2007). Thus, these studies set the basis for the efficacy of more innovative strategies such as NKT cell adjuvant-based vaccination strategies, and adoptive immunotherapy.

Tumor cell vaccines are a more recent immunotherapeutic strategy. It involves loading of α -GalCer into autologous tumors administered as an irradiated cellular vaccine. The rationale for this approach is that the irradiated tumor cells will act as a source of tumor-specific neoantigens, in addition to the agonistic α -GalCer, thus allowing for a robust immune response mediated largely by NKT cells. Moreover, this method is relatively simple, inexpensive (in comparison to genetically modified T cells), and patient-specific (Mattarollo and Smyth 2013). These vaccines have shown potential in the preclinical setting of hematological malignancies. In a murine mouse model of acute leukemia, C1498, one such study demonstrated anti-cancer efficacy with i.v. administered, irradiated α -

GalCer-loaded leukemic cells. The vaccine was highly effective as a prophylactic in preventing the onset of leukemia, but was unable to provide any protective benefit in established malignancies. However, if mice were treated with standard chemotherapy and the vaccine, the mice were protected upon rechallenge with second dose of leukemic cells. The lack of response to established cancers was found to be due to several immunosuppressive mechanisms including the presence of Tregs, and MDSCs, as well as tumor cell intrinsic means (Gibbins 2014). It would be interesting to determine if further modulation of NKT cells via adjuvants can render other cytotoxic cells resistant to immune suppression, as previously described in breast cancer (Kmieciak et al. 2011). Taken together, these findings have far-reaching clinical implications.

NKT cell adoptive immunotherapy in the preclinical setting has demonstrated potential in lymphoid neoplasms (Bagnara et al. 2009). Ex vivo expanded NKT cells were shown to limit the growth of a lymphoblastic CD1d⁺ cell line, but not CD1d⁻ or CD1c⁻ expressing negative controls. A significant reduction in tumor burden was observed after two sequential doses of α -GalCer and NKT cells. Moreover, these cells were present within the tumor after 24 h (Bagnara et al. 2009). These studies highlight a role for ex vivo expanded NKT cells as an effective therapeutic tool for treatment of CD1d-expressing hematologic malignancies.

Solid tumors

Preclinical studies examining NKT cell responses within solid tumors have been investigated extensively. Initial work assessing the role of NKT cells in immune regulation showed differential anti-tumor immunity of murine NKT cell subsets in methylcholanthrene (MCA)-induced sarcomas (Crowe et al. 2005). It was determined that liver-derived, but not spleen or thymus-derived, NKT cells are the predominant mediators of α -GalCer-induced rejection of established MCA tumors. Additionally, CD4⁻ and CD4⁺ liver-derived NKT cells were administered separately to tumor-bearing TCR J α 18^{-/-} mice. Tumor rejection was only observed in the CD4⁻ population, while the CD4⁺ population only partially slowed growth compared to PBS-injected control. Further investigation in this study found that IL-4^{-/-} mouse-derived NKT cells transferred to B16F10 (metastatic melanoma cell line)-inoculated mice conferred protection and decreased the number of lung metastases (Crowe et al. 2005). This investigation supports the notion that NKT cell subsets show varying levels of anti-tumor immune regulation and that the CD4⁺ subsets that produce IL-4 enhance tumor immune evasion.

Type II NKT cells have been shown to be the major contributor of NKT cell subsets to anti-cancer immune suppression (Renukaradhya et al. 2008; Terabe et al. 2005). Sulfatide has been established as an antigen specifically recognized by type II NKT cells, solely (Ambrosino et al. 2007). Therefore,

the use of sulfatide should abrogate an immune response to cancer, and tolerate tumor growth and metastasis. Consistent with this, treatment of CT26 lung metastases with α -GalCer was hindered by the simultaneous injection of sulfatide (Kato et al. 2014). Thus, various NKT cell subsets play distinct roles against cancer, and these subsets are highly dependent, and responsive, to antigenic stimuli.

