NKT CELLS IN CANCER IMMUNOTHERAPY, 2nd Edition

EDITED BY: Tonya J. Webb, Paolo Dellabona, Weiming Yuan and Everett Meyer

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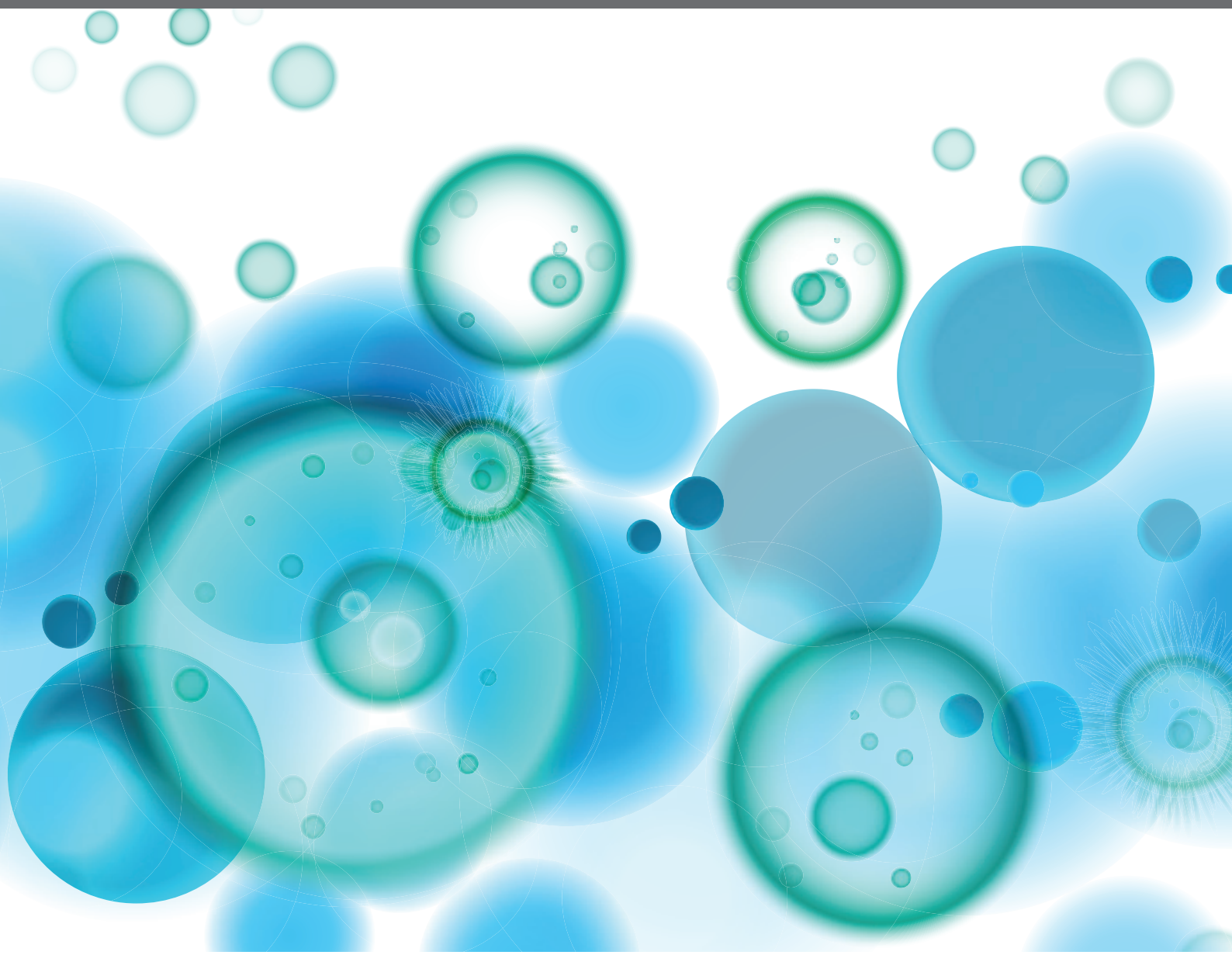

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NKT CELLS IN CANCER IMMUNOTHERAPY, 2nd Edition

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EDITORIAL
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Editorial: NKT Cells in Cancer Immunotherapy

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Keywords: iNKT, CD1d, dendritic cells, α-GalCer, cancer, immunotherapy

Editorial on the Research Topic

NKT Cells in Cancer Immunotherapy

Natural killer T (NKT) cells are a unique subset of T cells that recognize glycolipid antigens within the context of CD1d, a non-classical MHC class I-like molecule (1–3). NKT cells have the capacity to mount strong anti-tumor responses and have thus become a major focus in the development of effective cancer immunotherapy. Type I, invariant NKT (iNKT) cells, are the most well-characterized subset of CD1d-restricted T cells. NKT cells express an invariant Vαβ TCR and are known for their ability to rapidly produce copious amounts of Th1, Th2, and Th17-type cytokines following stimulation by CD1d-antigen complexes (4). α-Galactocylceramide (α-GalCer) is a potent activator of iNKT cells. Following treatment with α-GalCer, iNKT cells produce large amounts of cytokines, undergo clonal expansion, and subsequently activate NK cells, neutrophils, macrophages, dendritic cells (DC), B cells, and T cells. Moreover, activated NKT cells can directly induce cell death in tumor cells and infected cells. NKT cells have been shown to play a critical role autoimmune disease, infection, transplant immunology, and cancer. Therefore, it is important to understand how to effectively guide their effector functions in order to develop novel immunotherapeutic strategies (Lam et al.). The articles in this special issue are centered around our current understanding of NKT cell biology and address outstanding questions in the field.

α-GalCer has been utilized extensively due to its ability to induce potent activation of mouse and human iNKT cells. In this collection, Zhang et al. highlight different modalities for mobilizing iNKT cells for anti-cancer therapies. These studies are important because preclinical studies have shown that repeated exposure to α-GalCer can result in iNKT cell anergy. It is now appreciated that loading dendritic cells with glycolipid antigens can help avoid the induction of anergy. Fujii and Shimizu focused on NKT-mediated immunotherapy through selective DC targeting. Other approaches include using nanovectors/nanoparticle-based delivery systems, α-GalCer loaded exosomes, as well as treatment with IL-2 or using antibodies to block inhibitory signaling (Ghinnagow et al.; Lam et al.; Zhang et al.). Co-signaling molecules, such as CD28 and PD-1, can positively and negatively influence iNKT cell activation, and a review from the Webb lab details the costimulatory requirements for iNKT cell development and function (Shissler et al.).

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New approaches to improve preclinical platforms to study α-GalCer-based immunotherapies include the development of humanized CD1d/NKT mouse models by several groups (Zhang et al.). In addition, Dashtsoodo et al. has developed a therapy using a newly identified NKT cell agonist, RK. They found that RK-pulsed DCs resulted in the establishment of long-term T cell memory responses. Strikingly, treatment of B16-melanoma bearing mice with RK-pulsed DCs, resulted in a nearly complete elimination of the tumor, whereas treatment with a similar concentration of α-GalCer-pulsed DCs did not demonstrate any therapeutic benefit.

Studies by Wolf et al., discussed clinical trials focused on activating iNKT cells using α-GalCer pulsed antigen presenting cells, as well as adoptive iNKT cell therapies using blood-derived *ex vivo* expanded iNKT cells. This and other related work suggests that iNKT-CARs may be advantageous because their endogenous TCR has intrinsic anti-tumor activity, strong signaling through the TCR typically results in a Th1-type cytokine bias in NKT cells, and iNKT cells can migrate into non-lymphoid tissues; therefore, they could mediate anti-tumor immune responses in non-lymphoid tumors. Aside from the fact that iNKT-CAR could be developed more easily as a third-party cellular therapy, iNKT may behave differently than conventional CAR T for which exhaustion and anergy limit their efficacy in non-lymphoid tumors. In this vein, Zhang and Donda has developed bi-functional fusion proteins composed of extracellular CD1d and antibody scFv fragments specific to HER2 or CEA as a means of redirecting iNKT cells to the tumor site. Importantly, they found that treatment of tumor-bearing mice with their α-GalCer-loaded-CD1d anti-tumor fusion proteins resulted in the recruitment of iNKT, NK, and T cells to the tumor, leading to a significant reduction in tumor growth.

Mavers et al. highlights the critical role of NKT cells in reducing graft vs.-host-disease (GVHD) in preclinical and clinical studies of allogeneic hematopoietic stem cell transplantation and enhancing anti-tumor immune reactions (GVT). Their work also highlights two key areas needed to advance iNKT into clinical practice, including the need to better define and recapitulate NKT cell subsets and a better understanding of optimal drug delivery strategies for α-GalCer or other glycolipids for the activation and modulation of the appropriate NKT cell subset *in vivo*. It has been well-established that NKT cells can be activated following their recognition of lipid antigen presented in the context of CD1d or by cytokines. Modes of NKT cell activation are discussed by Cerundolo's lab as a means to understanding how to effectively harness their effector functions in cancer immunotherapy (Bedard et al.). Advances in technology, such as single cell RNA-seq and microfluidics, can help to provide a detailed description of the specific NKT subsets within the tumor microenvironment. Teyton et al. discusses how the implementation of these techniques can be used to gain a better understanding of NKT cell during tumorigenesis.

Type II NKT cells have been shown to play a suppressive role in many different disease settings; however, due to limitations in reagents and model systems it has been difficult to study this unique CD1d-restricted subpopulation. Herein, Kato et al. discuss experimental tools that can be used to analyze type II NKT cells, such as, 24αβ-TCR mice, 4get Jα18 $^{-/-}$ mice and CD1d tetramers. The review by Nair and Dhadapkar also discusses suppression of tumor immunity by type II NKT cells. Importantly, this comprehensive issue includes primary research investigating the impact of neurofibromin 1 on CD1d expression (Liu et al.) and discusses NKT cells from an ecological, evolutionary, and developmental biology "eco-evo-devo" perspective (Kumar et al.). The gut microbiota has been demonstrated to play a critical role determining responses to immune checkpoint inhibitor therapy, and NKT cells have been shown to regulate gut microbial ecology, thus future studies should investigate the impact of the gut microbiome and NKT cell number and function on immune responses and patient outcomes following treatment with cancer immunotherapy.

This special issue also describes models in which type I NKT cells have been shown to play a suppressive role in Th1 responses, thereby promoting tumor formation, specifically in the intestines (Wang and Cardell). Given the dual role that NKT cell can play in anti-tumor immunity, by either inducing tumor suppression or promoting tumor formation, it is important to investigate the role of the local tumor microenvironment and understand how this guides NKT cell responses (5, 6). Also, it is critical to identify and specific markers, such as PLZF, that can help define functional differences within particular NKT cell subsets. In closing, this special issue is a collection of reviews and research articles that showcases the pioneering techniques, unique model systems, and innovative therapeutic strategies being utilized to modulate NKT cells. This work in combination with future studies, will aid our understanding of how to effectively manipulate these potent effector cells in order to induce optimal anti-tumor immune responses.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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We would like to dedicate this special issue to Vincenzo Cerundolo (1959-2020).

REFERENCES

1. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol.* (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711
2. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nat Immunol.* (2010) 11:197–206. doi: 10.1038/ni.1841
3. Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. *Nat Rev Immunol.* (2002) 2:557–68. doi: 10.1038/nri854
4. Wang H, Hogquist KA. How lipid-specific T cells become effectors: the differentiation of iNKT subsets. *Front Immunol.* (2018) 9:1450. doi: 10.3389/fimmu.2018.01450

5. Liu D, Song L, Wei J, Courtney AN, Gao X, Marinova E, et al. IL-15 protects NKT cells from inhibition by tumor-associated macrophages and enhances antimetastatic activity. *J Clin Invest*. (2012) 122:2221–33. doi: 10.1172/JCI 59535
6. Cortesi F, Delfanti G, Grilli A, Calcinotto A, Gorini F, Pucci F, et al. Bimodal CD40/Fas-dependent crosstalk between iNKT cells and tumor-associated macrophages impairs prostate cancer progression. *Cell Rep*. (2018) 22:3006–20. doi: 10.1016/j.celrep.2018.02.058

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REVIEW
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Enhancement of Adjuvant Functions of Natural Killer T Cells Using Nanovector Delivery Systems: Application in Anticancer Immune Therapy

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Type I natural killer T (NKT) cells have gained considerable interest in anticancer immune therapy over the last decade. This "innate-like" T lymphocyte subset has the unique ability to recognize foreign and self-derived glycolipid antigens in association with the CD1d molecule expressed by antigen-presenting cells. An important property of these cells is to bridge innate and acquired immune responses. The adjuvant function of NKT cells might be exploited in the clinics. In this review, we discuss the approaches currently being used to target NKT cells for cancer therapy. In particular, we highlight ongoing strategies utilizing NKT cell-based nanovaccines to optimize immune therapy.

Keywords: natural killer T cells, adjuvant, α-galactosylceramide, CTL response, nanovaccines, dendritic cells, cancer

INTRODUCTION

Invariant or type I natural killer T cells (referred as NKT cells) represent a highly conserved subset of non-conventional T lymphocytes endowed with a remarkably broad range of immune effector and regulatory functions. These cells recognize foreign and self-derived glycolipid antigens presented by the monomorphic MHC/HLA class I-like molecule CD1d expressed by antigen-presenting cells, including dendritic cells (DCs) [for reviews, Ref. (1–5)]. NKT cells express on their surface a semi-invariant T cell receptor (TCR) composed by a unique TCR-α chain paired with a restricted number of β-chains. Rapidly after natural activation (inflammation, infection), NKT cells produce huge amounts of cytokines including T helper (Th)1-like (INF-γ), Th2-like (IL-4), Th17-like (IL-17, IL-22), and regulatory (IL-10) cytokines. This flexibility depends on the mode of stimulation, on the location and on the NKT cell subset challenged. Of note, NKT cells can be activated by direct TCR triggering and also *via* cytokines, without TCR engagement (5). With their ability to swiftly release cytokines, NKT cells have also the potential to lyse cellular targets following TCR recognition of lipid

Abbreviations: NKT, natural killer T; DCs, dendritic cells; TCR, T cell receptor; α-GalCer, α-galactosylceramide; CAR, chimeric antigen receptor; aAVC, artificial adjuvant vector; PLGA, polylactic-coglycolic acid; CTL, cytotoxic $CD8^+$ T lymphocyte; TLR, toll-like receptor; Trp2, tyrosinase-related protein 2.

antigens (6). This property is important in immune surveillance against tumor cells and could be exploited for immune-based therapy. The role of NKT cells in various pathologies including cancer, infection, acute, and chronic inflammation and autoimmune diseases has been evidenced in experimental models and in humans (5). Along with their natural (beneficial or detrimental) role in pathological settings, NKT cells can also be manipulated by means of specific CD1d-restricted ligands. For instance, exposure of antigen-presenting cells to α-galactosylceramide (α-GalCer) triggers potent innate and acquired immune responses. Of particular interest is the exquisite capacity of NKT cells to promote DC maturation and, as a consequence, to trigger potent T and B cell responses (7). This unique property, and given that the CD1d/NKT axis is conserved in humans (with no HLA restriction), could be used in clinical situations, including cancer. There is a strong interest to exploit the adjuvant effects of α-GalCer or related glycolipid derivatives to develop more efficient NKT cell-based vaccines (8–10). We herein review the effects of α-GalCer in preclinical and clinical studies and discuss ongoing and future strategies that aim to optimize NKT cell-based antitumor therapy with a particular focus on nanovector delivery systems. These systems, particularly those allowing encapsulation of tumor antigens and α-GalCer derivatives (adjuvant), might realize maximal therapeutic benefit with minimal toxicity.

FREE α-GalCer IN ANTITUMOR THERAPY: FROM PRECLINICAL STUDIES TO CLINICAL DEVELOPMENT

Alpha-GalCer is a marine sponge-derived glycosphingolipid originally discovered in a screen for antitumor compounds (11, 12). This seminal discovery has led to the development of synthetic α-GalCer derivatives as a family of powerful glycolipid agonists for NKT cells in order to promote protective immune responses against infections and cancers (13–15). α-GalCer triggers a mixed response by NKT cells including the production of IFN-γ, a cytokine important in tumor immune surveillance and inhibition of angiogenesis. Different agonists with Th1-promoting functions (which appear to be more adapted for anticancer therapies) have been described (13, 16). Preclinical studies have highlighted the potent antitumor effect of α-GalCer and α-GalCer derivatives against solid tumors (sarcoma, melanoma and colon, prostate, and lung carcinoma) and hematological malignancies (lymphoma) (12, 17–21). Mechanisms involved include early production of IFN-γ by NKT cells and NK cells and secretion of IL-12 by DCs (20). This success has led to clinical trials in patients with advanced lung cancer. Free soluble α-GalCer was used. Unfortunately, no or low clinical benefits were reported among patients (22–24). These disappointing results might be due to the lower number of NKT cells in patients relative to healthy individuals and/or to their diminished (but reversible) activation threshold capacity (22–32). Hence, one concern in NKT cell-based therapy is the diminished NKT cell count and/or function, although this cannot be generalized to all advanced cancer patients. Various means of circumventing this potential drawback are being developed including infusion of autologous *ex vivo*-expanded NKT cells. This approach can lead to clinically relevant antitumor responses (33–39). *In vivo* transfer of NKT cells expressing chimeric antigen receptor in order to redirect their cytotoxicity against tumor cells has also been explored in preclinical studies. This approach may provide potent antitumor activity (40, 41). Moreover, the reprogramming of NKT cells to induced pluripotent stem cells and their subsequent re-differentiation into more functional NKT cells (compared with the parental cells) is opening up new avenues in this field (42, 43). Another reason that might explain disappointing clinical data relates to the uncontrolled delivery of α-GalCer, which might lead to suboptimal primary and secondary activation of NKT cells. This later issue prompted researchers to inoculate α-GalCer in a vectorized (cellular or acellular systems) form in order to better control the delivery of the active principle and to generate more efficient innate and acquired immune-based antitumor responses.

VECTORIZATION OF α-GalCer IN CELLULAR SYSTEMS

Cellular systems in which α-GalCer is incorporated can act as potent (NKT cell-based) cellular adjuvants. As described below, these cellular systems include DCs, non-antigen presenting cells, and cancer cells. Studies in mice have demonstrated that α-GalCer loaded in DCs has a higher ability to activate NKT cells and to trigger antitumor responses relative to α-GalCer injected in a free (non-vectorized) form (18, 44). In the same line, adoptive transfer of α-GalCer-loaded autologous peripheral blood mononuclear cells or DCs induced clinical benefits in some patients (lung cancer and head and neck cancer), an effect that correlates with IFN-γ production (23, 33, 34, 36, 45–49). Of note, adoptive transfer of autologous NKT cells along with α-GalCer-pulsed mononuclear cells or DCs led to encouraging clinical results in term of prolonged median overall survival time (35, 36, 50). This effect was associated with a significant infiltration of NKT cells into the tumor (36) Hence, this combination therapy led to significant clinical efficacy, although technical and economic issues still persist.

Taniguchi's group was the first to exploit artificial adjuvant vectors (aAVCs) to enhance NKT cell-based antitumor responses (8). This system can induce both innate and long-term memory $CD8^+$ T cell responses against cancer. For instance, inoculation (single dose) of allogeneic fibroblasts (used as a vector cell) into which tumor antigen mRNA and CD1d with α-GalCer were introduced led to a long-lasting antitumor response (51–54). The same group has designed a human aAVC consisting of embryonic kidney cells transfected with the human melanoma MART-1 antigen and CD1d and pulsed with α-GalCer. This cellular system promoted antitumor response in humanized mice (55). Mechanistically, it is likely that allogeneic cells are selectively taken up by DCs and that the subsequent cross-presentation of tumor antigens to $CD8^+$ T cells and α-GalCer to NKT cell is critical in the promotion of strong and long-lasting tumor-specific cytotoxic $CD8^+$ T lymphocytes (CTL) responses.

Tumor cells are rich sources of tumor antigens. However, due to the low immunogenicity of tumor antigens, combined adjuvants are requisite in order to develop cancer vaccines. Shimizu and

collaborators were the first to evaluate the capacity of α-GalCer-pulsed tumor cells (melanoma) to act as a cellular adjuvant (56). Numerous studies have validated the efficacy of this strategy in therapeutical settings in the mouse system (solid tumor and hematological malignancies) (57–66). Mechanistically, inoculated α-GalCer-pulsed tumor cells are selectively taken up by DCs (as for aAVC), which have a unique capacity to cross-present antigens from dying cells. It is also possible that the killing of CD1d-expressing tumor cells by activated NKT cells leads to the release of tumor antigens and to their subsequent cross-presentation by DCs. Whatever the mechanism, it is likely that the presentation of both α-GalCer and tumor antigens by the same DC is critical in the development of the protective tumor-specific CTL-based antitumor response. Whether this strategy could be exploited in the human setting to harness cancer progression and recurrence, without inducing autoimmunity, is still unknown. Cooperative action of toll-like receptor (TLR) ligands and iNKT cells on DC function is a well-recognized phenomenon (67). Of interest, relative to inoculation of α-GalCer-loaded tumor cells alone, coadministration of α-GalCer-loaded tumor cells and TLR9 agonists augments the antitumor response (66).

Introduction of α-GalCer and tumor antigens in antigen-presenting cells has also been attempted in preclinical models. DCs expressing the mammary tumor-associated antigen Her-2 and pulsed with α-GalCer trigger potent antitumor responses (68). The use of different models of tumors revealed that this strategy was effective both in prophylactic and therapeutic settings (69). Of interest, vaccination with DCs transduced with OVA (used here as a model tumor antigen) plus CCL21, a chemokine that attracts both T cells and NKT cells, protects against OVA-expressing tumors (70). Finally, human embryonic stem cell-derived DCs genetically engineered to express CD1d can prime $CD8^+$ T cells against tumor antigens (71). The potential benefit of this latter strategy in cancer immunotherapy is being studied. In conclusion, cell-based vaccines to optimize α-GalCer activity *in vivo* are promising although technical, logistical, and financial difficulties might limit the development of such vaccines.

VECTORIZATION OF α-GalCer IN ACELLULAR SYSTEMS

Definition of Nanovectors

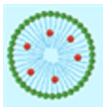
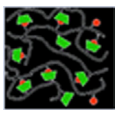
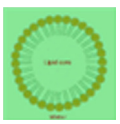
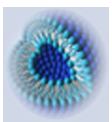
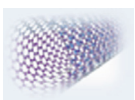
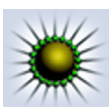
Development of nanovectors (<1 μm) holds great potential for cancer immunotherapy, including antitumor vaccines (72–74). The interest of using nanosized carriers able to incorporate α-GalCer (with or without tumor antigen) to optimize NKT cell-based anticancer therapy has recently emerged. Encapsulation of α-GalCer into nanovectors might offer several advantages relative to soluble α-GalCer. This includes preferential internalization by antigen-presenting cells (due to the size), slower and sustained release of α-GalCer in CD1d-containing endosomes, and minimal side effects (due to the lower amount required for a similar biological effect). Moreover, compared to cell-based vectorization, nanovectors are less invasive and costly (no adoptive transfer). Nanovectors offer the unique opportunity to deliver both adjuvant (including α-GalCer) and tumor antigens to the same antigen-presenting cells, especially DCs (75–77). Nanovectors represent an interesting class of delivery vehicles able to induce potent and long-lasting immune responses (78, 79). Surprisingly enough, few studies have exploited this unique property to enhance the antitumor functions of NKT cells.

Nanovectors include a multiple range of particulate systems including (among others) virus-like particles, dendrimers, silica microspheres, micelles, nanogels, nanoemulsions, liposomes, carbon nanotubes, metallic nanoparticles, and polymeric nanoparticles, which include nanospheres and nanocapsules (**Table 1** and not shown). The physical properties as well as the advantages and drawbacks of nanovectors are presented in **Table 1**. For vaccine development, a major goal is to target DCs. Uptake of nanovectors by DCs depends on several physicochemical properties including the size, shape, surface charge, hydrophobicity, and hydrophilicity of nanovectors. To target more selectively DCs, it is possible to arm nanovectors with ligands or antibodies on their surface. Among the different delivery systems for antigen encapsulation in vaccines, particularly for cancer therapy, polymeric nanoparticles have many advantages including low toxicity, high biodegradability, amenability to controlled release of the bioactive agents (antigen and adjuvant), preservation of their stability, and potential for surface functionalization (79, 80). Currently, there is a long list of polymers used to produce nanovectors including plasma albumin, chitosan, polyethyleneimine, polylactic acid, and polylactic-coglycolic acid (PLGA). PLGA is one of the most successful biocompatible and biodegradable polymers (approved for *in vivo* use by the United States Food and Drug Administration). PLGA-based nanoparticle systems are particularly interesting since they allow high antigen density, incorporation of different classes of molecules including proteins and lipids, ability to reach MHC I pathway after uptake by DCs, and slow release kinetics delivery (75, 81–85). Attempts have been made to incorporate α-GalCer in nanosized vectors, with or without tumor antigens (**Table 2**). Here, we detail the effect of vectorized α-GalCer in innate and acquired immune-based antitumor responses.

Vectorization of α-GalCer without Tumor Antigen

Preclinical studies suggest that α-GalCer vectorized in nanovectors is of potential interest. This relies mainly on passive (untargeted) and active (targeted) delivery of α-GalCer to antigen-presenting cells. For instance, silica microspheres coated with lipid bilayers plus α-GalCer target mouse CD169-expressing macrophages and DCs, both cell types being critical in the primary activation of NKT cells (86, 87). Others and we have demonstrated that PLGA-based nanoparticles are internalized by DCs to promote NKT cell activation (88, 89). Another study has shown that α-GalCer incorporated in octaarginine-modified liposomes are passively taken up by antigen-presenting cells and strongly activate NKT cells. This leads to therapeutic protection against B16F10 lung metastases (90). In order to optimize the targeting of α-GalCer to antigen-presenting cells, nanovectors can be armed with ligands or antibodies that bind to specific markers. For instance, liposomes decorated with oligomannose that binds to mannose receptor and DC-SIGN target DCs *in vivo* and potently

TABLE 1| Physical properties, advantages and drawbacks of nanovectors.

	Dendrimers	1.5–14.5 nm	Chemical homogeneity, high, degree of surface functionality and versatility, controlled degradation	Multistep syntheses, elevated cost
	Micelles	10–100 nm	Capacity and compatibility with the loaded drug, minimized cytotoxicity	Low drug loading, low drug incorporation stability, limited targeting ability
	Nanogels	20–200 nm	Large Surface area, high capacity to absorb water and other biological fluids, functional modification of the surfaces to prevent rapid clearance by phagocytic cells	Difficulties to remove the solvents and surfactants (toxicity)
	Nanoemulsions	≈100 nm	Stable structures. Large effective surface area (enhances the bioavailability of the active compound)	Special application techniques (high pressure homogenizers, ultrasonics), expensive equipment. Emulsions require large amounts of surfactants (toxic)
	Liposomes Multilamellar vesicles Large unilamellar vesicles Small unilamellar vesicles	400 nm to 5 μm 200 nm to 1 μm 20 nm to 200 nm	Controlled release of the active principle (reduced side effect relative to the free form), economical production, good tolerability, specific targeting, can transport up to 10,000 active compounds Approved for clinical use Possibility to incorporate PEG and antibodies/ligands onto the surface to lengthen blood circulation and target immune cells	Rapid clearance due to the reticuloendothelial system low-term stability
	Carbon nanotubes Single-walled Multi-walled (2–10 layers of graphene sheet)	Radius of up to 1 nm Diameter of >10 nm	Excellent chemical and thermal stability, ordered structure, high mechanical strength, high electrical and thermal conductivity, metallic or semimetallic behavior, high surface area, and bioavailability	Lack of solubility in aqueous media (may be solved by chemical modification and functionalization), potential toxic effects, aggregate formation (alteration of their general physico-chemical properties)
	Metallic nanoparticles	5–500 nm	Biological capacity to catalyze reactions in aqueous media at standard temperature and pressure, use in molecular imaging	Toxic chemicals, high-energy requirements of production
	Polymeric nanoparticles Nanospheres Continuous matrix systems in which loaded drugs are generally dispersed in and entrapped by different binding systems	10 nm to 1 μm	Slower and sustained release of the active principle (adjuvant, antigens), high physical stability, simple formulation, multifunctionality, incorporation (absorption or covalent conjugation) of hydrophilic polymers (e.g., PEG/PEO-chains, polysorbate 80 polysaccharides). Cationic systems enhance DC uptake, possibility to graft ligands or antibodies to enhance the targeting	Quickly eliminated from the bloodstream (need specific design to escape the reticuloendothelial system cells)
	Nanocapsules Core (hydrophobic or hydrophilic) structure surrounded by a polymeric shell in which the drugs are confined	10 nm to 1 μm	Natural polymers (dextran, Chitosan, albumin, gelatin, starch) Copolymers (PFLA, PGA, PLGA) approved by the FDA for clinical use, multiple functionalization (PLGA nanoparticles) for use in cancer immunotherapy	

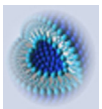
stimulate NKT cells toward a Th1 direction (91). Encapsulating α-GalCer in liposomes bearing on their surface glycans specific for the sialoadhesin CD169 strongly activates NKT cells *in vivo* (92). Our recent data demonstrate that, relative to non-vectorized α-GalCer, α-GalCer incorporated into antibody-armed PLGA nanoparticles that target DCs increases NKT cell-based innate immune responses (93).

Vectorization of α-GalCer and Tumor Antigens

Passive (Untargeted) Delivery

Very few studies have been devoted so far to study the potential benefit of encapsulating α-GalCer and tumor antigens in nano-sized vectors. A pioneer study from McKee and colleagues analyzed the consequences of α-GalCer and antigen co-encapsulation

TABLE 2 | Utilization of α-GalCer-encapsulated nanovectors to promote NKT cell activation and antitumor responses.

Nanovectors	Antigen	Targeting and NKT cell response	Antitumor response	Reference
Silica microspheres 	No	Targeting of dendritic cells (DCs) and CD169-expressing macrophages (NKT) cell activation	Not tested	(86, 87)
Virus-like particles 	Lymphocytic choriomeningitis virus-derived peptide gp33	NKT cell activation	gp33-specific CTL response Protection against melanoma (prophylactic setting)	(94)
Liposomes 	No	Targeting of DCs (Mannose receptor, DC-SIGN) *via* surface oligomannose NKT cell activation (Thl biais)	Not tested	(91)
	No	Targeting of macrophages (sialoadhesin CD169) *via* glycan ligands NKT cell activation (mouse and human)	Not tested	(92)
	No	Targeting of antigen-presenting cells (octaarginine-modified liposomes) strong NKT cell response	Antitumor effects (melanoma) Therapeutic setting	(90)
	Tyrosinase-related protein 2 (Trp2)	NKT cell activation	CTL response-antitumor effects Therapeutic setting	(97)
PLGA-based NPs (passive targeting) 	No	Better primary activation of NKT cells (IFN-γ)	Not tested	(88, 89)
	OVA	NKT cell activation	Higher CTL response relative to soluble OVA and α-GalCer and to TLR-based nanovaccine Protection against melanoma Prophylactic and therapeutic settings	(95, 96)
PLGA-based NPs (active targeting) 	No	Targeting of DEC205-expressing DCs Better primary activation of NKT cells compared to soluble α-GalCer Reduced unresponsiveness of NKT cells upon restimulation	Not tested	(93)
	OVA	Same extent of NKT cell activation relative to NPs without OVA	Robust OVA-specific CTL response Antitumor effects (melanoma, lymphoma) Prophylactic and therapeutic settings	(93)
	Trp2 Gp100	Targeting of Clec9a-expressing DCs NKT cell activation Better primary and secondary activation of NKT cells	CTL response against tumor self antigens Antitumor effects (melanoma) Prophylactic and therapeutic settings	(107)
	Melan A	Targeting of CLEC9a-expressing DCs Expansion and activation of human NKT cells (expanded from PBMCs)	Expansion of human Melan A-specific CD8+ T cells	(107)

in antitumor responses (94). In this work, α-GalCer and the gp33 peptide derived from lymphocytic choriomeningitis virus (used as a model antigen) were incorporated into virus-like particles. This composite particle system induced a 10-fold more active gp33-specific CTL response, compared to free α-GalCer and gp33, and prophylactically protected against gp33-expressing melanoma. Mechanistically, it is likely that α-GalCer and gp33 are delivered in the endosomal compartment of antigen-presenting cells to load to CD1d and MHC Class I, respectively, thus favoring cross-presentation by DCs. Dölen and collaborators have recently demonstrated that encapsulating α-GalCer and OVA in PLGA-based nanoparticles is efficient to trigger antitumor responses (95). Of interest was the observation that the response was superior compared to TLR agonist and OVA co-encapsulation. More recently, using a similar strategy, Li and colleagues showed that the immune responses triggered by α-GalCer and OVA encapsulated in PLGA nanoparticles was longer compared to that induced by its soluble counterparts (96). Of note, both intranasal and intraperitoneal injection of nanovaccine triggered robust antigen-specific $CD8^+$ T cell response. Of interest, Neumann and colleagues investigated the effect of α-GalCer and tumor antigens co-delivery on antitumor responses using a cationic liposome (97). The self-antigen tyrosinase-related protein 2 (Trp2) was used. The authors found that the liposomal formulation elicits potent antigen-specific CTL response and prevents tumor progression in a therapeutic setting. Collectively, encapsulation of α-GalCer and tumor antigens in nanovectors, including liposomes and PLGA NPs (**Table 2**), elicits antitumor responses in experimental models. In these settings (passive delivery), DCs and probably other antigen-presenting cells are critically important.

Active (Targeted) Delivery in DCs

Our work was the first to investigate the consequences of active α-GalCer and tumor antigen delivery to DCs by means of multifunctional nanovectors. In light of the literature showing the unique ability of cross-priming DCs ($CD8\alpha^+$ DCs and $BDCA3^+$ DCs in the mouse and human system, respectively) to initiate and maintain CTL responses (98–101), we decided to target this DC

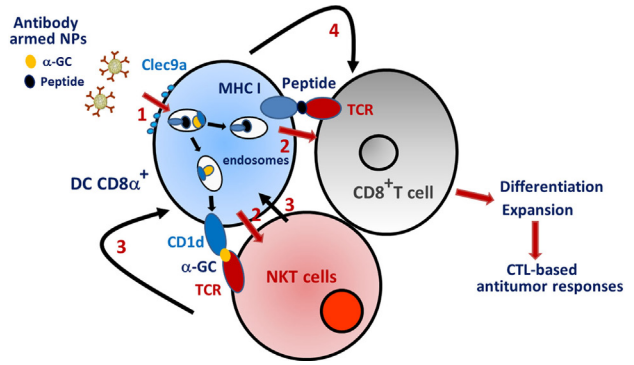


FIGURE 1 | Schematic "ménage à trois" between CD8α+ DC, natural killer T (NKT) cells, and naive CD8+ T cells. (1) Anti-Clec9a-armed nanoparticles that carry α-GalCer and tumor antigen are taken up by CD8α+ DC *via* the endocytic receptor Clec9a. (2) The active components are delivered in the endosomes and presented *via* MHC class I (peptide) and CD1d (α-GalCer) to naïve CD8+ T cells and NKT cells, respectively. (3) In response to TCR triggering, NKT cells activate the maturation of CD8α+ dendritic cells (DCs) through cytokines and costimulatory (CD40) molecules. (4) Mature DCs transmit signals to naïve CD8+ T cells, which, in turn, differentiate into CTLs. (5) CTLs destroy tumor cells. Note that α-GalCer is acquired and presented by DCs that are also actively engaged in presenting peptides to T cells. This scheme does not consider reciprocal interactions between NKT cells and CD8+ T cells.

subset. Moreover, we and others showed that CD8α+ DCs are very potent to stimulate primary and secondary NKT cell activation (93, 102). Finally, the fact that NKT cells can substitute "classical" CD4+ Th cells to license the DCs for cross-priming represents another reason explaining our devised strategy (103). Since cross-priming DCs express specific markers on their surface, we armed PLGA-based nanoparticles with antibodies in order to target these cells *in vivo*. Although DEC205 is not entirely specific for cross-priming CD8α+ DCs, nanoparticles armed with anti-DEC205 antibodies and carrying both α-GalCer and OVA successfully led to antigen cross-presentation and to potent antitumor responses (93). Of interest, this strategy also led to a long lasting antigen-specific antibody response. The C-type lectin Clec9a (also known as DNGR1) is almost exclusively expressed by cross-priming mouse and human DCs and is known to confer potent CTL responses (104–106). Our recent data indicate that PLGA-based nanoparticles armed with anti-Clec9a antibodies and incorporating both α-GalCer and OVA can confer protection against OVA-expressing tumors (lymphoma) (107). We also investigated whether our vectorization/targeting strategy might break tolerance to tumor self-antigens, an important challenge for optimal antitumor therapy [for reviews see Ref. (108–112)]. Indeed, co-incorporation of α-GalCer and tumor melanoma-derived self-antigens (including Trp2) triggered a potent CD8+ T cell-mediated antitumor response (107). Hence, our vaccine

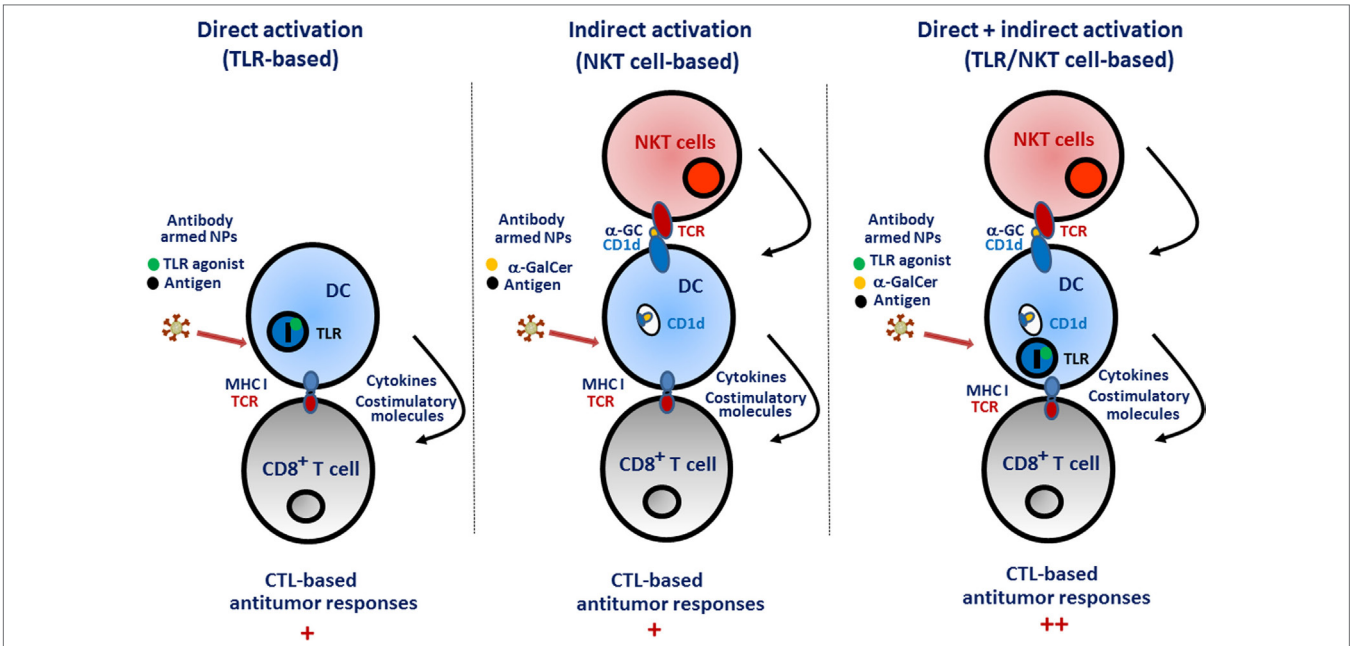


FIGURE 2 | Promotion of CD8+ T cell responses upon direct [toll-like receptor (TLR)-based] and/or indirect [natural killer T (NKT) cell based] dendritic cell (DC) activation. Direct activation. Nanoparticles bearing TLR agonists are internalized by DCs (i.e., those that excel in cross-presentation) and activate endosomal TLRs (such as TLR3, TLR7/8, or TLR9). This rapidly leads to DC maturation and to the production of inflammatory cytokines and costimulatory molecules that in turn promote the differentiation and expansion of naïve CD8+ T cells. Indirect activation. In this setting, the delivery of α-GalCer in DCs leads to the exposition of the glycolipid on the cell surface in association with the CD1d molecule (at this stage, the DC is still immature). TCR triggering in NKT cells leads to the release of cytokines and to the expression of costimulatory (CD40) molecules culminating in DC maturation. In turn, mature DCs activate naïve CD8+ T cells. Direct and indirect activation. One may suppose that the two effects are additive, or even synergistic, to promote optimal CD8+ T cell responses that control tumor progression.

strategy, probably by enhancing DC/NKT cell/naive CD8+ T cell interactions (**Figure 1**), abrogates self-tolerance and promotes effective antitumor CTL responses. Signals incorporated by DCs are critical to shape the functions of naive T lymphocytes, including CD8+ T cells. Because maturation processes of DCs due to direct innate sensor (such as TLR) signaling might be different to those triggered by NKT cells, it would be interesting to compare the efficacy of TLR-based and NKT cell-based targeted nanovaccines in cancer therapies (**Figure 2**). Co-administration of soluble α-GalCer and TLR agonists with antigen was shown to enhance the CD8+ T cell response with augmented effect on tumor progression, relative to antigen mixed with an adjuvant alone (113). Therefore, encapsulation of both TLR ligands and α-GalCer derivatives in antibody-armed nanovectors might additively or synergistically enhance the responses, a hypothesis that needs further investigations.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Growing evidences demonstrate that α-GalCer (or α-GalCer derivatives) might be successfully used in cancer therapy. However, innovative strategies to better manipulate the adjuvant properties and the antitumor potentials of NKT cells are required. Among them, optimization of delivery systems that contain α-GalCer and tumor antigens to optimally activate NKT cell-based immune responses remains an important goal. Cell-based vaccines that promote strong and long-lasting CTL responses offer an interesting immunotherapeutic strategy for the future although concerns still exist (cost, invasive procedure). Nanovectors that passively or actively target (cross-priming) DCs are also of clinical interest. Future studies will aim to enhance the efficacy of delivery systems in order to improve cell targeting and to optimize the delivery of the active principles (α-GalCer and tumor antigens) in the right cellular compartment. Such development will require the use of more sophisticated nanovectors to improve surgical strikes and possibly the targeting of other (DC expressed) specific molecules. Complementary approaches including strategies that boost the number/function of NKT cells in patients (transfer of functional NKT cells) and/or that aim to control immune suppression (e.g., check point blockers, immunomodulatory drugs) are of interest. Moreover, combination of NKT cell and TLR agonists might amplify the strength and the quality of the immune response in patients. An important area for future research is the development of humanized mouse models to accurately replicate the NKT cell response in humans. It is likely that, in a near future, the use of nanovector-based medicine will optimize antitumor responses for the sake of cancer patients, in combination with conventional immunotherapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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We apologize to colleagues whose works could not be cited due to space constraints.

