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REVIEW

Synthetic glycolipid activators of natural killer T cells as immunotherapeutic agents

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Certain types of glycolipids have been found to have remarkable immunomodulatory properties as a result of their ability to activate specific T lymphocyte populations with an extremely wide range of immune effector properties. The most extensively studied glycolipid reactive T cells are known as invariant natural killer T (iNKT) cells. The antigen receptors of these cells specifically recognize certain glycolipids, most notably glycosphingolipids with α-anomeric monosaccharides, presented by the major histocompatibility complex class I-like molecule CD1d. Once activated, iNKT cells can secrete a very diverse array of pro- and anti-inflammatory cytokines to modulate innate and adaptive immune responses. Thus, glycolipid-mediated activation of iNKT cells has been explored for immunotherapy in a variety of disease states, including cancer and a range of infections. In this review, we discuss the design of synthetic glycolipid activators for iNKT cells, their impact on adaptive immune responses and their use to modulate iNKT cell responses to improve immunity against infections and cancer. Current challenges in translating results from preclinical animal studies to humans are also discussed.

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NATURAL KILLER T (NKT) CELLS AND THEIR ROLE IN IMMUNITY

NKT cells are a specialized group of unconventional T-cell lymphocytes, characterized by the co-expression of T-cell antigen receptors (TCRs) together with multiple other surface receptors that are commonly expressed by NK cells (for example, CD161/NK1.1, NKG2D and members of the Ly-49 family).[1–8] NKT cells modulate the activation and phenotype of other immune cell types and hence affect the responses against a vast array of diseases, including cancer, infections, autoimmunity and allergy. This has led to substantial interest in these cells as targets for potential immunotherapeutic strategies.[5,6,8–10] In addition, they participate in the homeostasis of the immune system and under normal circumstances have been proposed to have a regulatory role.[11,12] As their name implies, NKT cells display features of both T cells and NK cells and have a range of effector functions that include the secretion of multiple cytokines and the ability to mediate cytotoxicity.

Unlike classical NK cells, NKT cells derive from the T-cell lineage and develop throughout a process that is dependent on thymic selection and specific TCR-mediated recognition. However, their ability to respond rapidly and strongly without prior antigen priming indicates that they also function as part of the innate immune system.[2,5,13] In contrast to conventional CD8 and CD4 T cells, whose TCRs recognize peptides bound to class I and class II major histocompatibility complex (MHC) molecules, respectively, TCRs of NKT cells recognize lipid antigens bound to CD1d, a non-polymorphic MHC-I-like molecule.[2,7] CD1d is expressed by all hematopoietic cells as well as some epithelia and other non-hematopoietic cell types, although expression levels are highest in immunologically relevant antigen-presenting cells, such as dendritic cells (DCs) and B lymphocytes.[2,14,15] Current classification schemes broadly define CD1d-dependent NKT cells into two broad classes, referred to as type I and type II NKT cells. Type I NKT cells express an invariant TCRα chain (Vα14Jα18 in mice and Vα24Jα18 in humans). These are paired with a moderately diverse repertoire of TCRβ chains using predominantly Vβ8, Vβ7 and Vβ2 in mice and Vβ11 in humans. Because of their characteristic invariant TCRα chain, the type I NKT cells are also known as invariant NKT cells (iNKT cells).[7,13] These cells recognize lipids and glycolipid antigens bound to CD1d,[7,13] and their activation has many potential effects on pro- and anti-inflammatory immune responses.[8,13] Although much less studied, type II NKT also respond to lipids and glycolipids presented by CD1d and have been shown to have a range of different immunomodulatory functions.[16–18] In contrast to iNKT cells, type II NKT cells express a diverse repertoire of TCRs, possibly as diverse as those of conventional T cells and thus are also referred to as diverse NKT cells (dNKT cells). Although less well studied than iNKT cells, dNKT cells appear to respond to different lipids than those recognized by iNKT cells and are likely to perform different roles in the immune system.[19,20] In this article, we focus exclusively on the immunomodulatory effects of iNKT cells and their glycolipid ligands.