Improved characterization of NKT cell-specific antigens has revealed several agonists with anti-tumor potential. Unlike α -GalCer, which protects against cancers in a largely IFN- γ -dependent manner, β -mannosylceramide (β -ManCer) demonstrates anti-tumor potential by way of an IFN- γ -independent mechanism. One study has reported that NKT cell anti-tumor immunity against CT26 lung metastasis induced by β -ManCer is nitric oxide and TNF- α -dependent. Moreover, β -ManCer can activate type I NKT cells without inducing the long-term anergy associated with α -GalCer activation demonstrated by PD-1 expression. This lack of functional anergy allows for a second dose to provide therapeutic benefit against lung metastasis (O'Konek et al. 2013). It will be interesting to see how targeting co-stimulation influences NKT cell anti-tumor responses (Parekh et al. 2009; Zaini et al. 2007).

Lastly, combination therapy of three mAbs incorporating NKT cell-specific stimulation (termed 1DMab) demonstrated eradication of a variety of established tumor cell lines in mice (Teng et al. 2009). These results strongly support the rational combination NKT cell-based immunotherapeutics, in the form of mAbs, vaccines, or ex vivo expansion for adoptive immunotherapy.

NKT cell-based immunotherapy

Numerous studies have shown that cancer patients have a deficiency in both NKT cell number and function (Fujii et al. 2003a; Kawano et al. 1999; Tahir et al. 2001), suggesting that in vivo NKT cell modulation may be ineffective in patients with low NKT cell numbers. In 2005, Molling et al. found that NKT cell numbers were 47 % lower in cancer patients compared to age and gender matched healthy controls among a large cohort of patients (Molling et al. 2005). This reduction in NKT cell numbers was independent of tumor type or stage/grade. Chang et al. showed that multiple injections of α -GalCer-loaded mature dendritic cells lead to sustained expansion of NKT cells and antigen-specific T cells. However, these expanded NKT cells from cancer patients still exhibited reduced capacity for IFN- γ secretion compared to NKT cells from healthy controls (Chang et al. 2005). Importantly, it was shown that lenalidomide enhances antigen-specific expansion of NKT cells in response to α -GalCer in both healthy donors and patients with myeloma (Chang et al. 2006). Studies by this group later demonstrated that treatment of patients with

asymptomatic myeloma with three cycles of combination of α -GalCer-loaded monocyte-derived dendritic cells and low-dose lenalidomide led to reduction in tumor-associated monoclonal immunoglobulin in three of four patients (Richter et al. 2013). Adoptive transfer of in vitro-activated NKT cells in patients with refractory lung cancer resulted in an increase in both the number of circulating NKT cells as well as the number of IFN- γ producing cells in the peripheral blood. The objective anti-tumor response rate, however, remained low. These studies indicate that cancer patients have a deficiency in both NKT cell number and function, suggesting that NKT cell modulation may be ineffective in patients with low NKT cells. Therefore, for these patients, adoptive immunotherapy using ex vivo-expanded NKT cells may be a more productive strategy (Motohashi et al. 2006).

Adoptive immunotherapy refers to the ex vivo stimulation and expansion of autologous T or NK cells, followed by reinfusion of the expanded lymphocyte population back into the patient. Thus far, the most successful efforts in adoptive immunotherapy have focused on the expansion of cancer-specific T cells isolated from tumors with a high mutation rate (i.e., melanoma) or ex vivo expansion of cancer-reactive T cells from the peripheral blood of patients. Rosenberg et al. were the first to demonstrate that ex vivo-expanded autologous tumor-specific cells are able to traffic to tumor sites and directly induce tumor shrinkage in vivo (Rosenberg et al. 1988). However, the efficacy of cancer immunotherapy using targeted T cells has been limited by tumor-induced immunosuppression mediated by myeloid-derived suppressor cells (MDSC) that interfere with T cell effector functions. To overcome MDSC-mediated immunosuppression, one strategy has focused on the conversion of MDSCs to APCs, which identified NKT cells as a key facilitator in the maturation of MDSCs into mature myeloid cells with anti-tumor immune stimulatory function (Ko et al. 2009b; Lee et al. 2012). A previous study using PBMCs of patients with early-stage breast cancer demonstrated that CD25⁺ NKT cells in combination with reprogrammed immune cells and cultured in the presence of MDSCs induced them to lose/downregulate CD11b, which was associated with HLA-DR up-regulation (Payne et al. 2013). More recent work by the same group demonstrated that concurrent administration of the epigenetic drug, decitabine, with adoptive transfer of tumor-sensitive T cells and CD25⁺ NKT cells rendered T cells resistant to remaining MDSCs and resulted in significantly prolonged survival of animals bearing metastatic tumor cells (Payne et al. 2016).