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REFERENCES

1. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* (2003) 21:483–513. doi:10.1146/annurev.immunol.21.120601.141057
2. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi:10.1146/annurev.immunol.25.022106.141711
3. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: natural killer T cell defects and human disease. *Nat Rev Immunol* (2011) 11:131–42. doi:10.1038/nri2904
4. Rossjohn J, Pellicci DG, Patel O, Gapin L, Godfrey DI. Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* (2012) 12:845–57. doi:10.1038/nri3328
5. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13:101–17. doi:10.1038/nri3369
6. Metelitsa LS. Anti-tumor potential of type-I NKT cells against CD1d-positive and CD1d-negative tumors in humans. *Clin Immunol* (2011) 140:119–29. doi:10.1016/j.clim.2010.10.005
7. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* (2003) 171:5140–7. doi:10.4049/jimmunol.171.10.5140
8. Fujii S-I, Shimizu K, Okamoto Y, Kunii N, Nakayama T, Motohashi S, et al. NKT cells as an ideal anti-tumor immunotherapeutic. *Front Immunol* (2013) 4:409. doi:10.3389/fimmu.2013.00409
9. Cerundolo V, Silk JD, Masri SH, Salio M. Harnessing invariant NKT cells in vaccination strategies. *Nat Rev Immunol* (2009) 9:28–38. doi:10.1038/nri2451
10. Mattarollo SR, Smyth MJ. NKT cell adjuvants in therapeutic vaccines against hematological cancers. *Oncoimmunology* (2013) 2:e22615. doi:10.4161/onci.22615
11. Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* (1995) 7:529–34.
12. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* (1997) 278:1626–9. doi:10.1126/science.278.5343.1626
13. Carreño LJ, Saavedra-Ávila NA, Porcelli SA. Synthetic glycolipid activators of natural killer T cells as immunotherapeutic agents. *Clin Transl Immunology* (2016) 5:e69. doi:10.1038/cti.2016.14
14. Kharkwal SS, Arora P, Porcelli SA. Glycolipid activators of invariant NKT cells as vaccine adjuvants. *Immunogenetics* (2016) 68:597–610. doi:10.1007/s00251-016-0925-y
15. East JE, Kennedy AJ, Webb TJ. Raising the roof: the preferential pharmacological stimulation of Th1 and th2 responses mediated by NKT cells. *Med Res Rev* (2014) 34:45–76. doi:10.1002/med.21276
16. Li X, Fujio M, Imamura M, Wu D, Vasan S, Wong C-H, et al. Design of a potent CD1d-binding NKT cell ligand as a vaccine adjuvant. *Proc Natl Acad Sci U S A* (2010) 107:13010–5. doi:10.1073/pnas.1006662107

17. Nakagawa R, Motoki K, Ueno H, Iijima R, Nakamura H, Kobayashi E, et al. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an alpha-galactosylceramide, KRN7000. *Cancer Res* (1998) 58:1202–7.
18. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* (1999) 163:2387–91.
19. Shin T, Nakayama T, Akutsu Y, Motohashi S, Shibata Y, Harada M, et al. Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Valpha14 NKT cells. *Int J Cancer* (2001) 91:523–8. doi:10.1002/1097-0215(20010215)91:4<523::AID-IJC1087>3.0.CO;2-L
20. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* (2002) 99:1259–66. doi:10.1182/blood.V99.4.1259
21. Nowak M, Arredouani MS, Tun-Kyi A, Schmidt-Wolf I, Sanda MG, Balk SP, et al. Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with α-galactosylceramide. *PLoS One* (2010) 5:e11311. doi:10.1371/journal.pone.0011311
22. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* (2002) 8:3702–9. doi:10.1155/2007/89017
23. Schneiders FL, Scheper RJ, von Blomberg BME, Woltman AM, Janssen HLA, van den Eertwegh AJM, et al. Clinical experience with α-galactosylceramide (KRN7000) in patients with advanced cancer and chronic hepatitis B/C infection. *Clin Immunol* (2011) 140:130–41. doi:10.1016/j.clim.2010.11.010
24. Exley MA, Nakayama T. NKT-cell-based immunotherapies in clinical trials. *Clin Immunol* (2011) 140:117–8. doi:10.1016/j.clim.2011.04.015
25. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, et al. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol* (2001) 167:4046–50. doi:10.4049/jimmunol.167.7.4046
26. Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. *J Immunol* (2002) 168:6494–9. doi:10.4049/jimmunol.168.12.6494
27. Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S-I, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J Exp Med* (2003) 197:1667–76. doi:10.1084/jem.20021650
28. Fujii S, Shimizu K, Klimek V, Geller MD, Nimer SD, Dhodapkar MV. Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br J Haematol* (2003) 122:617–22. doi:10.1046/j.1365-2141.2003.04465.x
29. Molling JW, Kölgen W, van der Vliet HJJ, Boomsma MF, Kruizenga H, Smorenburg CH, et al. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *Int J Cancer* (2005) 116:87–93. doi:10.1002/ijc.20998
30. Molling JW, Langius JAE, Langendijk JA, Leemans CR, Bontkes HJ, van der Vliet HJJ, et al. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. *J Clin Oncol* (2007) 25:862–8. doi:10.1200/JCO.2006.08.5787
31. Konishi J, Yamazaki K, Yokouchi H, Shinagawa N, Iwabuchi K, Nishimura M. The characteristics of human NKT cells in lung cancer – CD1d independent cytotoxicity against lung cancer cells by NKT cells and decreased human NKT cell response in lung cancer patients. *Hum Immunol* (2004) 65:1377–88. doi:10.1016/j.humimm.2004.09.003
32. Gorini F, Azzimonti L, Delfanti G, Scarfò L, Scielzo C, Bertilaccio MT, et al. Invariant NKT cells contribute to chronic lymphocytic leukemia surveillance and prognosis. *Blood* (2017) 129:3440–51. doi:10.1182/blood-2016-11-751065
33. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* (2005) 201:1503–17. doi:10.1084/jem.20042592
34. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* (2006) 12:6079–86. doi:10.1158/1078-0432.CCR-06-0114
35. Kunii N, Horiguchi S, Motohashi S, Yamamoto H, Ueno N, Yamamoto S, et al. Combination therapy of in vitro-expanded natural killer T cells and alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* (2009) 100:1092–8. doi:10.1111/j.1349-7006.2009.01135.x
36. Yamasaki K, Horiguchi S, Kurosaki M, Kunii N, Nagato K, Hanaoka H, et al. Induction of NKT cell-specific immune responses in cancer tissues after NKT cell-targeted adoptive immunotherapy. *Clin Immunol* (2011) 138:255–65. doi:10.1016/j.clim.2010.11.014
37. de Lalla C, Rinaldi A, Montagna D, Azzimonti L, Bernardo ME, Sangalli LM, et al. Invariant NKT cell reconstitution in pediatric leukemia patients given HLA-haploidentical stem cell transplantation defines distinct CD4+ and CD4- subset dynamics and correlates with remission state. *J Immunol* (2011) 186:4490–9. doi:10.4049/jimmunol.1003748
38. Casorati G, de Lalla C, Dellabona P. Invariant natural killer T cells reconstitution and the control of leukemia relapse in pediatric haploidentical hematopoietic stem cell transplantation. *Oncoimmunology* (2012) 1:355–7. doi:10.4161/onci.18399
39. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue SC, Sasada T, et al. Adoptive transfer of invariant NKT cells as immunotherapy for advanced melanoma: a phase 1 clinical trial. *Clin Cancer Res* (2017) 25:3510–19. doi:10.1158/1078-0432.CCR-16-0600
40. Heczey A, Liu D, Tian G, Courtney AN, Wei J, Marinova E, et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* (2014) 124:2824–33. doi:10.1182/blood-2013-11-541235
41. Tian G, Courtney AN, Jena B, Heczey A, Liu D, Marinova E, et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. *J Clin Invest* (2016) 126:2341–55. doi:10.1172/JCI83476
42. Kitayama S, Zhang R, Liu T-Y, Ueda N, Iriguchi S, Yasui Y, et al. Cellular adjuvant properties, direct cytotoxicity of re-differentiated Vα24 invariant NKT-like cells from human induced pluripotent stem cells. *Stem Cell Reports* (2016) 6:213–27. doi:10.1016/j.stemcr.2016.01.005
43. Yamada D, Iyoda T, Vizcardo R, Shimizu K, Sato Y, Endo TA, et al. Efficient regeneration of human Vα24(+) invariant natural killer T cells and their anti-tumor activity in vivo. *Stem Cells* (2016) 34:2852–60. doi:10.1002/stem.2465
44. Nishimura T, Kitamura H, Iwakabe K, Yahata T, Ohta A, Sato M, et al. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol* (2000) 12:987–94. doi:10.1093/intimm/12.7.987
45. Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, et al. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* (2004) 103:383–9. doi:10.1182/blood-2003-04-1155
46. Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, et al. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* (2005) 11:1910–7. doi:10.1158/1078-0432.CCR-04-1453
47. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* (2009) 182:2492–501. doi:10.4049/jimmunol.0800126
48. Nicol AJ, Tazbirkova A, Nieda M. Comparison of clinical and immunological effects of intravenous and intradermal administration of α-galactosylceramide (KRN7000)-pulsed dendritic cells. *Clin Cancer Res* (2011) 17:5140–51. doi:10.1158/1078-0432.CCR-10-3105
49. Nagato K, Motohashi S, Ishibashi F, Okita K, Yamasaki K, Moriya Y, et al. Accumulation of activated invariant natural killer T cells in the tumor microenvironment after α-galactosylceramide-pulsed antigen presenting cells. *J Clin Immunol* (2012) 32:1071–81. doi:10.1007/s10875-012-9697-9
50. Dellabona P, Casorati G, de Lalla C, Montagna D, Locatelli F. On the use of donor-derived iNKT cells for adoptive immunotherapy to prevent leukemia recurrence in pediatric recipients of HLA haploidentical HSCT for hematological malignancies. *Clin Immunol* (2011) 140:152–9. doi:10.1016/j.clim.2010.11.015

51. Fujii S, Goto A, Shimizu K. Antigen mRNA-transfected, allogeneic fibroblasts loaded with NKT-cell ligand confer antitumor immunity. *Blood* (2009) 113:4262–72. doi:10.1182/blood-2008-08-176446
52. Shimizu K, Asakura M, Shinga J, Sato Y, Kitahara S, Hoshino K, et al. Invariant NKT cells induce plasmacytoid dendritic cell (DC) cross-talk with conventional DCs for efficient memory CD8+ T cell induction. *J Immunol* (2013) 190:5609–19. doi:10.4049/jimmunol.1300033
53. Fujii S-I, Shimizu K. Immunotherapy with artificial adjuvant vector cells: harnessing both arms of the immune response. *Oncoimmunology* (2013) 2:e23432. doi:10.4161/onci.23432
54. Shimizu K, Yamasaki S, Shinga J, Sato Y, Watanabe T, Ohara O, et al. Systemic DC activation modulates the tumor microenvironment and shapes the long-lived tumor-specific memory mediated by CD8+ T cells. *Cancer Res* (2016) 76:3756–66. doi:10.1158/0008-5472.CAN-15-3219
55. Shimizu K, Mizuno T, Shinga J, Asakura M, Kakimi K, Ishii Y, et al. Vaccination with antigen-transfected, NKT cell ligand-loaded, human cells elicits robust in situ immune responses by dendritic cells. *Cancer Res* (2013) 73:62–73. doi:10.1158/0008-5472.CAN-12-0759
56. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S-I. Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *J Exp Med* (2007) 204:2641–53. doi:10.1084/jem.20070458
57. Shimizu K, Goto A, Fukui M, Taniguchi M, Fujii S. Tumor cells loaded with alpha-galactosylceramide induce innate NKT and NK cell-dependent resistance to tumor implantation in mice. *J Immunol* (2007) 178:2853–61. doi:10.4049/jimmunol.178.5.2853
58. Chung Y, Qin H, Kang C-Y, Kim S, Kwak LW, Dong C. An NKT-mediated autologous vaccine generates CD4 T-cell dependent potent antilymphoma immunity. *Blood* (2007) 110:2013–9. doi:10.1182/blood-2006-12-061309
59. Choi YS, Hoory T, Monie A, Wu A, Connolly D, Hung C-F. alpha-Galactosylceramide enhances the protective and therapeutic effects of tumor cell based vaccines for ovarian tumors. *Vaccine* (2008) 26:5855–63. doi:10.1016/j.vaccine.2008.08.027
60. Mattarollo SR, West AC, Steegh K, Duret H, Paget C, Martin B, et al. NKT cell adjuvant-based tumor vaccine for treatment of myc oncogene-driven mouse B-cell lymphoma. *Blood* (2012) 120:3019–29. doi:10.1182/blood-2012-04-426643
61. Hunn MK, Farrand KJ, Broadley KWR, Weinkove R, Ferguson P, Miller RJ, et al. Vaccination with irradiated tumor cells pulsed with an adjuvant that stimulates NKT cells is an effective treatment for glioma. *Clin Cancer Res* (2012) 18:6446–59. doi:10.1158/1078-0432.CCR-12-0704
62. Mattarollo SR, Steegh K, Li M, Duret H, Foong Ngiow S, Smyth MJ. Transient Foxp3(+) regulatory T-cell depletion enhances therapeutic anticancer vaccination targeting the immune-stimulatory properties of NKT cells. *Immunol Cell Biol* (2013) 91:105–14. doi:10.1038/icb.2012.58
63. Hong S, Lee H, Jung K, Lee SM, Lee S-J, Jun HJ, et al. Tumor cells loaded with α-galactosylceramide promote therapeutic NKT-dependent anti-tumor immunity in multiple myeloma. *Immunol Lett* (2013) 156:132–9. doi:10.1016/j.imlet.2013.10.002
64. Hunn MK, Hermans IF. Exploiting invariant NKT cells to promote T-cell responses to cancer vaccines. *Oncoimmunology* (2013) 2:e23789. doi:10.4161/onci.23789
65. Gibbins JD, Ancelet LR, Weinkove R, Compton BJ, Painter GF, Petersen TR, et al. An autologous leukemia cell vaccine prevents murine acute leukemia relapse after cytarabine treatment. *Blood* (2014) 124:2953–63. doi:10.1182/blood-2014-04-568956
66. Dong T, Yi T, Yang M, Lin S, Li W, Xu X, et al. Co-operation of α-galactosylceramide-loaded tumour cells and TLR9 agonists induce potent anti-tumour responses in a murine colon cancer model. *Biochem J* (2016) 473:7–19. doi:10.1042/BJ20150129
67. Hermans IF, Silk JD, Gileadi U, Masri SH, Shepherd D, Farrand KJ, et al. Dendritic cell function can be modulated through cooperative actions of TLR ligands and invariant NKT cells. *J Immunol* (2007) 178:2721–9. doi:10.4049/jimmunol.178.5.2721
68. Ko H-J, Kim Y-J, Kim Y-S, Chang W-S, Ko S-Y, Chang S-Y, et al. A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model. *Cancer Res* (2007) 67:7477–86. doi:10.1158/0008-5472.CAN-06-4639
69. Fukushima S, Hirata S, Motomura Y, Fukuma D, Matsunaga Y, Ikuta Y, et al. Multiple antigen-targeted immunotherapy with alpha-galactosylceramide-loaded and genetically engineered dendritic cells derived from embryonic stem cells. *J Immunother* (2009) 32:219–31. doi:10.1097/CJI.0b013e318194b63b
70. Matsuyoshi H, Hirata S, Yoshitake Y, Motomura Y, Fukuma D, Kurisaki A, et al. Therapeutic effect of alpha-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells. *Cancer Sci* (2005) 96:889–96. doi:10.1111/j.1349-7006.2005.00123.x
71. Zeng J, Shahbazi M, Wu C, Toh HC, Wang S. Enhancing immunostimulatory function of human embryonic stem cell-derived dendritic cells by CD1d overexpression. *J Immunol* (2012) 188:4297–304. doi:10.4049/jimmunol.1102343
72. Craparo EF, Bondì ML. Application of polymeric nanoparticles in immunotherapy. *Curr Opin Allergy Clin Immunol* (2012) 12:658–64. doi:10.1097/ACI.0b013e3283588c57
73. Smith DM, Simon JK, Baker JR. Applications of nanotechnology for immunology. *Nat Rev Immunol* (2013) 13:592–605. doi:10.1038/nri3488
74. Serda RE. Particle platforms for cancer immunotherapy. *Int J Nanomedicine* (2013) 8:1683–96. doi:10.2147/IJN.S31756
75. Cruz LJ, Tacken PJ, Fokkink R, Joosten B, Stuart MC, Albericio F, et al. Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN in vitro. *J Control Release* (2010) 144:118–26. doi:10.1016/j.jconrel.2010.02.013
76. Ghotbi Z, Haddadi A, Hamdy S, Hung RW, Samuel J, Lavasanifar A. Active targeting of dendritic cells with mannan-decorated PLGA nanoparticles. *J Drug Target* (2011) 19:281–92. doi:10.3109/1061186X.2010.499463
77. Waeckerle-Men Y, Groettrup M. PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines. *Adv Drug Deliv Rev* (2005) 57:475–82. doi:10.1016/j.addr.2004.09.007
78. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* (2010) 10:787–96. doi:10.1038/nri2868
79. Park Y-M, Lee SJ, Kim YS, Lee MH, Cha GS, Jung ID, et al. Nanoparticle-based vaccine delivery for cancer immunotherapy. *Immune Netw* (2013) 13:177. doi:10.4110/in.2013.13.5.177
80. Alyautdin R, Khalin I, Nafeeza MI, Haron MH, Kuznetsov D. Nanoscale drug delivery systems and the blood-brain barrier. *Int J Nanomedicine* (2014) 9:795–811. doi:10.2147/IJN.S52236
81. Fahmy TM, Demento SL, Caplan MJ, Mellman I, Saltzman WM. Design opportunities for actively targeted nanoparticle vaccines. *Nanomedicine (Lond)* (2008) 3:343–55. doi:10.2217/17435889.3.3.343
82. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. *J Control Release* (2008) 125:193–209. doi:10.1016/j.jconrel.2007.09.013
83. Lü J-M, Wang X, Marin-Muller C, Wang H, Lin PH, Yao Q, et al. Current advances in research and clinical applications of PLGA-based nanotechnology. *Expert Rev Mol Diagn* (2009) 9:325–41. doi:10.1586/erm.09.15
84. Lee Y-R, Lee Y-H, Im S-A, Kim K, Lee C-K. Formulation and characterization of antigen-loaded PLGA nanoparticles for efficient cross-priming of the antigen. *Immune Netw* (2011) 11:163–8. doi:10.4110/in.2011.11.3.163
85. Silva AL, Soema PC, Slütter B, Ossendorp F, Jiskoot W. PLGA particulate delivery systems for subunit vaccines: linking particle properties to immunogenicity. *Hum Vaccin Immunother* (2016) 12:1056–69. doi:10.1080/21645515.2015.1117714
86. Barral P, Polzella P, Bruckbauer A, van Rooijen N, Besra GS, Cerundolo V, et al. CD169(+) macrophages present lipid antigens to mediate early activation of iNKT cells in lymph nodes. *Nat Immunol* (2010) 11:303–12. doi:10.1038/ni.1853
87. Barral P, Sánchez-Niño MD, van Rooijen N, Cerundolo V, Batista FD. The location of splenic NKT cells favours their rapid activation by blood-borne antigen. *EMBO J* (2012) 31:2378–90. doi:10.1038/emboj.2012.87
88. Thapa P, Zhang G, Xia C, Gelbard A, Overwijk WW, Liu C, et al. Nanoparticle formulated alpha-galactosylceramide activates NKT cells without inducing anergy. *Vaccine* (2009) 27:3484–8. doi:10.1016/j.vaccine.2009.01.047
89. Macho Fernandez E, Chang J, Fontaine J, Bialecki E, Rodriguez F, Werkmeister E, et al. Activation of invariant Natural Killer T lymphocytes

in response to the α-galactosylceramide analogue KRN7000 encapsulated in PLGA-based nanoparticles and microparticles. *Int J Pharm* (2012) 423:45–54. doi:10.1016/j.ijpharm.2011.04.068
90. Nakamura T, Yamazaki D, Yamauchi J, Harashima H. The nanoparticulation by octaarginine-modified liposome improves α-galactosylceramide-mediated antitumor therapy via systemic administration. *J Control Release* (2013) 171:216–24. doi:10.1016/j.jconrel.2013.07.004
91. Ishii M, Kojima N. Effective stimulation of invariant natural killer T cells by oligomannose-coated liposomes. *Int Immunopharmacol* (2013) 15:685–92. doi:10.1016/j.intimp.2013.03.009
92. Kawasaki N, Vela JL, Nycholat CM, Rademacher C, Khurana A, van Rooijen N, et al. Targeted delivery of lipid antigen to macrophages via the CD169/sialoadhesin endocytic pathway induces robust invariant natural killer T cell activation. *Proc Natl Acad Sci U S A* (2013) 110:7826–31. doi:10.1073/pnas.1219888110
93. Macho-Fernandez E, Cruz LJ, Ghinnagow R, Fontaine J, Bialecki E, Frisch B, et al. Targeted delivery of α-galactosylceramide to CD8α+ dendritic cells optimizes type I NKT cell-based antitumor responses. *J Immunol* (2014) 193:961–9. doi:10.4049/jimmunol.1303029
94. McKee SJ, Young VL, Clow F, Hayman CM, Baird MA, Hermans IF, et al. Virus-like particles and α-galactosylceramide form a self-adjuvanting composite particle that elicits anti-tumor responses. *J Control Release* (2012) 159:338–45. doi:10.1016/j.jconrel.2012.02.015
95. Dölen Y, Kreutz M, Gileadi U, Tel J, Vasaturo A, van Dinther EAW, et al. Co-delivery of PLGA encapsulated invariant NKT cell agonist with antigenic protein induce strong T cell-mediated antitumor immune responses. *Oncoimmunology* (2015) 5:e1068493. doi:10.1080/2162402X.2015.1068493
96. Li B, Siuta M, Bright V, Koktysh D, Matlock BK, Dumas ME, et al. Improved proliferation of antigen-specific cytolytic T lymphocytes using a multimodal nanovaccine. *Int J Nanomedicine* (2016) 11:6103–21. doi:10.2147/IJN.S112432
97. Neumann S, Young K, Compton B, Anderson R, Painter G, Hook S. Synthetic TRP2 long-peptide and α-galactosylceramide formulated into cationic liposomes elicit CD8+ T-cell responses and prevent tumour progression. *Vaccine* (2015) 33:5838–44. doi:10.1016/j.vaccine.2015.08.083
98. Crozat K, Guiton R, Contreras V, Feuillet V, Dutertre C-A, Ventre E, et al. The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. *J Exp Med* (2010) 207:1283–92. doi:10.1084/jem.20100223
99. Bachem A, Güttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, et al. Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J Exp Med* (2010) 207:1273–81. doi:10.1084/jem.20100348
100. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen J-L, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J Exp Med* (2010) 207:1261–71. doi:10.1084/jem.20092618
101. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J Exp Med* (2010) 207:1247–60. doi:10.1084/jem.20092140
102. Arora P, Baena A, Yu KOA, Saini NK, Kharkwal SS, Goldberg MF, et al. A single subset of dendritic cells controls the cytokine bias of natural killer T cell responses to diverse glycolipid antigens. *Immunity* (2014) 40:105–16. doi:10.1016/j.immuni.2013.12.004
103. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, et al. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. *Nat Immunol* (2010) 11:313–20. doi:10.1038/ni.1848
104. Sancho D, Mourão-Sá D, Joffre OP, Schulz O, Rogers NC, Pennington DJ, et al. Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest* (2008) 118:2098–110. doi:10.1172/JCI34584
105. Picco G, Beatson R, Taylor-Papadimitriou J, Burchell JM. Targeting DNGR-1 (CLEC9A) with antibody/MUC1 peptide conjugates as a vaccine for carcinomas. *Eur J Immunol* (2014) 44:1947–55. doi:10.1002/eji.201344076
106. Tullett KM, Lahoud MH, Radford KJ. Harnessing human cross-presenting CLEC9A(+)XCR1(+) dendritic cells for immunotherapy. *Front Immunol* (2014) 5:239. doi:10.3389/fimmu.2014.00239
107. Ghinnagow R, De Meester J, Cruz LJ, Aspord C, Corgnac S, Macho-Fernandez E, et al. Co-delivery of the NKT agonist α-galactosylceramide and tumor antigens to cross-priming dendritic cells breaks tolerance to self-antigens and promotes antitumor responses. *Oncoimmunology* (in press).
108. Klebanoff CA, Acquavella N, Yu Z, Restifo NP. Therapeutic cancer vaccines: are we there yet? *Immunol Rev* (2011) 239:27–44. doi:10.1111/j.1600-065X.2010.00979.x
109. Andersen BM, Ohlfest JR. Increasing the efficacy of tumor cell vaccines by enhancing cross priming. *Cancer Lett* (2012) 325:155–64. doi:10.1016/j.canlet.2012.07.012
110. Palucka K, Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. *Immunity* (2013) 39:38–48. doi:10.1016/j.immuni.2013.07.004
111. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* (2013) 39:1–10. doi:10.1016/j.immuni.2013.07.012
112. Pol J, Bloy N, Buqué A, Eggermont A, Cremer I, Sautès-Fridman C, et al. Trial watch: peptide-based anticancer vaccines. *Oncoimmunology* (2015) 4:e974411. doi:10.4161/2162402X.2014.974411
113. Coelho-Dos-Reis JG, Huang J, Tsao T, Pereira FV, Funakoshi R, Nakajima H, et al. Co-administration of α-GalCer analog and TLR4 agonist induces robust CD8(+) T-cell responses to PyCS protein and WT-1 antigen and activates memory-like effector NKT cells. *Clin Immunol* (2016) 168:6–15. doi:10.1016/j.clim.2016.04.014

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MINI REVIEW
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Exploiting Antitumor Immunotherapeutic Novel Strategies by Deciphering the Cross Talk between Invariant NKT Cells and Dendritic Cells

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Immune checkpoint blockade therapy has prevailed for several types of cancer; however, its effectiveness as a single therapy is still limited. In principle, dendritic cells (DCs) should be able to control the post-therapy immune response, in particular since they can link the two major arms of the immune system: innate and adaptive immunity. Therefore, DCs would be a logical and ideal target for the development of immunotherapies. Since DCs are not activated in the steady state, an adjuvant to convert their function from tolerogenic to immunogenic would be desirable. Upon ligand activation, invariant natural killer T (iNKT) cells simultaneously activate NK cells and also energize the DCs, resulting in their full maturation. To utilize such iNKT-licensed "fully" matured DCs as adjuvants, mechanisms of both intercellular communication between DC subsets and iNKT cells and intracellular molecular signaling in DCs have to be clarified and optimized. To generate both innate and adaptive immunity against cancer, a variety of strategies with the potential to target iNKT-licensed DCs *in situ* have been studied. The benchmark of success in these studies, each with distinct approaches, will be the development of functional NK cells and cytotoxic T cells (CTLs) as well as generation of long-term, memory CTL. In this review, we provide a framework for NKT-mediated immunotherapy through selective DC targeting *in situ*, describe progress in the design of licensed therapies for iNKT cell targeting of DCs, and highlight the challenge to provide maximal benefit to patients.

Keywords: dendritic cells, invariant NKT cells, innate immunity, adaptive immunity, cross presentation, immunotherapy

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INTRODUCTION

Invariant natural killer T (iNKT) cells have several distinguishing characteristics, most notably they express an invariant TCRα chain, Vα14Jα18 in mice and Vα24Jα18 in human, paired with a TCRβ chain of limited diversity (1, 2). Unlike most αβTCRs, which recognize peptide MHC I/II complexes, iNKT TCRs recognize glycolipid antigens presented by the MHC class I-like molecule CD1d (3–5). The first NKT glycolipid ligand was identified by Kirin Pharmaceuticals (6). They extracted agelasphins as glycosphingolipid compounds from a marine sponge called *Agelas mauritianus*. They modified the structure of this compound and established a synthetic ligand with a branched galactosylceramide,

commonly referred to as α-GalCer (7, 8). In addition to α-GalCer, iNKT cells recognize certain microbial ligands, for example cell wall sphingolipids from *Sphingomonas*, *Borrelia*, or *Streptococcus* (9–11). iNKT cells recognize such natural or synthetic glycolipids and promptly produce a broad range of cytokines. iNKT cells are not only stimulated by these glycolipid ligands directly *via* their invariant TCR but also indirectly. Since iNKT cells express IL-12 receptors, they can be stimulated by IL-12 released from dendritic cells (DCs) or macrophages. For example, *Salmonella typhimurium* does not express a glycolipid ligand, but can stimulate iNKT cells *in vivo*. In this case, the iNKT cells can be activated by both IL-12 and recognition of endogenous glycosphingolipid ligand on DCs (1, 12).

In the course of establishment or recurrence of tumor cells, genetic factors or immune related pressure may mediate the selective outgrowth of tumor cell clones lacking MHC or potentially immunogenic tumor associated antigens (TAA), thus leading to heterogeneous tumor cell evolution (13). In terms of cancer immunity, tumors are in general composed of two types of cells, some are MHC positive, but others are MHC negative. Based on our current understanding, the former can be eliminated by cytotoxic T cells (CTLs) and the latter can be eliminated by NK cells, thus both innate and adaptive immune responses are required for complete elimination of tumors. It is well-known that DCs can play a crucial role in activating both innate and adaptive immune responses (14–16). We and others demonstrated that iNKT cells, as well as many toll-like receptor (TLR) ligands, can be used for activation of DCs to bridge innate and adaptive immunity (17–20). In this review, we detail the rationale for modulation or optimization of iNKT cell-licensed antigen-expressing DCs and also describe various attempts that have so far been made for developing antitumor therapeutic strategies.

NKT CELL SUBSETS—LOCALIZATION AND FUNCTION

When TCRs on iNKT cells are stimulated by a ligand such as α-GalCer, they are capable of producing IFN-γ and IL-4. These Vα14$^+$iNKT cells were recently shown to belong to three distinct functional subsets. The NKT1, NKT2, and NKT17 cells all express promyelocytic leukemia zinc finger, but can be distinguished because they are mainly regulated by transcription factors similar to those of helper T cells, i.e., T-bet, GATA-3, and RORγt, respectively (21, 22). Development of each type of iNKT cell is generally related to the cytokine milieu encountered upon activation (IFN-γ, IL-4, or IL-17). An intravenous injection of α-GalCer rapidly activates NKT1 cells in the red pulp of the spleen and liver, thus leading to systemic IFN-γ and IL-4 responses, but not iNKT cells in LN and thymus (23, 24). NKT2 cells are mainly located in the medullary area of the thymus and T cell zone of the spleen, as well as in mesenteric LNs. Oral administration of α-GalCer may induce the activation of NKT2 cells in mLN, resulting in local IL-4 production (23, 24). NKT17 cells which are capable of producing IL-17, but not IFN-γ, are particularly enriched in the lung and the subcapsular region of LNs (23, 24). Thus, the pattern and amount of cytokine production can be determined by the location of NKT cells, NKT cell type and routes of administration of NKT ligands.

INDUCTION OF NK CELL RESPONSES AS AN ADJUNCTIVE EFFECT OF iNKT CELL THERAPY

The critical role of iNKT cells in tumor immunosurveillance was shown in chemically induced spontaneous tumor models (25). The transfer of iNKT cells prevented the induction of methylcholanthrene sarcoma tumors in Jα18$^{-/-}$, iNKT cell-deficient mice. A similar protocol involving transfer of iNKT cells had demonstrated a significant antitumor effect in p53-deficient mice (26) and in the transgenic adenocarcinoma of the mouse prostate tumor model (27).

Our first approach to understanding iNKT cell immunotherapy was to compare cell therapy (e.g., administration of CD1d$^+$ cells loaded with α-GalCer) versus unbound glycolipid drug therapy (e.g., administration of soluble α-GalCer) (28). An injection of bone marrow-derived *ex vivo* DCs loaded with α-GalCer (BM-DC/Gal) induced iNKT cells capable of producing IFN-γ (28) (**Figure 1**), and this correlated with antitumor effects in B16 melanoma lung metastasis. In contrast, the iNKT cell response to unbound α-GalCer was more rapid, but transient and then the cells became anergic (28, 29). Thus, the glycolipid has different functional effects on iNKT cells when it is injected as a free glycolipid or in association with CD1d$^+$ cells. When activated by the iNKT cell ligand, IFN-γ and IL-2 production by iNKT cells enhances the activation of NK cells as iNKT–NK axis (30) (**Figure 2**). The interaction between iNKT cells and DCs can also enhance NK cell activity. After activation by NKT cells, DCs express NKG2D ligands and CD70, thus leading to the activation of NK cells (31). In addition, since NK cells also express IL-12R, IL-12 released from DCs enhances NK cell-mediated IFN-γ production (**Figure 2**). Thus, iNKT cells efficiently stimulate NK cells. The near synchronous activation of these iNKT and NK cell can account for innate resistance to susceptible tumors.