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Despite the great potential of NKT cells for immunomodulation, their relatively low frequency in the blood, lymphoid organs and tissues has made their study difficult in humans. On the other hand, mice display much higher frequencies of total NKT cells, a different tissue distribution and altered ratios of iNKT/dNKT cells as compared with humans, making them a useful but imperfect model of their human counterparts.[2,7,21] Although human and mouse NKT cells have many conserved features, the major difference in frequency makes it difficult to extrapolate findings from mouse to humans for NKT-cell-based immunotherapy. Some attempts to overcome this problem have considered the use of non-human primates, as they display NKT cell frequencies that are close to those seen in humans.[22,23] However, these studies are limited by sample size, available tools, high costs and the inability to perform genetic manipulations. These limitations have encouraged the development of humanized mouse models, such as the recently reported human CD1d knock-in mouse, which displays NKT cell frequencies and tissue distribution similar to humans.[24] The NKT cells in this mouse model show a ratio of iNKT to dNKT subsets that mirrors the ratio in normal humans and retain substantial immunomodulatory functions in a variety of *in vitro* and *in vivo* assays.[24,25]

EFFECTOR FUNCTIONS AND NATURAL GLYCOLIPID ANTIGENS OF iNKT CELLS

The activation of iNKT cells can be triggered by TCR ligation by glycolipid/CD1d complexes, or alternatively by inflammatory cytokines such as interleukin (IL)-12, or a combination of both.[26,27] Activation of iNKT cells lead to extremely rapid secretion of a very diverse array of both anti- and pro-inflammatory cytokines, including interferon-γ (IFNγ), tumor necrosis factor-α, IL-4, IL-5, IL-13, IL-17A and IL-22, among others (Figure 1). Because of the association of these cytokines with T helper type 1 (Th1)-, Th2- or Th17-type responses, it is common practice to refer to Th1-, Th2- and Th17-biased responses of iNKT cells.[26,28–31] Recently, a subset of IL-10-secreting iNKT cells with regulatory properties has also been identified,[32] expanding even further the potential of iNKT cell stimulation for immunomodulation. In comparison to conventional naive T cells, the activation of iNKT cells shows less dependence on costimulation and occurs very quickly after TCR engagement. Thus iNKT cells are said to constitutively display a partially activated phenotype, similar to conventional memory T cells, although in the case of iNKT cells this is independent of previous antigen exposure.[2,33]

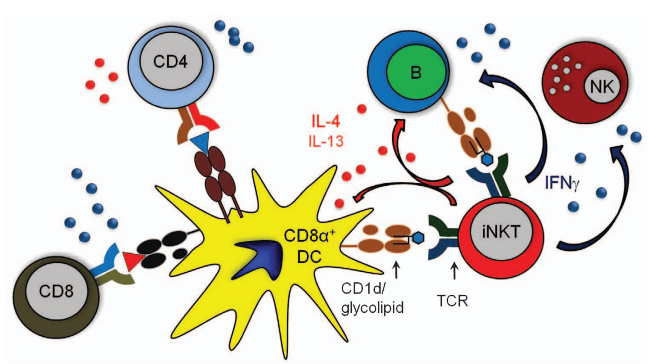

Figure 1 Immunomodulation by iNKT cells. iNKT cells are activated by ligation of their TCRs by cognate glycolipid–CD1d complexes expressed by antigen-presenting cells, particularly a subset of DCs (CD8α DCs in mice). After TCR ligation, iNKT cells rapidly secrete multiple Th1- and Th2-type cytokines, such as IFNγ and IL-4, influencing the activity of other immune cells, including NK cells, DCs, B cells and conventional CD4$^+$ and CD8$^+$ T cells.

Even though iNKT cells display cytotoxic capacity, their most important function seems to be more related to their rapid secretion of effector cytokines.[34] The rapid secretion of cytokines upon iNKT cell activation induces the subsequent activation of multiple other cell types, such as DCs, NK cells, B cells and CD4 and CD8 T cells, in a process known as transactivation[13,26,35–37] (Figure 1). This endows iNKT cells with a remarkable ability to bridge innate and adaptive immune responses. The transactivation induced by iNKT cells can greatly amplify their cytokine responses and profoundly affect the cytokine milieu during initiation of an adaptive immune response. For example, in the case of the Th1-like iNKT cell responses, the majority of IFNγ produced comes from NK cells following their transactivation by iNKT cells.[14]