A limitation of adoptive immunotherapy is that patients must have preexisting tumor reactive cells, which are difficult to identify in non-melanoma malignancies. In addition, conventional T cells are limited by their HLA restriction (Vivier et al. 2012). To address this issue, methods have been developed to engineer T cells to express genes encoding tumor antigen specific T cell receptors or chimeric antigen receptors.

Chimeric antigen receptors (CAR)

The chimeric antigen receptor (CAR) T cell field has historically focused on using autologous T cells to derive the therapeutic dose. Currently used CARs typically consist of an extracellular single chain variable fragment of an antibody for antigen binding and an intracellular T cell receptor zeta chain that mimics TCR activation as well as 1 or 2 co-stimulatory signaling domains, such as CD28 or 4-1BB (Imai et al. 2004; Maher et al. 2002; Sadelain et al. 2013). A promising target for CAR-modified T cells is CD19 for B cell malignancies, since CD19 is only expressed on B cells and perhaps follicular dendritic cells (Uckun et al. 1988) and destruction of normal B cells is well tolerated. Indeed, recent clinical trials demonstrated that T cells redirected against the CD19 antigen induce sustained complete responses in patients with B cell malignancies (Brentjens et al. 2013; Grupp et al. 2013; Kochenderfer et al. 2012; Porter et al. 2011).

The use of polyclonal T cells for CAR therapy, however, is associated with a concomitant risk of toxicity and GVHD. An alternate approach would be to use nonconventional donor T

cells, such as NKT cells, to generate CAR NKT cells. NKT cells are of particular interest since NKT-cell infiltration of primary tumors is associated with better outcomes in diverse tumors (Metelitsa et al. 2004; Tachibana et al. 2005). As outlined in Fig. 2, CAR NKT cells have distinct mechanistic advantages over CARs generated from bulk T cells. After activation, NKT cells exhibit NK-like MHC-independent cytotoxic activity against tumor cells through several mechanisms, including perforin and granzyme secretion, Fas ligand, or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (Kawano et al. 1999; Nieda et al. 2001). In addition, NKT cells can also rapidly produce large amounts of IFN- γ as well as other cytokines that can activate other immune cells such as NK cells, DCs, and CD8 T cells (Carnaud et al. 1999; Fujii et al. 2004; Fujii et al. 2003b), thereby serving as a bridge between innate and acquired immunity (Taniguchi et al. 2003). In addition, because NKT cells are CD1d restricted, they can be adoptively transferred to patients regardless of HLA allele expression. CD1d is expressed by only a few cell types (Brossay et al. 1998); thus, the potential toxicity of adoptively transferred NKT cells is limited.

