Review Article

Invariant Natural Killer T Cells in Immune Surveillance and Tumor Immunotherapy: Perspectives and Potentials

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Although various elements of the immune system are involved in detection and elimination of neoplastic cells, immune surveillance mechanisms are not always successful in eradicating cancer. Invariant natural killer T cells are potent immunomodulatory lymphocytes that secrete large amounts of Th-1 and/or Th-2 cytokines in response to glycolipid agonists. The observed role of invariant natural killer T cells in antitumor immunity in various animal models and the relatively recent identification and availability of Invariant natural killer T cell glycolipid ligands with anticancer properties such as α-galactosylceramide have led to several invariant natural killer T cell-based clinical trials in cancer patients with somewhat promising outcomes. The objective of this article is to provide a short overview on immunobiology of invariant natural killer T cells followed by their potential applications in treatment of cancer.

Introduction

Natural killer T cells: a brief overview

Natural killer T (NKT) cells are a unique subpopulation of lymphocytes that express T cell receptor (TCR)/CD3 complexes along with several markers characteristic of natural killer (NK) cells such as NK1.1 in certain mouse strains and NKR-P1A in humans.[1-3] In mice, NKT cells are highly abundant in the liver comprising up to 40% of all liver lymphocytes.[4] They are also found at low frequencies in the thymus, bone marrow, spleen, lymph nodes, and blood. Most NKT cells express an invariant or "canonical" TCRα chain [Vα14-Jα18 (formerly called Vα14-Jα281) in mice and Vα24-Jα18 in humans] paired with a limited set of TCRβ chains (Vβ8.2, Vβ2 or Vβ7 in mice and Vβ11 in humans).[5,6] These cells have been termed invariant, canonical, classical, or type I NKT cells. A smaller, less characterized subset of NKT cells express a diverse TCRαβ repertoire and are known as variant, noncanonical, or type II NKT cells.[7] I will, hereafter, refer to these two NKT cell subtypes as invariant NKT (iNKT) and variant NKT (vNKT) cells, respectively.

Unlike conventional T cells that are positively selected by thymic epithelial cells, NKT cells depend on CD1d+ double positive thymocytes for their positive selection in the thymus.[8,9] CD1 is a nonpolymorphic, β2-microglobulin-associated protein structurally related to major histocompatibility complex (MHC) class I molecules. Unlike classical MHC I molecules that accommodate 8-11-amino acid residue-long peptides, CD1 has a deep hydrophobic antigen (Ag)-binding pocket that is well suited for binding of endogenous or exogenous lipid molecules.[10]

Defective human NKT cell development is encountered in X-linked lymphoproliferative (XLP) syndrome, a fatal hereditary disorder caused by mutations in the gene coding for signaling lymphocytic activation molecule-associated protein (SAP).[11,12] SAP is an adaptor protein expressed in

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lymphocytes that recruits and activates the Src kinase FynT, which is essential for NKT cell development.[13,14]

iNKT cells, which will be the main focus of this review, exhibit a "pre-activated" phenotype in their steady state, which is evident even in germ free mice[15] and in human cord blood[16]. Both TCR-dependent and TCR-independent stimuli can signal iNKT cell activation. It is possible that self glycolipids presented by CD1d constantly trigger iNKT cells' TCR to maintain their activated state. Glycolipids derived from Ehrlichia and Sphingomonas[17] and mycobacterial phosphatidy-linositol mannoside[18] are examples of pathogen-derived TCR ligands for iNKT cells. A synthetic glycolipid agonist called α-galactosylceramide (α-GalCer) that binds to CD1d has been used experimentally to stimulate mouse or human iNKT cells in a TCR-dependent fashion.[19] Although not found in microbial pathogens or self tissues so far, α-GalCer is a powerful tool for studying iNKT cells in experimental models and a potential im-munotherapeutic agent in clinical settings. iNKT cells also respond to certain cytokines such as interleukin (IL)-12 and IL-18 in the absence of TCR cross-linking.[20,21]

A remarkable and unique feature of iNKT cells is their ability to swiftly produce copious amounts of Th1-type cytokines [e.g., interferon (IFN)-γ] and Th2-type cytokines (e.g., IL-4 and IL-13) following stimulation, and without de novo cytokine mRNA synthesis.[22,23] iNKT cells contain pre-formed mRNA for these cytokines, which could explain the rapidity with which they are secreted. Hence, iNKT cells appear to be responsible for the first wave of cytokine release early in immune responses that would not only contribute to innate host defense, but also shape the nature of the subsequent adaptive responses. The paradoxical ability of iNKT cells to either promote or suppress immune responses has earned them the moniker of "the double-edged sword" of the immune system.[4] This is at least in part owed to their ability to produce enormous quantities of Th1 cytokines, Th2 cytokines, or both.