The unique ability of iNKT cells to secrete diverse cytokines has led to an active search for specific glycolipids than can direct the secretion of an exclusive pattern of either pro- or anti-inflammatory cytokines. The basic premise is that glycolipids that induce predominantly pro-inflammatory cytokines can be beneficial in immunotherapy of cancer and infections, whereas glycolipids that induce mainly anti-inflammatory or tolerogenic cytokines may be more useful in controlling autoimmunity or other acute and chronic inflammatory disorders (Figure 1). Much effort has been focused on identifying the natural endogenous ligands that are recognized by iNKT cells, although this area of work has generated considerable controversy.[7,38,39] Initially, several cellular glycosylphosphatidylinositols, phospholipids and mammalian glycosphingolipids with β-anomeric glycosidic linkages were identified as potential natural iNKT cell ligands. In the case of glycosphingolipids, isoglobotrihexosylceramide (iGb3) was initially proposed as the principal endogenous iNKT cell ligand.[40–42] However, several subsequent studies have challenged the relevance of this compound as an endogenous ligand, given the fact that mice deficient in its production do not have any apparent iNKT cell defect.[43,44] In addition, humans lack iGb3 owing to a functional deficiency of the enzyme iGb3 synthase, and detailed studies of the three-dimensional structures of human CD1d and iNKT cell TCRs suggest structural constraints that may prevent efficient recognition of iGb3 by human iNKT cells.[45] Subsequently, it was reported that endogenous iNKT-cell-stimulating activity was mainly associated with cellular fractions enriched for β-glucosylceramides.[46] However, follow-up studies concluded that this activity was most likely due to a minor component of endogenous α-galactosyl or α-glucosyl ceramides.[38] This conclusion is strongly supported by recent immunochemical analyses providing direct evidence for the presence of α-glycosylceramides in mammalian tissues.[47]

Self-glycolipids bound to CD1d are thought to be recognized with weak reactivity by iNKT cells and contribute to tolerance by the induction of anti-inflammatory cytokines.[29,48] Naturally occurring glycolipid antigens for iNKT cells have also been identified in bacteria and parasites, including species of *Mycobacteria*, *Borrelia*, *Sphingomonas*, *Leishmania* and *Streptococcus*,[35,39,49–52] and several of these have been purified and their precise structures are determined. A comprehensive discussion of these ligands is beyond the scope of this review but can be found in another recently published review article.[39]

DESIGN OF SYNTHETIC GLYCOLIPIDS TO MODULATE iNKT CELL RESPONSES

A critical advance that had the greatest impact on the study of iNKT cell biology, and the development of specific glycolipid activators to harness their immunomodulatory potential, was the finding that α-galactosylceramides (αGalCers) extracted from the marine sponge *Agelas mauritianus* are potent activators of virtually all iNKT cells.[53] This led to the subsequent synthesis of the lipid compound αGalCer, also known as KRN7000, which bound to CD1d can potently activate iNKT cells.[53,54] This synthetic glycolipid is a remarkably powerful activator of both mouse and human iNKT cells. When administered to animals or humans, it stimulates the rapid production of a wide range of cytokines and transactivation of many lymphoid and myeloid cell types. However, the broad range of activities induced by KRN7000 has been considered to be problematic, as it induces both pro- and anti-inflammatory mediators that may lead to conflicting effects.[55] This mixed cytokine response induced by KRN7000 has been described as a Th0-like iNKT cell response[55,56] and is probably not ideal for precisely targeted immunotherapy.

In order to obtain glycolipid activators of iNKT cells with more restricted and predictable effects, including more narrowly focused patterns of cytokine secretion, a large range of derivatives of KRN7000 containing different chemical modifications has been synthesized and tested.[2,7,39,55,56] The structure of KRN700 consists of an α-galactose bound by a 1′-O-glycosidic bond to a C18 phytosphingosine base with an amide-linked, fully saturated C26 fatty acyl chain. Modifications made to this structure have included changes in the sphingosine chain, the *N*-acyl chain, the glycosidic bond and in the carbohydrate moiety[39,55,56] (Figure 2). Novel αGalCer analogs have been identified that have the ability to induce more restricted patterns of cytokines, often characterized as either a Th1- or a Th2-like cytokine bias[39] (Figure 2). Among analogs inducing markedly Th1-like responses, the C-glycoside derivative (α-C-GalCer) and the fluorophenyl fatty acid derivative (7DW8-5) have been the most extensively evaluated. These show enhanced potency as iNKT cell activators *in vivo* and have been found to be superior to KRN7000 for inducing anticancer immunity and as adjuvants for vaccination against infection when compared with KRN7000.[57–59] Conversely, αGalCer analogs inducing Th2-like cytokine responses have been also identified, such as the OCH analog with truncated acyl and sphingosine chains and αGalCer C20:2 with a truncated and unsaturated acyl chain. Both of these analogs have shown superior activity in mouse models for promoting tolerance and reducing inflammatory disease in autoimmune mouse models.[60,61]