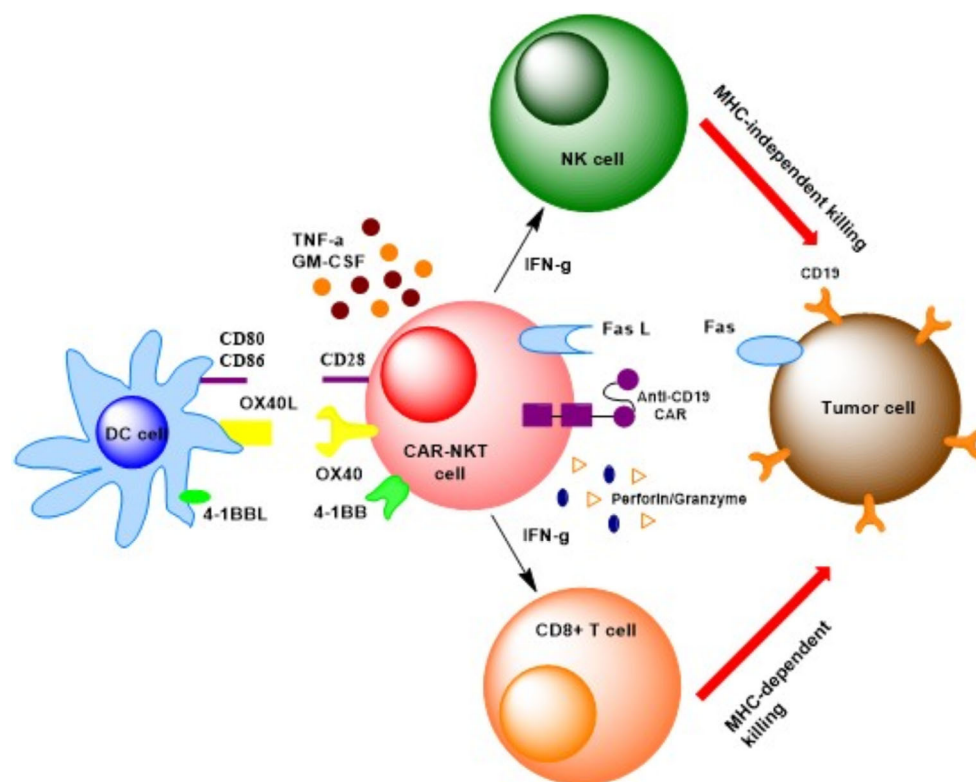


Fig. 2 NKT cells can mount strong anti-tumor responses and have become a major focus in the development of effective cancer immunotherapy. Following activation, NKT cells rapidly secrete cytokines (i.e., IFN- γ , TNF- α , and GM-CSF) and can directly mediate cytotoxicity through FAS/FASL, perforin, and granzyme. Cytokine production by NKT cells can lead to the activation of NK cells, CD8⁺ T cells, and in combination with CD40/CD40L interactions lead to the

maturation of dendritic cells (DC). NKT cells may be directed towards tumor cells expressing specific antigen through the transduction and expression of chimeric antigen receptors (CAR). CARs utilize an extracellular antigen-targeting domain (purple); thus, CARs are capable of binding their target antigen in an MHC-independent manner. Current research efforts are focused on harnessing the adaptability of CARs to enhance NKT cell targeting of tumors

Several studies have reported that donor-derived NKT cells may suppress GVHD while still maintaining anti-tumor function (Morris et al. 2005; Pillai et al. 2007). In agreement with these reports, a recent study demonstrated that reconstitution of NKT cells in peripheral blood is associated with long-term remission of pediatric leukemia patients receiving haplo-identical transplantation (Casorati et al. 2012; de Lalla et al. 2011; Dellabona et al. 2011). Moreover, NKT cells have been shown to colocalize with tumor-associated macrophages (TAMs) and can kill or inhibit these growth-promoting cells in a CD1d-dependent manner (Song et al. 2009).

Studies using CAR-modified NKT cells, however, have been relatively scant. Using a model of neuroblastoma, Heczey et al. demonstrated that the ex vivo expansion of human primary NKT modified with CARs specific for the GD2 ganglioside exhibited potent yet specific cytotoxicity against both GD2-positive tumor cells as well as CD1d-positive M2 macrophages in vitro (Heczey et al. 2014). Because the presence of TAMs with M2-like phenotype is associated with poor patient prognosis in neuroblastoma (Asgharzadeh et al. 2012), the elimination or inhibition of TAMs by CAR.GD2 NKT cells may sensitize tumor cells to CAR-mediated cytotoxicity and decrease the chances of tumor immune escape. CAR.GD2 NKT cells also effectively localized to the tumor site and had potent anti-tumor activity in a metastatic model of neuroblastoma without the induction of GVHD, successfully demonstrating the potential of NKT cells to serve as a safe and effective platform for CAR-redirectioned cancer immunotherapy (Heczey et al. 2014).

Artificial antigen-presenting cells

Effective adoptive T cell immunotherapy of cancer requires sufficient numbers of in vitro-generated, tumor-specific lymphocytes. The in vitro expanded T cells must also possess the capacity to engraft, proliferate, and persist in vivo after transfer. During and after the initial encounter with tumor antigens, the signals that T cell receive from antigen-presenting cells (APCs) can influence their programming and subsequent therapeutic efficacy (Harty and Badovinac 2008). Due to the inability to precisely regulate the signals and interactions provided during natural APC and T cell interactions, interest has increased in the use of artificial antigen-presenting cells (aAPC) in order to provide a greater degree of control to the generation of T cells for adoptive immunotherapy.