EFFICIENT INDUCTION OF ANTITUMOR CTLs BY iNKT CELL-LICENSED DCs

In situ DCs activated by iNKT cells act as a cellular adjuvant for T-cell priming. The licensing of DCs by iNKT cells occurs by several molecular mechanisms. When activated iNKT cells encounter DCs *in situ*, co-stimulatory molecules on DCs are upregulated, indicative of DC maturation (32, 33). Activated iNKT cells can promote conversion of DCs from a tolerogenic to an immunogenic state. The DC surface remodeling of these mature DCs is driven by two inflammatory cytokines, TNF-α and IFN-γ. Cytokine production by the innate lymphocytes is tightly regulated by the interactions among unique cell types. TNF-α is produced by endogenous DCs, whereas IFN-γ is produced by iNKT or NK cells (**Figure 2**). It was previously reported that IFN-γ-producing DCs are important for priming of the gut intraepithelial lymphocyte response against intracellular parasitic infection (34). We also tested the possibility of IFN-γ production

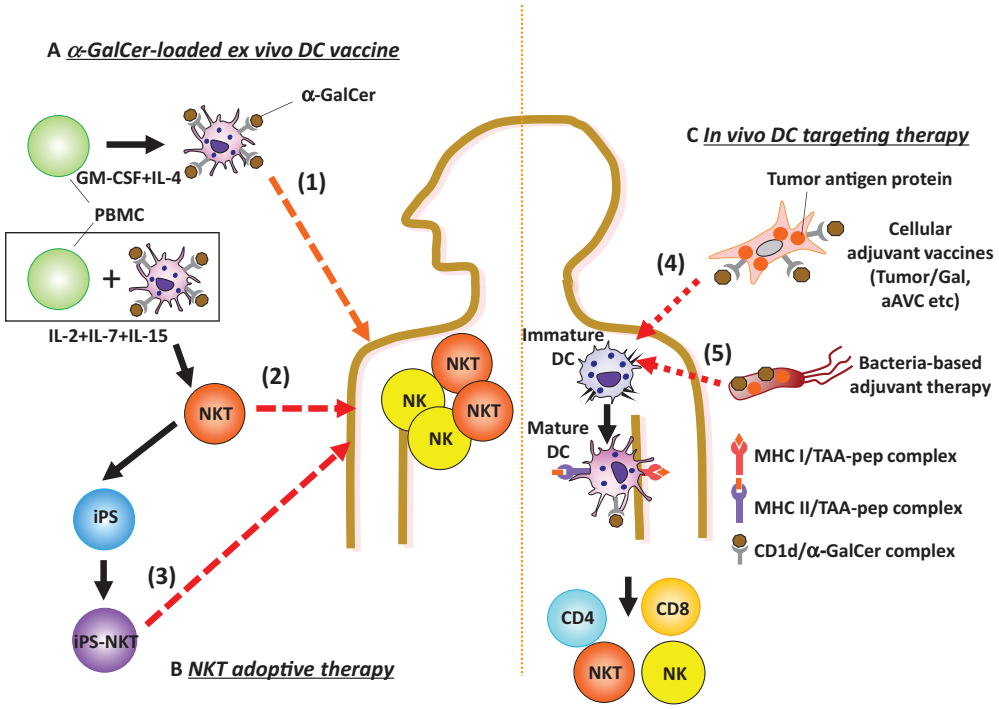

FIGURE 1 | *Ex vivo* or *in vivo* glycolipid-based dendritic cell (DC) immunotherapy. **(A,B)** *Ex vivo* glycolipid-based DC therapy and NKT transfer therapy have been studied. **(A)** (1) Active immunization with *ex vivo* DCs: monocyte-derived DCs loaded with α-GalCer (DCs/Gal) or autologous PBMCs pulsed with α-GalCer are administered intravenously to cancer patients. The invariant natural killer T (iNKT) and NK cells are promptly activated in lung, liver, and spleen. **(B)** As passive immunization, effector cells are adoptively transferred. (2) For this approach, *ex vivo* iNKT cells are harvested after coculturing with autologous DC/Gal and then injected into cancer patients. (3) In the future, iPS-reprogrammed iNKT cells may be applicable for adoptive transfer therapy. **(C)** As new strategies of *in vivo* DC targeting therapies, (4) adjuvant vector cells, including tumor cells loaded with α-GalCer (Tumor/Gal) or tumor antigen mRNA-transfected, allogeneic CD1d$^+$ cells loaded with α-GalCer (aAVC) or (5) non-somatic cell adjuvant (bacteria) will be candidates for the iNKT-triggered immunotherapy. When these agents are injected, both iNKT and NK cells will be activated. Host DCs can then prime antigen-specific CD4$^+$ and/or CD8$^+$ T cells.

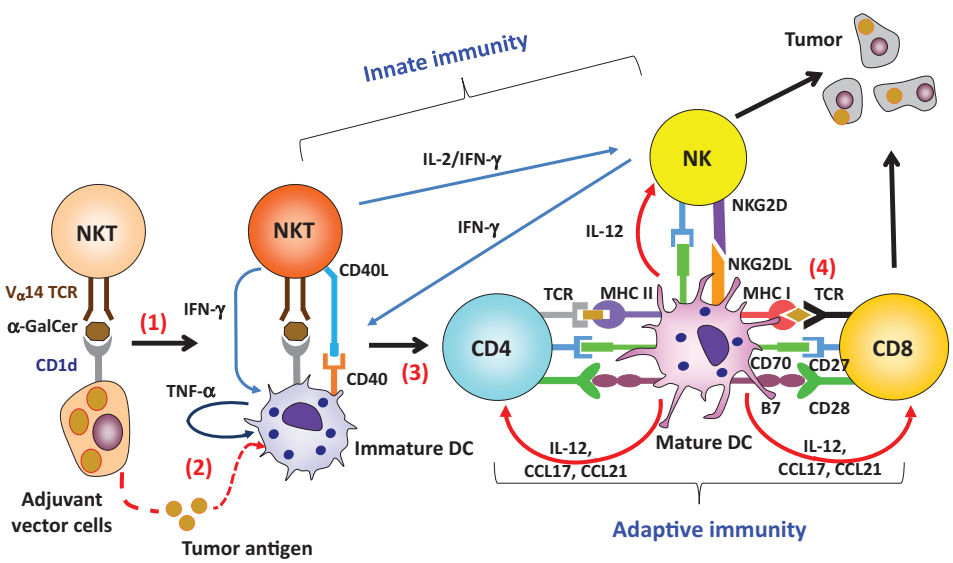

FIGURE 2 | Adjuvant effect by invariant natural killer T (iNKT) cell-triggered dendritic cells (DCs) on protective antitumor responses. (1) Administration of adjuvant vector cells, including Tumor/Gal or aAVC initially stimulate iNKT cells. (2) The adjuvant vector cells are killed by iNKT cells and NK cells, and then tumor antigen released from them can be captured by endogenous CD11c$^+$DCs. (3) The CD11c$^+$ DCs then undergo iNKT cell-induced maturation. (4) The activated DCs can then induce an antigen-specific T cell response in the lymphoid tissues. Thus, the CD11c$^+$DCs *in situ* are able to cross present tumor antigen, derived from phagocytosed adjuvant vector cells, to CD4$^+$ or CD8$^+$ T cells in an MHC-dependent manner.

by CD11c$^+$DCs after administration of α-GalCer and found that isolated CD11c$^+$cells produce IFN-γ, but that these were CD11c$^+$NK cells and not any DC subset (35).

Co-stimulatory molecules, CD40, CD80, and CD86 on DCs, which are important for priming T cells, are upregulated during the early phase (from 4 h). In addition, we and others recently demonstrated upregulation of CD70, 4-1-BBL, and IL-15Ra on DCs, which are important for generation of memory T cells, at the late phase (from 40 h) (36, 37).

Other innate lymphocytes, such as γδT cells (38, 39) and NK cells (40, 41) may also have adjuvant effects. They produce IFN-γ and TNF-α, thus promoting the maturation of DCs and help for the generation of CTLs. We also compared the magnitude of T cell responses after priming with the iNKT cell ligand α-GalCer versus NK cell ligands, such as retinoic acid early inducible-1ε (Rae1ε), Rae1γ, CD70 and murine UL16-binding protein-like transcript 1 (Mult-1). CTL induction triggered by iNKT cells is apparently more powerful than that triggering by NK cells (42).

An important difference between these other innate lymphocytes and iNKT cells lies in the CD40L signal to DCs. Bennett et al. reported that CD40L on helper T cells plays an crucial role in licensing DCs (43). Activated iNKT cells express CD40L transiently (44), but other lymphocytes do not. In fact, the adjuvant effect of DCs triggered by iNKT cells is eliminated when CD40$^{-/-}$ mice are used as recipients (44), even though the iNKT cell response is still present. In a reciprocal study, co-administration of antigen plus soluble TNF-α and soluble IFN-γ induces the phenotypic maturation of DCs *in situ*, but does not generate antigen-specific T cells. Thus, phenotypic maturation of DCs does not always correspond to an antigen-specific T cell response, whereas functional maturation of DCs does. CD40–CD40L interaction during DC-iNKT cell cross talk is critical for DC maturation, resulting in IL-12 production (**Figure 2**), whereas inflammatory cytokines (TNF-α and IFN-γ) serve as co-factors for full maturation of DCs. In addition, iNKT cell-licensed DCs apparently use a different mechanism rather than that used during TLR signaling. The adjuvant effect of TLR ligands depends on either MyD88 or TRIF (45), or both, but that by iNKT-licensed DCs does not (32). As discussed above, NKT licensed-DCs depend on CD40/CD40L signaling and inflammatory signals (TNF-α and IFN-γ). CD40/CD40L signaling may involve the TRAFs (TRAF1, 2, 3, 5, 6), whereas TNF-α and IFN-γ signaling may involve the TRAF2 and JAK1/2-STAT1 pathway respectively (46, 47).

The location of DCs and iNKT cells in spleen is another important factor in their mutual activation. After their activation, iNKT cells accumulate in the marginal zone, where they co-interact with DCs. After activation by iNKT cells, XCR1$^+$ DCs can traffic to the PALS area and then prime T cells (48). These responses are orchestrated by chemokines, cytokines, and cell surface molecules. iNKT cell-licensed DCs produce CCL17 (37, 49), which attracts CCR4$^+$ CD8$^+$ T cells for subsequent activation.

Several factors during the initiation of innate immunity determine the subsequent flavor of the adaptive immune response: (1) the number and function of iNKT cells and APCs, (2) the nature of the ligands (i.e., OCH, α-GalCer, or α-C-GalCer) (35), (3) the properties of host APCs (DC location or subset) (44), and (4) the level of CD1d expression (50). Thus, the magnitude of the innate immune response generated by all these factors can be directly correlated with the subsequent adaptive immune response.

DEVELOPMENT OF iNKT-TRIGGERED ANTITUMOR STRATEGIES LINKING INNATE AND ADAPTIVE THERAPY

Cellular Vaccines Acting As Immunological Adjuvant and Tumor Antigen Carrying Vector

We and others demonstrated that co-administration of antigen and iNKT ligand generates antigen-specific T cells, in addition to activating iNKT and NK cells (32, 33, 51). However, several optimal conditions are limited. It has been reported that DCs cannot phagocytize antigen after their maturation. Indeed, administration of tumor antigen 4 h after an administration of NKT cell ligand did not lead to a T cell response (32, 33). Therefore, tumor antigen and NKT cell ligands have to be delivered simultaneously to *in vivo* DCs (32, 33). In addition, we also found that this co-administration protocol generates CTL, but not memory T cells easily.

CD1d$^+$ cells loaded with α-GalCer can activate iNKT cells directly *in vitro*, but it was not known whether adaptive immunity was generated after initiating this innate immune response. We first showed conclusively that CD1d$^+$tumor/Gal can induce antigen-specific CD4$^+$ and CD8$^+$ T cell immunity (50) (**Figure 1**). From this finding, we proposed a strategy using all-in one cell type that expressed tumor antigen as well as CD1d that was loaded with the NKT cell ligand α-GalCer simultaneously, a cell that we reported an adjuvant vector cell (50, 52) (**Figure 1**). CD1d is generally expressed on most hematopoietic cells, e.g., DCs, B cells, T cells, and macrophages, and on some non-hematopoietic cells, e.g., intestinal epithelium and hepatocytes, including multiple tumor types (53). Therefore, approaches using adjuvant vector cells may be applicable not only for most hematological disorders, where they can be applied relatively easily, but also for many solid tumor cells, a more difficult therapeutic target. In fact, the therapeutic strategy using tumor/Gal has been extended to many types of CD1d$^+$ tumor cells, including in our own studies, e.g., B16 melanoma, EL4 thymoma, WEHI3B leukemia, and J558 plasmacytoma. In addition, Hunn et al. reported that irradiated Glioma/Gal was effective in a prophylactic setting and also in a therapeutic setting together with Treg depletion of intracranial glioma model (54). Kobayashi et al. have reported on an α-GalCer-loaded B cell lymphoma (Eμ-myc tumor) combined with an agonistic antibody targeting 4-1BB (CD137) (55). The studies as above demonstrated the antigen-specific effector T cell-mediated survival. These tumor/Gal vaccines would be useful in an autologous setting.

Instead of such syngeneic tumor/Gal therapy, we newly established the concept of an artificial adjuvant vector cell (aAVC) as a new type of cancer vaccine platform that incorporates *in vivo* iNKT-licensed DC therapy (**Figure 1**). These cells (aAVC), NIH3T3 cells for mouse and HEK293 for humans, have

been transfected with a CD1d and tumor antigen mRNA and then loaded with α-GalCer (37, 42, 56). The aAVC express the α-GalCer-CD1d complex on their surface and tumor antigen protein inside of the allogeneic cells. The aAVC treatment reduces the number of metastases, and eliminated grossly large tumors (37, 42, 56).

As the mechanism of adjuvant vector cells (tumor/Gal or aAVC), four immunological steps take place (**Figure 2**). Initially, these cells directly activate iNKT cells. iNKT cells producing IFN-γ can then simultaneously activate NK cells. These innate killer iNKT/NK cells capable of producing IFN- γ reject the adjuvant vector cells, but some of the killed adjuvant vector cells are taken up by DCs *in situ*, thereby several immunogenic features of DCs are engaged. The adjuvant vector cells-capturing DCs in lung, liver, and spleen become matured by their interaction with iNKT cells, resulting from CD40L–CD40 interactions and production of inflammatory cytokines. Next, the mature DCs present the TAAs to T cells on both MHC class I and II *in situ*. Particularly, the XCR1$^+$DCs are specialized to cross-present antigens on MHC class I. Notably, when mice were vaccinated with adjuvant vector cells, they became resistant to the parental tumor cells. In fact, administration of adjuvant vector cells induces CTL and long-term memory T cells efficiently *in vivo* (48, 50, 52).

Bacteria-Based Adjuvant Therapy

Listeria monocytogenes (LM) is a Gram-positive intracellular bacterium. Several groups have investigated whether recombinant LM lacking virulence genes, but expressing several TLR ligands such as lipoteichoic acid, would be useful for delivering TAA *in vivo* (57, 58). After infecting the target cells with LM, there was active phagocytosis and lysis of the bacteria in the phagosome. The recombinant LM allowed for the delivery of the TAA directly into macrophages and DCs, which can present TAA peptides to CD4$^+$ and CD8$^+$ T cells. In practice, a live attenuated, LM-based tumor vaccine expressing TAA-Mage-b (Mb) and α-GalCer has been studied (59) (**Figure 1**). The T cell-mediated antitumor efficacy resulting from direct incorporation of α-GalCer into live LM-Mb was found to be more powerful and safer than co-administration of the LM-Mb vaccine and α-GalCer, but the iNKT cell response was weaker.

Bacille Calmette–Guerin (BCG) was derived by attenuating *Mycobacterium bovis* and is widely used in many countries as a tuberculosis vaccine, although its efficacy has been contested. Recombinant BCG (rBCG) strains expressing either Listeriolysin-O from LM or perfringolysin from *Clostridium perfringens* have been investigated as candidate tumor vaccines (60). In simple rBCG-based vaccination models, skin CD11b^{high} DC subsets present antigen to CD4$^+$ T cells (61). Using an approach of incorporating glycolipids into rBCG strains, rBCG strains expressing an SIV Gag antigen (rBCG-SIV gag) together with α-GalCer enhanced CTL more efficiently compared to responses primed by simple rBCG-SIV gag (62) (**Figure 1**). Similar to the concept of adjuvant vector cells or aAVC, these two types of bacteria vaccine expressing antigen and NKT ligand showed CTL induction more efficiently than that of co-administration approach.

iNKT CELL TRANSFER IMMUNOTHERAPY

As the other option, complementation of iNKT cell therapy may be an approach for cancer patients with decreased iNKT cell frequencies in target organs. This type of iNKT cell-based immunotherapy may be able to take advantage of the recent iPS reprograming technology. We recently established a protocol to reprogram human Vα24$^+$iNKT cells and then to re-differentiate them into functional iNKT cells, so called iPS-iNKT cells (63). Similar to conventional human Vα24$^+$iNKT cells, iPS-iNKT cells can produce IFN-γ upon NKT ligand activation and kill several types of human tumor cell lines (leukemia, lung cancer, and head and neck cancers) *in vitro* and *in vivo*. As already discussed in this review, we demonstrated that NK cell activity but not T cell induction is induced after the iNKT cell activation, mainly through IFN-γ production as iNKT–NK axis. In fact, once activated *in vivo*, human iPS-iNKT cells have been shown, in "humanized" NOG mice with human peripheral blood cells, to mediate adjunctive activity by activating autologous NK cells (**Figure 1**). We, therefore, suggest that human iPS-Vα24$^+$iNKT cells could exert antitumor activity *in vivo*.

CONCLUSION

When iNKT cells are activated by the ligand, they subsequently have the power to activate NK cells by producing IFN-γ as iNKT–NK axis. In fact, several immunotherapies using autologous DC/Gal in clinical trials of solid tumor and hematological malignancies have indicated the importance of IFN-γ-producing iNKT cells and NK cell activation (**Figure 1**). Although iNKT cells can mature DCs *in vivo*, IFN-γ alone is not sufficient to DC maturation and T cell induction. Therefore, to further develop iNKT cell-mediated therapy, many groups have focused on the interaction between DC and iNKT cells. We summarized details of the mechanism of the interaction between these two cell types and also introduced several iNKT cell-triggered DC approaches that are candidates for potential new therapies for cancer treatment. Adjuvant vector cells, including Tumor/Gal or aAVC as well as non-somatic cell adjuvants (bacteria) are all candidates for iNKT-triggered DC mediated immunotherapy.

AUTHOR CONTRIBUTIONS

SF and KS conceptualized, wrote and edited the manuscript.

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REFERENCES

1. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13:101–17. doi:10.1038/nri3369
2. McEwen-Smith RM, Salio M, Cerundolo V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res* (2015) 3:425–35. doi:10.1158/2326-6066.CIR-15-0062
3. Bendelac A. CD1: presenting unusual antigens to unusual T lymphocytes. *Science* (1995) 269:185–6. doi:10.1126/science.7542402
4. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science* (1995) 268:863–5. doi:10.1126/science.7538697
5. Exley M, Garcia J, Balk SP, Porcelli S. Requirements for CD1d recognition by human invariant Valpha24+ CD4-CD8- T cells. *J Exp Med* (1997) 186:109–20. doi:10.1084/jem.186.1.109
6. Natori T, Morita M, Akimoto K, Koezuka Y. Agelasphins, novel antitumor and immunostimulatory cerebrosides from the sponge *Agelas mauritanus*. *Tetrahedron* (1994) 50:2771–84. doi:10.1016/S0040-4020(01)86991-X
7. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, et al. Structure-activity relationship of a-galactosylceramides against B16-bearing mice. *J Med Chem* (1995) 38:2176. doi:10.1021/jm00012a018
8. Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* (1995) 7:529–34.
9. Sriram V, Du W, Gervay-Hague J, Brutkiewicz RR. Cell wall glycosphingolipids of Sphingomonas paucimobilis are CD1d-specific ligands for NKT cells. *Eur J Immunol* (2005) 35:1692–701. doi:10.1002/eji.200526157
10. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* (2006) 7:978–86. doi:10.1038/ni1380
11. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol* (2011) 12:966–74. doi:10.1038/ni.2096
12. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* (2003) 4:1230–7. doi:10.1038/ni1002
13. McGranahan N, Swanton C. Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell* (2017) 168:613–28. doi:10.1016/j.cell.2017.01.018
14. Shimizu K, Fujii S. An adjuvant role of in situ dendritic cells (DCs) in linking innate and adaptive immunity. *Front Biosci* (2008) 13:6193–201. doi:10.2741/3147
15. Durai V, Murphy KM. Functions of murine dendritic cells. *Immunity* (2016) 45:719–36. doi:10.1016/j.immuni.2016.10.010
16. Corrales L, Matson V, Flood B, Spranger S, Gajewski TF. Innate immune signaling and regulation in cancer immunotherapy. *Cell Res* (2017) 27:96–108. doi:10.1038/cr.2016.149
17. Munz C, Steinman RM, Fujii S. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med* (2005) 202:203–7. doi:10.1084/jem.20050810
18. Fujii S, Shimizu K, Hemmi H, Steinman RM. Innate Va14$^+$ natural killer T cells mature dendritic cells, leading to strong adaptive immunity. *Immunol Rev* (2007) 220:183–98. doi:10.1111/j.1600-065X.2007.00561.x
19. Fujii S, Shimizu K, Okamoto Y, Kunii N, Nakayama T, Motohashi S, et al. NKT cells as an ideal anti-tumor immunotherapeutic. *Front Immunol* (2013) 4:409. doi:10.3389/fimmu.2013.00409
20. Faveeuw C, Trottein F. Optimization of natural killer T cell-mediated immunotherapy in cancer using cell-based and nanovector vaccines. *Cancer Res* (2014) 74:1632–8. doi:10.1158/0008-5472.CAN-13-3504
21. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol* (2013) 25:161–7. doi:10.1016/j.coi.2013.01.003
22. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol* (2016) 17:728–39. doi:10.1038/ni.3437
23. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity* (2015) 43:566–78. doi:10.1016/j.immuni.2015.06.025
24. Slauenwhite D, Johnston B. Regulation of NKT cell localization in homeostasis and infection. *Front Immunol* (2015) 6:255. doi:10.3389/fimmu.2015.00255
25. Crowe NY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med* (2002) 196:119–27. doi:10.1084/jem.20020092
26. Swann JB, Uldrich AP, van Dommelen S, Sharkey J, Murray WK, Godfrey DI, et al. Type I natural killer T cells suppress tumors caused by p53 loss in mice. *Blood* (2009) 113:6382–5. doi:10.1182/blood-2009-01-198564
27. Bellone M, Ceccon M, Grioni M, Jachetti E, Calcinotto A, Napolitano A, et al. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. *PLoS One* (2010) 5:e8646. doi:10.1371/journal.pone.0008646
28. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged interferon-g producing NKT response induced with a-galactosylceramide-loaded dendritic cells. *Nat Immunol* (2002) 3:867–74. doi:10.1038/ni827
29. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* (2005) 115:2572–83. doi:10.1172/JCI24762
30. Hayakawa Y, Takeda K, Yagita H, Kakuta S, Iwakura Y, Van Kaer L, et al. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol* (2001) 31:1720–7. doi:10.1002/1521-4141(200106)
31. Arora P, Baena A, Yu KO, Saini NK, Kharkwal SS, Goldberg MF, et al. A single subset of dendritic cells controls the cytokine bias of natural killer T cell responses to diverse glycolipid antigens. *Immunity* (2014) 40:105–16. doi:10.1016/j.immuni.2013.12.004
32. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by α-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a co-administered protein. *J Exp Med* (2003) 198:267–79. doi:10.1084/jem.20030324
33. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, et al. NKT cells enhance CD4$^+$ and CD8$^+$ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* (2003) 171:5140–7. doi:10.4049/jimmunol.171.10.5140
34. Moretto MM, Weiss LM, Combe CL, Khan IA. IFN-gamma-producing dendritic cells are important for priming of gut intraepithelial lymphocyte response against intracellular parasitic infection. *J Immunol* (2007) 179:2485–92. doi:10.4049/jimmunol.179.4.2485
35. Fujii S, Shimizu K, Hemmi H, Fukui M, Bonito AJ, Chen G, et al. Glycolipid a-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice. *Proc Natl Acad Sci U S A* (2006) 103:11252–7. doi:10.1073/pnas.0604812103
36. Taraban VY, Martin S, Attfield KE, Glennie MJ, Elliott T, Elewaut D, et al. Invariant NKT cells promote CD8$^+$ cytotoxic T cell responses by inducing CD70 expression on dendritic cells. *J Immunol* (2008) 180:4615–20. doi:10.4049/jimmunol.180.7.4615
37. Shimizu K, Yamasaki S, Shinga J, Sato Y, Watanabe T, Ohara O, et al. Systemic DC activation modulates the tumor microenvironment and shapes the long-lived tumor-specific memory mediated by CD8$^+$ T cells. *Cancer Res* (2016) 76:3756–66. doi:10.1158/0008-5472.CAN-15-3219
38. Leslie DS, Vincent MS, Spada FM, Das H, Sugita M, Morita CT, et al. CD1-mediated γ/δ T cell maturation of dendritic cells. *J Exp Med* (2002) 196:1575–84. doi:10.1084/jem.20021515
39. Conti L, Casetti R, Cardone M, Varano B, Martino A, Belardelli F, et al. Reciprocal activating interaction between dendritic cells and pamidronate-stimulated gammadelta T cells: role of CD86 and inflammatory cytokines. *J Immunol* (2005) 174:252–60. doi:10.4049/jimmunol.174.1.252
40. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med* (2002) 195:327–33. doi:10.1002/ijc.23962
41. Mocikat R, Braumuller H, Gumy A, Egeter O, Ziegler H, Reusch U, et al. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity* (2003) 19:561–9. doi:10.1016/S1074-7613(03)00264-4
42. Fujii S, Goto A, Shimizu K. Antigen mRNA-transfected, allogeneic fibroblasts loaded with NKT-cell ligand confer antitumor immunity. *Blood* (2009) 113:4262–72. doi:10.1182/blood-2008-08-176446
43. Bennett SRM, Carbone FR, Karamalis F, Flavell RA, Miller JFAP, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* (1998) 393:478–80. doi:10.1038/30996

44. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* (2004) 199:1607–18. doi:10.1084/jem.20040317
45. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011) 34:637–50. doi:10.1016/j.immuni.2011.05.006
46. Senhaji N, Kojok K, Darif Y, Fadainia C, Zaid Y. The contribution of CD40/CD40L axis in inflammatory bowel disease: an update. *Front Immunol* (2015) 6:529. doi:10.3389/fimmu.2015.00529
47. Summers deLuca L, Gommerman JL. Fine-tuning of dendritic cell biology by the TNF superfamily. *Nat Rev Immunol* (2012) 12:339–51. doi:10.1038/nri3193
48. Shimizu K, Asakura M, Shinga J, Sato Y, Kitahara S, Hoshino K, et al. Invariant NKT cells induce plasmacytoid dendritic cell (DC) cross-talk with conventional DCs for efficient memory CD8+ T cell induction. *J Immunol* (2013) 190:5609–19. doi:10.4049/jimmunol.1300033
49. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, et al. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. *Nat Immunol* (2010) 11:313–20. doi:10.1038/ni.1848
50. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S. Cross-presentation of glycolipid from tumor cells loaded with a-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *J Exp Med* (2007) 204:2641–53. doi:10.1084/jem.20070458
51. Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, Wilson JM, Schmieg J, Kronenberg M. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. *J Exp Med* (2002) 195:617–24. doi:10.1084/jem.20011889
52. Shimizu K, Goto A, Fukui M, Taniguchi M, Fujii S. Tumor cells loaded with α-galactosylceramide Induce innate NKT and NK cell-dependent resistance to tumor implantation in mice. *J Immunol* (2007) 178:2853–61. doi:10.4049/jimmunol.178.5.2853
53. Porcelli SA, Modlin RL. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu Rev Immunol* (1999) 17:297–329. doi:10.1146/annurev.immunol.17.1.297
54. Hunn MK, Farrand KJ, Broadley KW, Weinkove R, Ferguson P, Miller RJ, et al. Vaccination with irradiated tumor cells pulsed with an adjuvant that stimulates NKT cells is an effective treatment for glioma. *Clin Cancer Res* (2012) 18:6446–59. doi:10.1158/1078-0432.CCR-12-0704
55. Kobayashi T, Doff BL, Rearden RC, Leggatt GR, Mattarollo SR. NKT cell-targeted vaccination plus anti-4-1BB antibody generates persistent CD8 T cell immunity against B cell lymphoma. *Oncoimmunology* (2015) 4:e990793. doi:10.4161/2162402X.2014.990793
56. Shimizu K, Mizuno T, Shinga J, Asakura M, Kakimi K, Ishii Y, et al. Vaccination with antigen-transfected, NKT cell ligand-loaded, human cells elicits robust in situ immune responses by dendritic cells. *Cancer Res* (2013) 73:62–73. doi:10.1158/0008-5472.CAN-12-0759
57. Sun R, Liu Y. Listeriolysin O as a strong immunogenic molecule for the development of new anti-tumor vaccines. *Hum Vaccin Immunother* (2013) 9:1058–68. doi:10.4161/hv.23871
58. Wood LM, Paterson Y. Attenuated *Listeria monocytogenes*: a powerful and versatile vector for the future of tumor immunotherapy. *Front Cell Infect Microbiol* (2014) 4:51. doi:10.3389/fcimb.2014.00051
59. Singh M, Quispe-Tintaya W, Chandra D, Jahangir A, Venkataswamy MM, Ng TW, et al. Direct incorporation of the NKT-cell activator alpha-galactosylceramide into a recombinant *Listeria monocytogenes* improves breast cancer vaccine efficacy. *Br J Cancer* (2014) 111:1945–54. doi:10.1038/bjc.2014.486
60. Ng TW, Saavedra-Avila NA, Kennedy SC, Carreno LJ, Porcelli SA. Current efforts and future prospects in the development of live mycobacteria as vaccines. *Expert Rev Vaccines* (2015) 14:1493–507. doi:10.1586/14760584.2015.1089175
61. Bollampalli VP, Harumi Yamashiro L, Feng X, Bierschenk D, Gao Y, Blom H, et al. BCG skin infection triggers IL-1R-MyD88-dependent migration of EpCAMlow CD11bhigh skin dendritic cells to draining lymph node during $CD4^+$ T-Cell priming. *PLoS Pathog* (2015) 11:e1005206. doi:10.1371/journal.ppat.1005206
62. Venkataswamy MM, Ng TW, Kharkwal SS, Carreno LJ, Johnson AJ, Kunnath-Velayudhan S, et al. Improving *Mycobacterium bovis* bacillus Calmette-Guerin as a vaccine delivery vector for viral antigens by incorporation of glycolipid activators of NKT cells. *PLoS One* (2014) 9:e108383. doi:10.1371/journal.pone.0108383
63. Yamada D, Iyoda T, Vizcardo R, Shimizu K, Sato Y, Endo TA, et al. Efficient regeneration of human $Valpha24^+$ invariant natural killer T cells and their anti-tumor activity in vivo. *Stem Cells* (2016) 34:2852–60. doi:10.1002/stem.2465

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REVIEW
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Invariant Natural Killer T Cells As Suppressors of Graft-versus-Host Disease in Allogeneic Hematopoietic Stem Cell Transplantation

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Invariant natural killer T (iNKT) cells serve as a bridge between innate and adaptive immunity and have been shown to play an important role in immune regulation, defense against pathogens, and cancer immunity. Recent data also suggest that this compartment of the immune system plays a significant role in reducing graft-versus-host disease (GVHD) in the setting of allogeneic hematopoietic stem cell transplantation. Murine studies have shown that boosting iNKT numbers through certain conditioning regimens or adoptive transfer leads to suppression of acute or chronic GVHD. Preclinical work reveals that iNKT cells exert their suppressive function by expanding regulatory T cells *in vivo*, though the exact mechanism by which this occurs has yet to be fully elucidated. Human studies have demonstrated that a higher number of iNKT cells in the graft or in the peripheral blood of the recipient post-transplantation are associated with a reduction in GVHD risk, importantly without a loss of graft-versus-tumor effect. In two separate analyses of many immune cell subsets in allogeneic grafts, iNKT cell dose was the *only* parameter associated with a significant improvement in GVHD or in GVHD-free progression-free survival. Failure to reconstitute iNKT cells following allogeneic transplantation has also been associated with an increased risk of relapse. These data demonstrate that iNKT cells hold promise for future clinical application in the prevention of GVHD in allogeneic stem cell transplantation and warrant further study of the immunoregulatory functions of iNKT cells in this setting.

Keywords: invariant natural killer T cells, graft-versus-host disease, allogeneic hematopoietic stem cell transplantation, graft-versus-tumor effect, regulatory T cells

Abbreviations: α-GalCer, alpha-galactosylceramide; aGVHD, acute graft-versus-host disease; AML, acute myeloid leukemia; ATG, antithymocyte globulin; BMT, bone marrow transplantation; cGVHD, chronic graft-versus-host disease; DCs, dendritic cells; GCSF, granulocyte colony stimulating factor; GPFS, GVHD-free progression-free survival; GVHD, graft-versus-host disease; GVT, graft-versus-tumor; HCT, hematopoietic cell transplantation; IFN, interferon; IL, interleukin; iNKT, invariant natural killer T; MAC, myeloablative conditioning; MDSCs, myeloid-derived suppressor cells; MMRD, mismatched unrelated donor; MRD, matched related donor; MUD, matched unrelated donor; NRM, non-relapse mortality; OS, overall survival; PBSCT, peripheral blood stem cell transplantation; RIC, reduced intensity conditioning; STAT, signal transducer and activator of transcription; TBI, total body irradiation; TCR, T cell receptor; TGF, transforming growth factor; TLI, total lymphocyte irradiation; TNF, tumor necrosis factor; Tregs, regulatory T cells; UCB, umbilical cord blood.

INTRODUCTION

Hematopoietic cell transplantation (HCT) remains the only curative treatment option for patients with many hematologic malignancies and hematologic disorders. Despite many improvements to support patients following transplantation, graft-versus-host disease (GVHD) continues to be one of the leading causes of morbidity and mortality (1). GVHD is characterized by a dysregulation of donor immune cells leading to a massive alloreactive T cell proliferation and destruction of host tissues (2). To better understand the mechanisms underlying this dysregulation and potentially improve the outcome following HCT, different immune regulatory cell populations such as regulatory T cells (Tregs) and invariant natural killer T (iNKT) cells have been extensively studied. This review will focus on the immune biology of iNKT cells in the transplant setting.

Invariant natural killer T cells are a rare subset of T lymphocytes which are characterized by the coexpression of natural killer and T cell markers. They express a T cell receptor (TCR) which is semi-invariant (Vα14Jα18 pairing with a limited selection of beta chains in mice and Vα24Jα18 typically pairing with Vβ11 in humans) and which recognizes glycolipid antigens presented by the non-polymorphic MHC Class I-like molecule CD1d with high affinity (3).

Despite their rarity, iNKT cells exert potent immunomodulatory functions bridging the innate and adaptive immune systems by rapidly producing vast amounts of cytokines and chemokines. This results either in enhanced immune responses (i.e., defense against pathogens, immunosurveillance in cancer) via the production of Th1 cytokines such as interferon (IFN)-γ or in suppression of autoimmune and alloimmune reactions by the production of interleukin (IL)-4 and IL-10 (4, 5) (**Figure 1**).

MURINE STUDIES

Host iNKT Cells Protect from GVHD

During the last two decades multiple studies have focused on the role of iNKT cells in the context of allogeneic HCT utilizing different animal models. One approach from the group of Strober et al. revealed that reduced intensity conditioning (RIC) with total lymphoid irradiation (TLI) and antithymocyte globulin (ATG)

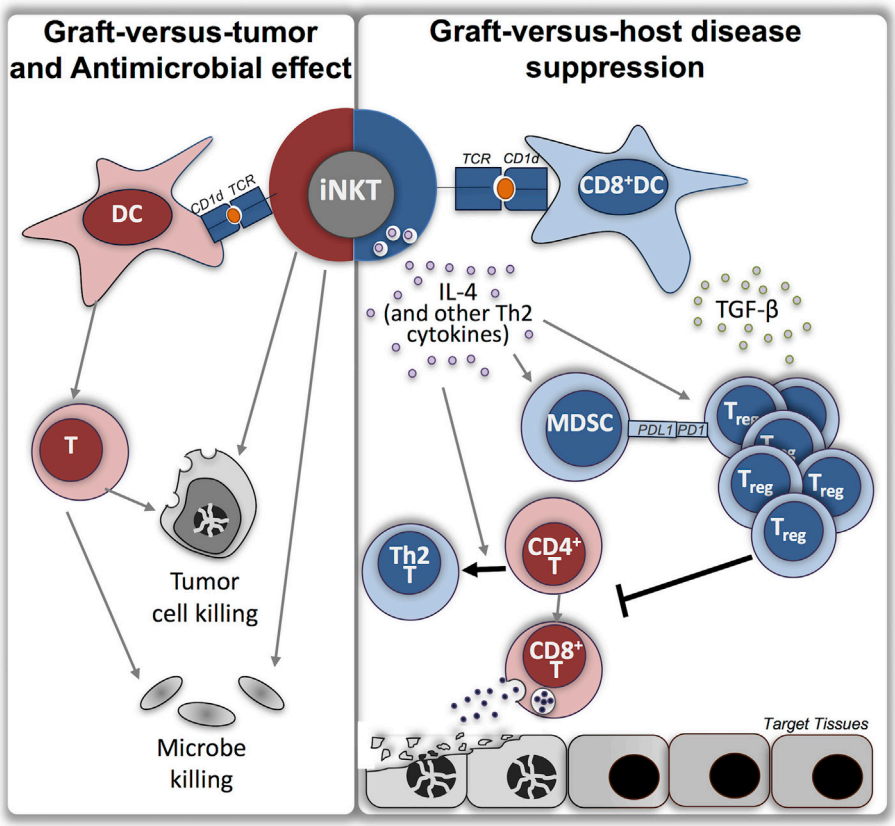

FIGURE 1 | Roles of invariant natural killer T cells in allogeneic stem cell transplantation. Depicted are the variety of roles for iNKT in allogeneic stem cell transplantation which have been demonstrated in multiple studies, including graft-versus-tumor effect, antimicrobial effect, and suppression of graft-versus-host disease (GVHD). Purported mechanisms by which iNKT may function to suppress GVHD are shown. Red color represents inflammatory phenotype, blue color represents suppressive or anti-inflammatory phenotype, orange circle represents endogenous lipid antigen, foreign lipid antigen, or exogenous synthetic lipid antigen [i.e., alpha-galactosylceramide (α-GalCer)]. iNKT, invariant natural killer T cells; TCR, T cell receptor; DC, dendritic cell; IL, interleukin; TGF, transforming growth factor; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; T, T lymphocyte.

results in a relative expansion of host iNKT cells which protect from GVHD, while preserving the graft-versus tumor effect (6, 7). The protection from GVHD was associated with a bias in donor T cell polarization to a Th2 cytokine pattern. The same group also demonstrated that host iNKT cells induce a donor Treg expansion and that these Tregs are crucial to protect from GVHD (8). The Treg proliferation was IL-4 dependent as it was lost when IL-4 deficient recipients were used. Another study investigating the effect of residual host NKT cells in a major mismatch transplantation model with total body irradiation (TBI) found that these cells are essential for the protection from GVHD but can also cause a delay in engraftment and, under certain conditions, graft rejection (9).