At least two phenotypically distinct subsets of iNKT cells exist in mice, namely CD4$^+$CD8$^-$ (CD4$^+$) and CD4$^-$CD8$^-$ [double negative (DN)] iNKT cells.[7] An additional subset called CD4$^-$CD8$^+$ (CD8$^+$) is found in humans.

Animal models for studying NKT cells

Mouse and human NKT cells share several characteristics and are functionally homologous to the extent that mouse NKT cells can recognize human CD1d, and vice versa.[24] It is therefore believed that further insight into the biology of human NKT cells can be obtained using in vivo mouse models. These models provide useful information regarding the function of NKT cells in the context of neoplasia and metastasis as well as their potential applications in anticancer immuno-therapy.

NKT cell-deficient mice

Complete absence of NKT cells is seen in mice with targeted mutation of the CD1 gene.[25] It is important to keep in mind that CD1d knockout (KO) mice are devoid of both iNKT and vNKT cells since the positive selection of both NKT subtypes during thymic ontogeny is strictly depen-dent on CD1d.[9] Therefore, one could not rule in or out the involvement of one subtype of NKT cells versus another in immune responses, simply using CD1d KO mice alone.

Jα18 KO mice that lack iNKT, but not vNKT cells are powerful experimental tools for studying NKT cells.[26] While the expression of CD1d in these mice is intact, the deletion of the Jα18 segment of iNKT cells' invariant TCRα chain leads to their selective deficiency of iNKT cells.

CD1d KO and Jα18 KO mice can therefore be used in parallel to distinguish between iNKT and vNKT cell contribution(s) to immune responses, including those elicited against cancer.

NKT cell-rich mice

The paucity of NKT cells in lymphoid tissues of wild-type mice poses an experimental challenge when large numbers of NKT cells are required, for instance, in adoptive transfer studies. Expanding NKT cells with glycolipid ligands is likely to alter their phenotype and activation state, and the resultant cell populations may not accurately represent NKT cells found in healthy animals. Using the liver as a rich source of NKT cells may not be an ideal solution either since liver contains functionally distinct NKT cells that are also different from NKT cells found in other sites. A recent investigation demonstrated that while liver CD4$^-$CD8$^-$ iNKT cells participate in tumor rejection, thymus-derived iNKT cells and liver-derived CD4$^+$ iNKT cells are far less potent in this capacity.[27]

The above limitations have led to the generation

of several lines of transgenic (Tg) mice in which NKT cells are much more abundant. For example, Vα14 Tg mice express the invariant Vα14 and Vβ8.2 transgenes on a recombination activating gene (RAG)-deficient background.[19] As a result, their lymphoid organs contain many more iNKT cells in comparison with their wild-type counterparts. Given the possibility that iNKT cells obtained from Vα14 Tg mice may not be perfectly similar to wild-type iNKT cells, it is often recommended that these mice be used in parallel with complementary approaches (e.g., using liver iNKT cells obtained from wild-type animals) when large numbers of iNKT cells are needed.

Modulation of antitumor immunity by iNKT cells

Immune surveillance refers to innate and adaptive immune mechanisms participating in early detection and destruction of malignant cells before they progress to invasive cancer. The tumoricidal activity of NK cells and CD8$^+$ cytotoxic T lymphocytes (CTLs) has been a major focus of intense investigation in the context of immune surveillance. Immune surveillance mechanisms, including those involving NK cells and CTLs are not always effective and can be overcome by tumor immune evasion strategies.[28,29] Understanding the cross talk between various arms, cells and molecules of the immune system is instrumental in our efforts to come up with novel immunotherapeutic approaches to cancer.