Originally, autologous antigen-presenting cells such as dendritic cells, monocytes, and activated B cells have been employed to generate tumor-specific T cells in vitro. However, the requirement to access cancer patients' blood to prepare autologous APC from each patient in a timely manner is burdensome and the quantity and quality of

prepared autologous APC varies between individuals. To overcome these issues, investigators have recently proposed the use of cell-based artificial APCs as a standardized reagent to reliably expand anti-tumor T cells in vitro. Cellular aAPCs derived from primary, transformed human, or xenogenic cells have been generated using retroviral or lentiviral transduction to introduce molecules that provide the necessary TCR, co-stimulatory, and adhesion molecules for synapse formation (Paulos et al. 2008).

Non-cellular aAPC can be utilized both for their potential clinical value in ex vivo T cell expansion as well as to investigate the basic requirements for T cell activation. Studies from Schneck's group have shown that MHC-Ig-based aAPCs can be used to effectively expand CMV and MART-1-specific CTL (Oelke et al. 2003). Monocyte-derived DCs from cancer patients express reduced levels of co-stimulatory molecules and produce less inflammatory cytokines (Onishi et al. 2002). To determine the effectiveness of bead-based approaches on NKT cell propagation and activation, CD1d-Ig-based aAPCs have been generated (Shiratsuchi et al. 2009; Webb et al. 2009). CD1d-based aAPC can be modified to systematically evaluate the role of a panel of potential co-stimulatory molecules on NKT cell proliferation and function.

Bispecific antibodies

The concept of bispecific antibodies was first introduced in the 1980s as a method to target multiple antigens by a single antibody (Raso and Griffin 1981). They are composed of fragments of two different monoclonal antibodies that can bind two different antigens. Recombinant bispecific antibodies can be generated with or without an Fc region (Kontermann and Brinkmann 2015). Bispecific antibodies with anti-CD3 and anti-CD19 components have been utilized in numerous preclinical studies, resulting in rapid and effective cytotoxicity specific for CD19⁺ B cells (Loffler et al. 2000; Schlereth et al. 2006). Blinatumomab, a bi-specific T cell engager (BiTE[®]) antibody construct made by fusing an anti-CD3 scFv to an anti-CD19 scFv via a short five residue peptide linker has performed impressively in multiple clinical trials as a single agent for relapsed/refractory B cell ALL (Topp et al. 2015; Topp et al. 2014). Although not as common, bi-specific antibodies have also been used for NKT cells. Using CD8⁺ NKT cells redirectioned with a bi-specific antibody against HER2 and CD3, Scheffold et al. demonstrated that administration of these NKT cells resulted in rapid, and in most instances sustained, eradication of HER2-expressing tumor cells in a *scid* mouse model, highlighting a promising strategy for NKT-based adoptive immunotherapy of neoplastic diseases (Scheffold et al. 2002; Table 1).

Table 1 Preclinical studies using NKT cell-based therapies

	Cancer type	Therapeutic used	Outcome	Reference
Hem.	Mantle cell lymphoma	aGC (single dose, 2 µg)	Delayed disease progression and increased survival	Li et al. (2014a)
	A20 B cell lymphoma	aGC (loaded tumor vaccine)	Regression, resistance to tumor rechallenge	Chung et al. (2007)
	C1498 acute leukemia	aGC (loaded into irradiated leukemic cells)	Highly preventative, no regression, and resistance to tumor rechallenge	Gibbins (2014)
	C1R B cell lymphoblast	aGC + ex vivo expanded NKT (2×)	Regression (found NKT TILs)	Bagnara et al. (2009)
Solid	MCA-induced sarcoma	aGC + CD4-hepatic NKT population	Tumor regression in NKT cells only	Crowe et al. (2005)
	CT26 colon carcinoma metastatic model	aGC + sulfatide	Blocked anti-metastatic effect of αGC and increased tumor metastasis	Kato et al. (2014)
	CT26 colon carcinoma metastatic model	β-ManCer	NO ₂ -TNFα-dependent regression and no long-term anergy	O’Konek et al. (2011)
	4T1 mammary carcinoma, renal, and CT26	Combination mAbs anti-DR5 + CD137 + CD1d (1DMab)	Tumor eradication	Teng et al. (2009)

Clinical studies using NKT cell-based therapies

Many studies have highlighted NKT cells as an ideal anti-tumor therapy (Fujii et al. 2013). Trials to date have included various types of solid and hematological malignancies (Dhodapkar and Richter 2011; Schneiders et al. 2011). Here, we will provide a brief overview of clinical trials using NKT cells in cancer patients, which reveal the potential of NKT cell-based therapeutics, refer to Tables 2 and 3.