Stimulation of iNKT Cells with Alpha-Galactosylceramide (α-GalCer) Protects from GVHD

Treatment of recipient mice with a synthetic iNKT TCR ligand α-GalCer (in aqueous or liposomal form) on the day of transplantation significantly reduced mortality and morbidity from GVHD (10–13). One study showed that this effect was abrogated if NKT cell-deficient CD1d knockout ($CD1d^{-/-}$) or $IL\text{-}4^{-/-}$ recipient mice were used, indicating that host iNKT cells and their production of IL-4 play a critical role in tolerance induction (11). Furthermore, in the same study it was shown that tolerance induction also failed if the donors were signal transducer and activator of transcription (STAT)6 deficient. This suggests that the protection from GVHD is dependent on the Th2 polarization of donor T cells mediated by STAT6-dependent mechanisms. Another study confirmed that the activation of iNKT with the liposomal form of α-GalCer and a CD40-CD40 ligand blockade lead to the establishment of a mixed chimerism and provides tolerance in a murine model with combined allogeneic bone marrow transplantation (BMT) and heart transplantation (13). Together with the protection from GVHD and the establishment of a mixed chimerism, an activation of Tregs and high amounts of Th2 cytokines in the recipient mice was observed. In line with this, Duramad et al. observed a dose-dependent expansion of Tregs in spleen, lymph nodes, and bone marrow after administration of α-GalCer on the day of HCT (12). Interestingly the protective effect of α-GalCer was abrogated if the donors were Treg depleted. Further, in a model of chronic GVHD (cGVHD) the administration of α-GalCer is capable of reversing and preventing cGVHD (14). As described below, treatment with α-GalCer for the protection of GVHD is the first approach that has been successfully translated into the clinic and is currently being investigated in clinical trials.

Adoptive Transfer of iNKT Cells Protects from GVHD

Another approach to harnessing the immunoregulatory abilities of iNKT cells is the adoptive transfer of either recipient-type, donor-type, or third party iNKT cells in the allogeneic transplantation setting. Several studies by our group and others convincingly demonstrated that the adoptive transfer of iNKT cells leads to a decrease in acute GVHD without losing the graft-versus-tumor (GVT) effect (7, 9, 15–17). In these studies, adoptively transferred donor $CD4^+$ iNKT expanded in secondary lymphoid organs and migrated to GvHD target organs, similar to conventional T cells (15). Interestingly, we have shown that extremely low iNKT cell numbers (i.e., 5×10^4) and even cells from a third-party source are capable of these effects (15–17). Additionally, the adoptive transfer of donor iNKT cells is able to prevent and even reverse cGVHD in a minor-mismatch BMT model. This effect was also dependent on the production of IL-4 by iNKT cells and the presence of Tregs (14).

In an effort to make iNKT cells more accessible in the clinic, *ex vivo* expansion before adoptive transfer has been explored. Several studies demonstrated that it is feasible to expand iNKT cells *in vitro* with a combination of α-GalCer and IL-2. In these studies, the expanded iNKT cells provide protection from GVHD which is dependent on the production of IL-4 by iNKT cells (18, 19).

Mechanisms of iNKT Cell Function in Murine HCT

Most of the studies described above had two intriguing findings in common. First, iNKT cells give rise to a bias in donor T cell polarization toward a Th2 cytokine pattern with significantly reduced production of IFN-γ and tumor necrosis factor (TNF)-alpha (6, 13, 15, 17), and some even showed that the proliferation of conventional T cells was diminished (6, 16). Second, the survival benefit of mice treated with TLI/ATG, donor iNKT cells, or α-GalCer was accompanied by an expansion of Tregs (12–14, 16, 17, 20). Tregs have been shown to function as potent immune suppressors in the context of allogeneic transplantation and are capable of both inhibiting GVHD as well as preserving the GVT effect (21–23). There is compelling evidence that the mechanism by which iNKT cells suppress GVHD is through the expansion of Tregs. Accordingly, it was shown in different murine transplantation models that cytokines such as IL-4 produced by iNKT cells play an important role in enhancing Treg function and that depletion of Tregs leads to a loss of function of iNKT cells (14, 17, 20). Interestingly, it was also demonstrated that Tregs are not capable of inducing tolerance in a model of combined marrow and organ transplantation if the recipient is iNKT cell deficient (20). Another hypothesis to support the latter findings is that other cell populations, such as myeloid-derived suppressor cells (MDSCs) or $CD8^+$ dendritic cells (DC), play an important role in the interplay between iNKT cells and Tregs.

Myeloid-derived suppressor cells are a heterogenous cell subset known to play a major role in the regulation of immune responses in cancer and other pathological conditions (24), and several studies have shown that they have the potential to inhibit GVHD (25, 26) and to induce Treg proliferation after HCT in PDL1-dependent manner (25, 26). Moreover, we demonstrated that MDSCs can work as a facilitator between iNKT cells and Tregs in a murine allogeneic BMT model with adoptive transfer of donor iNKT cells (17). In this model, certain subsets of MDSCs were shown to expand shortly after transplantation

and, if depleted, the protective effect of the transferred donor iNKT cells was lost. Furthermore, in the same model, MDSCs were also crucial to mediate the iNKT cell-induced expansion of Tregs as the depletion of MDSCs led to a depletion of Tregs (17). In another model with combined bone marrow and heart transplantation, MDSCs were crucial to promote tolerance and chimerism and their activation was dependent on host iNKT cells and their production of IL-4 (27).

The second cell population, which has recently come to attention, is CD8α$^+$ DCs. It was shown previously that these cells are the major DC subset to present a variety of glycolipids through the CD1d molecule to iNKT cells leading to their activation (28). Furthermore, it is known that CD8α$^+$205$^+$DCs induce Tregs in a transforming growth factor-beta (TGF-β) and retinoic acid-dependent manner (29) and that they can exert immunosuppressive characteristics in specific situations yet are crucial to promote the GVT effect (29, 30). One group also found that there is an aggravated course of GVHD if this subset of DCs is missing (31). In addition, the total number of Tregs and levels of TGF-β are significantly lower when CD8$^+$ DCs are not present. CD8$^+$ DCs induced iNKT cells to secret IL-4, IL-13, and IFN-γ with a Th-2 bias (32). In return these tolerogenic iNKT cells altered the differentiation of CD8$^+$ DCs and suppressed graft rejection *in vivo* demonstrating elegantly that interactions between tolerogenic CD8$^+$ DCs and iNKT cells are required to induce tolerance.

HUMAN STUDIES

In addition to these murine experiments, a number of human studies have also shown a role for iNKT in suppressing GVHD. Although largely correlative, these studies demonstrate the power of iNKT cells and the need for further research. In these reports, iNKT were defined as CD3$^+$Vα24$^+$Vβ11$^+$, except where indicated.

Persistence and Rapid Recovery of iNKT Cells following Allogeneic HCT Are Associated with Reduced GVHD

One of the earliest studies delineating iNKT reconstitution following allogeneic HCT demonstrated a correlation between increased peripheral blood iNKT count and reduced acute and chronic GHVD (33). In this study iNKT were enumerated in the peripheral blood starting on day +30 following transplantation of 106 patients undergoing HCT without T-depletion following myeloablative conditioning (MAC). Donors included a mix of matched unrelated donors (MUDs) and matched related donors (MRD). In a multivariate analysis, stem cell source was the only variable associated with iNKT content in the peripheral blood post-transplantation. Following peripheral blood stem cell transplantation (PBSCT), iNKT cells were reconstituted within 1 month, while after BMT, iNKT were not fully reconstituted within 1 year (and all iNKT were <10% recipient chimerism in four analyzed patients). In BMT, but not PBSCT, the number of total, CD4$^+$, and CD4$^-$ iNKT were significantly reduced in patients with aGVHD, and total and CD4$^+$ iNKT (as well as iNKT/T cell ratio) were significantly reduced in extensive cGVHD. Importantly, steroids had no impact on iNKT counts. Although limited by quite high numbers of missing values, this study represents the first human report to explore the link between iNKT recovery and development of GVHD.

An early human study from our group translated the encouraging murine TLI/ATG results in 37 patients receiving human leukocyte antigen-matched (MRD or MUD) granulocyte colony-stimulating factor-mobilized PBSCT for hematologic malignancies (34). Only two patients developed aGVHD (one was only grade I), considerably lower than previously reported for RIC regimens, and only seven developed extensive cGVHD. Importantly, 62% either converted to or remained in CR. Interestingly, production of IL-4 (but not IFN-γ, IL-2, or TNF-α) was markedly elevated in CD4$^+$ T cells from patients who underwent TLI/ATG conditioning as compared to conventional RIC or normal subjects. In the five patients for whom peripheral blood iNKT count was measured following conditioning, iNKT percentage was increased by a factor of 10. Building on this success, a second report of 111 additional patients, including a few mismatched unrelated donors (MMUD) demonstrated that the overall probability of aGVHD was substantially lower than typically observed with RIC at 5.4%, and slightly lower for extensive chronic GVHD at 28% (35). One limitation was that 19% of patients developed mixed chimerism, associated with higher risk of disease relapse or progression. The overall risk of relapse/progression was around 60%, though many were salvaged with donor lymphocyte infusion. Non-relapse mortality (NRM) was 3% suggesting excellent tolerability; indeed, death from infection was extremely low possibly related to less need for immunosuppression given the low GVHD rates. These findings were independently corroborated when TLI/ATG was used in a multicenter study of 45 patients (36) and when compared with TBI/fludarabine in a randomized study (37).

Interestingly, the effect of ATG on iNKT preservation and GVHD reduction does not appear to extend to the myeloablative setting. Immune cell reconstitution was analyzed starting at day +40 in 65 patients undergoing MAC prior to PBSCT with or without ATG (38). In this setting, the inclusion of ATG led to a significantly *decreased* number of iNKT cells (defined as CD3$^+$CD56$^+$Vα24Jα18$^+$Vβ11$^+$) at day +40, though by day +100 no difference was noted. The ATG group also had a dramatically lower incidence of aGVHD with no change in cGVHD. Another study failed to find an association of iNKT with GVHD in a MAC plus ATG setting (39). Similarly, a third study of MAC plus ATG conditioning evaluated iNKT after conditioning and starting at day +7 (40). Normalization of iNKT to healthy control levels had not occurred by day +730, though CD4$^-$ iNKT were recovering faster than CD4$^+$ iNKT. Interestingly, CD4$^-$ iNKT cells were significantly *higher* on d28 in patients who ultimately developed GVHD versus those did not.

However, in a study of 71 heterogenous patients (MAC and RIC, T cell-depleted and non-depleted, mix of donor types and cell sources), early post-transplantation iNKT recovery [defined as CD3$^+$CD1d tetramer (PBS57)$^+$] predicted aGVHD and overall survival (OS) (41). iNKT/T ratio was monitored post-transplant and a threshold of 1×10^{-3} appeared to

discriminate patients into low and high groups predictive of aGVHD. In multivariate analysis, high iNKT/T ratio was significantly associated with absence of aGVHD (hazard ratio 0.12), and OS was also significantly improved (90 versus 52% at 2 years). Significant reduction in NRM (6.1 versus 36.7%) was noted to be due to fewer deaths from aGVHD and infection. Impressively, the d15 ratio (with a cutoff of 0.58×10^{-3}) could discriminate the risk of aGVHD with an area under the curve of 0.812 (odds ratio 0.06 for aGVHD in high iNKT/T group). Interestingly, $CD4^+$ iNKT chimerism of five patients revealed all to be of donor origin.

Recovery of iNKT Cells Is Also Associated with Enhanced GVT Effect

One study evaluated iNKT reconstitution ($CD3^+V\alpha24J\alpha18^+$) in cord blood and following umbilical cord blood (UCB) transplantation in 33 patients with high-risk acute myeloid leukemia (AML) following RIC (42). While iNKT cells were reduced after conditioning compared to healthy controls, a transient increase in frequency was observed in the first 3 months post-transplant (which was not seen in other T cell populations). In the first few months, most iNKT were $CD4^+$, similar to that observed in UCB, which fell to healthy adult control levels by 12 months. CD45RO expression remained high throughout (with low CD45RA), suggesting a primed/memory phenotype similar to UCB, while CD62L and CCR7 fell over time, suggesting progression from a central memory phenotype toward more effector/memory tissue-homing. Early post-transplant iNKT cells were also enriched for CD69 which decreased over time, suggesting early activation. Production of IFN-γ and IL-4 were both substantially reduced and granulocyte–macrophage colony-stimulating factor was substantially increased in UCB and in early post-transplant samples compared to healthy adults, which all normalized by 6 months post-transplant. Although cytotoxicity of iNKT against CD1d-transfected AML cell lines and primary AML blasts was significantly reduced in UCB and early post-transplant, it reached healthy adult control levels within 6 months. This study highlights the reconstitution process of iNKT cells following UCB transplantation and begins to elucidate the mechanisms by which GVT may occur.

Invariant natural killer T cell reconstitution was further studied in a group of pediatric patients receiving T cell depleted haploidentical transplants for hematologic malignancies (43). Twenty-two patients were followed longitudinally from day +30 to 18 months; another 11 underwent cross-sectional analysis at 2–6 years post-transplant. In this setting, iNKT cells emerged in peripheral blood around 3 months post-transplant and reached levels similar to age-matched healthy children by 18 months. The iNKT cell profile initially resembled that of cord blood: predominantly $CD4^+CD161^-$. $CD4^-$ iNKT began to emerge about 2–4 months later than $CD4^+$ iNKT and $CD161^+$ cells emerged over time in both populations. IFN-γ production reached levels close to healthy adults by 6 months in $CD4^-$ iNKT, and both populations could produce IL-4 normally. This study also strikingly demonstrated that delayed iNKT reconstitution was significantly associated with relapse. iNKT remained essentially undetectable during the entire 18-month follow-up in the eight patients who experienced relapse, while absolute iNKT cell counts were significantly higher in all patients who maintained remission. Although a decreased absolute number of T cells was also significantly associated with relapse, in terms of frequency only a lower iNKT cell frequency was significantly associated with relapse (19 iNKT/10^6 T cells in patients who relapsed versus 107 iNKT cells/10^6 T cells in those who did not) (44). The authors suggest that monitoring of iNKT cell reconstitution post-transplant and adoptively transferring donor iNKT cells in those patients failing to reconstitute may be a method by which relapse could be prevented.

Increased Number of iNKT Cells in the Allogeneic Graft Product Is Associated with Reduced Acute GVHD

In addition to the above studies demonstrating that persistence and/or rapid recovery of iNKT cells following transplantation correlate with reduced GVHD, a number of studies have evaluated the graft content of various cell populations and potential impact on clinically meaningful outcomes. The frequency of lymphocyte subsets in cryopreserved samples of 78 sibling donor non-T-depleted PBSCT grafts for patients undergoing mostly MAC were analyzed (45). In multivariate regression analysis, $CD4^-$ iNKT cell dose and chronic myeloid leukemia diagnosis were the only factors associated with aGVHD, with a RR of 4.27 for $CD4^-$ iNKT dose below the median (0.031×10^6/kg). Interestingly, graft Treg dose did not predict for grades II–IV aGVHD. The impact of iNKT cell dose on chronic GVHD, relapse, or survival was not assessed. Further, $CD4^-$ iNKT cells consistently suppressed proliferation in a mixed lymphocyte reaction with autologous T cells and allogeneic stimulator cells in a dose-dependent manner, and suppressed IFN-γ secretion.

Another study characterized over 25 immune cell subsets within cryopreserved grafts of 80 patients undergoing PBSCT from a mix of MRD, MUD, and MMUDs (46). Most patients received RIC and the vast majority received *in vivo* T cell depletion with ATG. The dose of iNKT cells ($CD3^+V\alpha24^+$) was the only factor with a significant impact on GVHD-free progression-free survival (GPFS) in multivariate analysis. The 2-year GPFS was significantly increased in patients receiving greater than the median number of donor iNKT cells (0.11×10^6/kg) at 49% compared to 22% in patients receiving fewer than the median number, although OS was not significantly different. The findings were primarily due to an increased incidence of relapse in the patients receiving fewer than the median iNKT cell dose, as there was no significant difference in NRM, grades III–IV acute GVHD, or severe chronic GVHD. As noted in the manuscript, the use of ATG leading to very low rates of aGVHD overall (20–25%) may explain the lack of association of increased iNKT with reduction in acute GVHD.

A third study investigated 15 immune populations from a mix of fresh and cryopreserved graft products for 117 transplants from a variety of donors (MRD/MUD/MMUD), sources (PBSCT/BMT), and conditioning regimens (MAC/RIC, with about half receiving T-depletion with ATG) (47). Doses of

iNKT [$CD3^+CD1d$-tetramer$(PBS57)^+$] were noted to be higher in PBSC than BM grafts owing to the one log higher total T cell content. Although lower total and $CD4^-$ iNKT frequency was significantly associated with the occurrence of aGVHD in BMT and PBSCT, lower $CD4^-$ iNKT expansion factor was only associated with aGVHD in the PBSCT group. Importantly, this remained true when the expansion of iNKT cells was analyzed in donor peripheral blood *prior* to mobilization and at defined timepoints in patients post-transplant. The expansion factor was not predictive of relapse rate, cGVHD, NRM, or OS. Although, as the authors recognize, the ability to implement the expansion technique as a GVHD predictive tool across all centers is unlikely, this work highlights the critical need for high frequencies of functional iNKT cells for suppression of aGVHD. It also raises the question of whether iNKT cell dose or frequency relative to total T cells (or, indeed, expansion capacity) is the most important value in predicting aGVHD, which remains to be fully answered.

Interestingly, another study of immune cell content in 238 allografts for a very heterogenous patient population showed a significant association of increased iNKT content with *increased* risk of cGVHD in a univariate analysis (48). However, a non-standardized definition of iNKT cells was used ($CD3^+CD56^+CD16^-CD117^-$) and this finding did not pan out in multivariate analysis. The same study also found that increased iNKT cell content was associated with lower risk of relapse in AML/MDS patients.

Therapeutic Expansion of iNKT Compartment Is Well Tolerated with Lower GVHD Rates in Responders

Because of this strong evidence for GVHD suppression by iNKT cells, attempts to intentionally expand the iNKT cell compartment in allogeneic HCT patients have begun. A recently published phase 2A study explored pharmacologic expansion using a single dose of RGI-2001, the liposomal formulation of α-GalCer, on the day of transplantation with two dosing cohorts (identified from phase 1 testing) for a total of 29 patients (most undergoing RIC with PBSC grafts) (49). A total of 11 serious adverse events (two grade IV) were reported, regardless of causality. Immune cells were monitored prior to transplant and weekly for the first month with no significant changes in iNKT noted (remained <3% of total lymphocytes). However, similar to the iNKT-mediated Treg expansion noted in mice (16), four patients from each cohort had significant expansion of absolute number and frequency of Tregs ($CD4^+CD25^{hi}CD127^{low}$). These responders were enriched for patients who had received sirolimus with their GVHD prophylaxis (though not statistically significant), suggesting a synergistic effect (although an isolated effect of sirolimus cannot be entirely ruled out). Importantly, rates of grades II–IV aGVHD were 12.5% in responders and 52.4% in non-responders, though as noted by the authors based on the study design conclusions cannot be drawn as to whether RGI-2001 reduces GVHD incidence. Future work optimizing the dosing strategy, comparing patients receiving RGI-2001 to those who do not, and investigating why some respond and others do not will be required. Despite these limitations, this study represents a critical first step in the intentional boosting of iNKT cells to suppress GVHD.

CONCLUSION AND FUTURE DIRECTIONS

As demonstrated in the aforementioned murine and human studies, iNKT cells may be the holy grail sought in stem cell transplantation that is capable both of suppressing allogeneic immune reactions (GVHD) and enhancing anti-tumor immune reactions (GVT). The murine studies all demonstrated a strong benefit of iNKT cells on GVHD reduction. Although a minority of human studies (primarily in the MAC plus ATG setting) did not find a significant association between iNKT and GVHD, or even found higher iNKT associated with worse GVHD (38–40), most found that increased iNKT cell content of the graft or of peripheral blood post-transplantation was associated with GVHD suppression. One should keep in mind, however, that these are correlative studies. Indeed, cause-and-effect cannot be determined for those studies evaluating iNKT content of peripheral blood, and the possibility of evolving GVHD causing lowered iNKT numbers in peripheral blood (perhaps due to increased target tissue homing) cannot be excluded. However, the studies revealing an association between higher iNKT counts (in the graft or peripheral blood) and improved NRM and survival (41, 46) suggest that, in general, iNKT cells exert an overall protective effect. The extensive murine data support this as well. Indeed, given the broad functions of iNKT cells, increased numbers may be beneficial not just for GVHD suppression, but also infection control and reduction in relapse (**Figure 1**).

Despite these promising results, however, a number of questions remain unanswered. First is whether donor iNKT, recipient iNKT, or both are critical for these functions. Although the results from TLI/ATG conditioning studies suggest a role for host iNKT in suppression of GVHD, the importance of donor graft iNKT cell content in other studies, as well as iNKT chimerism revealing donor origin, implicate the role of donor iNKT cells. One could envision that donor iNKT cells are most critical in a MAC setting, while both donor and recipient iNKT play a role in RIC, but more study is needed to tease apart their individual contributions. Second, is whether certain iNKT cell subsets are more functional in the transplant setting and/or whether different subsets serve different (and yet equally critical) functions in transplant patients. The results of studies included in this review are not always congruous in this regard. In the murine adoptive transfer studies, our group found that $CD4^+$ iNKT suppressed aGVHD in a major mismatch model (16, 17). However, two human studies have found in multivariate analyses that $CD4^-$, not $CD4^+$, iNKT graft content was significantly associated with reduced aGVHD (45, 47). Conversely, an increased number of $CD4^+$ iNKT in post-transplant peripheral blood was significantly associated with reduced cGVHD (increased $CD4^-$ iNKT were also associated with reduced cGVHD, but did not reach significance) (33). The discrepancy between mouse and human studies (and indeed, among human data) as to whether the $CD4^+$ or $CD4^-$ iNKT population is best suited to reduce GVHD

(and possible differences between acute and chronic GVHD) highlights the need for further study. Recently, several subsets of iNKT cells have been described skewing toward phenotypes resembling Th1 cells, Th2 cells, and Th17 cells [reviewed in Ref. (5)], and even Treg-like iNKT which constitutively produce IL-10 (50). $CD4^{-}CD8^{+}$ iNKT have also been described in humans, but not mice, and exhibit potent cytotoxic functions (51). The contribution of each of these subsets in the setting of stem cell transplantation remains to be determined, and a better understanding of their physiologic and pathologic functions, as well as their degree of plasticity, will inform future work. In addition, the ability of $CD4^{+}$ iNKT cells to produce some Th1-type cytokines and vice versa (51) calls into question whether distinguishing subsets of iNKT based on expression of CD4 alone and thereby assigning differing functions is appropriate. A third question is how to boost iNKT numbers in patients receiving grafts containing low iNKT numbers or with poor post-transplantation iNKT reconstitution, and indeed these cutoffs remain to be clearly established (and may differ depending on transplant conditions). However, these studies begin to identify an iNKT cell dose in the graft, or in peripheral blood as the population is reconstituting, that is associated with lower GVHD risk. This will aid clinical trial design for the adoptive transfer of iNKT cells. Several approaches have been investigated in mice to intentionally use iNKT cells to dampen GVHD while preserving the GVT effect. For instance, iNKT cells were expanded and their immunoregulatory abilities enhanced by treating recipient mice with α -GalCer on the day of transplantation (10–12). In addition, iNKT cells from different origins (host, donor, third party) were each shown to be effective in reducing acute and chronic GVHD when adoptively transferred together with the graft (7, 9, 14–16). Furthermore, iNKT cells can be expanded *ex vivo* with IL-2 and α-GalCer and adoptively transferred for GVHD suppression (18, 19). Most of these studies also demonstrated a conservation of the GVT effect. There is convincing evidence in mice that iNKT cells work through the expansion of Tregs, and the underlying biological mechanism is currently under investigation. IL-4 production by iNKT cells or facilitators such as MDSCs or $CD8^{+}$ DCs are possibilities. The findings that third party iNKT cells are as functional as donor-derived cells for the suppression of GVHD in mice and that the ability to expand these cells has been demonstrated sets the stage for clinical translation.

The first approach being investigated in humans utilizes delivery of α-GalCer to recipients at the time of transplantation (49). Though the choice of most appropriate transplant setting and optimal dosing strategy remains to be determined, phases 1 and 2A trials have revealed this approach to be safe. Given the results with other approaches in mice, it would be exciting to pursue these additional strategies in humans. The non-polymorphic nature of the CD1d molecule, as well as the ability of iNKT cells to be activated in non-TCR-dependent ways, make adoptive transfer of iNKT cells a feasible approach. Correlative biologic evaluations must accompany all clinical trial designs to determine the effect that any approach has not only on iNKT cell number, but also functional skewing.

Despite these lingering questions, harnessing the power of iNKT cells remains an attractive approach to improving HCT outcomes. Although extremely rare in number, iNKT cells may represent the most versatile and critical cell population for suppressing GVHD, fighting infection, and reducing relapse in patients undergoing allogeneic hematopoietic cell transplantation.

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MM and KB contributed equally to this manuscript. MM and KB did the literature study, wrote the manuscript, and critically revised the manuscript. RN provided senior supervision, helped in writing, and critically revised the manuscript. All coauthors approved the final manuscript.

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REFERENCES

1. Magenau J, Reddy P. Next generation treatment of acute graft-versus-host disease. *Leukemia* (2014) 28(12):2283–91. doi:10.1038/leu.2014.195
2. Ferrara JLM, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373(9674):1550–61. doi:10.1016/S0140-6736(09)60237-3
3. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med* (1994) 180(3):1097–106. doi:10.1084/jem.180.3.1097
4. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi:10.1146/annurev.immunol.25.022106.141711
5. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13(2):101–17. doi:10.1038/nri3369
6. Lan F, Zeng D, Higuchi M, Higgins JP, Strober S. Host conditioning with total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host disease: the role of CD1-reactive natural killer T cells. *Biol Blood Marrow Transplant* (2003) 9(6):355–63. doi:10.1016/S1083-8791(03)00108-3
7. Pillai AB, George TI, Dutt S, Teo P, Strober S. Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol* (2007) 178(10):6242–51. doi:10.4049/jimmunol.178.10.6242
8. Pillai AB, George TI, Dutt S, Strober S. Host natural killer T cells induce an interleukin-4-dependent expansion of donor CD4+CD25+Foxp3+ T regulatory cells that protects against graft-versus-host disease. *Blood* (2009) 113(18):4458–67. doi:10.1182/blood-2008-06-165506
9. Haraguchi K, Takahashi T, Matsumoto A, Asai T, Kanda Y, Kurokawa M, et al. Host-residual invariant NK T cells attenuate graft-versus-host immunity. *J Immunol* (2005) 175(2):1320–8. doi:10.4049/jimmunol.175.2.1320
10. Morecki S, Panigrahi S, Pizov G, Yacovlev E, Gelfand Y, Eizik O, et al. Effect of KRN7000 on induced graft-vs-host disease. *Exp Hematol* (2004) 32(7):630–7. doi:10.1016/j.exphem.2004.04.005
11. Hashimoto D, Asakura S, Miyake S, Yamamura T, Van Kaer L, Liu C, et al. Stimulation of host NKT cells by synthetic glycolipid regulates acute

graft-versus-host disease by inducing Th2 polarization of donor T cells. *J Immunol* (2005) 174(1):551–6. doi:10.4049/jimmunol.174.1.551
12. Duramad O, Laysang A, Li J, Ishii Y, Namikawa R. Pharmacologic expansion of donor-derived, naturally occurring CD4+Foxp3+ regulatory T cells reduces acute graft-versus-host disease lethality without abrogating the graft-versus-leukemia effect in murine models. *Biol Blood Marrow Transplant* (2011) 17(8):1154–68. doi:10.1016/j.bbmt.2010.11.022
13. Hirai T, Ishii Y, Ikemiyagi M, Fukuda E, Omoto K, Namiki M, et al. A novel approach inducing transplant tolerance by activated invariant natural killer T cells with costimulatory blockade. *Am J Transplant* (2014) 14(3):554–67. doi:10.1111/ajt.12606
14. Du J, Paz K, Thangavelu G, Schneidawind D, Baker J, Flynn R, et al. Invariant natural killer T cells ameliorate murine chronic GVHD by expanding donor regulatory T cells. *Blood* (2017) 129(23):3121–5. doi:10.1182/blood-2016-11-752444
15. Leveson-Gower DB, Olson JA, Sega EI, Luong RH, Baker J, Zeiser R, et al. Low doses of natural killer T cells provide protection from acute graft-versus-host disease via an IL-4-dependent mechanism. *Blood* (2011) 117 (11):3220–9. doi:10.1182/blood-2010-08-303008
16. Schneidawind D, Pierini A, Alvarez M, Pan Y, Baker J, Buechele C, et al. CD4+ invariant natural killer T cells protect from murine GVHD lethality through expansion of donor CD4+CD25+FoxP3+ regulatory T cells. *Blood* (2014) 124(22):3320–8. doi:10.1182/blood-2014-05-576017
17. Schneidawind D, Baker J, Pierini A, Buechele C, Luong RH, Meyer EH, et al. Third-party CD4+ invariant natural killer T cells protect from murine GVHD lethality. *Blood* (2015) 125(22):3491–500. doi:10.1182/blood-2014-11-612762
18. Kuwatani M, Ikarashi Y, Iizuka A, Kawakami C, Quinn G, Heike Y, et al. Modulation of acute graft-versus-host disease and chimerism after adoptive transfer of in vitro-expanded invariant Vα14 natural killer T cells. *Immunol Lett* (2006) 106(1):82–90. doi:10.1016/j.imlet.2006.05.001
19. Yang J, Gao L, Liu Y, Ren Y, Xie R, Fan H, et al. Adoptive therapy by transfusing expanded donor murine natural killer T cells can suppress acute graft-versus-host disease in allogeneic bone marrow transplantation. *Transfusion* (2010) 50(2):407–17. doi:10.1111/j.1537-2995.2009.02395.x
20. Hongo D, Tang X, Dutt S, Nador RG, Strober S. Interactions between NKT cells and Tregs are required for tolerance to combined bone marrow and organ transplants. *Blood* (2012) 119(6):1581–9. doi:10.1182/blood-2011-08-371948
21. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4+CD25+ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* (2002) 196(3):389–99. doi:10.1084/jem.20020399
22. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4+CD25+ immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* (2002) 99(10):3493–9. doi:10.1182/blood.V99.10.3493
23. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* (2003) 9(9):1144–50. doi:10.1038/nm915
24. Bronte V, Brandau S, Chen S-H, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun* (2016) 7:12150. doi:10.1038/ncomms12150
25. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood* (2010) 116(25):5738–47. doi:10.1182/blood-2010-06-287839
26. van der Merwe M, Abdelsamed HA, Seth A, Ong T, Vogel P, Pillai AB. Recipient myeloid-derived immunomodulatory cells induce PD-1 ligand-dependent donor CD4+Foxp3+ regulatory T cell proliferation and donor-recipient immune tolerance after murine nonmyeloablative bone marrow transplantation. *J Immunol* (2013) 191(11):5764–76. doi:10.4049/jimmunol.1302191
27. Hongo D, Tang X, Baker J, Engleman EG, Strober S. Requirement for interactions of natural killer T cells and myeloid-derived suppressor cells for transplantation tolerance. *Am J Transplant* (2014) 14(11):2467–77. doi:10.1111/ajt.12914
28. Arora P, Baena A, Yu KO, Saini NK, Kharkwal SS, Goldberg MF, et al. A single subset of dendritic cells controls the cytokine bias of natural killer T cell responses to diverse glycolipid antigens. *Immunity* (2014) 40(1):105–16. doi:10.1016/j.immuni.2013.12.004
29. Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, Bonito AJ, Inaba K, et al. CD8+CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol* (2008) 181(10):6923–33. doi:10.4049/jimmunol.181.10.6923
30. Toubai T, Mathewson N, Oravecz-Wilson K, Reddy P. Host CD8α+ dendritic cells may be a key factor for separating graft-versus-host disease from graft-versus-leukemia. *Biol Blood Marrow Transplant* (2015) 21(4):775–6. doi:10.1016/j.bbmt.2015.01.005
31. Weber M, Rudolph B, Stein P, Yogev N, Bosmann M, Schild H, et al. Host-derived CD8+ dendritic cells protect against acute graft-versus-host disease after experimental allogeneic bone marrow transplantation. *Biol Blood Marrow Transplant* (2014) 20(11):1696–704. doi:10.1016/j.bbmt.2014.08.005
32. Hongo D, Tang X, Zhang X, Engleman EG, Strober S. Tolerogenic interactions between CD8+ dendritic cells and NKT cells prevent rejection of bone marrow and organ grafts. *Blood* (2017) 129(12):1718–28. doi:10.1182/blood-2016-07-723015
33. Haraguchi K, Takahashi T, Hiruma K, Kanda Y, Tanaka Y, Ogawa S, et al. Recovery of Valpha24+ NKT cells after hematopoietic stem cell transplantation. *Bone Marrow Transplant* (2004) 34(7):595–602. doi:10.1038/sj.bmt.1704582
34. Lowsky R, Takahashi T, Liu YP, Dejbakhsh-Jones S, Grumet FC, Shizuru JA, et al. Protective conditioning for acute graft-versus-host disease. *N Engl J Med* (2005) 353(13):1321–31. doi:10.1056/NEJMoa050642
35. Kohrt HE, Turnbull BB, Heydari K, Shizuru JA, Laport GG, Miklos DB, et al. TLI and ATG conditioning with low risk of graft-versus-host disease retains antitumor reactions after allogeneic hematopoietic cell transplantation from related and unrelated donors. *Blood* (2009) 114(5):1099–109. doi:10.1182/blood-2009-03-211441
36. Messina G, Giaccone L, Festuccia M, Irrera G, Scortechini I, Sorasio R, et al. Multicenter experience using total lymphoid irradiation and antithymocyte globulin as conditioning for allografting in hematological malignancies. *Biol Blood Marrow Transplant* (2012) 18(10):1600–7. doi:10.1016/j.bbmt.2012.03.012
37. Baron F, Zachée P, Maertens J, Kerre T, Ory A, Seidel L, et al. Non-myeloablative allogeneic hematopoietic cell transplantation following fludarabine plus 2 Gy TBI or ATG plus 8 Gy TLI: a phase II randomized study from the Belgian Hematological Society. *J Hematol Oncol* (2015) 8(1):4. doi:10.1186/s13045-014-0098-9
38. Servais S, Menten-Dedoyart C, Beguin Y, Seidel L, Gothot A, Daulne C, et al. Impact of pre-transplant anti-T cell globulin (ATG) on immune recovery after myeloablative allogeneic peripheral blood stem cell transplantation. *PLoS One* (2015) 10(6):e0130026. doi:10.1371/journal.pone.0130026
39. Podgorny PJ, Liu Y, Dharmani-Khan P, Pratt LM, Jamani K, Luider J, et al. Immune cell subset counts associated with graft-versus-host disease. *Biol Blood Marrow Transplant* (2014) 20(4):450–62. doi:10.1016/j.bbmt.2014.01.002
40. Bosch M, Dhadda M, Hoegh-Petersen M, Liu Y, Hagel LM, Podgorny P, et al. Immune reconstitution after antithymocyte globulin-conditioned hematopoietic cell transplantation. *Cytotherapy* (2012) 14(10):1258–75. doi:10.3109/14653249.2012.715243
41. Rubio MT, Moreira-Teixeira L, Bachy E, Bouillie M, Milpied P, Coman T, et al. Early posttransplantation donor-derived invariant natural killer T-cell recovery predicts the occurrence of acute graft-versus-host disease and overall survival. *Blood* (2012) 120(10):2144–54. doi:10.1182/blood-2012-01-404673
42. Beziat V, Nguyen S, Exley M, Achour A, Simon T, Chevallier P, et al. Shaping of iNKT cell repertoire after unrelated cord blood transplantation. *Clin Immunol* (2010) 135(3):364–73. doi:10.1016/j.clim.2010.01.010
43. de Lalla C, Rinaldi A, Montagna D, Azzimonti L, Bernardo ME, Sangalli LM, et al. Invariant NKT cell reconstitution in pediatric leukemia patients given HLA-haploidentical stem cell transplantation defines distinct CD4+ and CD4- subset dynamics and correlates with remission state. *J Immunol* (2011) 186(7):4490–9. doi:10.4049/jimmunol.1003748

44. Dellabona P, Casorati G, de Lalla C, Montagna D, Locatelli F. On the use of donor-derived iNKT cells for adoptive immunotherapy to prevent leukemia recurrence in pediatric recipients of HLA haploidentical HSCT for hematological malignancies. *Clin Immunol* (2011) 140(2):152–9. doi:10.1016/j.clim.2010.11.015
45. Chaidos A, Patterson S, Szydlo R, Chaudhry MS, Dazzi F, Kanfer E, et al. Graft invariant natural killer T-cell dose predicts risk of acute graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. *Blood* (2012) 119(21):5030–6. doi:10.1182/blood-2011-11-389304
46. Malard F, Labopin M, Chevallier P, Guillaume T, Duquesne A, Rialland F, et al. Larger number of invariant natural killer T cells in PBSC allografts correlates with improved GVHD-free and progression-free survival. *Blood* (2016) 127(14):1828–35. doi:10.1182/blood-2015-12-688739
47. Rubio MT, Bouillie M, Bouazza N, Coman T, Trebeden-Negre H, Gomez A, et al. Pre-transplant donor CD4- invariant NKT cell expansion capacity predicts the occurrence of acute graft-versus-host disease. *Leukemia* (2017) 31(4):903–12. doi:10.1038/leu.2016.281
48. Vasu S, Geyer S, Bingman A, Auletta JJ, Jaglowski S, Elder P, et al. Granulocyte colony-stimulating factor-mobilized allografts contain activated immune cell subsets associated with risk of acute and chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2016) 22(4):658–68. doi:10.1016/j.bbmt.2015.12.015
49. Chen Y-B, Efebera YA, Johnston L, Ball ED, Avigan D, Lekakis LJ, et al. Increased Foxp3+helios+ regulatory T cells and decreased acute graft-versus-host disease after allogeneic bone marrow transplantation in patients receiving sirolimus and RGI-2001, an activator of invariant natural killer T cells. *Biol Blood Marrow Transplant* (2017) 23(4):625–34. doi:10.1016/j.bbmt.2017.01.069
50. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124(9):3725–40. doi:10.1172/JCI72308
51. O'Reilly V, Zeng SG, Bricard G, Atzberger A, Hogan AE, Jackson J, et al. Distinct and overlapping effector functions of expanded human CD4+, CD8α+ and CD4-CD8α- invariant natural killer T cells. *PLoS One* (2011) 6(12):e28648. doi:10.1371/journal.pone.0028648

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REVIEW
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Natural Killer T Cells in Cancer Immunotherapy

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Natural killer T (NKT) cells are specialized CD1d-restricted T cells that recognize lipid antigens. Following stimulation, NKT cells lead to downstream activation of both innate and adaptive immune cells in the tumor microenvironment. This has impelled the development of NKT cell-targeted immunotherapies for treating cancer. In this review, we provide a brief overview of the stimulatory and regulatory functions of NKT cells in tumor immunity as well as highlight preclinical and clinical studies based on NKT cells. Finally, we discuss future perspectives to better harness the potential of NKT cells for cancer therapy.