To gain an understanding of the molecular mechanisms responsible for controlling iNKT cell responses and enable the rational design of targeted immunotherapies, the structure–activity relationship of αGalCer glycolipids has been extensively studied. Although TCR affinity for variant glycolipids may contribute to the range of different functional outcomes, this is not sufficient to explain many of the observed effects on iNKT cell activation.[55,62–66] Several studies have suggested differences in kinetics and the cellular pathways involved in generating complexes of glycolipids bound to CD1d have a major role

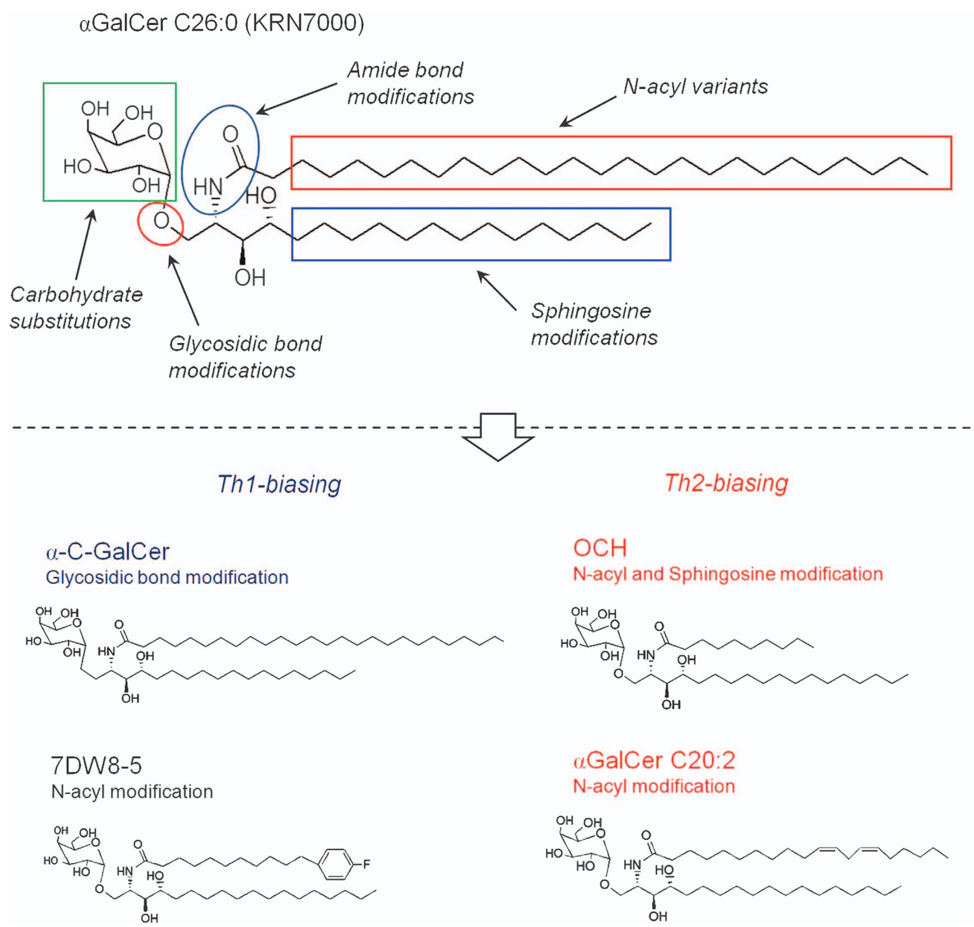

Figure 2 Structure of KRN7000 and structural analogs. KRN7000 is composed of an α-galactose bound by a 1′-O-glycosidic bond to a C18 phytosphingosine base with an amide linked, fully saturated C26 fatty acyl chain. The main modifications made in its structure, in order to obtain analogs with different properties, are depicted in the figure. Some examples of representative Th1- and Th2-biasing analogs and their chemical modifications relative to KRN7000 are shown below.