Findings in animal models

While vNKT cells reportedly suppress immune responses in several mouse tumor models,[30] iNKT cells have been implicated in immune surveillance against neoplastic transformation and metastasis. iNKT cells were required for rejection of methylcholanthrene-induced sarcoma in mice.[31] Established sarcoma cell lines also grew better in iNKT-deficient mice, and adoptive transfer of wild-type iNKT cells into these mice led to tumor rejection.[32] Similar findings were reported in a model of pulmonary sarcoma metastasis.[33]

Administration of iNKT cell-stimulating glycolipids has been demonstrated to offer antitumor and antimetastatic benefits in animal models of cancer. These include liver metastasis of colon adenocarcinoma[34] and melanoma[35] and lung metastasis of thymoma[36] and Lewis carcinoma cells[37] in mice, as well as liver metastasis of pancreatic

cancer in Syrian hamsters.[38]

Potential mechanisms

In vitro studies have revealed the expression of cytotoxic effector molecules by human iNKT cells as well as CD1d-dependent killing of leukemic cells from patients with T cell acute lymphoblastic leukemia (T-ALL),[39] myelomonocytic leukemia,[40] and B cell chronic lymphocytic leukemia (B-CLL).[41] In addition, iNKT cell-mediated cytotoxicity was enhanced by loading the leukemic cells with α-GalCer. Whether these findings are translatable to human iNKT cell properties *in vivo* remain to be established. It is possible that NKT cells recognize endogenous glycolipid ligands found on tumor cells. In fact, some lipid components of tumor cell membranes were demonstrated to bind to CD1d and stimulate NKT hybridoma cells.[42]

Activation of iNKT cells *in vivo* may also contribute to antitumor immunity indirectly by augmenting nonspecific and/or tumor-specific CD8$^+$ CTL responses. A fairly recent study found a marked numerical increase in hepatic CD8$^+$ T cells shortly after α-GalCer administration into mice with B16 melanoma.[43] These cells were not only cytotoxic towards B16 target cells, but also against irrelevant tumor cells (EL-4 thymoma cells). In addition, highly purified CD8$^+$ T cells isolated from the liver of α-GalCer-treated mice exhibited *ex vivo* lytic activity against B16 cells regardless of prior tumor inoculation, further suggesting nonspecific bystander activation of CD8$^+$ T cells by this compound. Notwithstanding, α-GalCer treatment of melanoma-bearing mice resulted in rejection of B16, but not EL-4 cells injected 14 days later. This seemingly tumor-specific response was partly dependent on CD8$^+$ T cells, but not on NK cell function.

The adjuvanticity of α-GalCer is believed to stem from its ability to provoke iNKT cell activation under the conditions that favor Th1-type cytokine production (e.g., IFN-γ). These cytokines promote cell-mediated cytotoxicity and mature dendritic cells (DCs) that are involved in the development of cognate T cell responses.[44]

iNKT cells in immunotherapy of human cancer

Following the identification of highly specific glycolipid ligands for iNKT cells and their anticancer properties in rodent models, several groups have used these compounds as immuno-

therapeutic agents in human malignancies. KRN7000, the prototype iNKT cell ligand with a unique α-GalCer structure, was in fact discovered in a screen for novel anticancer agents.[45,46] Isolated from the marine sponge *Agelas mauritianus*, KRN7000 has been the most commonly used agonist ligand for both mouse and human iNKT cells.

The idea of expanding iNKT cells by treatment with α-GalCer is particularly appealing given the observed reduction in iNKT cell numbers in numerous types of cancer, including prostate cancer,[47] lung cancer,[48] and hematologic malignancies.[49] The residual iNKT cells in many cancer patients are also functionally impaired, most notably in their capacity to produce IFN-γ.[47] Importantly, the pool size and function of iNKT cells may have some prognostic values. For instance, low numbers of tumor-infiltrating iNKT cells in patients with colorectal carcinoma were correlated with poor prognosis.[50] It was also demonstrated that iNKT cells from the blood circulation or tumor bed of patients with progressive myeloma, but not nonprogressive myeloma or premalignant gammopathy, were defective in producing IFN-γ.[51] However, this defect could be reversed by *in vitro* exposure to α-GalCer loaded on DCs. It is important to note that although iNKT cells may infiltrate some (but not all) tumors, their effectiveness in the context of tumor immunity may also be influenced by the type, complexity, and composition of tumor micro-environment in which they interact with neoplastic cells and with other immune cells. Tumor-infiltrating immunocytes may release inflammatory mediators and effector molecules that either promote or suppress angiogenesis and tumor growth.[52] tumor-infiltrating iNKT cells are likely to play a role in modulation of these responses.