Keywords: natural killer T, glycolipid antigens, CD1d, dendritic cells, innate immunity

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INTRODUCTION

Both innate and adaptive immune systems respond to tumor cells and participate in immune-surveillance against tumor (1). Defined immune interactions in the context of cancer include recognition of tumor-associated antigens or cues by innate cell populations such as antigen-presenting cells (APCs) [macrophages and dendritic cells (DCs), neutrophils, and natural killer (NK) cells (2)]. Innate immune cells rely on germline encoded pattern recognition receptors to recognize and elicit prompt response against cancer-associated danger signals, and also augment components of the adaptive immune system, composed of antigen-specific B and T cells (1). One of the key players that link the innate and adaptive immune systems is the natural killer T (NKT) cells (3–5). NKT cells are innate-like T lymphocytes that possess ability to quickly respond to antigenic stimulation and rapidly produce copious amounts of cytokines and chemokines (6). This rapid effect can modulate both innate and adaptive immunity and is important in influencing host immune responses to cancer (7).

Natural killer T cells are a heterogeneous subset of specialized T cells (8). These cells exhibit innate cell-like feature of quick response to antigenic exposure in combination with adaptive cell's precision of antigenic recognition and diverse effector responses (9). Like conventional T cells, NKT cells undergo thymic development and selection and possess T cell receptor (TCR) to recognize antigens (10). However, unlike conventional T cells, TCR expressed by NKT cells recognize lipid antigens presented by the conserved and non-polymorphic MHC class 1 like molecule CD1d (11). In addition to TCRs, NKT cells also possess receptors for cytokines such as IL-12, IL-18, IL-25, and IL-23 similar to innate cells such as NK and innate lymphoid cells (12). These cytokine receptors can be activated by steady state expression of these inflammatory cytokines even in the absence of TCR signals. Thus, NKT cells can amalgamate signals from both TCR-mediated stimulations and inflammatory cytokines to manifest prompt release of an array of cytokines (13). These cytokines can in turn modulate different immune cells present in the tumor microenvironment (TME) thus influencing host immune responses to cancer. Their predominant tissue localization and ability to sense cancer-mediated changes in host lipid metabolism or breach in tissue integrity *via* recognition of endogenous lipids, makes NKT cells an ideal candidate for cancer immunotherapy (14).

TYPE I NKT CELLS

Broadly, CD1d-restricted NKT cells can be divided into two main subsets based on their TCR diversity and antigen specificities. Type I (invariant) NKT cells, so named because of their limited TCR repertoire, express a semi-invariant TCR (iTCR) α chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) paired with a heterogeneous Vβ chain repertoire (V β 2,7 or 8.2 in mice and V β 11 in humans) (8, 9). The prototypic antigen for type I NKT cells is galactosylceramide (α-GalCer or KRN 7000), which was isolated from a marine sponge as part of an antitumor screen (15). α-GalCer is a potent activator of type I NKT cells, inducing them to release large amounts of interferon-γ (IFN-γ), which helps activate both $CD8^+$ T cells and APCs (16, 17). The primary techniques used to study type I NKT cells include staining and identification of type I NKT cells using CD1d-loaded α-GalCer tetramers, administering α-GalCer to activate and study the functions of type I NKT cells and finally using CD1d deficient mice (that lack both type I and type II NKT) or Jα18-deficient mice (lacking only type I NKT) (10). Recent published study reported that Jα18-deficient mice in addition to having deletion in the *Traj18* gene segment (essential for type I NKT cell development), also exhibited overall lower TCR repertoire caused by influence of the transgene on rearrangements of several Jα segments upstream *Traj18*, complicating interpretations of data obtained from the Jα18-deficient mice (18). To overcome this drawback, a new strain of Jα18-deficient mice lacking type I NKT cells while maintaining the overall TCR repertoire has been generated, which should facilitate future studies on type I NKT cells (19). Type I NKT cells can be further subdivided based on the surface expression of CD4 and CD8 into $CD4^+$ and $CD4^-CD8^-$ (DN) subsets and a small fraction of $CD8^+$ cells found in humans (6, 20–24). Type I NKT cells are present in different tissues in both mice and humans but at higher frequency in mice (25, 26). Two very unique characteristics of type I NKT cells are that they possess dual reactivity to both self and foreign lipids, and that even at steady state type I NKT cell have an activated/memory phenotype (6, 27, 28). Functionally distinct subsets of NKT cells analogous to Th1, Th2, Th17, and TFH subsets of conventional T cells have been described. These subsets express the corresponding cytokines, transcription factors and surface markers of their conventional T cell counterparts (29–31). Type I NKT cells have a unique developmental program that is regulated by a number of transcription factors (32). Transcriptionally, one of the key regulators of type I NKT cell development and activated memory phenotype is the transcription factor promyelocytic leukemia zinc finger (PLZF). In fact, PLZF deficient mice show profound deficiency of type I NKT cells and cytokine production (33, 34). Other transcription factors that are known to impact type I NKT cell differentiation are c-Myc (35, 36), RORγt (37), c-Myb (38), Elf-1 (39), and Runx1 (40). Furthermore, transcription factors that control conventional T cell differentiation such as Th1 lineage specific transcription factor T-bet and Th2 specific transcription factor GATA-3 can also affect type I NKT cell development (41–43). Aside from transcription factors, SLAM-associated protein (SAP) signaling pathway can also selectively control expansion and differentiation of type I NKT cell (44, 45). Type I NKT cells have been shown to respond to both self and foreign α and β linked glycosphingolipids (GSL), ceramides, and phospholipids (46). Type I NKT cells have been reported to mostly aid in mounting an effective immune response against tumor (3, 5, 47–49).

TYPE II NKT CELLS

Type II NKT cells also called diverse or variant NKT cells, are CD1d-restricted T cells that express more diverse alpha-beta TCRs and do not recognize α-GalCer (50). Type II NKT cells are major subset in humans with higher frequency as compared to type I NKT cells (51). Due to absence of specific markers and agonistic antigens to identify all type II NKT cells, characterization of these cells has been challenging. Different methodologies employed to characterize type II NKT cells include, comparing immune responses between $J\alpha18^{-/-}$ (lacking only type I NKT) and $CD1d^{-/-}$ (lacking both type I and type II NKT) mice, using 24 αβ TCR transgenic mice (that overexpresses Vα3.2/Vβ9 TCR from type II NKT cell hybridoma VIII24), using a Jα18-deficient IL-4 reporter mouse model, staining with antigen-loaded CD1d tetramer and asses binding to type II NKT hybridomas [reviewed in Ref. (46)]. The first major antigen identified for self-glycolipid reactive type II NKT cells in mice was myelin derived glycolipid sulfatide (25, 26, 52). Subsequently, sulfatide and lysosulfatide reactive CD1d-restricted human type II NKT cells have been reported (53, 54). Sulfatide specific type II NKT cells predominantly exhibit an oligoclonal TCR repertoire (V α 3/V α 1-J α 7/J α 9 and V β 8.1/V β 3.1-J β 2.7) (25). Other self-glycolipids such as β GlcCer and β GalCer have been shown to activate murine type II NKT cells (55–57). Our group recently reported that two major sphingolipids accumulated in Gaucher disease (GD), β-glucosylceramide (β GlcCer) and its deacylated product glucosylsphingosine, are recognized by murine and human type II NKT cells (57). In an earlier study, we have also shown that lysophosphatidylcholine (LPC), lysophospholipid markedly upregulated in myeloma patients was an antigen for human type II NKT cells (58). Type II NKT cells can be distinguished from type I NKT cells by their predominance in humans versus mice, TCR binding and distinct antigen specificities (59). Crystal structures of type II NKT TCR-sulfatide/CD1d complex and type I NKT TCR-α-GalCer/CD1d complex provided insights into the mechanisms by which NKT TCRs recognize antigen (60). The type I NKT TCR was found to bind α-GalCer/CD1d complex in a rigid, parallel configuration mainly involving the α-chain. The key residues within the CDR2β, CDR3α, and CDR1α loops of the semi-iTCR of type I NKT cells were determined to be involved in the detection of the α-GalCer/CD1d complex (61). On the other hand, type II NKT TCRs contact their ligands primarily *via* their CDR3β loop rather than CDR3 α loops in an antiparallel fashion very similar to binding observed in some of the conventional MHC-restricted T cells (62). Ternary structure of sulfatide-reactive TCR molecules revealed that CDR3 α loop primarily contacted CD1d and the CDR3β determined the specificity of sulfatide antigen (63). The flexibility in binding of type II NKT TCR to its antigens akin to TCR–peptide–MHC complex

resonates with its greater TCR diversity and ability to respond to wide range of ligands. However, despite striking difference between the two subsets, similarities among the two subsets have also been reported. For example, both type I and type II NKT cells are autoreactive and depend on the transcriptional regulator PLZF and SAP for their development (55, 64, 65). Although, many type II NKT cells seem to have activated/memory phenotype like type I NKT cells, in other studies including ours, a subset of type II NKT cells also displayed naïve T cell phenotype ($CD45RA^+$, $CD45RO^-$, $CD62^{high}$, and $CD69^{-/low}$) (66, 67). Type II NKT cell is activated mainly by TCR signaling following recognition of lipid/CD1d complex (56, 68) independent of either TLR signaling or presence of IL-12 (65, 69).

In tumor and autoimmune disease models, type II NKT cells are typically associated with immunosuppression (70–72).

HOW DO NKT CELL TARGET TUMOR CELLS?

Several clues exist attributing a significant role of type I NKT cells in mediating protective immune response against tumors. Decreased frequency and function of type I NKT cells in the peripheral blood of different cancer patients is suggestive of their role in effective antitumor immunity (73–78). Increased frequency of peripheral blood type I NKT cells in cancer patients predicts a more favorable response to therapy (79, 80). Furthermore, recent studies found an association between number of tumor-infiltrating NKTs with better clinical outcome (79, 81). Notably, α-GalCer, the prototypic NKT ligand, was first discovered in a screen for antitumor agents (82). Many studies using genetic knockouts and murine models of tumor have been useful to discern the role of NKT cells in malignancy (83, 84). Type I NKT cells can lead to effective antitumor immunity by three mechanisms: (a) direct tumor lysis, (b) recruitment and activation of other innate and adaptive immune cells by initiating Th1 cytokine cascade, and (c) regulating immunosuppressive cells in TME (**Figure 1**).

DIRECT CYTOTOXICITY AGAINST TUMOR CELLS

Natural killer T cells can eliminate CD1d-expressing transformed cells by direct cytolysis using either perforin (85, 86), granzyme B, Fas ligand (FasL) (87, 88), or TNF-α-mediated cytotoxic pathways (89). Tumor cells expressing CD1d are mainly of myelomonocytic and B-cell lineages origin (90), and very few solid tumors have also been found to be CD1d-positive (91–95). Surface expression of CD1d on tumor cells is assumed to directly correlate with NKT cell-mediated cytotoxicity (96). With higher expression of CD1d, resulting in higher tumor cell lysis and thereby lower metastasis rates (92, 97), while lack of CD1d expression in tumors leads to their escape from recognition by NKT cells, and tumor progression in some models (90, 98, 99). These studies postulate that loss or downregulation of surface expression of CD1d favors tumor survival and permits tumor escape from NKT cell-mediated immunosurveillance. This concept is further strengthened by observations that downregulation of CD1d in human breast cancer and multiple Myeloma correlated with increased metastatic potential and disease progression (92, 99). Similarly, downregulation of CD1d by human papillomavirus in infected cervical epithelial cells was linked to their progression to cervical carcinoma (100). Another means by which tumor cells escape NKT cell-mediated antitumor response was shown in a mouse model of lymphoma, where shedding of tumor-associated glycolipids was shown to inhibit CD1-mediated presentation to NKT cells (101). Interestingly, in chronic lymphocytic leukemia (CLL), CD1d expression was found to increase during disease progression, counteracting the suggested role of CD1d as an anti-survival factor in cancer (102, 103). However, a recent study has shown that higher CD1d expression on CLL cells associated with disease progression actually led to impairment in both function and numbers of type I NKT cells (104). CD1d independent cytotoxic effect of NKT cells on various hematopoietic tumor cell lines have also been reported (98, 105, 106). Although, the mechanisms or tumor specific CD1d—glycolipid complex that helps NKT cells recognize and kill only CD1d-positive tumor cells and not normal cells is still enigmatic. Membrane glycolipids especially GSL such as globotriaosyl-ceramide (Gb3Cer/CD77), gangliosides (GD2, GD3, and GM2) have been shown to be overexpressed and altered in a range of cancers compared to normal tissue (107, 108). Shedding of some of the gangliosides and GSL into the TME have also been reported. Recognition of these overexpressed GSL and gangliosides on the surface of tumor cells may lead to differential recognition and killing of tumor cells by NKT cells.

CYTOKINE-MEDIATED MODULATIONS OF EFFECTOR CELLS

In addition to direct tumor lysis, type I NKT cells can activate and recruit both innate and adaptive immune cells, such as DCs, NK cells, B cells, and T cells through rapid secretion of cytokines on activation (109). This is underscored by the observed increase in NK cells, $CD8^+$ T cells and macrophages among tumor-infiltrating leukocytes brought about by α-GalCer injection (110). Owing to partially activated state and the presence of preformed cytosolic mRNA for various cytokines, type I NKT cells can rapidly produce broad spectrum of Th1 and Th2 cytokines on activation (111–113). The nature and magnitude of the type I NKT cell cytokine response is contingent on a number of variables that include the glycolipid antigen, subsets of NKT, and tissue location. For example, while α-GalCer-activated type I NKT cell primarily elicits an IFN-γ, a synthetic analog of α-GalCer with a truncated lipid chain OCH elicits majorly elicits IL-4 production (114). Further, DN liver subset of type I NKT was found to confer protection as compared to $CD4^+$ liver subset or IL-4 inducing thymic type I NKT cells in MCA-induced fibrosarcoma model (115). Type I NKT cells play a crucial role in induction of early immune responses to tumor by influencing DC maturation (116). Mostly DCs found in TME are immature and inept at activating specific T cells (117). Maturation and differentiation of DCs is important in shaping the magnitude and polarization of T cell-mediated response (118).

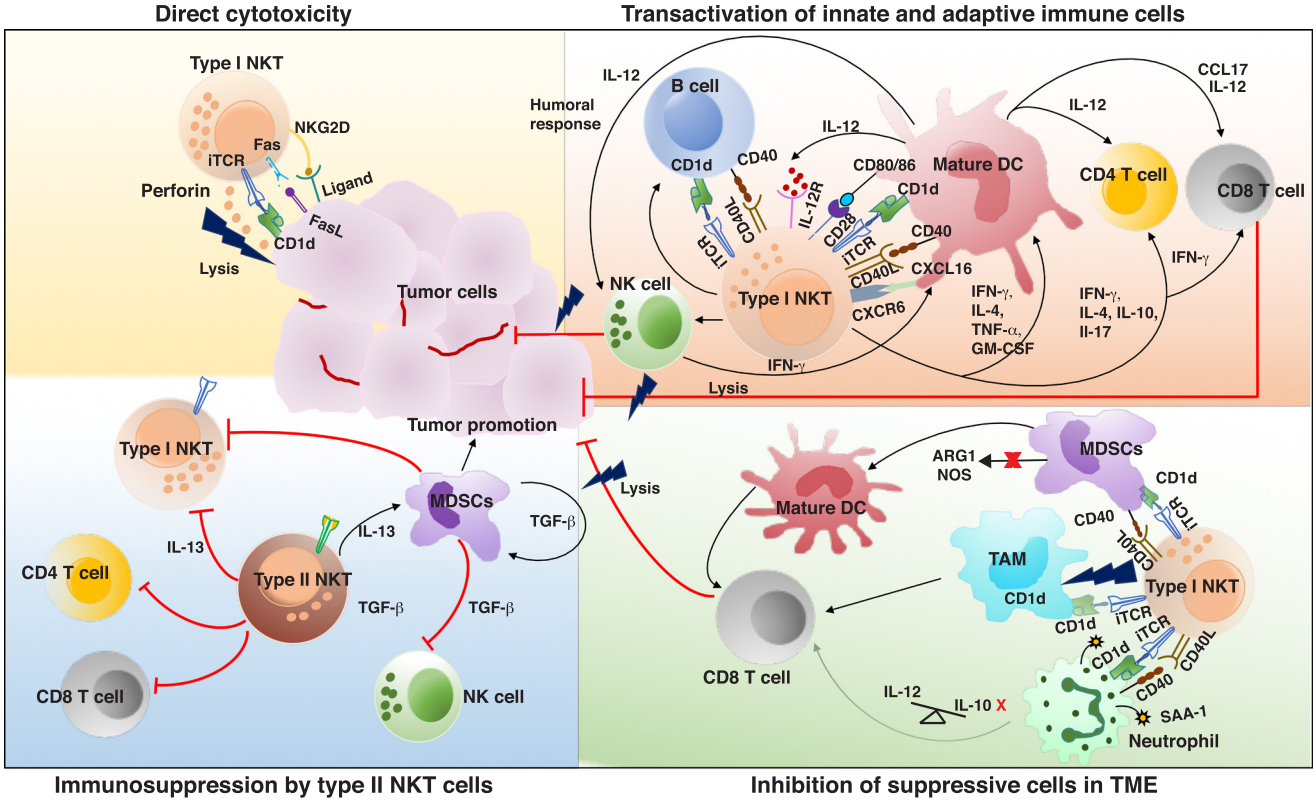

FIGURE 1 | Interactions and cross talk between different subsets of natural killer T (NKT) cells and other immune cells in tumor microenvironment (TME). Antigenic activated type I NKT cells can promote antitumor immunity by directly killing tumor cells in a CD1d-dependent and -independent mechanism. Type I NKT cells can recognize self or foreign lipid antigens presented by different CD1d-expressing antigen-presenting cells (APCs) in TME such as dendritic cells (DCs), TAMs, B cells, and neutrophils. On activation type I NKT cells can produce various Th1 and Th2 cytokines leading to reciprocal activation and or modulation of the APCs as well as other effector lymphocytes. Major type I NKT cytokine that helps activate DCs and $CD8^+$ T cells is interferon-γ (IFN-γ). Type I NKT cells and DCs reciprocally activate each other *via* CD1d-TCR/lipid antigen and CD40–CD40L interactions. IL-12 produced by type I NKT cell matured DCs stimulates natural killer (NK), NKT, and MHC-restricted T cells to produce more IFN-γ which can secondarily activate other antitumor-promoting effector lymphocytes. Mature DCs derived factors as well as costimulatory receptors can activate $CD8^+$ T cells to promote adaptive immunity. Type I NKT cells enhance tumor immunity by subduing the actions of tumor supporting cells such as TAMs, MDSCs, and suppressive neutrophils. In some instances, type II NKT cells have been shown to suppress the activation of type I NKT cells, T cells, NK cells and enhance development of tumor-associated MDSCs, aiding in tumor growth. iTCR, invariant TCR; IL-12, interleukin 12; IL-12R, IL-12 receptor; CXCL16, chemokine ligand 16; CXCR6, chemokine receptor 6; MDSCs, myeloid-derived suppressor cell; TAM, tumor-associated macrophages; ARG1, arginase 1; NOS, nitrous oxide synthase; SAA-1, serum amyloid A1; TCR, T cell receptor.

A mutually costimulatory interaction between DC and type I NKT cells ensues following encounter with CD1d/antigen complexes displayed by immature DCs. Ligation of APC-expressed CD40 with upregulated CD40L on type I NKT cells induces DCs' maturation with higher surface expression of MHC class II, the costimulatory molecules CD40, CD80, CD86, CD70 and the endocytic receptor DEC205 and potent IL-12 production (119, 120). Sustained IL-12 secretion by mature DCs induces IFN-γ production by NKT cells (121–126). Mature DCs reciprocally enhance expression of CD40L and IL-12 receptor on type I NKT cells providing a strong feed forward signal that amplifies IFN-γ responses (119, 127). Ligation of chemokine receptor CXCR6 on the surface of type I NKT cells by its ligand CXCL16 expressed on APCs can also provide costimulatory signal resulting in robust α-GalCer-induced type I NKT activation (128, 129). α-GalCer-induced type I NKT cells can provide cognate licensing for cross-priming CD8 alpha $^+$ DCs to produce CCL17, which attracts $CCR4^+CD8^+$ T cells for subsequent activation (130, 131). Presence of phenotypic maturation ligands, suitable cytokines (IFN-γ), other functional immunostimulatory factors on type I NKT licensed DC can induce activation of CD8 T cells and their polarization toward antitumor effector function (119, 132–134). Release of various cytokines such as IL-2, IL-12, and IFN-γ by type I NKT cells leads to activation and expansion of NK cells into lymphokine-activated killer (LAK) cells. These LAK cells upregulate the effectors or adhesion molecules such as perforin, NKp44, granzymes, FasL, and TRAIL and secrete IFN-γ to adhere and lyse tumor cells (135, 136). Type I NKT cells can form bidirectional interactions with B cells, wherein B cells can present lipid antigens to type I NKT cells through CD1d (137) and NKT cells can license B cells to effectively prime and activate antitumor CTL responses (138, 139) and can also directly provide B cell help to enhance and sustain humoral response (57, 140–143).

ALTERING THE EFFECTS OF IMMUNOSUPPRESSIVE CELLS IN TME

Tumor-associated macrophages (TAMs) are prominent immunosuppressive immune cells present in the TME (144). TAMs contribute to tumor progression by enhancing angiogenesis, tumor cell invasion, suppression of NK, and T cell responses (145, 146). Type I NKT cells were found to co-localize with CD1d-expressing TAMs in neuroblastoma and kill TAMs in an IL-15 and CD1d-restricted manner (90, 147). Besides TAMs, type I NKTs can alter the effects of $CD1d^+$ myeloid-derived suppressor cells (MDSCs)-mediated immune suppression. MDSCs are heterogeneous population of cells of myeloid origin, which often accumulate during tumor growth and contribute to immune escape and tumor progression (148). In a model of influenza A viral infection, adoptive transfer of type I NKTs inhibited arginase 1 and nitrous oxide synthase-mediated suppressive activity of MDSCs. The ability of type I NKT cells to abolish the suppressive activity of MDSCs was found to be dependent on CD1d and CD40 interactions (149). In a tumor model, α-GalCer-loaded MDSCs facilitate conversion of immature MDSCs to mature APCs capable of eliciting cytotoxic NK and T cell immune response against cancer cells (150). De Santo et al. reported type I NKT cell-mediated reversal of immunosuppressive activity of neutrophils in melanoma, serum amyloid A1 (SAA-1) derived as consequence of tumor-associated inflammation induced differentiation of IL-10-producing neutrophils causing suppression of antigen-specific T cell responses. Conversely, SAA-1 also enhanced CD1d-CD40 dependent interaction between the suppressive neutrophils and type I NKT cells. This crosstalk lead to dephosphorylation of Erk, p38, and phosphatidylinositol-3-OH kinase, which in turn lead to inhibition of IL-10 secretion and promotion of IL-12 production by neutrophils, reinstating the proliferation of antigen-specific $CD8^+$ T cells (151).

SUPPRESSION OF TUMOR IMMUNITY BY TYPE II NKT CELLS

In contrast to the established protective role of type I NKT in most murine tumor models, type II NKT cells have been shown to possess a more suppressive/regulatory role in tumor immunity (4, 59, 65, 152). Comparison of antitumor response in Jα18-deficient mice (which lack only type I NKT) with CD1d deficient mice (which lack both type I and II NKT cell) revealed that type II NKT cells were responsible for suppression of antitumor responses in several murine tumor models (152–154). Furthermore, sulfatide-reactive type II NKT cells was shown to antagonize the protective antitumor immune responses mounted by α-GalCer-stimulated type I NKT cells (47). Sulfatide activated murine type II NKT cells were reported to inhibit proinflammatory functions of type I NKT cells, conventional T cells and DCs and also induce tolerization of myeloid DCs (155). A major attribute of type II NKT-mediated suppression of tumor immunity is elevated production of IL-13 and IL-4 cytokines capable of skewing the cytokine response predominantly toward tumor-promoting Th2 type. In a mouse model of transformed recurrent fibrosarcoma, type II NKT cells was shown to suppress cytotoxic T cells through IL-13 production *via* IL4R and STAT6 axis and also induce MDSCs producing immunosuppressive cytokine TGF-β (71). Similarly, LPC reactive type II NKT cells have been shown to preferentially produce IL-13 and exhibit immunoregulatory role in myeloma patients (58). Concentration of LPC, a phospholipid associated with inflammation, was found to be elevated in myeloma sera. Progressive myeloma disease is associated with a decline as well as dysfunctional activation of type I NKT cells and increased frequency of type II NKT cells (58, 78). The preferential production of IL-13, a cytokine implicated in promoting tumor growth, by LPC specific type II NKT cells suggests their role in disease progression (58). Recently, we have shown a possible implication of type II NKT cells in the development of B-cell malignancies associated with GD. GD is uniquely associated with increased cancer risk particularly with multiple myeloma (156). GD is a lysosomal storage disorder caused due to an inherited deficiency of the acidic β-glucosidase enzyme, resulting in marked accumulation of β-glucosylceramide (β GlcCer) and its deacylated product, glucosylsphingosine (LGL1). Increased frequency of LGL1-specific type II NKT cells with reduced frequency of type I NKT cells was observed in murine model and patients of GD. Interestingly, LGL1 reactive type II NKT cells demonstrated follicular helper T cell phenotype and were able to provide help to germinal center B cells to produce lipid-reactive antibodies (57). In both patients and mice with GD having monoclonal gammopathy, the monoclonal immunoglobulin was found to be reactive to Gaucher lipids (157). Though studies described earlier hint to pro- and antitumor functional dichotomy between type I and type II NKT, respectively, there are several emerging evidences challenging this paradigm, and the pro/antitumor roles of these cells may be context or activation-dependent. While type I NKT cells have been shown to assume immune-suppressive role in several tumor settings (158–161), a recent study showed that CpG-activated type II NKT cells secreted IFN-γ rather than IL-13, which in turn enhanced the activation and function of $CD8^+$ T cells and contributed to the antitumor effect of CpG in the B16 melanoma model (162).

PRECLINICAL STUDIES

There are several theoretical advantages for harnessing type I NKT cells against cancer. NKT cell can simultaneously target both MHC positive and negative tumor cells due to ability to activate both antigen-specific $CD8^+$ T cells and NK cells. Second, type I NKT cells show strong adjuvant activity thereby activating both innate and adaptive immune cells. Finally, NKT cells have the ability to convert immature and or tolerogenic DCs found in tumor bed into mature DCs capable of initiating tumor specific $CD8^+$ T cell response. However, major limitations in targeting NKT cell for tumor treatment are the cancer-mediated reversible defect in the number and function of type I NKT cells (73, 74, 76–78, 80, 163, 164). Circulating type I NKT cell deficiency leads to decreased proliferation and IFN-γ production by type I NKT cells, consequently skewing immune response to a pro-tumor Th2 cytokine profile (73, 74, 76–78, 80, 163, 164). In line with this observation, reduced type I NKT cell frequency was shown to correlate with poor survival, while increased type I

NKT cell numbers capable of making IFN-γ have positive prognostic value for survival in cancer patients (74, 80, 163–167). To restore the numbers and function of type I NKT cells in cancer patients and murine models, several approaches like administration of α-GalCer either alone or with IL-12, administration of APCs (DC or irradiated tumor cells) with α-GalCer, adoptive transfer of *ex vivo* expanded and/or activated type I NKT cells, and finally a combination of α-GalCer with antibodies or fusion proteins have been exploited. Data from numerous studies on variety of experimental and spontaneous murine tumor models have shown significant role for NKT cells in launching of powerful antitumor immune responses (**Table 1**).

Type I NKT cells were shown to be indispensable in mediating IL-12-mediated antitumor effects in low- and moderate-dose IL-12 treatment models (91, 169, 204). IL-12 was found to activate the NKT cell-mediated lysis of tumor cells and also induce IFN-γ production by type I NKT cells. Administration of soluble α-GalCer leads to activation and expansion of type I NKT cells, creating a milieu of immune-stimulatory cytokines including IFN-γ and costimulatory molecules, resulting in maturation of host APC thus enhancing antitumor T cell response. IFN-γ production by type I NKT cell was found to be pivotal in inducing NK cell activation, proliferation of memory $CD4^+$ and $CD8^+$ T cell effector functions, and inhibiting angiogenesis, all contributing to effective immune response against tumor. One of the major drawbacks of administering soluble free α-GalCer is that it causes type I NKT cell to adopt an anergic state causing unresponsiveness to sequential stimulation with α-GalCer (205). To circumvent this problem, mice were administrated DCs loaded with either α-GalCer alone or in combination with tumor antigens (180, 182, 187, 190, 206). α-GalCer-pulsed APCs induced a more prolonged cytokine response as well as powerful antitumor immune response than α-Galcer alone (180, 207). Another recent immunotherapeutic approach has been to load autologous irradiated tumors, which act as source of tumor antigens with α-GalCer (121, 182, 187, 188). A big improvement of this approach is CD1d-mediated cross-presentation of endogenous glycolipids and or α-GalCer from tumor cells to NKT cells, leading to DC maturation and consequently effective long-term T cell resistance to the tumor (128). Another approach involved adoptive transfer of *ex vivo* expanded and or activated type I NKT cells to restore type I NKT cell numbers in preclinical models of melanoma and lymphoid neoplasms (194, 196, 208). This approach has been shown to be more effective compared to the i.v. administration of α-GalCer (194). Finally, combination therapy using monoclonal Abs targeting CD1d alone or in combination with tumor cell death inducing and immunomodulating mAbs has emerged as promising immunotherapeutic candidate against CD1d-negative cancers (199). Stirnemann and Corgnac et al. attempted to target α-GalCer to tumor site by using constructs consisting of either α-GalCer/CD1d molecules alone or fused to tumor Ag specific scFv fragments in a colon carcinoma and murine melanoma model, respectively, and reported specific tumor localization of type I NKT activating potent antitumor responses compared to α-GalCer alone (200, 201). Preclinical studies obtained using chimeric antigen receptors (CARs) with engineered type I NKT cells have yielded promising result. CAR-bearing type I NKT cells effectively localized to the tumor sites, eliminating tumor cells, and exhibited potent and specific cytotoxicity against TAMs without producing graft-versus-host disease (202). Recently, $CD62L^+CD19^-$specific CAR-engineered NKT cells have been shown to possess superior therapeutic activity in a B-cell lymphoma model (203).

CLINICAL TRIALS OF NKT CELLS

Based on the preponderance of data from preclinical mice models, showing that activation of type I NKT cells plays a substantial role in providing protection against tumor growth and metastasis of several tumors, different clinical trials have been initiated to harness NKT cell's antitumor potential (**Table 2**). However, while direct administration of soluble α-GalCer in cancer patients was well tolerated, it failed to yield any clinical response (209). Potential reasons for the low efficacy in human trials could be attributed to insufficient drug delivery, inter-individual variability and very low type I NKT cell numbers at baseline, induction of anergy or regulatory IL-10-producing type I NKT cells (205, 210, 211). To overcome these limitations of soluble α-GalCer administration and improve NKT-mediated antitumor responses, multiple clinical trials were performed using autologous α-GalCer-pulsed APCs in patients with advanced and recurrent non-small cell lung cancer, head and neck squamous cell carcinoma (**Table 2**). Different types of APCs and alternative routes to efficiently target activated NKT cells directly to cancer region were optimized to achieve objective antitumor responses. Though promising, this strategy too suffers from certain caveats like the treatment is again dependent on the baseline NKT levels, which are inevitably low in most cancer patients. Second, it is difficult to obtain large number of autologous monocyte-derived DCs (moDCs) from immune suppressed cancer patients and also cumbersome for *ex vivo* generation of DCs in compliance with good manufacturing practices regulations. Another strategy involves adoptive transfer of *in vitro*-expanded autologous type I NKT populations. Clinical trials using this approach in non-small cell lung cancer and advanced melanoma do show increase in type I NKT expansion and elevated serum IFN-γ levels *in vivo*; however, further optimization of the protocols and perhaps combination approaches such as combining with immune checkpoint blockade may be needed to obtain a significant clinical response. Remarkably, combining activated type I NKT cells and α-GalCer-pulsed APCs has been reported to enhance the low antitumor response observed with monotherapy employing either NKT or APCs alone in head and neck squamous cell carcinoma patients (212, 213). Similarly, combining regimen of α-GalCer-pulsed DCs and the immune-modulatory drug lenalidomide in treating multiple myeloma patients leads to type I NKT expansion with downstream activation of NK, monocytes and decrease in tumor-associated M spikes (214).

EMERGING APPROACHES

Adoptive Transfer of Type I NKT Cells

Advanced cancer patients with low NKT cell numbers may benefit from development of *in vitro* methods for generation of large

TABLE 1 | Preclinical studies on natural killer T (NKT) cell-targeted immunotherapeutics.