Giaccone et al. described one of the first clinical trials utilizing α-GalCer (KRN7000) therapy (Giaccone et al. 2002). Although KRN7000 was well tolerated in cancer patients over a wide range of doses, no objective clinical response was observed. They observed a transient decrease in NKT cells following injection of α-GalCer. Importantly, increased serum cytokine levels (TNF-α and GM-CSF) were observed in patients with relatively normal NKT cell numbers, suggesting that efficacy depends on pretreatment NKT cell numbers.

Studies by Nicol’s group evaluated the clinical and immunologic effects of α-GalCer-pulsed monocyte-derived DCs administered at a 2-week interval to 12 human subjects with metastatic malignancy. It was found that activation of NKT cells results in increased serum IFN-γ and IL-12 (Nieda et al. 2004). In another study, this group examined the percentage, absolute number, and function (responsiveness to α-GalCer stimulation) of peripheral blood NKT cells in healthy donors and cancer patients (Crough et al. 2004). Similar to other studies, it was found that NKT cells were significantly reduced in melanoma and breast cancer patients. They performed a phase I clinical trial with α-GalCer-pulsed DC and found that the treatment could induce immune responses, and that the responses elicited are highly dependent on cell dose and administration route (Nicol et al. 2011).

Ishikawa et al. performed a phase 1 clinical trial to target endogenous NKT cell activation. Patients with advanced and recurrent non-small cell lung cancer were treated with α-

Table 2 Clinical trials using NKT cell-based therapy

Reference	Phase	Type of cancer	Number of patients	Country
Giaccone et al. (2002)	I	Solid tumor	24	The Netherlands (1998–2000)
Ishikawa et al. (2005)	I	Non-small cell lung cancer	11	Japan (2001–2002)
Motohashi et al. (2006)	I	Non-small cell lung cancer	23	Japan (2003–2004)
Motohashi et al. (2009)	I–II	Non-small cell lung cancer	6	Japan (2004–2006)
Zhang et al., unpublished	I–II	Solid tumor	120 (estimated)	China (2015–2018)
Guo et al. (2014)	I	Melanoma	20 (estimated)	China (2015–2017)

Table 3 Patient profiles following treatment with NKT cell therapy

Reference	Number of patients	Adverse events			Immunologic response	Anti-tumor response
		Grade 1	Grade 2	Grade 3		
Giaccone et al. (2002)	24	Flush (1) and sneezing (1)	None	Fever (1)	Increase in serum GM-CSF and TNF- α	SD (7)
Ishikawa et al. (2005)	11	Hot flashes (1), headache (1), and elevation of Cr (1) and Bil (1)	Hot flashes (1), headache (1), and elevation of Cr (1) and Bil (1)	None	Increased IFN- γ production and NKT cell expansion	SD (3)
Motohashi et al. (2006)	6	Hot flashes (1), headache (2), malaise (1), and fever (1)	Facial paralysis (1)	None	Increased IFN- γ production and NKT cell expansion	SD (5)
Motohashi et al. (2009)	23	Fever (4)	Fever (1) and anemia (1)	DVT (1)	Increased IFN- γ production and NKT cell expansion	SD (4)

Adverse events and changes in laboratory values were graded according to the National Cancer Institute Common Toxicity Criteria version 2.0
Bil bilirubin, *Cr* creatinine, *SD* stable disease

GalCer (KRN7000)-pulsed dendritic cells (Ishikawa et al. 2005). Each patient received four doses of α -GalCer-pulsed DCs by intravenous injection. The DCs used in this study were prepared by stimulation with GM-CSF and IL-2. No severe adverse events were observed. After the first and second injection of α -GalCer-pulsed DCs, a dramatic increase in NKT cells (>20-fold) was observed in one case and significant responses were seen in two cases receiving the level 3 dose. This study indicates that α -GalCer-pulsed DC therapy was well tolerated and it can be done safely, even in patients with advanced disease. Motohashi et al. conducted a phase I study in advanced and recurrent non-small cell lung cancer patients using autologous in vitro activated NKT cells (Motohashi et al. 2006). Similar to their previous study, no severe adverse events were observed. While the number of IFN- γ -producing cells in peripheral blood mononuclear cells (PBMC) increased after the administration of activated NKT cells, no patient was found to meet the criteria for either a partial or a complete response.