Several studies in animal models have demons-trated that α-GalCer-coated DCs prevent tumor growth and metastasis or even eradicate already metastasized tumors[37,53,54] and may therefore be superior to directly injected α-GalCer. Mature DCs pulsed with α-GalCer were more potent than monocytes and immature DCs in activating human peripheral blood iNKT cells *in vitro*[55] and induced sustained, rather than transient, human iNKT cell expansion *in vivo*.[56]

The idea of expanding the patient's own (autologous) iNKT cells ex vivo before use in im-munotherapeutic regimens has also been explored

and led to a recent clinical trial.[57] However, two potential problems may arise. First, transferring iNKT cells in their activated state may result in their rapid clearance from the system, before they can efficiently participate in antitumor immune responses. Secondly, the numerical and functional inadequacy of iNKT cells in cancer patients are frequently accompanied by Ag-presenting cells' (APCs') dysfunction.[58] Therefore, generating bona fide iNKT cells using autologous APCs may prove difficult. By the same token, it is not always accurate to compare the potency of iNKT cells and other effector cells obtained from different patients or even use iNKT cell responsiveness to autologous APCs as a prognostic factor when the function of both cell types may be affected by the patient's underlying disease. Consistent with this notion, Shimizu et al.[58] were unable to detect IFN-γ synthesis by iNKT cells of patients with chronic myeloid leukemia (CML) using sensitive enzyme-linked immunospot (ELISPOT) assays. However, iNKT cell responses were readily detectable in a group of patients when these investigators used murine DCs as xenogeneic APCs instead of autologous APCs. The success of this novel approach is at least in large part due to the ability of human iNKT cells to recognize murine CD1d and vice versa.[24] In addition, NKT cells from both species can be activated by the same glycolipid compounds presented by CD1d+ APCs, including α-GalCer and its analogues.[58]

DCs are the most potent professional APCs and the only APCs capable of activating naïve T cells. However, they are scarce in circulating blood and lymphoid organs and their *ex vivo* expansion is relatively difficult. Whether more readily obtainable cell types (e.g., B cells) can be used as potential APCs in immunotherapeutic protocols has attracted considerable interest. Although B cells are abundant in the body and home into lymphoid tissues, they are poorly immunogenic or even tolerogenic due to their low expression levels of co-stimulatory molecules. A recent study found that splenic mouse B cells loaded with α-GalCer induce iNKT cell activation and upregulate the B7-2 co-stimulatory molecule after injection into syngeneic mice.[59] When also pulsed with MHC I-restricted peptides derived from the model Ag ovalbumin or the HER-2/neu tumor Ag, these B cells were almost as potent as DCs in inducing cognate CTL responses to the immunizing peptides and protective immunity to tumors expressing

these Ags. It remains to be elucidated whether these findings are applicable to human B cells. Given that B cells are not as efficient as DCs in capturing Ags to be processed for cross-presentation to CD8^{+} T cells, using α-GalCer-loaded B cells is likely to be most useful in peptide-based vaccination, and not in protein Ag-based therapies. It will be interesting to examine whether loading α-GalCer on CD1d^{+} malignant tumor cells of B cell origin would result in CTL responses to their naturally processed tumor Ags.

Clinical trials targeting iNKT cells

Several clinical trials targeting iNKT cells have been conducted on cancer patients. Some of the findings of these trials are summarized in Table 1.

1. Giaccone et al. (2002)[60]: The first phase I clinical trial of α-GalCer in cancer was conducted in 2002. In this study, 24 patients with advanced solid tumors received α-GalCer intravenously (i.v.). α-GalCer was well-tolerated over a wide range of doses and did not cause adverse side effects. Although α-GalCer administration did not result in any clinical responses, elevated serum levels of tumor necrosis factor (TNF)-α and granulocyte/macrophage colony-stimulating factor (GM-CSF) were observed in five patients, suggesting immune activation in these subjects. Interestingly, all these five subjects were part of a subgroup of patients who had relatively high numbers of iNKT cells in their peripheral blood prior to α-GalCer treatment, and subjects with drastically low iNKT cell numbers demonstrated no evidence of immune activation.