Therapy regimen	Murine model/ cancer type	Outcome	Immunological response	Reference
Injection of α-GalCer/IL-12				
IL-12 injection	FBL-3 erythroleukemia, B16 melanoma	Inhibition of tumor growth and metastasis	1. NKT cell produced IL-12-mediated tumor rejection 2. NKT cell-mediated direct cytotoxicity	(168)
α-GalCer (i.v.)	Colon 26 hepatic metastasis adenocarcinoma model	1. Regression of Colon 26 nodules 2. Inhibition of tumor growth in liver	Activation of natural killer (NK) cells, T cells, and $NK1^+$ T cells	(169)
α-GalCer (i.p.)	B16 melanoma cells	Prevented liver metastasis	NK cell-mediated killing	(170)
α-GalCer (i.v.)	Spontaneous liver metastasis of reticulum cell sarcoma (M5076)	Suppressed growth of established liver metastases, prolonged survival time	Increased IFN-γ and IL-12 production by liver NKT cells	(171)
α-GalCer + OVA (i.v.) or OCH + OVA (i.v.)	C57BL/6 mice s.c. injected with murine thymoma that express OVA	Slower growth of tumor up until 10 days followed by rapid regression	Induction of cytotoxic effector cells with potent antitumor activity	(172)
α-GalCer (i.v.) + IL-12 i.p.	BL6-B16 melanoma	Effective against metastatic tumor	NKT activation with induction of Th1 immunity and $CD4^+$, $CD8^+$ T cells, and B cells activation	(173)
α-GalCer (i.v.) + IL-12 i.p.	BL6-B16-HM melanoma	Prevention of tumor at early stages	NKT and NK activation	(174)
α-GalCer (i.p.) 2 μg single dose	B16-BL6 melanoma cells	Subcutaneous tumor growth and tumor-induced angiogenesis at early time points	1. IFN-γ-dependent inhibition of tumor angiogenesis by α-GalCer 2. α-GalCer-activated NKT cells and secondarily activated NK cells contributed to the inhibition of endothelial cell proliferation *via* their IFN-γ production	(175)
α-GalCer (i.p.)	MCA induced sarcoma, mammary carcinomas in Her-2/neu transgenic mice, spontaneous sarcomas in $p53^{-/-}$mice	Inhibition of primary tumor formation	1. NK cell and T cell activation 2. Higher serum levels of IFN-γ and IL-4 3. TRAIL-dependent antimetastatic activity	(176)
α-GalCer (i.p.) + IL-12 i.p.	TRAMP prostate tumor	Reversion of prostrate tumor-mediated IFN-γ secretion by type I NKT cells	α-GalCer and IL-12 bypasses tumor cell-induced block of IFN-γ production	(91)
α-GalCer (i.v.) single dose	Mantle cell lymphoma	1. Inhibition of disease development 2. Delayed disease progression	NKT activation	(177)
α-GalCer (i.p.) 2 μg	5T33 multiple myeloma	Significant reduction in micro vessel density	Possible role of IFN-γ from stimulated type I NKT cells in the antiangiogenic process	(178)
Priming with DNA vaccine expressing human papillomavirus type 16 $E7^+$ α-GalCer and boosting with E7-pulsed DC-1	E7-expressing tumor model TC-1	Prolonged survival of vaccinated animals	E7-specific $CD8^+$ T-cell responses	(179)
***Ex vivo*-generated dendritic cell (DC) loaded with α-GalCer/dying tumor cells**				
α-GalCer-loaded DC	B16 melanoma cells, LLC (lung metastatic model)	1. Inhibition of tumor metastasis in liver and lung 2. Eradication of established tumor metastasis	Activation of NKT cells	(180)
α-GalCer-loaded ES DC genetically engineered to express a model antigen OVA + SLC/CCL21	MO4 (ova expressing melanoma)	1. Protection against tumor 2. Enhanced antitumor activity, rejection of tumor cell	Synergic activation of antigen reactive CTL and α-GalCer-activated NKT cells	(181)

(*Continued*)

TABLE 1 | Continued

Therapy regimen	Murine model/ cancer type	Outcome	Immunological response	Reference
α-GalCer + CD4-hepatic NKT	MCA-induced sarcoma	Tumor regression	NA	(115)
α-GalCer-loaded irradiated tumor cells	A20 lymphoma, Meth A sarcoma, J558	Long-lived tumor immunity	1. Type I NKT, $CD8^+$ T cells, $CD4^+$ T cells contribute to tumor resistance 2. Activation and proliferation of antigenic specific T cells 3. Secretion of IFN-γ and IL-2	(182)
α-GalCer-loaded DC	Ductal pancreatic adenocarcinoma	Decrease in tumor growth and prolonged survival	Expansion of IFN-γ-producing NKT	(183)
α-GalCer-loaded tumor cell	A20 lymphoma	Tumor regression, resistance to tumor challenge	$CD4^+$ T cells mediate antitumor activity	(184)
α-GalCer-loaded tumor cell	B16 melanoma cells, WEHI-3B myelomonocytic leukemia, EL4 thymoma tumor cells transfected with CD1d	Better survival with metastatic development thwarted	NKT and NK cell activation with induction of IFN-γ and IL-12p70 secretion	(185)
BM DC loaded with combination of tumor Ag and α-GalCer and anti-CD25 Ab	B16 melanoma cells	Delayed onset of tumor growth	Prolonged proliferative burst of responding $CD8^+$ T cells	(186)
α-GalCer-loaded irradiated tumor cells	VK*Myc mice, AML-ET09G, Eu-myc lymphoma	Reduction in tumor load, resistance to rechallenge	1. Expansion NKT and NK cells 2. IL-12-dependent IFN-γ production by NKT and NK cells	(187)
α-GalCer-loaded mature DC	5T33 multiple myeloma	Increased survival	Increased IFN-γ and Th1 response that tapers off at the end of disease	(178)
α-GalCer-loaded irradiated tumor cells	Multiple myeloma (MOPC315BM)	1. Retarded tumor growth 2. Regression of established tumors 3. Protection of surviving mice from tumor rechallenge	1. Expansion and activation of NKT cell *in vivo* 2. Induction of strong myeloma specific antibodies and $CD8^+$ CTL and memory T cells 3. Decreased Treg frequency	(188)
α-GalCer delivery to $CD8a^+$ DCs with anti-DEC205 decorated nanoparticles	B16 F10 melanoma cells expressing Ova	Potent antitumor responses	Type I NKT-mediated transactivation of NK cells, DCs, and gDT cells	(189)
α-GalCer-loaded irradiated tumor cells	C1498 leukemia model	1. Prevention of new leukemia development however no protective benefit in established leukemia	NKT cells are activated by $langerin^+CD8^+$ DC leading to generation of $CD4^+CD8^+$ T cells	(190)
α-GalCer loaded in $CXCL16^{hi}$ BMDCs	B16 melanoma model	Inhibition of metastasis	Increased IFN-γ^+ and $Tbet^+$ type I NKT cells, enhanced serum IFN-γ levels	(191)
α-GalCer-loaded tumor cell + TLR9 agonist (CpG1826)	Colon cancer	1. Inhibition of established tumor 2. Prolonged survival of tumor bearing mice 3. Resistance to tumor rechallenge	1. Type I NKT activation and DC maturation 2. IFN-γ secretion by type I NKT and NK cells 3. Redirection of Th2 response toward Th1 immune response by DC produced IL-12	(192)
α-GalCer-loaded DCs + tumor cells	B-cell lymphoma	Potent long-lasting tumor-specific antitumor immune response	1. Type I NKT cells secreting IFN-γ 2. T cells and NK cell-mediated antitumor effect	(193)
Adoptive transfer of *ex vivo*-expanded NKT cells				
IL-12-activated NKT i.v. injection (4 times)	B16 melanoma cells	Inhibition of tumor metastasis	Strong cytotoxic activity by activated NKT on metastasized tumor cells in liver	(194)
In vitro-expanded $CD8^+$ NKT cells redirected with humanized bispecific antibody F(ab′)2HER2xCD3	HER2-expressing ovarian carcinoma	Rapid tumor regression with prolonged survival	High efficacy of target cell killing by $CD8^+$ NKT	(195)
α-GalCer + *ex vivo*-expanded NKT	C1R B-cell lymphoblasts	Reduced growth of $CD1d^+$ leukemic cells and eradication of neoplastic clone	1. NKT cell-mediated cytotoxicity on $CD1d^+$ nodules 2. Presence of NKT cells infiltrating lymphoid nodules	(196)

(*Continued*)

TABLE 1 | Continued

Therapy regimen	Murine model/ cancer type	Outcome	Immunological response	Reference
Tumor-sensitive T cells + $CD25^+$ NKT cells + epigenetic drug decitabine	Carcinoma	Prolonged survival of animals bearing metastatic tumor cells	1. Decitabine functioned to induce the expression of highly immunogenic cancer testis antigens in the tumor, while also reducing the frequency of myeloid-derived suppressor cells (MDSCs) 2. The presence of $CD25^+$ NKT cells rendered T cells resistant to remaining MDSCs	(197)
Monoclonal antibodies stimulating NKT and α-GalCer with fusion proteins				
Anti-CD1d mAbs	4T1 mammary carcinoma, R331 renal carcinoma and CT26L5 colon adenocarcinoma	Suppression of established tumor growth	1. Activation of $CD1d^+$ antigen-presenting cell to produce tumor inhibiting IFN-γ and IL-12 2. Blocking of type II NKT cells activity in these models	(198)
Combination mAbs anti-$DR5^+$ $CD137^+CD1d$ (1DMab)	4T1 mammary carcinoma, R331 renal carcinoma, and CT26L5 colon adenocarcinoma	Suppression and or eradication of established tumors	Tumor rejection was dependent on $CD8^+$ T cells, IFN-γ, and CD1d and partially dependent on NK cells and IL-12	(199)
α-GalCer-loaded recombinant soluble (sCD1d) + HER2-specific scFv antibody fragment	HER2-expressing B16 melanoma model	Potent inhibition of lung metastasis	Specific localization to tumor site and accumulation of type I NKT, NK, and T cells at tumor site	(200)
α-GalCer-loaded sCD1d fusion proteins	MC38 colon carcinoma transfected with human CEA	Inhibition of tumor growth	1. Strong and prolonged reactivity of type I NKT cells 2. IFN-γ production by NK and NKT cells 3. Direct lysis by NKT cells	(201)
Type I NKT chimeric antigen receptor (CAR)				
CAR.GD2 NKT with CD28, 4-1BB	Metastatic neuroblastoma	Potent antitumor activity and long-term survival	1. Potent dose dependent cytotoxicity against GD2-positive neuroblasts 2. Enhanced *in vivo* persistence of NKT cells with systemic elevation of Th1 cytokines 3. Effective localization to tumor site without inducing GVHD	(202)
$CD62L^+$ CAR.CD19 NKT	B-cell lymphoma	Prolonged survival of tumor bearing mice and sustained tumor regression	$CD62L^+$ NKTs have prolonged persistence *in vivo*	(203)

numbers of functional NKT cells which can be further used for adoptive transfers. NKT cells have been generated from $CD34^+$ cells isolated from cord blood using IL-15 and stem cell factor (flt-3 ligand) in liquid culture system. Watarai et al. successfully differentiated murine induced pluripotent stem cells (iPSCs) into functional NKT cells *in vitro* that secreted large amounts of Th1 cytokine IFN-γ acting as adjuvant and antitumor agent (223). Recently, protocol to generate human type I NKT cells *in vitro* from iPSC that are competent in eliciting antitumor activity has been generated (224). Human type I NKT cells can also be reprogrammed to pluripotency followed by redifferentiation back to type I NKT cells *in vitro* using an IL-7/IL-15-based cytokine combination (225). The immunological features of re-differentiated type I NKT cells and their unlimited availability from iPSCs offer a potentially effective immunotherapy against cancer. Functionally mature human NKT cells have been also generated from bone marrow-derived adult hematopoietic stem-progenitor cells by expansion with CD1d-Ig-based artificial-presenting cells (226). Owing to the feasibility of producing large quantities of competent NKT cells, stem cell-derived type I NKT cells offer a promising strategy for effective anticancer immunotherapy.

ALTERNATE LIGANDS

As discussed earlier, while α-GalCer is a potent activator of type I NKT cells, α-GalCer suffers from few drawback that limits its use as effective cancer immunotherapeutic. For example, α-GalCer induces anergy in type I NKT cells. This has led to preclinical exploration of several alternate ligands that are now poised to enter the clinic. Synthetic glycolipids or α-GalCer analogs chemically modified to induce more precise and predictable cytokine profile than α-GalCer have been synthesized and tested. These analogs as compared α-GalCer, show superior anticancer immunity in tumor mouse models and therefore hold great potential as an alternative vaccine adjuvant (227–229). As compared to α-GalCer, alternative non-glycosidic type I NKT-cell agonist threitol ceramide promoted stronger activation of human and mouse type I NKT cells and stronger antitumor responses in comparison to α-GalCer, making it potential candidate for NKT cell-based clinical trials (230). Another interesting prospect is encapsulating α-GalCer or other lipids in nanoparticle carriers or liposomes decorated with Abs or ligands to target specific APCs. These approaches have several advantages like slower release of α-GalCer, specific targeting

TABLE 2 | Clinical studies using natural killer T (NKT) cell-targeted immunotherapeutics.

Treatment	Injection site, number of injections/cycles	Tumor type	Number of patients	Safety	Clinical outcome	Immunological response	Reference
Direct α-GalCer injection							
α-GalCer	i.v., 50–4,800 μg/m^2; 3 days 4 weekly cycle	Solid tumors	24	No dose limiting toxicity	1. 7/24 patient stable disease for 123 days 2. No clinical response	1. Transient decrease in type I NKT and natural killer (NK) cells from circulation 2. Increased serum cytokine levels of IFN-γ and GM-CSF in 5/24 patients 3. Cytotoxicity in 7/24 patients. 4. The effect was dependent on pretreatment type I NKT cell numbers.	(209)
***Ex vivo*-generated dendritic cell (DC) pulsed with α-GalCer**							
α-GalCer-pulsed CD1d-expressing immature monocyte-derived DCs (moDCs)	i.v., 2 doses over 2-week cycle	Metastatic malignancy	12	No severe toxicity	1. 2/12 patients had decreased serum tumor markers 2. 1 subject developed extensive necrosis of tumor-infiltrating bone marrow 3. 2 patients with hepatic infiltration had reduction in serum hepatocellular enzyme levels. 4. Clinically apparent treatment specific inflammatory response at tumor sites	1. NKT cell, T cell activation 2. Increase in NK cell numbers, activation and enhanced cytotoxicity 3. Increased IFN-γ (10/10) and IL-12 (6/9) levels in serum	(215)
α-GalCer-pulsed IL-2/GM-CSF cultured PBMCs	i.v., 4 doses, 5×10^7 cells (level 1) 5 patients, 2.5×10^8 cells (level 2) 3 patients, 3×10^9 cells (level 3) 3 patients	Non-small cell lung cancer	11	No severe toxicity	Stable disease in 3 patients	1. Expansion of type I NKT cells in 3/11 patients 2. Elevated IFN-γ mRNA levels in 1/11 patients	(216)
α-GalCer-pulsed immature moDCs	i.v., 4 injections of 1×10^9 cells	Non-small cell lung cancer	17	No severe toxicity	Stable disease in 5 patients, median survival time 18.6 months	1. Expansion of type I NKT cells in 16/17 patients 2. Elevated IFN-γ-producing cells by ELISPOT in 10/17 patients	(217)
α-GalCer-pulsed immature moDCs	4 treatments total with iv., 2 treatments, and intradermal (i.d.) 2 treatments, doses ranging from 5×10^5, 5×10^6, and $2–5 \times 10^7$ cells	Metastatic solid tumor	12	Safe and well tolerated	1. Stable disease in 6/10 patients 2. 3 patients show minor objective defined as reduction in tumor mass/marker 3. 9/12 had transient therapy related tumor inflammation	Dose of 5×10^6 *via* i.v. route gave the most reproducible result of NKT activation resulting in increased circulating type I NKT cells levels with NK and T cell activation and increased serum IFN-γ levels	(218)
α-GalCer-pulsed IL-2/GM-CSF cultured PBMCs	i.v., 1 injection	Non-small cell lung cancer	4	No serious toxicity	NA	1. Increased mobilization of type I NKT cells into primary site of the lung cancer 2. Augmented IFN-γ-producing ability of tumor-infiltrating type I NKT cells	(219)
α-GalCer-pulsed antigen-presenting cell (APCs)	Nasal sub-mucosal injections, 2 treatments with 1-week interval	Head and neck squamous cell carcinoma	9	Safe and well tolerated	1 patient showed partial response, 7 patients showed stable disease	1. Increase in circulating type I NKT numbers (4/9) 2. Expansion of α-GalCer reactive IFN-γ-producing cells in PBMCs (8/9)	(220)

(*Continued*)

TABLE 2 | Continued

Treatment	Injection site, number of injections/cycles	Tumor type	Number of patients	Safety	Clinical outcome	Immunological response	Reference
α-GalCer-pulsed mature moDCs	i.v. 2 injections	Advanced cancer	5	Safe and well tolerated	Patients had stable disease. 3 patients had decreased M spike levels in serum and urine	1. >100-fold expansion of type I NKT cell subsets sustained up to 5 months after vaccination 2. Type I NKT cell activation was associated with increased serum levels of IL-12p40, IP-10, and MIP-1β	(221)
Adoptive transfer of autologous *ex vivo*-expanded NKT cells							
Ex vivo-expanded NKT cells with autologous α-GalCer-pulsed PBMCs	i.v., 2 doses, 1×10^7 cells (level 1) 6 patients, 2.5×10^7 cells (level 2) 3 patients	Non-small cell lung cancer	9	No adverse effects	1. No tumor regression 2. Stable disease in 2/9 patients	1. Absolute number of circulating type I NKT cells increased in 2/3 case receiving level 2 dose 2. IFN-γ production augmented in all 3 cases receiving level 2 dose	(222)
Ex vivo-expanded NKT cells	i.v., 3 infusions of 25×10^7 cells/infusion spaced 2 weeks apart with pretreatment of GM-CSF before cycle 2 and 3 to enhance DC functions	Advanced melanoma	9	No adverse effects	1. Patients deceased (3/9) 2. Patients progressed (3/9). Median follow-up for 63 months	1. Type I NKT infusions appeared to cause transient peak of circulating type I NKT cells that were enhanced by GM-CSF pretreatment 2. Increased number of activated monocytes 3. Elevated IFN-γ production (5/8)	(208)
Combination therapies							
Ex vivo-expanded NKT cells (intra-arterial) and autologous α-GalCer-pulsed PBMCs (*via* nasal submucosal)	1×10^8 α-GalCer-loaded APCs submucosal injections (2 injections) followed by *in vitro* activated type I NKT cells (i.a) into tumor feeding artery (1 injection)	Head and neck squamous cell carcinoma	8	Serious adverse event (1). Mild adverse events (7)	1. Partial response (3/8) 2. Stable disease (4/8) 3. Progressive disease (1/8)	1. Increase in circulating type I NKT numbers (6/8) 2. Expansion of α-GalCer reactive IFN-γ-producing cells in PBMCs (7/8)	(212)
Ex vivo-expanded NKT cells (intra-arterial) and autologous α-GalCer-pulsed PBMCs (*via* nasal submucosal)	1×10^8 α-GalCer-loaded APCs submucosal injections (1 injection) followed by *in vitro* activated type I NKT cells (i.a) into tumor feeding artery (1 injection)	Head and neck squamous cell carcinoma	10	No adverse effects	1. Objective tumor regression (5/10) 2. Stable disease (5/10) 3. Antitumor effects (8/10)	1. Expansion of type I NKT in PBMC (7/10) and TIL correlating with partial response (6/6) 2. Elevated expansion of IFN-γ spot forming cells in PBMCs (8/10) and in tumor tissue	(213)
α-GalCer-pulsed mature moDCs + LEN	i.v., LEN (oral 10 mg/day), 28 day ×3 cycles	Multiple myeloma	6	Safe and well tolerated	3/4 patients show reduction in tumor-associated M spike after therapy	Activation of NKT, NK, monocyte, and eosinophils	(214)

of APC subset, lower amounts of α-GalCer required to activate NKT cells than soluble α-GalCer (231). Positive therapeutic effect of α-GalCer-loaded octa-arginine modified liposomes was reported in melanoma murine model (232). Administration of α-GalCer and ovalbumin coencapsulated PLGA nanoparticles provided significant prophylactic and therapeutic responses in mouse melanoma model by enhancing activation and tumor infiltration of the antigen-specific $CD8^+$ T cell (233).

COMBINATION APPROACHES

A major limitation of the initial studies targeting NKT cells in cancer is that these studies were conducted using single agent strategies and did not account for blockade of immune checkpoints or other immune-suppressive factors. PD-1:PD-L pathway has been shown to play an important role in mediating αGalCer-induced anergy in NKT cells. Antibody-mediated blockade of PD-1:PD-L interactions at the time of α-GalCer treatment prevent the induction of type I NKT anergy and also enhance the antitumor activities of αGalCer. Therefore, combination of NKT-targeted therapies with PD-1:PD-L blockade should be considered (234). Synthetic lipopeptide vaccines based on conjugation of MHC-binding peptide epitopes to α-GalCer displayed promising antitumor activity in a melanoma model. The principle behind these vaccines is to simultaneously provide both adjuvant and antigen to the same cell in a controlled fashion. Application of this vaccine technology using different tumor antigens might serve as a novel strategy for diverse malignancies (235). Combination of type I NKT-targeted DC vaccine with low dose of lenalidomide led to promising clinical activity in myeloma (214). Therefore, there is an unmet need to pursue combination approaches targeting type I NKT cells to better harness the antitumor properties of type I NKT cells in the clinic.

CONCLUDING REMARKS

Natural killer T cells are an important component of the TME and play key roles in regulating antitumor immunity. Although preclinical studies with NKT cell-targeted therapies in murine tumor models have been positive, clinical translation of these results has proven challenging. Translational challenge could be attributed to incomplete knowledge of human NKT subsets. Generation of improved preclinical models that replicate human NKT cell response is needed to gain insights into the cross talk between APCs and NKT subsets and to improve the efficacy of NKT cell-targeting therapies.

AUTHOR CONTRIBUTIONS

Both SN and MVD participated in conceptualization and drafting of the article as well as critical revision of the article for important intellectual content. Both authors gave final approval of the submitted publication.

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REFERENCES

1. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* (2013) 14:1014–22. doi:10.1038/ni.2703
2. Marcus A, Gowen BG, Thompson TW, Iannello A, Ardolino M, Deng W, et al. Recognition of tumors by the innate immune system and natural killer cells. *Adv Immunol* (2014) 122:91–128. doi:10.1016/B978-0-12-800267-4.00003-1
3. McEwen-Smith RM, Salio M, Cerundolo V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res* (2015) 3:425–35. doi:10.1158/2326-6066.CIR-15-0062
4. Terabe M, Berzofsky JA. The role of NKT cells in tumor immunity. *Adv Cancer Res* (2008) 101:277–348. doi:10.1016/S0065-230X(08)00408-9
5. Robertson FC, Berzofsky JA, Terabe M. NKT cell networks in the regulation of tumor immunity. *Front Immunol* (2014) 5:543. doi:10.3389/fimmu.2014.00543
6. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi:10.1146/annurev.immunol.25.022106.141711
7. Godfrey DI, Pellicci DG, Patel O, Kjer-Nielsen L, Mccluskey J, Rossjohn J. Antigen recognition by CD1d-restricted NKT T cell receptors. *Semin Immunol* (2010) 22:61–7. doi:10.1016/j.smim.2009.10.004
8. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13:101–17. doi:10.1038/nri3369
9. Salio M, Silk JD, Jones EY, Cerundolo V. Biology of CD1- and MR1-restricted T cells. *Annu Rev Immunol* (2014) 32:323–66. doi:10.1146/annurev-immunol-032713-120243
10. Berzins SP, Uldrich AP, Pellicci DG, Mcnab F, Hayakawa Y, Smyth MJ, et al. Parallels and distinctions between T and NKT cell development in the thymus. *Immunol Cell Biol* (2004) 82:269–75. doi:10.1111/j.0818-9641.2004.01256.x
11. Godfrey DI, Uldrich AP, Mccluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol* (2015) 16:1114–23. doi:10.1038/ni.3298
12. Cohen NR, Brennan PJ, Shay T, Watts GF, Brigl M, Kang J, et al. Shared and distinct transcriptional programs underlie the hybrid nature of iNKT cells. *Nat Immunol* (2013) 14:90–9. doi:10.1038/ni.2490
13. Kohlgruber AC, Donado CA, Lamarche NM, Brenner MB, Brennan PJ. Activation strategies for invariant natural killer T cells. *Immunogenetics* (2016) 68:649–63. doi:10.1007/s00251-016-0944-8
14. Fan X, Rudensky AY. Hallmarks of tissue-resident lymphocytes. *Cell* (2016) 164:1198–211. doi:10.1016/j.cell.2016.02.048
15. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* (1997) 278:1626–9. doi:10.1126/science.278.5343.1626
16. Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. *Nat Rev Immunol* (2002) 2:557–68. doi:10.1038/nri854
17. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. *Immunol Today* (2000) 21:573–83. doi:10.1016/S0167-5699(00)01735-7
18. Bedel R, Matsuda JL, Brigl M, White J, Kappler J, Marrack P, et al. Lower TCR repertoire diversity in Traj18-deficient mice. *Nat Immunol* (2012) 13:705–6. doi:10.1038/ni.2347
19. Chandra S, Zhao M, Budelsky A, De Mingo Pulido A, Day J, Fu Z, et al. A new mouse strain for the analysis of invariant NKT cell function. *Nat Immunol* (2015) 16:799–800. doi:10.1038/ni.3203
20. Bendelac A, Killeen N, Littman DR, Schwartz RH. A subset of CD4+ thymocytes selected by MHC class I molecules. *Science* (1994) 263:1774–8. doi:10.1126/science.7907820
21. Gadola SD, Dulphy N, Salio M, Cerundolo V. Valpha24-JalphaQ-independent, CD1d-restricted recognition of alpha-galactosylceramide by human CD4(+) and CD8alphabeta(+) T lymphocytes. *J Immunol* (2002) 168:5514–20. doi:10.4049/jimmunol.168.11.5514
22. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* (2002) 195:625–36. doi:10.1084/jem.20011786

23. Lee H, Hong C, Shin J, Oh S, Jung S, Park YK, et al. The presence of CD8+ invariant NKT cells in mice. *Exp Mol Med* (2009) 41:866–72. doi:10.3858/emm.2009.41.12.092
24. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med* (2002) 195:637–41. doi:10.1084/jem.20011908
25. Arrenberg P, Halder R, Kumar V. Cross-regulation between distinct natural killer T cell subsets influences immune response to self and foreign antigens. *J Cell Physiol* (2009) 218:246–50. doi:10.1002/jcp.21597
26. Jahng AW, Maricic I, Pedersen B, Burdin N, Naidenko O, Kronenberg M, et al. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* (2001) 194:1789–99. doi:10.1084/jem.194.12.1789
27. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nat Immunol* (2010) 11:197–206. doi:10.1038/ni.1841
28. Wu L, Van Kaer L. Natural killer T cells and autoimmune disease. *Curr Mol Med* (2009) 9:4–14. doi:10.2174/156652409787314534
29. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol* (2013) 25:161–7. doi:10.1016/j.coi.2013.01.003
30. Coquet JM, Chakravarti S, Kyparissoudis K, Mcnab FW, Pitt LA, Mckenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci U S A* (2008) 105:11287–92. doi:10.1073/pnas.0801631105
31. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity* (2015) 43:566–78. doi:10.1016/j.immuni.2015.06.025
32. Das R, Sant'Angelo DB, Nichols KE. Transcriptional control of invariant NKT cell development. *Immunol Rev* (2010) 238:195–215. doi:10.1111/j.1600-065X.2010.00962.x
33. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, et al. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol* (2008) 9:1055–64. doi:10.1038/ni.1641
34. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* (2008) 29:391–403. doi:10.1016/j.immuni.2008.07.011
35. Dose M, Sleckman BP, Han J, Bredemeyer AL, Bendelac A, Gounari F. Intrathymic proliferation wave essential for Valpha14+ natural killer T cell development depends on c-Myc. *Proc Natl Acad Sci U S A* (2009) 106:8641–6. doi:10.1073/pnas.0812255106
36. Mycko MP, Ferrero I, Wilson A, Jiang W, Bianchi T, Trumpp A, et al. Selective requirement for c-Myc at an early stage of V(alpha)14i NKT cell development. *J Immunol* (2009) 182:4641–8. doi:10.4049/jimmunol.0803394
37. Michel ML, Mendes-Da-Cruz D, Keller AC, Lochner M, Schneider E, Dy M, et al. Critical role of ROR-gammat in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation. *Proc Natl Acad Sci U S A* (2008) 105:19845–50. doi:10.1073/pnas.0806472105
38. Hu T, Simmons A, Yuan J, Bender TP, Alberola-ILA J. The transcription factor c-Myb primes CD4+CD8+ immature thymocytes for selection into the iNKT lineage. *Nat Immunol* (2010) 11:435–41. doi:10.1038/ni.1865
39. Choi HJ, Geng Y, Cho H, Li S, Giri PK, Felio K, et al. Differential requirements for the ETS transcription factor ELF-1 in the development of NKT cells and NK cells. *Blood* (2011) 117:1880–7. doi:10.1182/blood-2010-09-309468
40. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, et al. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* (2005) 22:705–16. doi:10.1016/j.immuni.2005.03.011
41. Kim PJ, Pai SY, Brigl M, Besra GS, Gumperz J, Ho IC. GATA-3 regulates the development and function of invariant NKT cells. *J Immunol* (2006) 177:6650–9. doi:10.4049/jimmunol.177.10.6650
42. Matsuda JL, Zhang Q, Ndonye R, Richardson SK, Howell AR, Gapin L. T-bet concomitantly controls migration, survival, and effector functions during the development of Valpha14i NKT cells. *Blood* (2006) 107:2797–805. doi:10.1182/blood-2005-08-3103
43. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* (2004) 20:477–94. doi:10.1016/S1074-7613(04)00076-7
44. Nichols KE, Hom J, Gong SY, Ganguly A, Ma CS, Cannons JL, et al. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med* (2005) 11:340–5. doi:10.1038/nm1189
45. Pasquier B, Yin L, Fondaneche MC, Relouzat F, Bloch-Queyrat C, Lambert N, et al. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J Exp Med* (2005) 201:695–701. doi:10.1084/jem.20042432
46. Macho-Fernandez E, Brigl M. The extended family of CD1d-restricted NKT cells: sifting through a mixed bag of TCRs, antigens, and functions. *Front Immunol* (2015) 6:362. doi:10.3389/fimmu.2015.00362
47. Ambrosino E, Terabe M, Halder RC, Peng J, Takaku S, Miyake S, et al. Cross-regulation between type I and type II NKT cells in regulating tumor immunity: a new immunoregulatory axis. *J Immunol* (2007) 179:5126–36. doi:10.4049/jimmunol.179.8.5126
48. Pilones KA, Aryankalayil J, Demaria S. Invariant NKT cells as novel targets for immunotherapy in solid tumors. *Clin Dev Immunol* (2012) 2012:720803. doi:10.1155/2012/720803
49. Altman JB, Benavides AD, Das R, Bassiri H. Antitumor responses of invariant natural killer T cells. *J Immunol Res* (2015) 2015:652875. doi:10.1155/2015/652875
50. Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med* (1995) 182:993–1004. doi:10.1084/jem.182.4.993
51. Exley MA, Tahir SM, Cheng O, Shaulov A, Joyce R, Avigan D, et al. A major fraction of human bone marrow lymphocytes are Th2-like CD1d-reactive T cells that can suppress mixed lymphocyte responses. *J Immunol* (2001) 167:5531–4. doi:10.4049/jimmunol.167.10.5531
52. Blomqvist M, Rhost S, Teneberg S, Lofbom L, Osterbye T, Brigl M, et al. Multiple tissue-specific isoforms of sulfatide activate CD1d-restricted type II NKT cells. *Eur J Immunol* (2009) 39:1726–35. doi:10.1002/eji.200839001
53. Fuss IJ, Joshi B, Yang Z, Degheidy H, Fichtner-Feigl S, De Souza H, et al. IL-13Ralpha2-bearing, type II NKT cells reactive to sulfatide self-antigen populate the mucosa of ulcerative colitis. *Gut* (2014) 63:1728–36. doi:10.1136/gutjnl-2013-305671
54. Yang SH, Lee JP, Jang HR, Cha RH, Han SS, Jeon US, et al. Sulfatide-reactive natural killer T cells abrogate ischemia-reperfusion injury. *J Am Soc Nephrol* (2011) 22:1305–14. doi:10.1681/ASN.2010080815
55. Rhost S, Sedimbi S, Kadri N, Cardell SL. Immunomodulatory type II natural killer T lymphocytes in health and disease. *Scand J Immunol* (2012) 76:246–55. doi:10.1111/j.1365-3083.2012.02750.x
56. Roy KC, Maricic I, Khurana A, Smith TR, Halder RC, Kumar V. Involvement of secretory and endosomal compartments in presentation of an exogenous self-glycolipid to type II NKT cells. *J Immunol* (2008) 180:2942–50. doi:10.4049/jimmunol.180.5.2942
57. Nair S, Boddupalli CS, Verma R, Liu J, Yang R, Pastores GM, et al. Type II NKT-TFH cells against Gaucher lipids regulate B-cell immunity and inflammation. *Blood* (2015) 125:1256–71. doi:10.1182/blood-2014-09-600270
58. Chang DH, Deng H, Matthews P, Krasovsky J, Ragupathi G, Spisek R, et al. Inflammation-associated lysophospholipids as ligands for CD1d-restricted T cells in human cancer. *Blood* (2008) 112:1308–16. doi:10.1182/blood-2008-04-149831
59. Dhodapkar MV, Kumar V. Type II NKT cells and their emerging role in health and disease. *J Immunol* (2017) 198:1015–21. doi:10.4049/jimmunol.1601399
60. Girardi E, Zajonc DM. Molecular basis of lipid antigen presentation by CD1d and recognition by natural killer T cells. *Immunol Rev* (2012) 250:167–79. doi:10.1111/j.1600-065X.2012.01166.x
61. Pellicci DG, Patel O, Kjer-Nielsen L, Pang SS, Sullivan LC, Kyparissoudis K, et al. Differential recognition of CD1d-alpha-galactosyl ceramide by the V beta 8.2 and V beta 7 semi-invariant NKT T cell receptors. *Immunity* (2009) 31:47–59. doi:10.1016/j.immuni.2009.04.018
62. Girardi E, Maricic I, Wang J, Mac TT, Iyer P, Kumar V, et al. Type II natural killer T cells use features of both innate-like and conventional T cells to recognize sulfatide self antigens. *Nat Immunol* (2012) 13:851–6. doi:10.1038/ni.2371
63. Patel O, Pellicci DG, Gras S, Sandoval-Romero ML, Uldrich AP, Mallevaey T, et al. Recognition of CD1d-sulfatide mediated by a type II natural killer T cell antigen receptor. *Nat Immunol* (2012) 13:857–63. doi:10.1038/ni.2372
64. Zhao J, Weng X, Bagchi S, Wang CR. Polyclonal type II natural killer T cells require PLZF and SAP for their development and contribute to CpG-mediated

antitumor response. *Proc Natl Acad Sci U S A* (2014) 111:2674–9. doi:10.1073/pnas.1323845111
65. Marrero I, Ware R, Kumar V. Type II NKT cells in inflammation, autoimmunity, microbial immunity, and cancer. *Front Immunol* (2015) 6:316. doi:10.3389/fimmu.2015.00316
66. Arrenberg P, Halder R, Dai Y, Maricic I, Kumar V. Oligoclonality and innate-like features in the TCR repertoire of type II NKT cells reactive to a beta-linked self-glycolipid. *Proc Natl Acad Sci U S A* (2010) 107:10984–9. doi:10.1073/pnas.1000576107
67. Kadri N, Korpos E, Gupta S, Briet C, Lofbom L, Yagita H, et al. CD4(+) type II NKT cells mediate ICOS and programmed death-1-dependent regulation of type 1 diabetes. *J Immunol* (2012) 188:3138–49. doi:10.4049/jimmunol.1101390
68. Tatituri RV, Watts GF, Bhowruth V, Barton N, Rothchild A, Hsu FF, et al. Recognition of microbial and mammalian phospholipid antigens by NKT cells with diverse TCRs. *Proc Natl Acad Sci U S A* (2013) 110:1827–32. doi:10.1073/pnas.1220601110
69. Zeissig S, Blumberg RS. Primary immunodeficiency associated with defects in CD1 and CD1-restricted T cells. *Ann N Y Acad Sci* (2012) 1250:14–24. doi:10.1111/j.1749-6632.2011.06380.x
70. Moodycliffe AM, Nghiem D, Clydesdale G, Ullrich SE. Immune suppression and skin cancer development: regulation by NKT cells. *Nat Immunol* (2000) 1:521–5. doi:10.1038/82782
71. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* (2000) 1:515–20. doi:10.1038/82771
72. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* (2003) 198:1741–52. doi:10.1084/jem.20022227
73. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, et al. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol* (2001) 167:4046–50. doi:10.4049/jimmunol.167.7.4046
74. Molling JW, Kolgen W, Van Der Vliet HJ, Boomsma MF, Kruizenga H, Smorenburg CH, et al. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *Int J Cancer* (2005) 116:87–93. doi:10.1002/ijc.20998
75. Yoneda K, Morii T, Nieda M, Tsukaguchi N, Amano I, Tanaka H, et al. The peripheral blood Valpha24+ NKT cell numbers decrease in patients with haematopoietic malignancy. *Leuk Res* (2005) 29:147–52. doi:10.1016/j.leukres.2004.06.005
76. Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. *J Immunol* (2002) 168:6494–9. doi:10.4049/jimmunol.168.12.6494
77. Fujii S, Shimizu K, Klimek V, Geller MD, Nimer SD, Dhodapkar MV. Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br J Haematol* (2003) 122:617–22. doi:10.1046/j.1365-2141.2003.04465.x
78. Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J Exp Med* (2003) 197:1667–76. doi:10.1084/jem.20021650
79. Metelitsa LS, Wu HW, Wang H, Yang Y, Warsi Z, Asgharzadeh S, et al. Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2. *J Exp Med* (2004) 199:1213–21. doi:10.1084/jem.20031462
80. Schneiders FL, De Bruin RC, Van Den Eertwegh AJ, Scheper RJ, Leemans CR, Brakenhoff RH, et al. Circulating invariant natural killer T-cell numbers predict outcome in head and neck squamous cell carcinoma: updated analysis with 10-year follow-up. *J Clin Oncol* (2012) 30:567–70. doi:10.1200/JCO.2011.38.8819
81. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, et al. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res* (2005) 11:7322–7. doi:10.1158/1078-0432.CCR-05-0877
82. Natori T, Koezuka Y, Higa T. Novel antitumor and immunostimulatory cerebrosides from the marine sponge *Agelas mauritianus*. *Tetrahedron Lett* (1993) 34:5591–2. doi:10.1016/S0040-4039(00)73889-5
83. Shissler SC, Bollino DR, Tiper IV, Bates JP, Derakhshandeh R, Webb TJ. Immunotherapeutic strategies targeting natural killer T cell responses in cancer. *Immunogenetics* (2016) 68:623–38. doi:10.1007/s00251-016-0928-8
84. van der Vliet HJ, Molling JW, Von Blomberg BM, Nishi N, Kolgen W, Van Den Eertwegh AJ, et al. The immunoregulatory role of CD1d-restricted natural killer T cells in disease. *Clin Immunol* (2004) 112:8–23. doi:10.1016/j.clim.2004.03.003
85. Bassiri H, Das R, Guan P, Barrett DM, Brennan PJ, Banerjee PP, et al. iNKT cell cytotoxic responses control T-lymphoma growth in vitro and in vivo. *Cancer Immunol Res* (2014) 2:59–69. doi:10.1158/2326-6066.CIR-13-0104
86. Dao T, Mehal WZ, Crispe IN. IL-18 augments perforin-dependent cytotoxicity of liver NK-T cells. *J Immunol* (1998) 161:2217–22.
87. Leite-De-Moraes MC, Hameg A, Arnould A, Machavoine F, Koezuka Y, Schneider E, et al. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J Immunol* (1999) 163:5871–6.
88. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol* (2010) 185:2721–9. doi:10.4049/jimmunol.1001018
89. Hagihara M, Gansuvd B, Ueda Y, Tsuchiya T, Masui A, Tazume K, et al. Killing activity of human umbilical cord blood-derived TCRValpha24(+) NKT cells against normal and malignant hematological cells in vitro: a comparative study with NK cells or OKT3 activated T lymphocytes or with adult peripheral blood NKT cells. *Cancer Immunol Immunother* (2002) 51:1–8. doi:10.1007/s00262-001-0246-2
90. Metelitsa LS. Anti-tumor potential of type-I NKT cells against CD1d-positive and CD1d-negative tumors in humans. *Clin Immunol* (2011) 140:119–29. doi:10.1016/j.clim.2010.10.005
91. Nowak M, Arredouani MS, Tun-Kyi A, Schmidt-Wolf I, Sanda MG, Balk SP, et al. Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with alpha-galactosylceramide. *PLoS One* (2010) 5:e11311. doi:10.1371/journal.pone.0011311
92. Hix LM, Shi YH, Brutkiewicz RR, Stein PL, Wang CR, Zhang M. CD1d-expressing breast cancer cells modulate NKT cell-mediated antitumor immunity in a murine model of breast cancer metastasis. *PLoS One* (2011) 6:e20702. doi:10.1371/journal.pone.0020702
93. Chong TW, Goh FY, Sim MY, Huang HH, Thike AA, Lim WK, et al. CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. *J Clin Pathol* (2015) 68:200–5. doi:10.1136/jclinpath-2014-202735
94. Dhodapkar KM, Cirignano B, Chamian F, Zagzag D, Miller DC, Finlay JL, et al. Invariant natural killer T cells are preserved in patients with glioma and exhibit antitumor lytic activity following dendritic cell-mediated expansion. *Int J Cancer* (2004) 109:893–9. doi:10.1002/ijc.20050
95. Liu D, Song L, Brawley VS, Robison N, Wei J, Gao X, et al. Medulloblastoma expresses CD1d and can be targeted for immunotherapy with NKT cells. *Clin Immunol* (2013) 149:55–64. doi:10.1016/j.clim.2013.06.005
96. Haraguchi K, Takahashi T, Nakahara F, Matsumoto A, Kurokawa M, Ogawa S, et al. CD1d expression level in tumor cells is an important determinant for anti-tumor immunity by natural killer T cells. *Leuk Lymphoma* (2006) 47:2218–23. doi:10.1080/10428190600682688
97. Fallarini S, Paoletti T, Orsi Battaglini N, Lombardi G. Invariant NKT cells increase drug-induced osteosarcoma cell death. *Br J Pharmacol* (2012) 167:1533–49. doi:10.1111/j.1476-5381.2012.02108.x
98. Nicol A, Nieda M, Koezuka Y, Porcelli S, Suzuki K, Tadokoro K, et al. Human invariant valpha24+ natural killer T cells activated by alpha-galactosylceramide (KRN7000) have cytotoxic anti-tumour activity through mechanisms distinct from T cells and natural killer cells. *Immunology* (2000) 99:229–34. doi:10.1046/j.1365-2567.2000.00952.x
99. Spanoudakis E, Hu M, Naresh K, Terpos E, Melo V, Reid A, et al. Regulation of multiple myeloma survival and progression by CD1d. *Blood* (2009) 113:2498–507. doi:10.1182/blood-2008-06-161281
100. Miura S, Kawana K, Schust DJ, Fujii T, Yokoyama T, Iwasawa Y, et al. CD1d, a sentinel molecule bridging innate and adaptive immunity, is downregulated