Notably, Motohashi and colleagues have extended the study of α -GalCer-pulsed DC to test immunological safety and clinical responses (Motohashi et al. 2009). α -GalCer (KRN7000)-pulsed PBMC cultured in the presence of IL-2 and GM-CSF were given to patients with advanced and recurrent non-small cell lung cancer. Twenty-three patients were enrolled and 17 cases completed the study. After the injection of α -GalCer-pulsed IL-2/GM-CSF cultured PBMC, an increased number of IFN- γ -producing cells in the peripheral blood was detected in ten patients. Five cases remained as stable disease, and the remaining 12 cases were evaluated as progressive disease. Similar to the phase I trial, α -GalCer-pulsed DC injection was well tolerated and accompanied by the

successful induction of NKT cells. Ten patients with increased IFN- γ -producing cells (>2-fold), following α -GalCer stimulation, showed prolonged median survival time (MST) of 31.9 months as compared to the control group (MST of 9.7 months). The authors proposed that the increased number of IFN- γ -producing cells in PBMCs may serve as a biological marker for predicting the clinical course in response to α -GalCer-pulsed IL-2/GM-CSF-cultured PBMC administration. There are other ongoing trials exploring NKT cell immunotherapy in cancer patients. Zhang and colleagues have initiated a phase I–II study of NKT cell infusion in patients with advanced solid tumor (non-small cell lung cancer, gastric cancer, hepatocellular carcinoma, and colorectal cancer; NCT02562963) to determine whether NKT cells are effective and safe in the treatment of patients with unresectable advanced solid tumor. Another ongoing phase I clinical trial of adoptive transfer with autologous NKT cells in metastatic melanoma patients (NCT02619058) is investigating the maximum tolerated dose (MTD) and dose limiting toxicity (DLT) of intravenous administration of autologous NKT cells in metastatic melanoma patients.

Future directions

While NKT cell-based immunotherapy for the treatment of cancer has shown promise, issues remain. Many immunotherapies work under the assumption of a normally functioning immune system. Yet it is known that cancer patients frequently have impaired immune responses, including exhausted NKT cells. We have developed qPCR-

based diagnostic (Sohn et al. 2014) that can be used to rapidly determine which patients have functional NKT cells and would benefit from antibody or adjuvant-based immunotherapy and which patients have defective NKT cell responses and would need the more robust treatment of adoptive cell transfer (Fig. 3).

Current studies focused on inducing the activation of endogenous NKT cells are utilizing nanoparticle-based therapy. Nanoparticles, typically poly(lactic-co-glycolic acid) (PLGA)-based, are extremely versatile and can be modified to include adjuvants, tumor-derived antigens and targeting mechanisms. Like CD1d antibody fusion proteins, nanoparticle-delivered α -GalCer does not induce energy (Thapa et al. 2009). Anti-DEC-205 coated nanoparticles loaded with α -GalCer and antigen were shown to specifically target DCs that are highly efficient at cross-presentation and promote significant anti-tumor immunity (Macho-Fernandez et al. 2014). Recent work has shown that co-encapsulation of α -GalCer and antigen produces a stronger anti-tumor and CTL response than either co-encapsulated TLR agonists or antigen-nanoparticles mixed