The above study was followed by three clinical trials in which α-GalCer-pulsed DCs were used to expand and activate iNKT cells. This approach was based on the findings in preclinical animal studies that robust iNKT cell responses and antitumor effects can be elicited following *in vivo* administration of α-GalCer-coated DCs.[37,53,54]

2. Nieda et al. (2004)[61]: These investigators treated 12 patients with various types of metastatic cancer with autologous α-GalCer-pulsed monocyte-derived DCs (MoDCs) administered at two-week intervals. The usage of immature MoDCs in this study was justified based on previous *in vitro* experiments indicating the higher expression levels of CD1d on immature vs. mature MoDCs. The trafficking pattern of α-GalCer-pulsed MoDCs was found to be similar to antigenic peptide-pulsed MoDCs that were prepared using identical proto-

cols and administered in a smaller clinical trial. This suggests that treating MoDCs with α-GalCer does not affect their trafficking and homing properties. Following treatment, tumor-related symptoms deteriorated in the majority of patients, possibly as a consequence of inflammatory responses to the tumor. These included tender enlargement of palpable tumor deposits or affected lymph nodes in all five patients with nodal metastases, bone pain, and respiratory syndromes in patients with pulmonary metastases. The observed flares were only temporary and dissipated after the study period. Nine of 12 subjects also experienced minor systemic side effects (Table 1). The serum levels of IFN-γ increased transiently but markedly in all evaluable cases. In addition, four patients showed increased levels of IL-12 following treatment with α-GalCer-pulsed DCs. One very interesting observation in this study was that a "priming" treatment was needed for induction of increases in serum IFN-γ and IL-12 levels such that substantially high levels of these cytokines were detectable in peripheral blood only after the second dose was administered. In comparison with the first injection, the second treatment led to the activation of large numbers of NK and T cells, higher and more sustained peak levels of NKT and NK cells following the initial drop in their numbers, and more frequent occurrence of systemic symptoms suggestive of immune activation. Faster and more robust secondary immune activation points to the existence of some sort of memory for induction and/or consequences of iNKT cell activation. It will be interesting to explore whether more vigorous secondary immune activation in the context of iNKT cell responses can be seen in similar therapeutic regimens involving longer-term intervals. Finally, although antitumor clinical responses were not a focus of this study, decreased levels of tumor markers in two patients with adenocarcinoma, extensive necrosis of bone marrow-infiltrating tumor cells in one subject with renal cell carcinoma, and reduction in blood hepatic enzyme levels in two patients with liver-infiltrating tumors were noticed.

3. Ishikawa et al. (2005)[48]: In a dose-escalation clinical trial, autologous APCs containing immature MoDCs were pulsed with α-GalCer and administered i.v. to patients with advanced non-small cell lung cancer. Unlike in common protocols that use GM-CSF and IL-4 to generate MoDCs, APCs used in this study were grown in

Table 1. A summary of clinical trials targeting iNKT cells in cancer.

Clinical trial investigators	No. of patients	Tumor type	Therapeutic agent/cells	Adverse events in some or all patients	Clinical tumor responses (some or all patients)
Giaccone et al., 2002[60]	24	Various refractory solid tumors	KRN7000	None or minor	None observed
Nieda et al., 2004[61]	12	Various metastatic tumors	KRN7000-pulsed immature MoDCs	Transient tumor-associated flares; minor side effects (fever, lethargy, malaise, headache)	↓ Serum tumor markers; Tumor necrosis; ↓ Liver enzymes
Ishikawa et al., 2005[48]	11 enrolled; 9 completed	Advanced or recurrent nonsmall cell lung cancer	KRN7000-pulsed APCs rich in immature MoDCs	None or minor	None observed
Chang et al., 2005[56]	5	Myeloma; anal squamous cell carcinoma; renal cell cancer	KRN7000-pulsed mature MoDCs	None or minor	Not evaluated
Motohashi et al., 2006[57]	6	Advanced or recurrent nonsmall cell lung cancer	*ex vivo* stimulated autologous PBMCs rich in iNKT cells	None or minor (transient flush and headache, arrhythmia, fever)	None observed