by the human papillomavirus (HPV) E5 protein: a possible mechanism for immune evasion by HPV. *J Virol* (2010) 84:11614–23. doi:10.1128/JVI.01053-10
101. Sriram V, Cho S, Li P, O'Donnell PW, Dunn C, Hayakawa K, et al. Inhibition of glycolipid shedding rescues recognition of a CD1+ T cell lymphoma by natural killer T (NKT) cells. *Proc Natl Acad Sci U S A* (2002) 99:8197–202. doi:10.1073/pnas.122636199
102. Anastasiadis A, Kotsianidis I, Papadopoulos V, Spanoudakis E, Margaritis D, Christoforidou A, et al. CD1d expression as a prognostic marker for chronic lymphocytic leukemia. *Leuk Lymphoma* (2014) 55:320–5. doi:10.3109/10428194.2013.803222
103. Bojarska-Junak A, Hus I, Chocholska S, Tomczak W, Wos J, Czubak P, et al. CD1d expression is higher in chronic lymphocytic leukemia patients with unfavorable prognosis. *Leuk Res* (2014) 38:435–42. doi:10.1016/j.leukres.2013.12.015
104. Gorini F, Azzimonti L, Delfanti G, Scarfo L, Scielzo C, Bertilaccio MT, et al. Invariant NKT cells contribute to chronic lymphocytic leukemia surveillance and prognosis. *Blood* (2017) 129:3440–51. doi:10.1182/blood-2016-11-751065
105. Kawano T, Nakayama T, Kamada N, Kaneko Y, Harada M, Ogura N, et al. Antitumor cytotoxicity mediated by ligand-activated human V alpha24 NKT cells. *Cancer Res* (1999) 59:5102–5.
106. Gansuvd B, Hagihara M, Yu Y, Inoue H, Ueda Y, Tsuchiya T, et al. Human umbilical cord blood NK T cells kill tumors by multiple cytotoxic mechanisms. *Hum Immunol* (2002) 63:164–75. doi:10.1016/S0198-8859(01)00382-2
107. Durrant LG, Noble P, Spendlove I. Immunology in the clinic review series; focus on cancer: glycolipids as targets for tumour immunotherapy. *Clin Exp Immunol* (2012) 167:206–15. doi:10.1111/j.1365-2249.2011.04516.x
108. Hakomori S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res* (1996) 56:5309–18.
109. Parekh VV, Lalani S, Van Kaer L. The in vivo response of invariant natural killer T cells to glycolipid antigens. *Int Rev Immunol* (2007) 26:31–48. doi:10.1080/08830180601070179
110. Nakagawa R, Serizawa I, Motoki K, Sato M, Ueno H, Iijima R, et al. Antitumor activity of alpha-galactosylceramide, KRN7000, in mice with the melanoma B16 hepatic metastasis and immunohistological study of tumor infiltrating cells. *Oncol Res* (2000) 12:51–8. doi:10.3727/096504001108747521
111. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* (2003) 198:1069–76. doi:10.1084/jem.20030630
112. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol* (2008) 20:358–68. doi:10.1016/j.coi.2008.03.018
113. Monteiro M, Graca L. iNKT cells: innate lymphocytes with a diverse response. *Crit Rev Immunol* (2014) 34:81–90. doi:10.1615/CritRevImmunol.2014010088
114. Sullivan BA, Nagarajan NA, Wingender G, Wang J, Scott I, Tsuji M, et al. Mechanisms for glycolipid antigen-driven cytokine polarization by Valpha14i NKT cells. *J Immunol* (2010) 184:141–53. doi:10.4049/jimmunol.0902880
115. Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J Exp Med* (2005) 202:1279–88. doi:10.1084/jem.20050953
116. Keller CW, Freigang S, Lunemann JD. Reciprocal crosstalk between dendritic cells and natural killer T cells: mechanisms and therapeutic potential. *Front Immunol* (2017) 8:570. doi:10.3389/fimmu.2017.00570
117. Dudek AM, Martin S, Garg AD, Agostinis P. Immature, semi-mature, and fully mature dendritic cells: toward a DC-cancer cells interface that augments anticancer immunity. *Front Immunol* (2013) 4:438. doi:10.3389/fimmu.2013.00438
118. de Jong EC, Smits HH, Kapsenberg ML. Dendritic cell-mediated T cell polarization. *Springer Semin Immunopathol* (2005) 26:289–307. doi:10.1007/s00281-004-0167-1
119. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, et al. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* (1999) 189:1121–8. doi:10.1084/jem.189.7.1121
120. Tomura M, Yu WG, Ahn HJ, Yamashita M, Yang YF, Ono S, et al. A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J Immunol* (1999) 163:93–101.
121. Shimizu K, Hidaka M, Kadowaki N, Makita N, Konishi N, Fujimoto K, et al. Evaluation of the function of human invariant NKT cells from cancer patients using alpha-galactosylceramide-loaded murine dendritic cells. *J Immunol* (2006) 177:3484–92. doi:10.4049/jimmunol.177.5.3484
122. Munz C, Steinman RM, Fujii S. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med* (2005) 202:203–7. doi:10.1084/jem.20050810
123. Fujii S, Shimizu K, Hemmi H, Fukui M, Bonito AJ, Chen G, et al. Glycolipid alpha-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice. *Proc Natl Acad Sci U S A* (2006) 103:11252–7. doi:10.1073/pnas.0604812103
124. Fujii S, Shimizu K, Hemmi H, Steinman RM. Innate Valpha14(+) natural killer T cells mature dendritic cells, leading to strong adaptive immunity. *Immunol Rev* (2007) 220:183–98. doi:10.1111/j.1600-065X.2007.00561.x
125. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, et al. Nkt cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* (2003) 171:5140–7. doi:10.4049/jimmunol.171.10.5140
126. Brigl M, Tatituri RV, Watts GF, Bhowruth V, Leadbetter EA, Barton N, et al. Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. *J Exp Med* (2011) 208:1163–77. doi:10.1084/jem.20102555
127. Hayakawa Y, Godfrey DI, Smyth MJ. Alpha-galactosylceramide: potential immunomodulatory activity and future application. *Curr Med Chem* (2004) 11:241–52. doi:10.2174/0929867043456115
128. Shimaoka T, Seino K, Kume N, Minami M, Nishime C, Suematsu M, et al. Critical role for Cxc chemokine ligand 16 (SR-PSOX) in Th1 response mediated by NKT cells. *J Immunol* (2007) 179:8172–9. doi:10.4049/jimmunol.179.12.8172
129. Germanov E, Veinotte L, Cullen R, Chamberlain E, Butcher EC, Johnston B. Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. *J Immunol* (2008) 181:81–91. doi:10.4049/jimmunol.181.1.81
130. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, et al. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. *Nat Immunol* (2010) 11:313–20. doi:10.1038/ni.1848
131. Gottschalk C, Mettke E, Kurts C. The role of invariant natural killer T cells in dendritic cell licensing, cross-priming, and memory CD8(+) T cell generation. *Front Immunol* (2015) 6:379. doi:10.3389/fimmu.2015.00379
132. Vincent MS, Leslie DS, Gumperz JE, Xiong X, Grant EP, Brenner MB. CD1-dependent dendritic cell instruction. *Nat Immunol* (2002) 3:1163–8. doi:10.1038/ni851
133. Taraban VY, Martin S, Attfield KE, Glennie MJ, Elliott T, Elewaut D, et al. Invariant NKT cells promote CD8+ cytotoxic T cell responses by inducing CD70 expression on dendritic cells. *J Immunol* (2008) 180:4615–20. doi:10.4049/jimmunol.180.7.4615
134. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* (2003) 198:267–79. doi:10.1084/jem.20030324
135. Trinchieri G. Natural killer cells wear different hats: effector cells of innate resistance and regulatory cells of adaptive immunity and of hematopoiesis. *Semin Immunol* (1995) 7:83–8. doi:10.1006/smim.1995.0012
136. Smyth MJ, Swann J, Kelly JM, Cretney E, Yokoyama WM, Diefenbach A, et al. NKG2D recognition and perforin effector function mediate effective cytokine immunotherapy of cancer. *J Exp Med* (2004) 200:1325–35. doi:10.1084/jem.20041522
137. Chaudhry MS, Karadimitris A. Role and regulation of CD1d in normal and pathological B cells. *J Immunol* (2014) 193:4761–8. doi:10.4049/jimmunol.1401805
138. Chung Y, Kim BS, Kim YJ, Ko HJ, Ko SY, Kim DH, et al. CD1d-restricted T cells license B cells to generate long-lasting cytotoxic antitumor immunity in vivo. *Cancer Res* (2006) 66:6843–50. doi:10.1158/0008-5472.CAN-06-0889

139. Kim YJ, Ko HJ, Kim YS, Kim DH, Kang S, Kim JM, et al. alpha-Galactosylceramide-loaded, antigen-expressing B cells prime a wide spectrum of antitumor immunity. *Int J Cancer* (2008) 122:2774–83. doi:10.1002/ijc.23444
140. Barral P, Eckl-Dorna J, Harwood NE, De Santo C, Salio M, Illarionov P, et al. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. *Proc Natl Acad Sci U S A* (2008) 105:8345–50. doi:10.1073/pnas.0802968105
141. Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, et al. NK T cells provide lipid antigen-specific cognate help for B cells. *Proc Natl Acad Sci U S A* (2008) 105:8339–44. doi:10.1073/pnas.0801375105
142. Dellabona P, Abrignani S, Casorati G. iNKT-cell help to B cells: a cooperative job between innate and adaptive immune responses. *Eur J Immunol* (2014) 44:2230–7. doi:10.1002/eji.201344399
143. Galli G, Nuti S, Tavarini S, Galli-Stampino L, De Lalla C, Casorati G, et al. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *J Exp Med* (2003) 197:1051–7. doi:10.1084/jem.20021616
144. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* (2017) 14:399–416. doi:10.1038/nrclinonc.2016.217
145. Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? *Science* (2013) 339:286–91. doi:10.1126/science.1232227
146. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* (2010) 141:39–51. doi:10.1016/j.cell.2010.03.014
147. Song L, Asgharzadeh S, Salo J, Engell K, Wu HW, Sposto R, et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. *J Clin Invest* (2009) 119:1524–36. doi:10.1172/JCI37869
148. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* (2009) 9:162–74. doi:10.1038/nri2506
149. De Santo C, Salio M, Masri SH, Lee LY, Dong T, Speak AO, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest* (2008) 118:4036–48. doi:10.1172/JCI36264
150. Ko HJ, Lee JM, Kim YJ, Kim YS, Lee KA, Kang CY. Immunosuppressive myeloid-derived suppressor cells can be converted into immunogenic APCs with the help of activated NKT cells: an alternative cell-based antitumor vaccine. *J Immunol* (2009) 182:1818–28. doi:10.4049/jimmunol.0802430
151. De Santo C, Arscott R, Booth S, Karydis I, Jones M, Asher R, et al. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat Immunol* (2010) 11:1039–46. doi:10.1038/ni.1942
152. Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, et al. A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med* (2005) 202:1627–33. doi:10.1084/jem.20051381
153. Berzofsky JA, Terabe M. NKT cells in tumor immunity: opposing subsets define a new immunoregulatory axis. *J Immunol* (2008) 180:3627–35. doi:10.4049/jimmunol.180.6.3627
154. Berzofsky JA, Terabe M. The contrasting roles of NKT cells in tumor immunity. *Curr Mol Med* (2009) 9:667–72. doi:10.2174/156652409788970706
155. Halder RC, Aguilera C, Maricic I, Kumar V. Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. *J Clin Invest* (2007) 117:2302–12. doi:10.1172/JCI31602
156. Mistry PK, Taddei T, Vom Dahl S, Rosenbloom BE. Gaucher disease and malignancy: a model for cancer pathogenesis in an inborn error of metabolism. *Crit Rev Oncog* (2013) 18:235–46. doi:10.1615/CritRevOncog.2013006145
157. Nair S, Branagan AR, Liu J, Boddupalli CS, Mistry PK, Dhodapkar MV. Clonal immunoglobulin against lysolipids in the origin of myeloma. *N Engl J Med* (2016) 374:555–61. doi:10.1056/NEJMoa1508808
158. Bjordahl RL, Gapin L, Marrack P, Refaeli Y. iNKT cells suppress the CD8+ T cell response to a murine Burkitt's-like B cell lymphoma. *PLoS One* (2012) 7:e42635. doi:10.1371/journal.pone.0042635
159. Renukaradhya GJ, Sriram V, Du W, Gervay-Hague J, Van Kaer L, Brutkiewicz RR. Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* (2006) 118:3045–53. doi:10.1002/ijc.21764
160. Yang W, Li H, Mayhew E, Mellon J, Chen PW, Niederkorn JY. NKT cell exacerbation of liver metastases arising from melanomas transplanted into either the eyes or spleens of mice. *Invest Ophthalmol Vis Sci* (2011) 52:3094–102. doi:10.1167/iovs.10-7067
161. Pilones KA, Kawashima N, Yang AM, Babb JS, Formenti SC, Demaria S. Invariant natural killer T cells regulate breast cancer response to radiation and CTLA-4 blockade. *Clin Cancer Res* (2009) 15:597–606. doi:10.1158/1078-0432.CCR-08-1277
162. Zhao J, Bagchi S, Wang CR. Type II natural killer T cells foster the antitumor activity of CpG-oligodeoxynucleotides. *Oncoimmunology* (2014) 3:e28977. doi:10.4161/onci.28977
163. Exley MA, Lynch L, Varghese B, Nowak M, Alatrakchi N, Balk SP. Developing understanding of the roles of CD1d-restricted T cell subsets in cancer: reversing tumor-induced defects. *Clin Immunol* (2011) 140:184–95. doi:10.1016/j.clim.2011.04.017
164. Molling JW, Langius JA, Langendijk JA, Leemans CR, Bontkes HJ, Van Der Vliet HJ, et al. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. *J Clin Oncol* (2007) 25:862–8. doi:10.1200/JCO.2006.08.5787
165. Motohashi S, Okamoto Y, Yoshino I, Nakayama T. Anti-tumor immune responses induced by iNKT cell-based immunotherapy for lung cancer and head and neck cancer. *Clin Immunol* (2011) 140:167–76. doi:10.1016/j.clim.2011.01.009
166. Najera Chuc AE, Cervantes LA, Retiguin FP, Ojeda JV, Maldonado ER. Low number of invariant NKT cells is associated with poor survival in acute myeloid leukemia. *J Cancer Res Clin Oncol* (2012) 138:1427–32. doi:10.1007/s00432-012-1251-x
167. Shaulov A, Yue S, Wang R, Joyce RM, Balk SP, Kim HT, et al. Peripheral blood progenitor cell product contains Th1-biased noninvariant CD1d-reactive natural killer T cells: implications for posttransplant survival. *Exp Hematol* (2008) 36:464–72. doi:10.1016/j.exphem.2007.12.010
168. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* (1997) 278:1623–6. doi:10.1126/science.278.5343.1623
169. Nakagawa R, Motoki K, Ueno H, Iijima R, Nakamura H, Kobayashi E, et al. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an alpha-galactosylceramide, KRN7000. *Cancer Res* (1998) 58:1202–7.
170. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, et al. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci U S A* (1998) 95:5690–3. doi:10.1073/pnas.95.10.5690
171. Fuji N, Ueda Y, Fujiwara H, Toh T, Yoshimura T, Yamagishi H. Antitumor effect of alpha-galactosylceramide (KRN7000) on spontaneous hepatic metastases requires endogenous interleukin 12 in the liver. *Clin Cancer Res* (2000) 6:3380–7.
172. Silk JD, Hermans IF, Gileadi U, Chong TW, Shepherd D, Salio M, et al. Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. *J Clin Invest* (2004) 114:1800–11. doi:10.1172/JCI200422046
173. Nishimura T, Watanabe K, Yahata T, Ushaku L, Ando K, Kimura M, et al. Application of interleukin 12 to antitumor cytokine and gene therapy. *Cancer Chemother Pharmacol* (1996) 38(Suppl):S27–34. doi:10.1007/s002800051033
174. Nakui M, Ohta A, Sekimoto M, Sato M, Iwakabe K, Yahata T, et al. Potentiation of antitumor effect of NKT cell ligand, alpha-galactosylceramide by combination with IL-12 on lung metastasis of malignant melanoma cells. *Clin Exp Metastasis* (2000) 18:147–53. doi:10.1023/A:1006715221088
175. Hayakawa Y, Takeda K, Yagita H, Smyth MJ, Van Kaer L, Okumura K, et al. IFN-gamma-mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, alpha-galactosylceramide. *Blood* (2002) 100:1728–33.
176. Hayakawa Y, Rovero S, Forni G, Smyth MJ. Alpha-galactosylceramide (KRN7000) suppression of chemical- and oncogene-dependent carcinogenesis. *Proc Natl Acad Sci U S A* (2003) 100:9464–9. doi:10.1073/pnas.1630663100
177. Li J, Sun W, Subrahmanyam PB, Page C, Younger KM, Tiper IV, et al. NKT cell responses to B cell lymphoma. *Med Sci (Basel)* (2014) 2:82–97. doi:10.3390/medsci2020082
178. Nur H, Rao L, Frassanito MA, De Raeve H, Ribatti D, Mfopou JK, et al. Stimulation of invariant natural killer T cells by alpha-galactosylceramide activates the JAK-STAT pathway in endothelial cells and reduces angiogenesis in the 5T33 multiple myeloma model. *Br J Haematol* (2014) 167:651–63. doi:10.1111/bjh.13092

179. Kim D, Hung CF, Wu TC, Park YM. DNA vaccine with alpha-galactosylceramide at prime phase enhances anti-tumor immunity after boosting with antigen-expressing dendritic cells. *Vaccine* (2010) 28:7297–305. doi:10.1016/j.vaccine.2010.08.079
180. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* (1999) 163:2387–91.
181. Matsuyoshi H, Hirata S, Yoshitake Y, Motomura Y, Fukuma D, Kurisaki A, et al. Therapeutic effect of alpha-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells. *Cancer Sci* (2005) 96:889–96. doi:10.1111/j.1349-7006.2005.00123.x
182. Liu K, Idoyaga J, Charalambous A, Fujii S, Bonito A, Mordoh J, et al. Innate NKT lymphocytes confer superior adaptive immunity via tumor-capturing dendritic cells. *J Exp Med* (2005) 202:1507–16. doi:10.1084/jem.20050956
183. Nagaraj S, Ziske C, Strehl J, Messmer D, Sauerbruch T, Schmidt-Wolf IG. Dendritic cells pulsed with alpha-galactosylceramide induce anti-tumor immunity against pancreatic cancer in vivo. *Int Immunol* (2006) 18:1279–83. doi:10.1093/intimm/dxl059
184. Chung Y, Qin H, Kang CY, Kim S, Kwak LW, Dong C. An NKT-mediated autologous vaccine generates CD4 T-cell dependent potent antilymphoma immunity. *Blood* (2007) 110:2013–9. doi:10.1182/blood-2006-12-061309
185. Shimizu K, Goto A, Fukui M, Taniguchi M, Fujii S. Tumor cells loaded with alpha-galactosylceramide induce innate NKT and NK cell-dependent resistance to tumor implantation in mice. *J Immunol* (2007) 178:2853–61. doi:10.4049/jimmunol.178.5.2853
186. Petersen TR, Sika-Paotonu D, Knight DA, Dickgreber N, Farrand KJ, Ronchese F, et al. Potent anti-tumor responses to immunization with dendritic cells loaded with tumor tissue and an NKT cell ligand. *Immunol Cell Biol* (2010) 88:596–604. doi:10.1038/icb.2010.9
187. Mattarollo SR, West AC, Steegh K, Duret H, Paget C, Martin B, et al. NKT cell adjuvant-based tumor vaccine for treatment of myc oncogene-driven mouse B-cell lymphoma. *Blood* (2012) 120:3019–29. doi:10.1182/blood-2012-04-426643
188. Hong S, Lee H, Jung K, Lee SM, Lee SJ, Jun HJ, et al. Tumor cells loaded with alpha-galactosylceramide promote therapeutic NKT-dependent anti-tumor immunity in multiple myeloma. *Immunol Lett* (2013) 156:132–9. doi:10.1016/j.imlet.2013.10.002
189. Macho-Fernandez E, Cruz LJ, Ghinnagow R, Fontaine J, Bialecki E, Frisch B, et al. Targeted delivery of alpha-galactosylceramide to CD8alpha+ dendritic cells optimizes type I NKT cell-based antitumor responses. *J Immunol* (2014) 193:961–9. doi:10.4049/jimmunol.1303029
190. Gibbins JD, Ancelet LR, Weinkove R, Compton BJ, Painter GF, Petersen TR, et al. An autologous leukemia cell vaccine prevents murine acute leukemia relapse after cytarabine treatment. *Blood* (2014) 124:2953–63. doi:10.1182/blood-2014-04-568956
191. Veinotte L, Gebremeskel S, Johnston B. CXCL16-positive dendritic cells enhance invariant natural killer T cell-dependent IFNgamma production and tumor control. *Oncoimmunology* (2016) 5:e1160979. doi:10.1080/2162402X.2016.1160979
192. Dong T, Yi T, Yang M, Lin S, Li W, Xu X, et al. Co-operation of alpha-galactosylceramide-loaded tumour cells and TLR9 agonists induce potent anti-tumour responses in a murine colon cancer model. *Biochem J* (2016) 473:7–19. doi:10.1042/BJ20150129
193. Escriba-Garcia L, Alvarez-Fernandez C, Tellez-Gabriel M, Sierra J, Briones J. Dendritic cells combined with tumor cells and alpha-galactosylceramide induce a potent, therapeutic and NK-cell dependent antitumor immunity in B cell lymphoma. *J Transl Med* (2017) 15:115. doi:10.1186/s12967-017-1219-3
194. Shin T, Nakayama T, Akutsu Y, Motohashi S, Shibata Y, Harada M, et al. Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Valpha14 NKT cells. *Int J Cancer* (2001) 91:523–8. doi:10.1002/1097-0215(20010215)91:4<523::AID-IJC1087>3.0.CO;2-L
195. Scheffold C, Kornacker M, Scheffold YC, Contag CH, Negrin RS. Visualization of effective tumor targeting by CD8+ natural killer T cells redirected with bispecific antibody F(ab')(2)HER2xCD3. *Cancer Res* (2002) 62:5785–91.
196. Bagnara D, Ibatici A, Corselli M, Sessarego N, Tenca C, De Santanna A, et al. Adoptive immunotherapy mediated by ex vivo expanded natural killer T cells against CD1d-expressing lymphoid neoplasms. *Haematologica* (2009) 94:967–74. doi:10.3324/haematol.2008.001339
197. Payne KK, Keim RC, Graham L, Idowu MO, Wan W, Wang XY, et al. Tumor-reactive immune cells protect against metastatic tumor and induce immunoediting of indolent but not quiescent tumor cells. *J Leukoc Biol* (2016) 100:625–35. doi:10.1189/jlb.5A1215-580R
198. Teng MW, Yue S, Sharkey J, Exley MA, Smyth MJ. CD1d activation and blockade: a new antitumor strategy. *J Immunol* (2009) 182:3366–71. doi:10.4049/jimmunol.0802964
199. Teng MW, Sharkey J, Mclaughlin NM, Exley MA, Smyth MJ. CD1d-based combination therapy eradicates established tumors in mice. *J Immunol* (2009) 183:1911–20. doi:10.4049/jimmunol.0900796
200. Stirnemann K, Romero JF, Baldi L, Robert B, Cesson V, Besra GS, et al. Sustained activation and tumor targeting of NKT cells using a CD1d-anti-HER2-scFv fusion protein induce antitumor effects in mice. *J Clin Invest* (2008) 118:994–1005. doi:10.1172/JCI33249
201. Corgnac S, Perret R, Derre L, Zhang L, Stirnemann K, Zauderer M, et al. CD1d-antibody fusion proteins target iNKT cells to the tumor and trigger long-term therapeutic responses. *Cancer Immunol Immunother* (2013) 62:747–60. doi:10.1007/s00262-012-1381-7
202. Heczey A, Liu D, Tian G, Courtney AN, Wei J, Marinova E, et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* (2014) 124:2824–33. doi:10.1182/blood-2013-11-541235
203. Tian G, Courtney AN, Jena B, Heczey A, Liu D, Marinova E, et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. *J Clin Invest* (2016) 126:2341–55. doi:10.1172/JCI83476
204. Smyth MJ, Crowe NY, Hayakawa Y, Takeda K, Yagita H, Godfrey DI. NKT cells – conductors of tumor immunity? *Curr Opin Immunol* (2002) 14:165–71. doi:10.1016/S0952-7915(02)00316-3
205. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* (2005) 115:2572–83. doi:10.1172/JCI24762
206. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* (2002) 3:867–74. doi:10.1038/ni827
207. Nishimura T, Kitamura H, Iwakabe K, Yahata T, Ohta A, Sato M, et al. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol* (2000) 12:987–94. doi:10.1093/intimm/12.7.987
208. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, et al. Adoptive transfer of invariant NKT cells as immunotherapy for advanced melanoma: a phase I clinical trial. *Clin Cancer Res* (2017) 23:3510–9. doi:10.1158/1078-0432.CCR-16-0600
209. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* (2002) 8:3702–9.
210. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124:3725–40. doi:10.1172/JCI72308
211. Sullivan BA, Kronenberg M. Activation or anergy: NKT cells are stunned by alpha-galactosylceramide. *J Clin Invest* (2005) 115:2328–9. doi:10.1172/JCI26297
212. Kunii N, Horiguchi S, Motohashi S, Yamamoto H, Ueno N, Yamamoto S, et al. Combination therapy of in vitro-expanded natural killer T cells and alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* (2009) 100:1092–8. doi:10.1111/j.1349-7006.2009.01135.x
213. Yamasaki K, Horiguchi S, Kurosaki M, Kunii N, Nagato K, Hanaoka H, et al. Induction of NKT cell-specific immune responses in cancer tissues after NKT cell-targeted adoptive immunotherapy. *Clin Immunol* (2011) 138:255–65. doi:10.1016/j.clim.2010.11.014
214. Richter J, Neparidze N, Zhang L, Nair S, Monesmith T, Sundaram R, et al. Clinical regressions and broad immune activation following combination therapy targeting human NKT cells in myeloma. *Blood* (2013) 121:423–30. doi:10.1182/blood-2012-06-435503
215. Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, et al. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* (2004) 103:383–9. doi:10.1182/blood-2003-04-1155

216. Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, et al. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* (2005) 11:1910–7. doi:10.1158/1078-0432.CCR-04-1453
217. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* (2009) 182:2492–501. doi:10.4049/jimmunol.0800126
218. Nicol AJ, Tazbirkova A, Nieda M. Comparison of clinical and immunological effects of intravenous and intradermal administration of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells. *Clin Cancer Res* (2011) 17:5140–51. doi:10.1158/1078-0432.CCR-10-3105
219. Nagato K, Motohashi S, Ishibashi F, Okita K, Yamasaki K, Moriya Y, et al. Accumulation of activated invariant natural killer T cells in the tumor microenvironment after alpha-galactosylceramide-pulsed antigen presenting cells. *J Clin Immunol* (2012) 32:1071–81. doi:10.1007/s10875-012-9697-9
220. Uchida T, Horiguchi S, Tanaka Y, Yamamoto H, Kunii N, Motohashi S, et al. Phase I study of alpha-galactosylceramide-pulsed antigen presenting cells administration to the nasal submucosa in unresectable or recurrent head and neck cancer. *Cancer Immunol Immunother* (2008) 57:337–45. doi:10.1007/s00262-007-0373-5
221. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* (2005) 201:1503–17. doi:10.1084/jem.20042592
222. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* (2006) 12:6079–86. doi:10.1158/1078-0432.CCR-06-0114
223. Watarai H, Fujii S, Yamada D, Rybouchkin A, Sakata S, Nagata Y, et al. Murine induced pluripotent stem cells can be derived from and differentiate into natural killer T cells. *J Clin Invest* (2010) 120:2610–8. doi:10.1172/JCI42027
224. Yamada D, Iyoda T, Vizcardo R, Shimizu K, Sato Y, Endo TA, et al. Efficient regeneration of human Valpha24+ invariant natural killer T cells and their anti-tumor activity in vivo. *Stem Cells* (2016) 34:2852–60. doi:10.1002/stem.2465
225. Kitayama S, Zhang R, Liu TY, Ueda N, Iriguchi S, Yasui Y, et al. Cellular adjuvant properties, direct cytotoxicity of re-differentiated Valpha24 invariant NKT-like cells from human induced pluripotent stem cells. *Stem Cell Reports* (2016) 6:213–27. doi:10.1016/j.stemcr.2016.01.005
226. Sun W, Wang Y, East JE, Kimball AS, Tkaczuk K, Kesmodel S, et al. Invariant natural killer T cells generated from human adult hematopoietic stem-progenitor cells are poly-functional. *Cytokine* (2015) 72:48–57. doi:10.1016/j.cyto.2014.12.009
227. Carreno LJ, Saavedra-Avila NA, Porcelli SA. Synthetic glycolipid activators of natural killer T cells as immunotherapeutic agents. *Clin Transl Immunology* (2016) 5:e69. doi:10.1038/cti.2016.14
228. Venkataswamy MM, Porcelli SA. Lipid and glycolipid antigens of CD1d-restricted natural killer T cells. *Semin Immunol* (2010) 22:68–78. doi:10.1016/j.smim.2009.10.003
229. Carreno LJ, Kharkwal SS, Porcelli SA. Optimizing NKT cell ligands as vaccine adjuvants. *Immunotherapy* (2014) 6:309–20. doi:10.2217/imt.13.175
230. Jukes JP, Gileadi U, Ghadbane H, Yu TF, Shepherd D, Cox LR, et al. Non-glycosidic compounds can stimulate both human and mouse iNKT cells. *Eur J Immunol* (2016) 46:1224–34. doi:10.1002/eji.201546114
231. Faveeuw C, Trottein F. Optimization of natural killer T cell-mediated immunotherapy in cancer using cell-based and nanovector vaccines. *Cancer Res* (2014) 74:1632–8. doi:10.1158/0008-5472.CAN-13-3504
232. Nakamura T, Moriguchi R, Kogure K, Harashima H. Incorporation of polyinosine-polycytidylic acid enhances cytotoxic T cell activity and antitumor effects by octaarginine-modified liposomes encapsulating antigen, but not by octaarginine-modified antigen complex. *Int J Pharm* (2013) 441:476–81. doi:10.1016/j.ijpharm.2012.11.006
233. Dolen Y, Kreutz M, Gileadi U, Tel J, Vasaturo A, Van Dinther EA, et al. Co-delivery of PLGA encapsulated invariant NKT cell agonist with antigenic protein induce strong T cell-mediated antitumor immune responses. *Oncoimmunology* (2016) 5:e1068493. doi:10.1080/2162402X.2015.1068493
234. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* (2009) 182:2816–26. doi:10.4049/jimmunol.0803648
235. Compton BJ, Tang CW, Johnston KA, Osmond TL, Hayman CM, Larsen DS, et al. Synthesis and activity of 6"-deoxy-6"-thio-alpha-GalCer and peptide conjugates. *Org Lett* (2015) 17:5954–7. doi:10.1021/acs.orglett.5b02836

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Natural Killer T Cell-Targeted Immunotherapy Mediating Long-term Memory Responses and Strong Antitumor Activity

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Current tumor therapies, including immunotherapies, focus on passive eradication or at least reduction of the tumor mass. However, cancer patients quite often suffer from tumor relapse or metastasis after such treatments. To overcome these problems, we have developed a natural killer T (NKT) cell-targeted immunotherapy focusing on active engagement of the patient's immune system, but not directly targeting the tumor cells themselves. NKT cells express an invariant antigen receptor α chain encoded by *Trav11* (Vα14)-*Traj18* (Jα18) gene segments in mice and *TRAV10* (Vα24)-*TRAJ18* (Jα18) in humans and recognize glycolipid ligand in conjunction with a monomorphic CD1d molecule. The NKT cells play a pivotal role in the orchestration of antitumor immune responses by mediating adjuvant effects that activate various antitumor effector cells of both innate and adaptive immune systems and also aid in establishing a long-term memory response. Here, we established NKT cell-targeted therapy using a newly discovered NKT cell glycolipid ligand, RK, which has a stronger capacity to stimulate both human and mouse NKT cells compared to previous NKT cell ligand. Moreover, RK mediates strong adjuvant effects in activating various effector cell types and establishes long-term memory responses, resulting in the continuous attack on the tumor that confers long-lasting and potent antitumor effects. Since the NKT cell ligand presented by the monomorphic CD1d can be used for all humans irrespective of HLA types, and also because NKT cell-targeted therapy does not directly target tumor cells, this therapy can potentially be applied to all cancer patients and any tumor types.

Keywords: natural killer T cell, tumor immunology, CD1d, immunotherapy, neoglycolipid, adjuvant activity, long-term memory responses

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INTRODUCTION

Immunotherapy, which acts by harnessing the power of patient's own immune system, has recently emerged as a treatment option to combat cancer in addition to conventional treatment options such as surgery, chemotherapy, and radiation therapy (1). Despite encouraging results, tumor relapse and metastasis still remain a major problem for any of the current anticancer therapies.

A common and significant limitation of current anticancer immunotherapies is that they often target only one type of antitumor effector cell. For example, in the immunotherapy approaches using tumor peptide-specific CTL, dendritic cells (DCs), engineered CAR-T cells, tumor-infiltrating lymphocytes, or antibodies against PD-1, the target is the effector T cell, which kills MHC-positive, but not MHC-negative, tumor cells, resulting in recurrence of MHC-negative tumor cells (2). Similarly, in the case of lymphokine-activated NK cells or NK cells generated by the enforced expression of NK receptor ligands, such as Rae1/H60/Mult-1 (NKG2D-L), the effector cells eliminate only MHC-negative tumor cells, resulting in the relapse by MHC-positive tumor cells (3). Moreover, tumor cells often undergo mutational changes that render them resistant to these therapies.

In contrast to the current immunotherapy approaches described above, natural killer T (NKT) cells (4–6), but not other effector cell types, have the potential to simultaneously activate various effector cell types, including both CD8 T and NK cells that, in turn, eliminate both MHC-positive and MHC-negative tumor cells (7). In addition, activated NKT cells can interact with immature DCs in the presence of their agonist ligand and induce maturation of DCs, thereby overcoming of the immunodeficiency status often seen in cancer patients, and also in establishment of long-term antitumor immunity (8). Therefore, NKT cell-targeted therapy is thought to be an ideal treatment approach for combating cancer and preventing tumor relapse and metastasis. Moreover, as an NKT cell ligand is presented by the monomorphic CD1d molecule (9), the ligand itself could be used as a drug that could be administered to any patient, no matter what their HLA haplotype. As the antitumor effect of NKT cell-targeted immunotherapy largely depends on activating other innate and adaptive immune cells of patient's own immune system, which theoretically contains clones of tumor-specific effector cells that, however, cannot be effectively activated due to the immunosuppressive mechanisms mediated by tumor cells, the NKT cell-targeted therapy could be used to harness the immune system to fight any tumors type.

Based on exceptional results from preclinical studies using the potent NKT cell agonistic glycolipid ligand α-galactosylceramide (GC) (10–14), NKT cell-targeted cancer therapy in human clinical trials was started in patients with advanced or recurrent stages of various cancers. The results from these clinical trials were fairly promising, e.g., prolonged median survival time (MST) of 18.6 months in all treated patients (17 cases) with advanced non-small lung cancer refractory to the conventional therapies such as chemotherapy, radiation or surgery upon treatment with GC-pulsed peripheral blood mononuclear cells (PBMCs) compared to the MST of 4.6 months in the best supportive care patient group (15, 16). However, there is still a need for further improvements aimed to increase the efficacy of NKT cell-targeted immunotherapy. It is also important to mention that the efficacy of this NKT cell therapy is almost equivalent to that using checkpoint inhibitor such as a PD-1 mAb treatment, where the reported MST of advanced non-squamous non-small-cell lung cancer patients was 12.2 months (17).

One of approaches aimed to increase the efficacy of NKT cell-targeted antitumor immunotherapy is the optimization of NKT cell-activating ligands (18). The antitumor effect of NKT cell-based immunotherapy depends primarily on potent secretion of IFN-γ, a cytokine that actually mediates the activation of downstream cellular networks, resulting in a strong adjuvant action *via* NK cells, CD8 cytotoxic T cells, and other cell types (19), and also establishment of long-term memory responses (8). Thus, the search for a ligand capable of stimulating human NKT cells with a strong T_H1 cytokine profile is an important objective.

In this study, we developed NKT cell-targeted cancer therapy using a newly synthesized glycolipid, termed RK, which is recognized by both mouse and human NKT cells, thereby resulting in the superior antitumor responses compared to GC. In addition, RK shows stronger activity in inducing IFN-γ release from both human and mouse NKT cells compared with the prototypical ligand GC when presented by DCs. We also demonstrate that RK-pulsed DCs have remarkable potential for induction of NKT cell-mediated adjuvant activity by activating downstream cell types such as NK and CD8 T cells, and in the establishment of long-term memory responses against a model antigen ovalbumin. Taken together, we believe that RK has a potential use in human translational studies in anticancer immunotherapy applications targeting NKT cells.

MATERIALS AND METHODS

Human Samples and Animal Studies

All experiments involving human samples were performed with authorization from the Institutional Review Board for Human Research at RIKEN IMS. Umbilical cord blood samples were obtained from RIKEN BRC Cord Blood Bank collected with written informed consent. PBMCs from healthy donors were purchased from Astarte Biologics, LLC (USA).

Mice

Wild-type (WT) C57BL/6 (B6) mice were purchased from Charles River Laboratories; B6.CD45.1 mice were from The Jackson Laboratory; the new *Traj18*$^{-/-}$ mice expressing undisturbed TCRα chain repertoire, except for Jα18, on B6 background were described (20). Mice were maintained in the animal facility of RIKEN IMS under specific pathogen-free conditions and were used at 8–10 weeks of age. All animal experiments were approved by RIKEN Animal Care and Use Committee.

Neoglycolipid

The structure and the synthesis method of RK were described previously (21). In brief, reduction of an azide prepared by modification of the 6-hydroxy group of the known alcohol (2*S*,3*R*,4*E*)-2-Azido-3,4-di-*O*-benzyl-1-*O*-[2,3,4-tri-*O*-benzyl-6-*O*-(*tert*-butyldimethylsilyl)-D-galactopyranosyl]octadecane-1,3,4-triol by using Staudinger reaction gave an amine (22). The amine was acylated with cerotyl chloride to give an amide. Deprotection of all of the benzyl groups of the amide by hydrogenolysis afforded RK as white powder.

Cell-Free Antigen Presentation Assay

Flat bottom 96-well culture plates were coated with 2 μg of soluble dimeric mouse CD1d:Ig fusion protein (BD Biosciences) in

50 µL PBS. After incubation for 6 h at 4°C, 50 µl of the indicated lipid antigens diluted in PBS were added, and the plate was incubated overnight at 37°C. The next day, the wells were washed with PBS and incubated with complete culture medium before the addition of the NKT cell hybridoma 2E10 at 1×10^5 cells per well. Culture supernatants were collected after 16 h for the cytokine measurement assay by ELISA.