in determining the type of immune response generated, particularly with respect to the pattern of cytokines produced.[39,55,56,67] In general, modifications that enhance glycolipid solubility in aqueous environments result in an anti-inflammatory or Th2-like cytokine-biased response, while modifications that increase hydrophobicity are pro-inflammatory and lead to prominent Th1-like bias. This effect of lipid solubility has been linked to differences in requirements for cellular uptake and intracellular loading of the glycolipids onto CD1d. Thus, αGalCer analogs inducing a Th1-like bias require internalization by antigen-presenting cells for presentation, and their association with CD1d depends on the acidic pH of endosomal compartments and on a variety of intracellular lipid transfer proteins.[42,68,69] As a result, these ligands undergo endosomal-dependent presentation and form complexes with CD1d that have been found to accumulate in plasma membrane microdomains known as lipid rafts.[55,56] In contrast, due to their lower hydrophobicity, Th2-like cytokine-biasing glycolipids can be loaded directly into cell surface CD1d molecules, bypassing endosomal presentation and localizing mainly outside of lipid rafts. Neutralization of endosomal pH can enhance endosomal loading of these Th2-like biasing αGalCer analogs, resulting in their presentation by lipid raft-localized CD1d molecules and a shift toward stimulation of more Th1-like cytokine production.[70] Thus, lipid raft localization is likely to be a key factor to favor the cell signaling required to induce a Th1-like or pro-inflammatory cytokine bias.

ENHANCEMENT OF ADAPTIVE IMMUNE RESPONSES BY SYNTHETIC GLYCOLIPIDS

A major effect of iNKT cell activation by glycolipids is its effects on DCs, T cells and B cells during priming of adaptive immune responses.[13,35–37,71] The shaping of these adaptive responses can have a profound effect on the development of T-cell effector properties and the magnitude and quality of antibody responses.[23,72–76] As DCs are considered the master regulators of T-cell priming, the modulation of their activity can be crucial for T-cell fates during generation of immune responses. It has been reported that administration of KRN7000 *in vivo* induces the upregulation of costimulatory molecules in DCs, such as CD40, CD80 and CD86, and also an increase in the secretion of pro-inflammatory cytokines, such as IL-12 and tumor necrosis factor-α.[72,73] This enhancement of DC activation is dependent on the interaction between CD40 and CD40L expressed by the DC and NKT cell, respectively.[77] Such enhancement of DC activity leads to a consequent enhancement of the priming of CD4 and CD8 T cells.[72,73] Similarly, KRN7000 and the pro-inflammatory Th1-like biasing analogs, α-C-GalCer and 7DW8-5, significantly enhance the cross-priming of CD8 T cells.[23,25,74] Whether different glycolipids that induce differential cytokine responses in iNKT cells can affect not only the magnitude of T-cell activation but also the effector fate of CD4 T cells (that is, their differentiation into Th1, Th2, Th17, follicular helper T cells or regulatory T cells) remains to be determined.

B-cell activity is also enhanced by glycolipid-dependent activation of iNKT cells. The administration of KRN700 *in vivo* not only increases the levels of immunoglobulin G antibodies, and also immunoglobulin E levels in some mouse models of allergic disease, but also the number and persistence of plasma cells.[75,76,78] This B-cell enhancement is strictly dependent on a direct interaction through CD1d expressed on B cells and the TCRs of iNKT cells but is maximized when DCs also present the glycolipid to iNKT cells, as mice with selective deficiency of CD1d on DCs display a less prominent enhancement of B-cell immunity by iNKT cell activation.[79,80] Although the potential of these agents has yet to be fully realized, numerous preclinical studies and a few early clinical studies support the continued study of iNKT cell activating glycolipids in the prevention and treatment of cancer, infectious and inflammatory diseases.[81–87]

GLYCOLIPID ACTIVATORS OF iNKT CELLS FOR CANCER IMMUNOTHERAPY

The initial discovery of the potent antitumor effects of αGalCer in mice was made >20 years ago,[54,88] even before it was determined that these glycolipids specifically activate iNKT cells in a CD1d-dependent manner.[53] The role of iNKT cells and their TCR interactions with CD1d molecules were identified afterwards using iNKT-cell-deficient mice (Jα18$^{-/-}$ and CD1d$^{-/-}$mice), which showed enhanced tumor growth as compared with wild-type mice.[89,90] Subsequent studies of human iNKT cells also showed significant antitumor activities, including direct cytotoxicity for tumor cells *in vitro* that was in some cases dependent on direct recognition of CD1d.[91] Administration of αGalCer by different routes has been found to inhibit tumor metastases and increase survival in various murine tumor models, such as B16 melanoma,[92] spontaneous sarcomas in p53$^{-/-}$ mice,[93] C26GM colon carcinoma[94] and 4T1 breast carcinoma.[95] In addition, in humans there is a decrease in the iNKT cell frequency in patients with different types of cancers in comparison with healthy volunteers,[96,97] and a larger number of iNKT cells among tumor-infiltrating lymphocytes has been associated with a better prognosis.[98]