No.=number; MoDCs=monocyte-derived dendritic cells; *i*NKT cells=invariant natural killer T cells; PBMCs=peripheral blood mononuclear cells.

the presence of GM-CSF and IL-2 and contained various CD1d$^+$ cell types, including but not restricted to DCs. Cell types other than DCs could thus also participate in α-GalCer presentation to iNKT cells. The therapy was well tolerated and could be safely done even in outpatients. No major adverse effects were observed as a result of the treatment, although minor problems such as headache and general fatigue were occasionally recorded. Finally, out of nine patients who could be evaluated by chest X-ray and computed tomography (CT) at the end of the study period, five exhibited no changes and four showed signs of disease progression.

4. Chang et al. (2005)[56]: This clinical trial involved the use of mature rather than immature MoDCs as cellular vehicles for α-GalCer. Five patients with various types of cancer received unpulsed autologous mature MoDCs followed by two subsequent doses of α-GalCer-coated MoDCs in four-week intervals. The treatment was tolerable and nontoxic. The administration of unpulsed DCs did not result in increased peripheral blood iNKT cell numbers in patients at any time. Unlike previous studies that induced relatively transient iNKT cell activation, α-GalCer-pulsed mature DCs led to a dramatic (>100 fold) increase in circulating iNKT cell numbers that remained above the

baseline for more than 12 weeks in all patients, and over six months in two patients with follow-up. Cytofluorimetric analysis of the expanded peripheral blood iNKT cells also revealed that DC-mediated mobilization of iNKT cells in humans is associated with expansion of all three human iNKT cell subsets, namely CD4$^+$, CD4$^-$CD8$^-$, and CD8$^+$ iNKT cells, albeit with different kinetics. The sustained expansion of iNKT cells was also evident in the marrow tumor bed in one patient with myeloma whose tumor-associated iNKT cells were enumerated. Extensive cytokine and chemokine analyses revealed elevated blood levels of IL-12, IFN-γ inducible protein-10 (IP-10), and macrophage inflammatory protein (MIP)-1β following treatment with α-GalCer-loaded MoDCs (the third injection), but not unpulsed MoDCs in all subjects tested. These soluble mediators are generally believed to be derived from myeloid APCs.

Importantly, the *in vivo* expanded iNKT cells failed to produce IFN-γ upon *ex vivo* re-exposure to α-GalCer in comparison with blood iNKT cells obtained from healthy donors. This is similar to what is seen in unexpanded iNKT cells found in patients with myeloma and some, but not all other types of cancer.[47,51,62-64] In contrast, treatment with α-GalCer-pulsed DCs was able to induce IFN-γ

synthesis by CD8^{+} memory T cells specific for a peptide epitope of the persistent and ubiquitous virus, cytomegalovirus (CMV).[56] This intriguing finding on one hand indicates that IFN-γ production in cancer patients is not globally impaired and on the other hand suggests a link between human iNKT and cognate CD8^{+} T cell responses that merits further investigation.

5. Motohashi et al. (2006)[57]: This clinical trial was the first of its kind in that it used adoptive transfer of *ex vivo* expanded autologous iNKT cells in patients with recurrent nonsmall cell adeno-carcinoma or squamous cell carcinoma of the lung. These adoptively transferred cells were prepared by culturing leukapheresed peripheral blood cells in the presence of α-GalCer (KRN7000) and IL-2. The usage of mixed cell populations in peripheral blood mononuclear cells (PBMCs) eliminated the need to separately grow DCs and use them as APCs for α-GalCer presentation to iNKT cells. Although this method led to dramatic expansion of iNKT cells reaching purity levels up to around 25%, the transferred cells inevitably contained cell types other than iNKT cells. Injecting *ex vivo* expanded iNKT cells led to increased numbers of circulating iNKT in several patients. The number of IFN-γ-producing cells among PBMCs increased after the iNKT cell administration in some patients, the majority of which were NK and NKT cells. Several immunologic changes such as increased cytotoxic activity against two human tumor cell lines as well as IFN-γ secretion were reported for PBMCs stimulated with α-GalCer *ex vivo*. Although interesting, these findings need to be interpreted with caution since some of the observed changes following therapy cannot be ascribed with 100% certainty to the adoptively transferred iNKT cells. Adoptively transferred cells were tolerated without any severe adverse effects. However, no antitumor clinical responses were observed. All six patients were evaluated at the end of the clinical trial. Based on the chest X-ray and CT findings, there were no cases of complete or partial response.