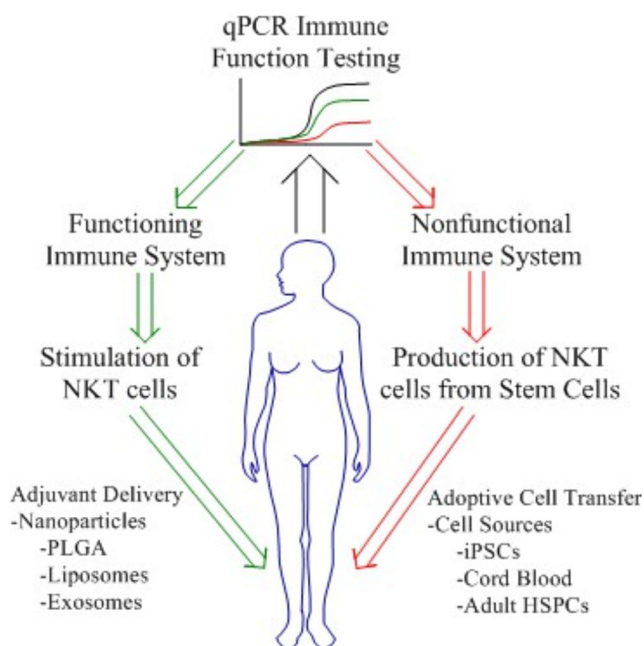


Fig. 3 Future directions for NKT cell-mediated cancer therapy. While NKT cell immunotherapies have shown promise, it is unclear which patient populations are most likely to benefit from this treatment. We have developed a qPCR-based diagnostic to assess NKT cell function in cancer patients. This assay will allow one to determine which patients have functional NKT cells and may suggest the need for checkpoint inhibiting therapy or adoptive transfer of NKT cells in patients with dysfunctional or exhausted NKT cell populations. Studies from our group and others have shown that human NKT cells can be generated from stem cells. In contrast, patients with high IFN- γ producing NKT cells may benefit from treatment with NKT cell agonists, used as adjuvants and delivered using a variety of platforms

with adjuvant (Dölen et al. 2016). R8-peptide-coated liposomes selectively target APCs and when loaded with α -GalCer and administered with IL-12 demonstrate the benefits of combination therapy by allowing lower doses of liposome to produce a more potent result (Abdelmegeed et al. 2016). Finally, adipose-derived mesenchymal stem cell exosomes have been shown to stimulate anti-tumor immunity via NKT cells (Ko et al. 2015).

For patients with low immune function, adoptive cell transfer is an appealing remedy. Previous options involved ex vivo expansion of autologous NKT isolated from the blood or tumor. Current research is moving towards the generation of NKT cells from stem cells using various methods. CD4⁺CD8 α ⁺ NKT cells have been generated from CD34⁺ cells isolated from cord blood using a liquid culture system, dependent on IL-15 and flt-3 ligand or stem cell factor (SCF). While double positive NKT cells are not a standard population found in vivo, these cells produced both Th1 and Th2 cytokines (Woo et al. 2003). Another group has differentiated murine induced pluripotent stem cells (iPSCs) into NKT cells using the OP9/DL-1 culture system and has shown that replacing the OP9/DL-1 culture system at day 14 with a WT OP-9 culture system generated Th1 skewed NKT cells that would be more useful in cancer immunotherapy (Watarai et al. 2010a; Watarai et al. 2010b; Watarai et al. 2012). It was shown that human NKT cells generated from iPSCs could be redifferentiated in vitro using a IL-7/IL-15-based cytokine co-culture system (Kitayama et al. 2016) Human NKT cells have been generated from bone marrow-derived adult hematopoietic stem-progenitor cells using the OP-9/DL-1 culture system in the presence of IL-7 and flt-3 ligand for 21 days followed by expansion with CD1d-based aAPC. The resulting cells were shown to be functionally mature and responsive to α -GalCer stimulation (Sun et al. 2015).

Advancements in the study of NKT cells are necessary and important. Recent studies examining the efficacy of cancer therapeutics have utilized zebrafish models. Zebrafish are an attractive model for multiple reasons: short generation time, large progeny, ease of care, transparency for in vivo imaging, genetically tractable, and ease of chemical screening (Yen et al. 2014). A transgenic hepatocellular carcinoma zebrafish model has shown that their immune system plays a similarly prominent role in tumor progression (Li et al. 2014b). They possess innate and adaptive immune systems that are comparable to higher organisms (Rauta et al. 2012). While NKT cells have not yet been discovered in zebrafish, amphibians have a comparable subset called invariant T (iT) cells that play a conserved role in immunity (Robert and Edholm 2014). Collectively, these studies demonstrate the feasibility of targeting NKT cells for cancer immunotherapy and reveal innovative strategies that can be employed to increase our understanding of this important population of T cells.

Acknowledgments Given the wide breadth of studies examining the role of NKT cells in cancer, only recent reviews and closely related articles have been cited and we apologize to those whose work may have been omitted due to space considerations. The authors have no competing financial interest. This work was supported by grants from the NIH/NCI to TJW.

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