Despite the well-established antitumor activities of iNKT cells,[99] their use in cancer therapy remains challenging for several reasons. First, iNKT cells are less abundant in humans than in mice, making preclinical assessment in mouse models imprecise.[100] Second, direct intravenous administration of αGalCer in humans has been associated with relatively weak immune activation,[81,101] and this is compounded by the finding that cancer patients frequently show a decreased iNKT cell frequency.[96,97] Third, after a single dose of αGalCer there is a short period of strong iNKT activation followed by long-term anergy.[101,102] Consistent with this, subsequent administrations of αGalCer resulted in blunted immune responses in a phase I clinical study.[81] Finally, it has also been observed that administration of the free glycolipid generates liver injury in mice,[103] and recently, a phase I clinical trial have shown liver toxicity in humans.[104] As discussed above and recently reviewed in detail by Laurent *et al.*,[105] hundreds of compounds based on modifications of αGalCer have been synthesized with the objective of generating more potent and selective iNKT cell agonists.[39,56] In mouse models, a small number of compounds have been identified that show enhanced anticancer effects in direct comparison with KRN7000, although it remains unclear whether these observations will be validated in humans.[106] In addition, the problem of iNKT cell depletion or anergy induction by injection of free αGalCer glycolipids remains a major hurdle. This has motivated recent efforts at the development of alternate delivery methods that circumvent such issues.

CELL-BASED THERAPY USING αGALCER-PULSED DCS

The use of autologous DCs loaded *ex vivo* with αGalCer glycolipids has been explored as a strategy that offers potential advantages over injections of free glycolipids. DCs can efficiently present αGalCer to NKT cells in culture, and this is associated with strong production of IL-12.[77] The production of this cytokine is correlated with the induction of tumor antigen-specific cytotoxic lymphocytes[37] and other antitumor effects, such as reduced angiogenesis.[107] Furthermore, αGalCer-pulsed DCs modify the responses of iNKT cells as compared with the administration of the free glycolipid, with increased expansion of iNKT cells and stimulation of IFNγ secretion that is stronger and more prolonged.[108] Also of great significance is the observation in some experiments that administration of αGalCer in this way is less likely to generate anergy or apoptosis of iNKT cells than free glycolipid injections.[102,103,109,110]

Several phase I clinical trials using DCs pulsed *ex vivo* with KRN7000 have been reported in cancer patients, demonstrating that this approach is well tolerated and leads to measurable activation of iNKT cells *in vivo*.[82,84,85,111–114] Moreover, these studies have assessed different routes of administration for αGalCer-pulsed DCs, showing that both intravenous injection and transfer directly into the nasal submucosa can activate iNKT cells and other cell types, including NK, T and B cells.[85,111–114] Another study compared intravenous and intradermal administration, finding the former to be more effective,[114] most likely because the injected immature DCs did not traffic out of the injection site in the skin to regional lymph nodes nor did they migrate to the lungs, liver or spleen. Furthermore, Chang *et al.*[111] have developed a protocol using administration of mature DCs, in comparison with the immature counterpart, which results in a substantial expansion of iNKT cells that is sustained for up to 6 months after vaccination. Finally, considering the relatively low frequency of peripheral iNKT cells in humans, which is even further reduced in cancer patients, another approach that is being considered is combination cellular therapy involving transfer of both *ex vivo* expanded autologous iNKT cells and αGalCer-pulsed DCs.[84,97]