Future directions on the clinical front

Although the feasibility and safety of using KRN7000, KRN7000-coated DCs, or *ex vivo* expanded iNKT cells in cancer patients have been confirmed in several clinical trials, significant clinical responses to tumors were not elicited.

As antitumor immunity is believed to be most robust under Th1-favoring conditions, therapeutic approaches to reverse impaired IFN-γ synthesis encountered in some types of cancer need to be explored. New versions of α-GalCer that promote IFN-γ secretion and skew immune responses towards a Th1 phenotype are emerging. A recent study found that a C-glycoside analogue of α-GalCer (α-C-GalCer) is a more potent inducer of IFN-γ and IL-12 production in mice in comparison with α-GalCer.[65] Moreover, α-C-GalCer-coated DCs elicited more robust and sustained iNKT cell responses *in vivo* and were more effective in reducing the size and number of lung metastases of the B16 melanoma in mice. The efficiency of this glycolipid compound as a therapeutic agent for cancer warrants future clinical trials.

Patients with cancer frequently exhibit APC dysfunction along with numerical and functional deficits in their iNKT cell compartment. Combination immunotherapeutic protocols aiming at manipulation of APCs to rescue their function along with mobilization of iNKT cells would be worthy of future investigation.

APCs co-pulsed with tumor lysate (or tumor-derived peptides) and α-GalCer are another attractive candidate for immunotherapy of cancer. Thus, the effectiveness of α-GalCer-loaded CD1d-expressing tumor cells, for instance tumors of hematopoietic origin, in inducing antitumor immunity could be tested in preclinical and clinical studies. CD1d gene transfection into CD1d^{-} tumor cells followed by α-GalCer treatment may also be considered an option in development of tumor vaccines.

The availability of bona fide APCs is instrumental in expanding iNKT cells *ex vivo*. The impairment of APCs in cancer patients justifies the need to assess the properties of various types of potential APCs, including xenogeneic DCs in an effort to replace autologous APCs for iNKT cell expansion. Once iNKT cells are optimally and sufficiently expanded, adoptive transfer of these cells followed by treatment with α-GalCer or α-GalCer-pulsed DCs that are simultaneously coated with tumor Ags could be performed.

The distinct properties of different iNKT cell subsets need to be explored thoroughly before these subsets can be used in human studies. While CD4^{+} iNKT cells produce both Th1- and Th2-type cytokines, CD4^{-}CD8^{-} and CD8^{+} iNKT cells preferentially produce Th1-type cytokines.[66-68]

Finally, combining iNKT cell-based therapies

with potentially additive or synergistic immuno- or chemo-therapeutic strategies may prove beneficial to cancer patients. A recent study found that the novel thalidomide analogue, lenalidomide augments iNKT cell expansion and IFN-γ synthesis in response to α-GalCer.[69] This immunomodulatory agent is used in treatment of patients with myelodysplastic syndrome. Importantly, treatment with lenalidomide and thalidomide led to an impressive increase in iNKT cell numbers in patients with myeloma and del(5q) myelodysplastic syndrome, respectively. Collectively, these findings support the feasibility of combining lenalidomide, thalidomide, and possibly other pharmacologic agents with iNKT-targeting regimens to maximize iNKT cell responsiveness in cancer patients.

Closing remarks

Invaluable information has been obtained from several clinical studies conducted in patients with various types of cancer. The feasibility and safety of the administration of α-GalCer or *ex vivo* expanded iNKT cells have been confirmed. Although the role of iNKT cells in modulation of various aspects of immunity, including antitumor immune responses has been a subject of intense investigation, much remains to be learned about the functions fulfilled by iNKT cells in health and disease. Apart from the outstanding questions discussed above, future clinical studies will have to include more patients undergoing longer monitoring periods following iNKT cell-targeting immunotherapy to yield more convincing results. Optimal activation of functional iNKT cells in tumor hosts under the conditions that promote the development of a Th-1-dominated phenotype alone or in combination with other potentially additive or synergistic therapeutic approaches holds promise of boosting immune responses that could ideally lead to protective immunity to tumors.

Acknowledgment

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