ANTIBODY TARGETING OF CD1D/GLYCOLIPID COMPLEXES TO CANCERS

Another promising approach has been developed using cancer-specific antibodies in association with CD1d/αGC complexes. Examples studied to date have used the variable fragments of antibodies recognizing human tumor-associated antigens, specifically HER-2 and carcinoembryonic antigen, which are expressed on the surface of several types of cancers, including some breast, lung and colon carcinomas. *In vitro* analyses showed that single-chain variable fragments of these tumor-specific antibodies in translational fusion with CD1d can effectively target CD1d–αGalCer complexes to tumor cells, enabling their direct recognition by iNKT cells and leading to potent tumor cell lysis. In addition, *in vivo* studies in tumor-bearing mice using this approach demonstrated the potential to inhibit tumor growth with activation of a variety of antitumor immune mechanisms that are orchestrated by direct iNKT cell stimulation, including activation of NK cells, release of Th1 cytokines and priming of tumor antigen-specific cytotoxic CD8$^+$ T cells.[115–117] Cancer-specific antibodies fused with CD1d/glycolipid complexes also have the advantage of stimulating activation and expansion of iNKT cells while preserving their ability to respond to multiple subsequent stimulations.[116] Efforts in this area are currently directed at identifying the optimal αGalCer analogs for *ex vivo* loading of targeted CD1d single-chain variable fragment proteins. One recent approach has used photoactivatable derivatives of αGalCer that can be covalently bound to the targeted CD1d, thus stabilizing and extending the half-life of the complex while potentially avoiding unwanted off-target effects.[118]

LIVE BACTERIA AS αGALCER DELIVERY VECTORS

Methods for incorporation of αGalCer analogs directly into live bacteria have recently been developed as another approach for delivery of these compounds to stimulate anticancer or antimicrobial immunity. One vector that has been used in this manner is *Listeria monocytogenes* (Lm), an intracellular bacterium that has the capacity to deliver antigens through efficient infection of phagocytic antigen-presenting cells. Of particular note is the observation that Lm directly infects tumor cells and causes cytolytic effects through induction of high levels of reactive oxygen species.[119] This direct killing of tumor cells has the important secondary effect of releasing tumor-derived antigens in an environment that efficiently primes tumor-specific CD8$^+$ T cells. As a vaccine vector, Lm has proven to effectively deliver tumor-associated antigens such as Mage-b, which is abundantly expressed by a variety of human carcinomas. For example, recombinant strains of Lm-expressing Mage-b can dramatically reduce the number of metastases in the mouse 4T1 model of breast cancer if administered before or shortly after initiating a primary tumor. This prophylactic effect of Lm-based vaccination and immunotherapy was not as evident if administered after the primary tumor had become well established, likely owing to the immune suppression that is characteristic of tumor-bearing hosts.[120] This underscores the belief that to increase the therapeutic effect and overcome the immune suppression in subjects already having clinically apparent cancers, immunological adjuvants are needed. Relevant to this, it has been shown that direct incorporation of αGC into a recombinant Lm vaccine strain expressing Mage-b results in nearly complete elimination of metastases 4T1 breast carcinoma cells, even if the vaccination is delayed until after establishment of primary tumors.[95] This approach to antitumor vaccination may thus overcome immune suppression and does so without apparent major toxicity even with repetitive treatments. Ongoing work seeks to optimize the design of synthetic αGC analogs and the methods for their administration to optimize the antitumor effects of Lm-based vaccines. Incorporation of glycolipids into other bacterial vectors, specifically in *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), has also been studied to improve the activation of iNKT cells and favor a Th1 profile that could be beneficial for cancer immunotherapy.[39,121]

USE OF SYNTHETIC αGALCER GLYCOLIPIDS AS VACCINE ADJUVANTS

In addition to their potential applications for cancer immunotherapy, several αGalCer glycolipids have also been used effectively as vaccine adjuvants for prevention of infections in mouse models.[25,57–59,122–127] Several experimental vaccines, including examples targeting malaria, tuberculosis, influenza and HIV, have been shown to give improved protective immunity when administered with KRN7000. For example, when used in combination with several antimalaria vaccines, including irradiated sporozoites and viral vectors expressing malaria antigens, KRN7000 enhanced the protection and improved immune responses against malaria parasites in a process dependent on iNKT-derived IFNγ production.[122] Also, KRN7000 has been shown to enhance the immunogenicity of several antiviral vaccines, including experimental vaccines against HIV and influenza. The administration of KRN7000 with an HIV-1 DNA vaccine encoding viral envelope and Gag proteins greatly enhanced both CD4- and CD8-specific T-cell responses in mice and also antibody responses against these proteins.[127] Similar enhancement is observed for influenza, where the immunization with KRN7000 in combination with either influenza hemagglutinin or inactivated influenza virus results in an increase of antibody response against the virus, as well as improved viral clearance and survival.[123,124] These effects appear to be influenced by structural variations in αGalCer, with superior effects with the use of pro-inflammatory, Th1-like biasing analogs.[126]

Immune responses against *Mycobacterium tuberculosis* (TB) are also known to be significantly enhanced by incorporation of KRN7000 into the live, attenuated BCG vaccine, especially at the level of CD8 T-cell cross-priming. However, protection against TB is not consistently enhanced by KRN7000 in mouse models of infection.[74] This emphasizes limitations of the use of KRN7000 for optimal iNKT-cell-dependent adjuvant effects. However, when the pro-inflammatory, Th1-like cytokine biasing compound α-C-GalCer was incorporated into BCG, a significantly higher protection against TB challenge was observed, compared with immunization with unmodified BCG. Moreover, the enhancement of TB-specific $CD8^+$ T-cell cross-priming was higher compared with KRN7000.[74] Of note, the combination of an influenza vaccine with α-C-GalCer is also very effective at inducing anti-influenza T-cell responses and enhancing protection against challenge in mouse models,[125] and α-C-GalCer in combination with an antimalaria vaccine markedly increased its immunogenicity compared with KRN7000.[57] These findings underscore the crucial role of a pro-inflammatory cytokine milieu during priming by these vaccines, and thus αGalCer analogs that promote pro-inflammatory cytokines such as IFNγ with relatively less production of Th2-like cytokines are needed.

Although the C-glycoside analog α-C-GalCer seems, from studies in mice, to have great potential as a vaccine adjuvant, there are data suggesting that this analog may not perform nearly as well in primates or humans. This may reflect differences in structure between mouse and human CD1d, iNKT TCRs or both. Studies using mice expressing human CD1d (human CD1d knock-in mice) as a partially humanized model for studying iNKT cell activators have suggested a relatively poor activity of α-C-GalCer with presentation by human CD1d molecules in this context.[24,25] However, another novel pro-inflammatory cytokine biasing glycolipid, 7DW8-5, displayed a favorable adjuvant profile when incorporated into BCG-based vaccines in the hCD1d-KI mouse model.[25] This αGalCer analog has been shown to be up to 100-fold more active at stimulating both human and mouse NKT cells and has also shown superior effects as a malaria vaccine adjuvant when compared directly to KRN7000 in mouse models.[58,59] A very promising potential application of this glycolipid for human immunotherapy comes from studies showing that 7DW8-5 combined with a human malaria vaccine candidate can significantly enhance malaria-specific immune responses in non-human primates.[23]

ADDITIONAL APPLICATIONS OF iNKT-CELL-BASED IMMUNOTHERAPIES

Extensive studies have also been carried out on iNKT-cell-based therapy in multiple inflammatory and autoimmune diseases, including inflammatory bowel disease, experimental autoimmune encephalomyelitis and type-1 diabetes.[32,128–131] This area of research has not yet led to clinical studies, and its potential for contributing to new therapeutics remains uncertain. Details of research and preclinical studies in this area can be found in recent extensive literature reviews published elsewhere.[128,132]

FUTURE DIRECTIONS AND CONCLUDING REMARKS

The discovery of iNKT cells and of αGalCer analogs to modulate their activities has created a new research area aimed at the use of these cells for immunotherapy against infectious diseases and cancer. Although many of the results obtained in mouse models are promising, the translation of these results to non-human primates and humans has been challenging, and early phase clinical studies have yet to show clear evidence of efficacy for cancer therapy or other applications. Discrepancies between the results obtained in tumor-bearing mice and in human cancer patients treated with αGC may be related to the differences between human and mouse CD1d molecules and related differences in CD1d-restricted iNKT cells. The generation of better humanized mouse models that can allow more accurate replication of the human iNKT cell responses remains a major objective, as such models will permit a more rapid and accurate selection of novel glycolipids for use as immunotherapeutic adjuvants for several diseases, including cancer and infectious diseases. Newer mouse models such as hCD1d-KI mice may provide much better opportunities for preclinical testing and screening of novel αGalCer analogs and delivery methods.[24] The method for delivery of glycolipids as immunomodulators *in vivo* also represents a critical challenge, with an emphasis on approaches that limit or reduce unwanted effects on iNKT cells such as anergy induction or stimulation of suppressive functions. New approaches, including some already under development, offer novel strategies for safe and efficient delivery of iNKT-cell-activating glycolipids that will ensure continuing interest and progress in this area of immunotherapy.

CONFLICT OF INTEREST

S Porcelli is a paid consultant for Vaccines, Inc., which has commercial interest in the area of iNKT cell-based therapeutics and vaccines. The remaining authors declare no conflict of interest.

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