Cell Preparation and Flow Cytometry

Single-cells from designated mouse organs and mononuclear cells from umbilical cord blood samples were prepared as described previously (23, 24). Surface antigen staining was performed after Fc receptor blocking using TruStain fcX™ or human TruStain FcX™ (BioLegend). Forward light-scatter gating and 7-AAD staining (BD Biosciences) were used to gate out doublets and dead cells. Samples were acquired on FACS Canto II (BD Biosciences), and data were analyzed with FlowJo 10.0.8r1 software (Tree Star). Anti-mouse antibodies were as follows: CD3ε$^+$ or -PE (145-2C11), CD8α$^+$ or -PE (53-6.7), CD19-PerCP-Cy5.5 (1D3), CD45.1-FITC (A20), CD45.2-PE (104), NK1.1-APC (PK136), and TCRβ$^+$ (H57-597). Anti-human antibodies were as follows: TCR Vα24-Jα18-Brilliant Violet 421 (6B11), CD3-FITC, or -APC (OKT3). Above mAbs were from BD Biosciences or BioLegend. Vehicle-, RK-, or GC-loaded soluble dimeric mouse or human CD1d:Ig fusion protein (BD Biosciences) were used with APC-anti-mouse IgG1 (X56; BD Biosciences) to detect mouse or human NKT cells, respectively (23).

Intracellular Cytokine Staining

Splenocytes were seeded at 2×10^7 cells/mL in a complete medium supplemented with GolgiPlug (BD Biosciences) and cultured for 1 h at 37°C. After cell surface staining, cells were fixed, permeabilized and stained with PE-labeled anti-IFN-γ (XMG1.2) from BioLegend using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's instructions.

In Vitro Cell Culture Conditions

The NKT cell hybridoma 2E10 was cultured as described (25). Bone marrow-derived DCs from B6 mice were prepared as described (23, 26), where after 6 days of culture in a complete RPMI-1640 medium (ThermoFisher Scientific) supplemented with 5 ng/mL mGM-CSF (R&D), DCs were purified with AutoMACS and anti-mouse CD11c microbeads (Miltenyi Biotec). Human DCs were prepared as described (27), where CD14$^+$ monocytes were purified from PBMNCs with a MACS LS column and anti-human CD14 microbeads (Miltenyi Biotec) and cultured for 6 days in a DendriMACS GMP medium containing 800 U/mL hGM-CSF and 250 U/mL hIL-4 (all from Miltenyi Biotec). Human umbilical cord blood derived mononuclear cells were prepared by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare), and NKT cell cultures were performed as reported (23) with a minor modification, where the culture medium consisted of 50% AIM-V medium (ThermoFisher Scientific), 45% RPMI-1640, 5% heat-inactivated fetal bovine sera (Sigma), 1 × NEAA, 1 mM sodium pyruvate, 55 µM 2-ME, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin (all from ThermoFisher Scientific) and supplemented with 100 U/mL hIL-2 (Shionogi, Japan).

CD40 Ligation and Real-time Quantitative PCR

Human PBMNC-derived DCs (1×10^5 per well) were cultured in the presence or absence of histidine-tagged recombinant human CD40 Ligand (0.1 µg/mL; from R&D) and His Tag Antibody (10 µg/mL; from R&D) in 96-well culture plates for 12 h. RNA was purified using an RNeasy Plus Micro kit (Qiagen), and cDNA was prepared with Superscript VILO cDNA Synthesis Kit (Life Technologies). Quantitative real-time PCR was performed with the ABI PRISM 7900HT system (Applied Biosystems) using FastStart Universal Probe Master Mix (Roche). Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method, where the *GAPDH* expression level served as an internal control. Taqman® Gene Expression Assays for *IL12B* (Hs01011518_m1) and *GAPDH* (Hs02758991_g1) were from Applied Biosystems.

Cytokine Measurements

IFN-γ concentrations in plasma or culture supernatants were quantified with ELISA kits for mouse IFN-γ (R&D) or human IFN-γ (BD Biosciences). The levels of mouse IL-12p70, mouse IL-4, and human IL-4 were measured with a cytometric bead array (CBA) (BD Biosciences).

Induction of T Cell-Mediated Immunity against OVA Antigen

To identify OVA-specific T cells expanded upon NKT stimulation *in vivo*, splenocytes were prepared according to a published report (28) with some minor modifications. In brief, splenocytes (2×10^7 cells per mouse) pulsed with OVA peptide (Worthington Biochemical) were administered intravenously together with 5×10^4 DCs that had been pulsed overnight with RK or GC at 100 ng/mL. The mice were sacrificed after 7 days, and liver mononuclear cells were stained for the presence of OVA-specific CD8 T cells using T-Select H-2Kb OVA Tetramer-SIINFEKL-APC (MBL). For the experiments to assess the induction of long-term OVA-specific immunity, OVA-pulsed, osmotically shocked splenocytes (2×10^7 cells per mouse) were intravenously injected together with unpulsed or RK-pulsed DCs (1×10^6 cells per mouse). These mice received another injection of unpulsed or RK-pulsed DCs (1×10^6 cells per mouse) 4 days after the initial immunization. At the indicated time points, mice were sacrificed and splenocytes were directly assessed for the presence of OVA-specific CD8 T cells using T-Select H-2Kb OVA Tetramer-SIINFEKL-APC (MBL), or were primed *in vitro* with or without 1 µM SIINFEKL ($OVA_{257–264}$) peptide (Abbiotec) for 6 h in the presence of GolgiPlug (BD Biosciences). The cells were then stained for cell surface markers, fixed with the Cytofix/Cytoperm Plus permeabilization kit (BD Biosciences), and stained with PE-labeled anti-IFN-γ (XMG1.2) from BioLegend.

B16 Melanoma Metastasis Model

Mice were anesthetized and the spleen was surgically removed on day 0 after intrasplenic inoculation of B16 melanoma

(5×10^5) cells. Four days after the B16 inoculation, mice were injected intravenously with either RK- or GC-pulsed bone marrow-derived DCs (3×10^4 or 1×10^5). The mice were sacrificed after 2 weeks, and the liver was visually or quantitatively evaluated for B16 metastases.

Measurement of B16 Melanoma Metastases in a Liver Tissue with Visible Light Absorption Spectrophotometry

Liver tissues were homogenized in 10 mL of a sodium hydroxide (1 M) solution with IKA Ultra-Turrax tube disperser workstation system using DT-20 dispersing tube with rotor–stator for 30 s. Then 1 mL of homogenate was heated at 75°C for 1.5 h, diluted further with sodium hydroxide, and 0.2 mL of diluted homogenate was used for measurement of absorbance at 405 nm with a Wallace 1420 ARVO MX multi-label plate reader (PerkinElmer). A standard curve was generated using serially diluted B16 melanoma cells, and normalized absorbance values were calculated relative to the B16 only (untreated) controls.

Statistical Analysis

Where indicated, two-tailed unpaired *t* tests were done using PRISM 6 software (GraphPad). $P < 0.05$ was considered statistically significant.

RESULTS

RK Is Recognized by Mouse NKT Cells and Elicits Strong IFN-γ Secretion

In search of a potent NKT cell-activating antigen with superior antitumor characteristics compared to the widely used glycolipid ligand α-GC, we have newly synthesized a series (172) of neoglycolipids and screened these compounds for their potential to activate NKT cells. For the initial screening assay to measure the NKT cell activating potential of the newly synthesized compounds, we used a cell-free antigen presentation assay, in which soluble CD1d was coated on a culture plate, pulsed with glycolipid antigens, and IFN-γ release from the NKT cell hybridoma 2E10 expressing an invariant Vα14Jα18 TCRα paired with Vβ8 was used as a readout (25). Results showed that one of the newly synthesized glycolipid, termed RK, is a much more potent activator of the NKT cell hybridoma than GC in a glycolipid antigen dose-dependent manner (**Figure 1A**).

Next, we sought to verify and extend the *in vitro* screening result with a more physiologically relevant *in vivo* antigen presentation assay, where WT B6 mice were intravenously injected with RK-pulsed mouse bone marrow-derived dendritic cells (RK-pulsed DCs). Compared with GC-pulsed DCs, the RK-pulsed DCs showed a significantly higher (>4-fold) ability to elicit IFN-γ secretion in the sera collected at both early (6 h) and later (24, 36 h) time points. Conversely, the serum levels of IL-4 were similarly lower in mice injected with RK-pulsed or GC-pulsed DCs compared to IFN-γ levels (**Figure 1B**). In addition, as expected, IFN-γ was not detected in the sera of NKT cell-deficient *Traj18*$^{-/-}$ mice, verifying the NKT cell specificity of both RK- and GC-pulsed DCs (data not shown). These results demonstrated that RK-pulsed DCs induce stronger T_H1-type cytokine secretion compared with GC-pulsed DCs.

RK Is Recognized by Human NKT Cells and Elicits Strong IFN-γ Secretion

Because of its importance for therapeutic application, we wished to extend our mouse experiments into a human system to assess

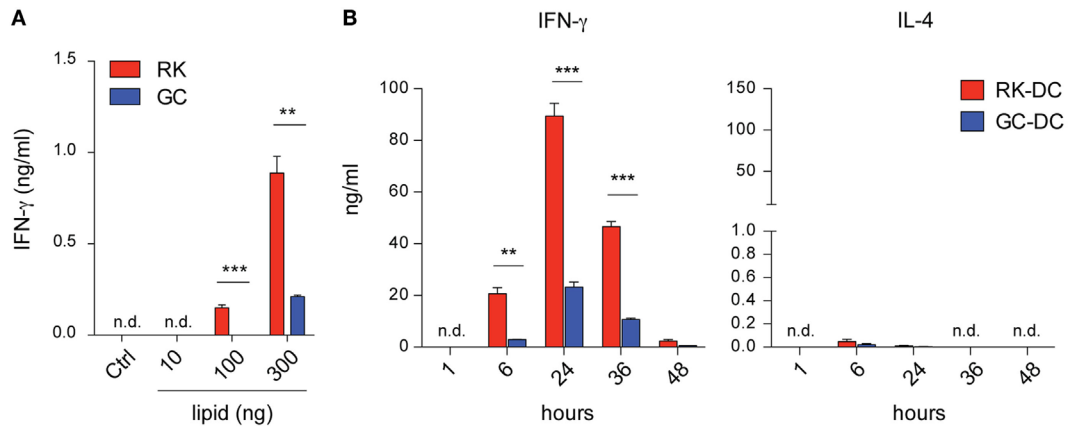

FIGURE 1 | IFN-γ release from mouse Vα14$^+$ natural killer T (NKT) cells activated with RK or galactosylceramide (GC) glycolipids. **(A)** NKT cell hybridoma E210 cells were plated 1×10^5 cells per well into 96-well culture plates, which were previously coated with soluble CD1d and incubated with RK or GC at the indicated concentrations overnight. Vehicle was used as a control in the NKT cell hybridoma activation assay. The culture supernatants were collected after 16 h and IFN-γ levels were measured by ELISA. Data are mean ± SEM from triplicate wells. The data are representative from two independent experiments with similar results. **(B)** *In vivo* antigen presentation assay with RK- or GC-pulsed dendritic cells (DCs). B6 mice were injected intravenously with 5×10^5 RK- or GC-pulsed DCs per mouse, and levels of IFN-γ and IL-4 in the sera collected at the indicated time points were measured by ELISA or cytometric bead array, respectively. Data are mean ± SEM from three mice and repeated three times with similar results. $^{**}P < 0.01$; $^{***}P < 0.001$, unpaired Student's *t*-test; n.d., not detected.

whether the RK antigen is recognized by human NKT cells. To this end, we first loaded human CD1d dimers with RK or GC according to the previously published method (23), and stained a human NKT-iPSC line described previously (29). Flow cytometry results clearly demonstrated that RK-loaded human CD1d molecules efficiently stain human NKT cells, where GC-loaded CD1d and unloaded CD1d were used as positive and negative staining controls, respectively (**Figure 2A**). Of note, the mean fluorescence intensity (MFI) of RK-loaded CD1d was significantly higher than GC-loaded CD1d (**Figure 2B**), which mirrored results obtained by staining of mouse NKT cells with mouse RK-loaded CD1d (Figures S1A–C in Supplementary Material). The elevated MFI levels could be best explained by the higher affinity of the RK-loaded CD1d complex for the invariant TCR of NKT cells.

Next, we examined whether RK can activate human NKT cells using proliferative responses upon *in vitro* culture as the readout. To this end, we cultured umbilical cord derived mononuclear

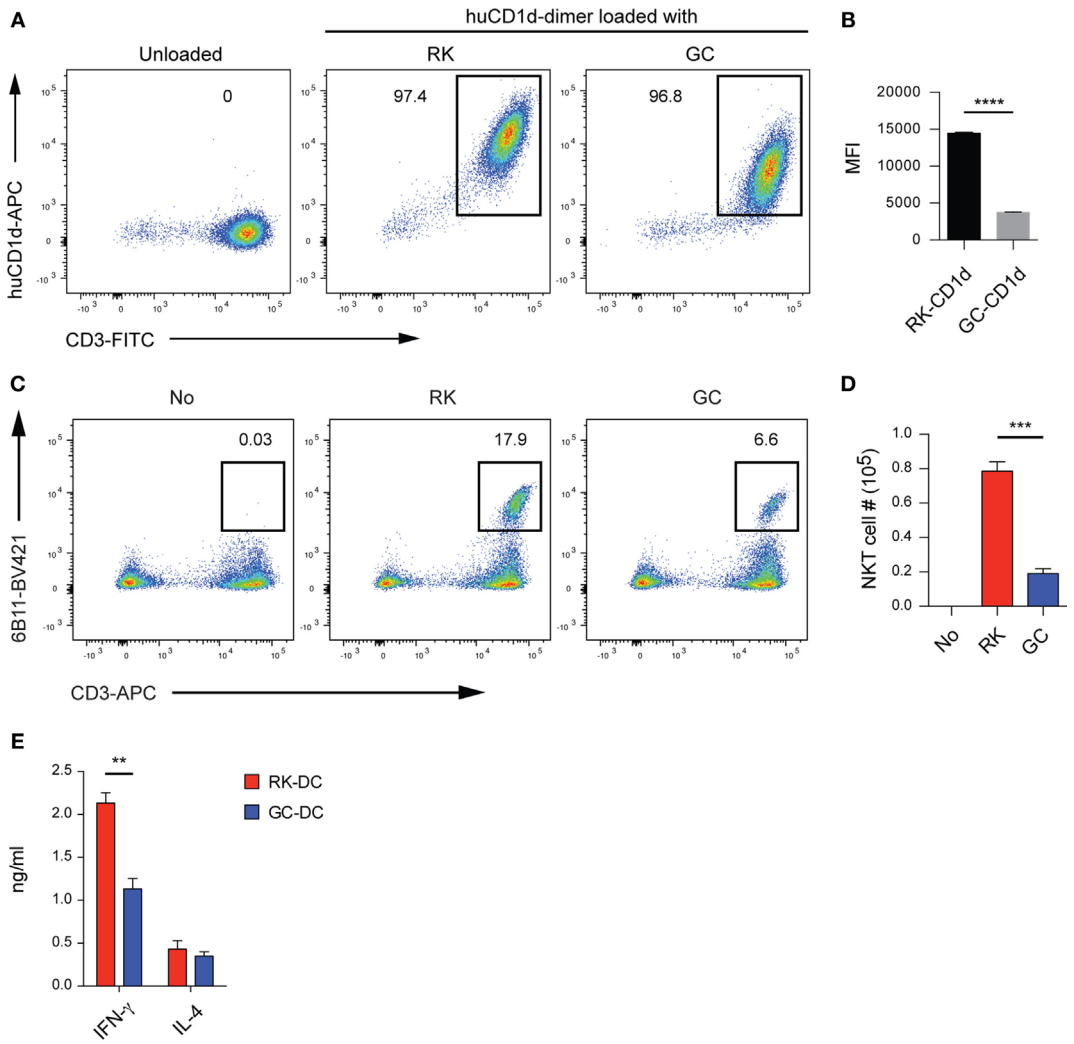

FIGURE 2 | The novel glycolipid RK is recognized by human natural killer T (NKT) cells. **(A)** Staining of an NKT-iPSC line with RK- or galactosylceramide (GC)-loaded human CD1d dimers. Flow cytometry plots are representative from triplicate samples per group. Numbers indicate the percentage of human CD1d dimer$^+$CD3$^+$ cells within the 7-AAD$^-$ viable lymphocyte gates. **(B)** The mean fluorescence intensity (MFI) levels of human CD1d dimers loaded with RK- or GC, which were gated as shown in panel **(A)**. Data are mean ± SEM from triplicate samples per group. Experiments were repeated three times with similar results. **(C)** Human NKT cell expansion upon culturing with RK. Umbilical cord blood mononuclear cells were cultured in the presence or absence of the indicated glycolipids (100 ng/mL) for 19 days in complete media supplemented with 100 U/mL hIL-2. The cultures were re-stimulated with fresh glycolipid-pulsed antigen-presenting cells on culture day 9. Numbers on flow cytometry plots show frequencies (mean ± SEM, $n = 3$ samples per group) of Vα24$^+$CD3$^+$ human NKT cells within gated viable lymphocytes. **(D)** Absolute NKT cell numbers (mean ± SEM, $n = 3$ samples per group) of Vα24$^+$CD3$^+$ human NKT cells as shown in panel **(C)**. Experiments were repeated with two different donors with similar results. **(E)** IFN-γ and IL-4 release from human NKT cells activated with RK-pulsed dendritic cells (DCs). Human NKT cells (5×10^4 per well) were co-cultured for 48 h with the same numbers of peripheral blood monocyte-derived DCs that were pulsed overnight with RK or GC at 100 ng/mL. IFN-γ and IL-4 levels in culture supernatants were measured with ELISA or cytometric bead array, respectively. Data are mean ± SEM from triplicate wells. Experiments were repeated three times with similar results. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, unpaired Student's *t*-test.

cells in the presence of RK or GC for a total of 19 days. Flow cytometry analyses demonstrated that RK induces significantly higher expansion of human NKT cells compared with GC (**Figures 2C,D**). These results indicate that RK not only strongly stimulates human NKT cells but also is efficiently processed and presented by human antigen-presenting cells (APCs).

To investigate whether RK presented by human DCs has the capacity to induce increased T_H1 cytokine release from human NKT cells, we carried out an antigen presentation assay using peripheral blood monocyte-derived DCs as APCs together with a human NKT cell line as effector cells. The IFN-γ but not IL-4 levels in 48 h co-culture supernatants were significantly higher in RK-pulsed DCs compared with GC-pulsed DCs (**Figure 2E**).

In summary, these results demonstrate that RK is efficiently presented by human DCs and that these RK-pulsed DCs show superior activity compared to GC-pulsed DCs in promoting T_H1 cytokine production from human NKT cells.

The *In Vivo* Dynamics of RK-Pulsed DCs

It is well known that GC-pulsed DCs mediate strong antitumor activities upon systemic administration (14, 20). For pharmacokinetic characterization of RK-pulsed DCs, we investigated their tissue distribution and dynamics. To this end, we prepared RK-pulsed DCs from B6.CD45.2 donors and then injected these cells intravenously into B6.CD45.1 congenic recipient mice. $CD45.2^+$ DCs of donor origin were enumerated at 1, 24, and 72 h time points after injection. Flow cytometry analyses of recipient mice demonstrated that injected DCs of donor origin are eliminated within 72 h when administered *via* the intravenous route (**Figure 3**). The distribution pattern of donor DCs suggests that the majority are located in the lung and liver at the 1-h time point and to a lesser extent in the spleen. The number of donor DCs gradually decreased until they were no longer detectable at the 72-h time point. These results imply that the RK-pulsed DCs are quickly eliminated from the organism, soon after these cells have done their job to activate NKT cells.

Adjuvant Effects on Immune Cell Types upon RK-Pulsed DC Administration

We went on to investigate *in vivo* adjuvant effects of RK-pulsed DCs on both innate and adaptive immune cell types, such as NKT, NK, and CD8 T cells of the host. Flow cytometry analyses of splenocytes from B6 mice on day 6 post-injection revealed that both the frequency and absolute number of splenic NKT cells was clearly augmented upon RK-pulsed DC administration compared with control mice injected with unpulsed DCs (**Figures 4A,B**). These results suggest that adoptive transfer of RK-pulsed DCs induces a robust expansion of NKT cells *in vivo*.

To directly assess the NKT cell-mediated trans-activation of NK cells (30, 31), we intravenously injected RK-pulsed DCs and then investigated IFN-γ production by splenic NK cells at 16 h post-injection. Flow cytometry analysis of splenic NK cells, which were detected as $NK1.1^+CD3\varepsilon^-$ cells, revealed that frequency and number of IFN-γ^+ NK cells from RK-pulsed DC-injected animals were significantly increased compared to mice injected with unpulsed DCs (**Figures 4C,D**). These data suggest that RK-pulsed DCs induce strong trans-activation of NK cells, which in turn leads to the long-lasting IFN-γ secretion.

To directly assess the NKT cell-mediated adjuvant activity on adaptive immune responses, we immunized B6 mice with OVA antigen together with unpulsed or RK-pulsed DCs and analyzed liver mononuclear cells 7 days later for the presence of OVA-specific CD8 T cells. Flow cytometry analysis showed that the RK-pulsed DC injection resulted in greatly increased frequencies and numbers of OVA tetramer-positive CD8 T cells compared with those of unpulsed DCs (**Figures 4E,F**). These results indicate that RK-pulsed DCs show strong adjuvant effects in the induction of antigen-specific immunity.

Then, we wished to test whether *in vivo* administration of RK-pulsed DCs results in IL-12 release, which in turn provides a positive feedback effect on IFN-γ production from NK and NKT cells (32–34). To this end, we assessed sera samples collected 6 h after injection with RK-pulsed DCs or unpulsed DCs. The CBA results clearly revealed strong IL-12p70 release from mice injected with RK-pulsed DCs, while no IL-12p70 was detected in mice that received unpulsed DCs (**Figure 4G**). Given that the mechanism of IL-12 release from DCs is thought to be a consequence of NKT cell-mediated maturation of glycolipid-presenting immature DCs through CD40/CD40L interactions (35), we wished to further extend these results using human PBMNC-derived DCs pulsed with RK and assess the IL-12 induction mimicking the CD40/CD40L interaction *in vitro*. Real-time PCR analyses of human RK-pulsed DCs cultured with or without CD40 ligand clearly demonstrated a significant

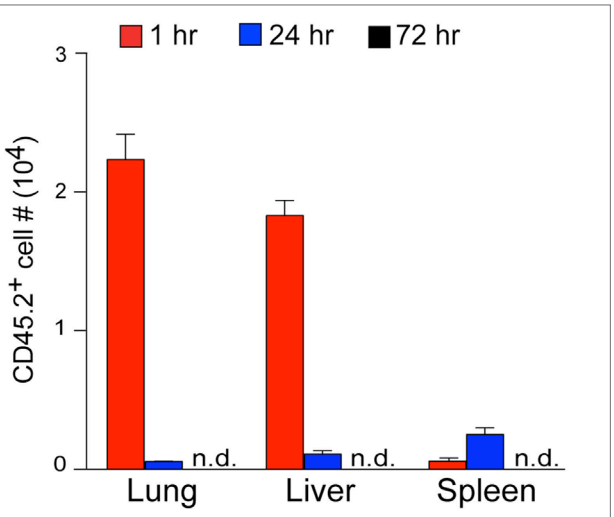

FIGURE 3 | The *in vivo* tissue dynamics of RK-pulsed dendritic cells (DCs). RK-pulsed DCs prepared from B6.CD45.2 donors (1×10^7) were adoptively transferred *via* the tail veins of B6.CD45.1 congenic recipient mice, and $CD45.2^+$ DCs of donor origin were assessed by flow cytometry at 1, 24, and 72 h time points after injection. Absolute numbers of $CD45.2^+$ donor-derived cells found within the indicated organs are shown (mean numbers ± SEM, $n = 3$ recipient mice). Experiments were repeated two times with similar results; n.d., not detected.

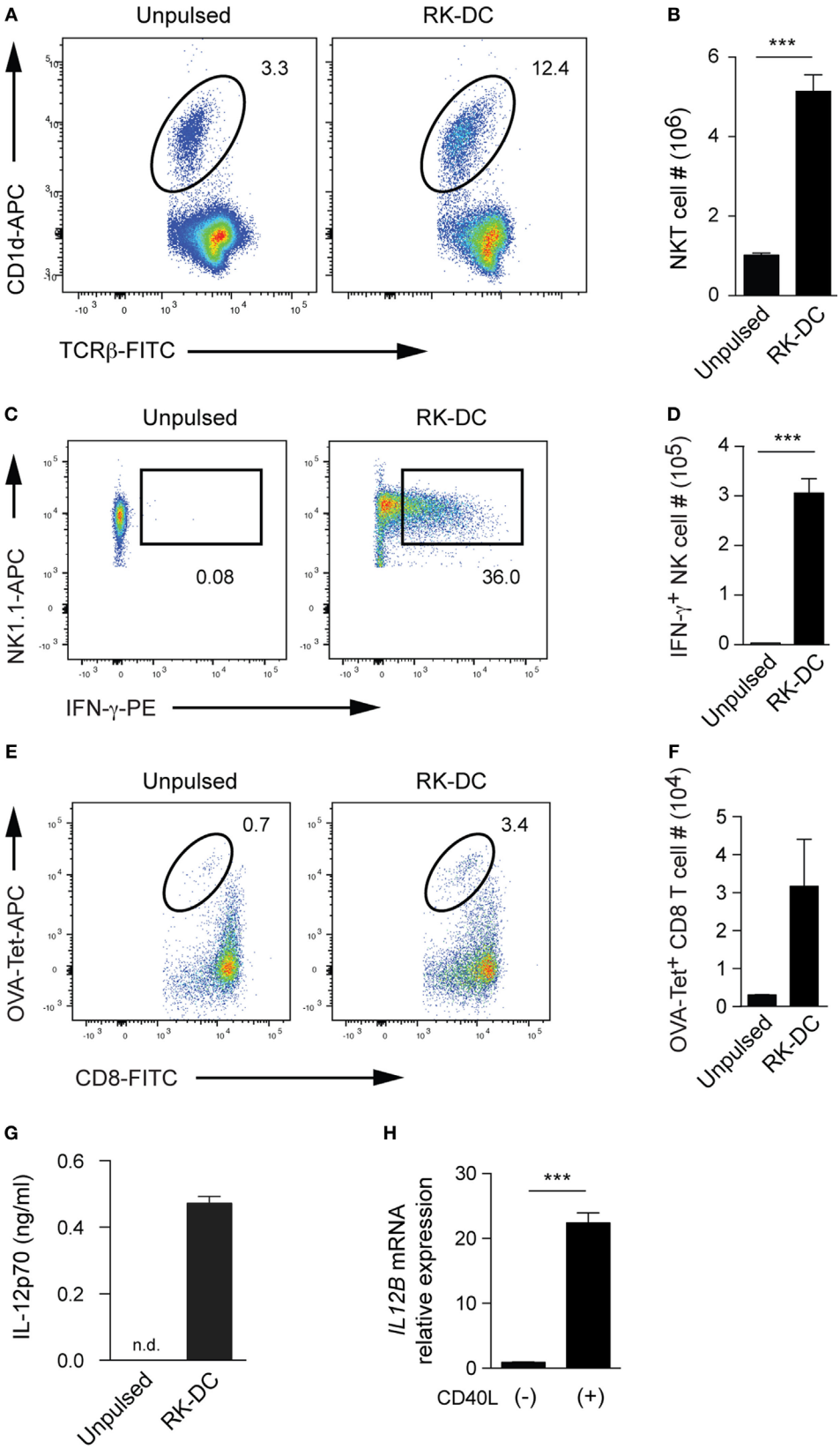

FIGURE 4 | Continued

FIGURE 4 | Continued
The *in vivo* adjuvant effects of RK-pulsed dendritic cells (DCs). **(A)** *In vivo* expansion of natural killer T (NKT) cells upon injection of RK-DCs. Flow cytometry of wild-type B6 splenocytes 6 days after intravenous injection of 5×10^4 unpulsed DCs or RK-DCs. Numbers indicate percentages of CD1d dimer$^+$TCRβ$^+$ NKT cells within the 7-AAD$^-$CD8$^-$CD19$^-$ viable splenocyte gate. Data are representative of 3 mice per group. **(B)** Absolute numbers of splenic NKT cells as shown in panel **(A)**. Data are mean ± SEM from three mice per group. **(C)** Intracellular analysis of IFN-γ production by splenic NK cells. Unpulsed- or RK-pulsed DCs (5×10^4 per mouse) were injected intravenously into B6 mice. Splenocytes were isolated after 16 h and cultured in complete media containing GolgiPlug (BD Biosciences) for 1 h. Numbers on flow cytometry plots show percentages of NK1.1$^+$IFN-γ$^+$ cells among NK1.1$^+$CD3ε$^-$ gated cells. The data are representative of $n = 3$ mice per group. **(D)** Absolute cell numbers of IFN-γ$^+$ NK cells. Data are mean ± SEM from $n = 3$ mice per group. **(E)** Detection of OVA-specific tetramer-positive CD8 effector T cells. B6 mice were immunized with OVA antigen together with unpulsed- or RK-pulsed DCs (5×10^4 per mouse) on day 0 and liver mononuclear cells were analyzed 7 days later. Numbers on flow cytometry plots indicate percentages of OVA tetramer-positive cells among viable CD8 T cells. **(F)** Absolute numbers of OVA tetramer + CD8 T cells (mean ± SEM, $n = 3$ mice per group) gated as shown in panel **(E)**. Experiments shown in panels **(A–F)** were repeated two times with similar results. **(G)** Detection of IL-12p70 in the sera collected 6 h after intravenous injection of 5×10^4 unpulsed- or RK-pulsed DCs into B6 mice. Serum IL-12p70 levels were measured by cytometric bead array. Data are mean ± SEM from three mice, and experiments were repeated three times with similar results. **(H)** Detection of *IL12B* mRNA encoding IL-12p40 by real-time quantitative PCR. RK-pulsed human PBMNC-derived DCs (1×10^5 per well) were cultured for 12 h with or without recombinant human CD40 ligand. Bars depict the relative gene expression (mean ± SEM from triplicate wells) with *GAPDH* used as internal control. The experiment was repeated with three different donors with essentially similar results. ***$P < 0.001$, unpaired Student's *t*-test; n.d., not detected.

induction of *IL12B* mRNA encoding IL-12p40 upon CD40 ligation (**Figure 4H**).

Taken together, these results suggest a scenario where adoptively transferred RK-pulsed DCs, in addition to eliciting strong IFN-γ production by NKT cells, mediate positive feedback to further enhance IFN-γ production through their ability to release IL-12 upon interaction with endogenous NKT cells of the host through CD40/CD40L. These cellular interactions presumably lead to observed remarkably strong adjuvant effects, resulting in the activation of players associated with innate and adaptive immune systems such as NK cells and antigen-specific effector CD8 T cells.

RK-Pulsed DCs Promote an Establishment of Long-term T Cell Memory Responses

It was previously reported that NKT cells were required for the establishment of long-term protective immunity against several tumor cell lines that lasted even after one year of vaccination with GC-loaded tumor cells used as vaccine delivery vectors (8). Here, we investigated whether NKT activation with RK-pulsed DCs can induce long-term antigen-specific T cell-mediated memory responses. To test this, we made use of experimental model with OVA as an artificial antigen, where B6 mice were immunized with osmotically shocked splenocytes pulsed with OVA together with unpulsed or RK-pulsed DCs by the intravenous route. When we assessed the immunized mice 3 months later, the *in vitro* functional assay results clearly demonstrated the presence of clonotypically expanded IFN-γ producing OVA-specific CD8 T cells in mice that had received RK-pulsed DCs, whereas these OVA-specific CD8 T cells were virtually undetectable in mice that had received unpulsed DCs (**Figures 5A,B**). When we assessed similarly immunized mice after 9 months, we could still detect significant numbers of OVA-specific T cells (**Figures 5C,D**), where the numbers of both CD44$^+$CD62L$^+$ central memory and CD44$^+$CD62L$^-$ effector memory OVA tetramer$^+$ CD8 T cells were expanded in mice treated with RK-pulsed DCs compared with those treated with unpulsed DCs (**Figures 5E,F**). These results demonstrate that RK-pulsed DCs have strong adjuvant effects that confer the induction of long-term antigen-specific T cell memory, which presumably plays a prime role in the long-lasting T cell-mediated immunity against the MHC-positive tumors.

RK-Pulsed DCs Possess Superior Antitumor Activity

Since the *in vivo* biological effects of RK-pulsed DCs were compatible with their being promising antitumor immunotherapy therapeutics, we investigated the antitumor properties of RK-pulsed DCs. For this purpose, we used a B16 melanoma liver metastasis model in which GC-pulsed DCs have been shown to completely eradicate the tumor (14, 20). Therefore, in order to directly compare the antitumor potential of RK- and GC-pulsed DCs, we modified our previously reported method. We established a liver metastasis model by intrasplenic injection of B16 melanoma cells at day 0, and then after 4 days we injected RK- or GC-pulsed DCs into the melanoma-bearing hosts. Macroscopic observation of liver tissues obtained after two weeks of injection with DCs clearly showed that RK-pulsed DCs have much stronger antitumor activity compared with GC-pulsed DCs (**Figure 6A**). Strikingly, as few as 3×10^4 RK-pulsed DCs almost completely eradicated the tumor, while the same number of GC-pulsed DCs did not show any therapeutic effect. Moreover, the effect from injection of 3×10^4 RK-pulsed DCs was almost comparable to the effect from injection of 1×10^5 RK-DCs. In addition to the macroscopic evaluation, we also attempted to quantitatively measure the B16 melanoma burden in the liver by homogenizing the liver tissue and analyzing the melanin concentration in samples by visible light spectroscopy, based on a standard curve obtained with serially diluted melanoma cells (**Figure 6B**). Results from quantitative analyses of B16 melanoma burden confirmed the results derived from macroscopic observation (**Figure 6C**). Collectively, these results clearly demonstrate that RK-pulsed DCs have superior antitumor activity compared with the widely used GC-pulsed DCs.

DISCUSSION

In this study, we have developed NKT cell-targeted cancer therapy using a newly synthesized glycolipid, termed RK, which is recognized by both mouse and human NKT cells, mediates strong adjuvant activity on various cell types in the innate and acquired immune systems, establishes long-term T cell memory responses (lasting more than 9 months), and shows superior antitumor responses.

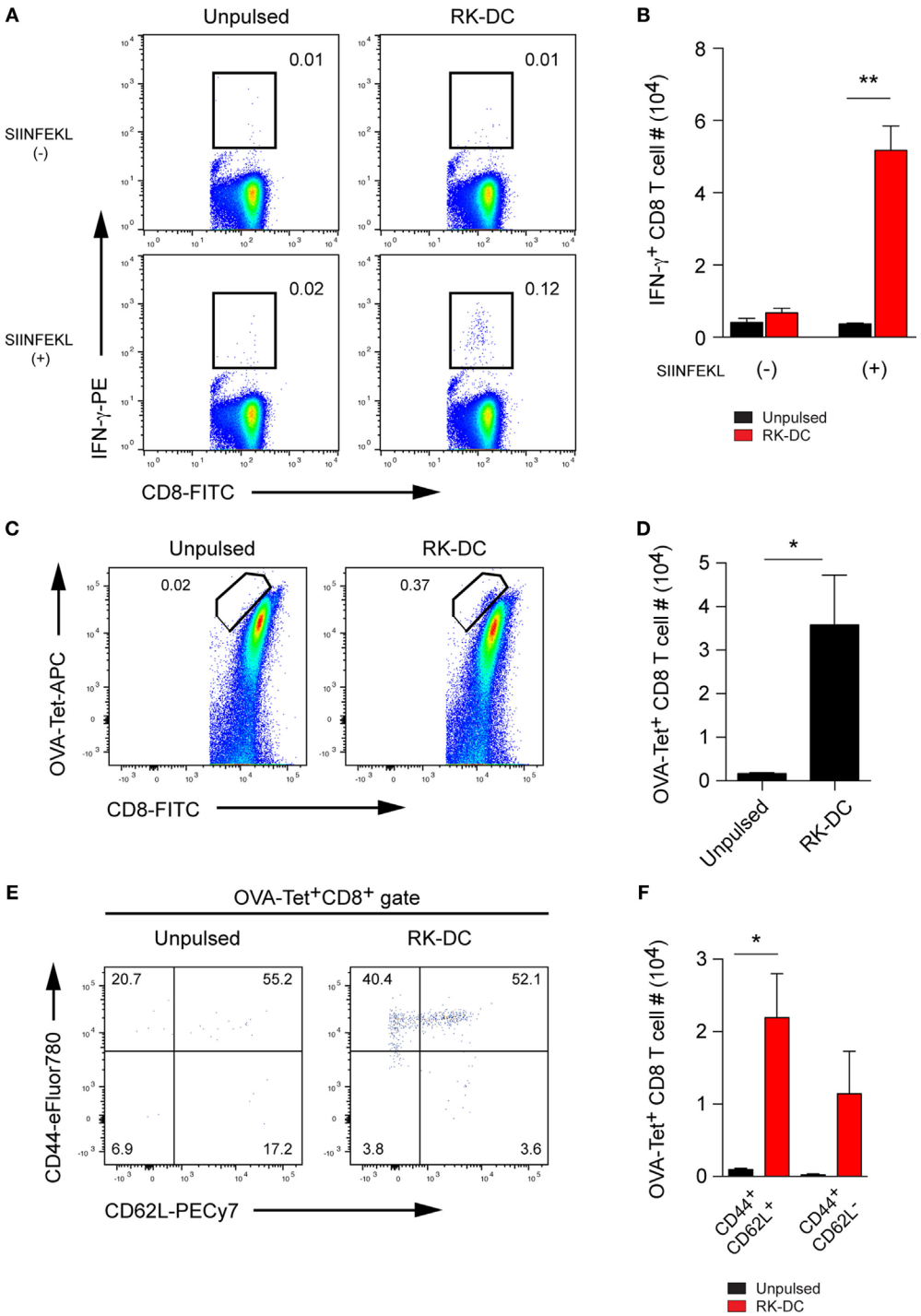

FIGURE 5 | Evaluation of long-term T cell memory responses. **(A)** Detection of IFN-γ-producing memory CD8 T cells. B6 mice were immunized with OVA antigen and injected with unpulsed- or RK-pulsed dendritic cells (DCs) (1×10^6 cells per injection) on day 0 and day 4 by the intravenous route. Splenocytes were harvested 3 months later and were activated *in vitro* with or without SIINFEKL ($OVA_{257-264}$) peptide for 6 h in the presence of GolgiPlug Protein Transport Inhibitor. Cells were stained with anti-IFN-γ mAb using a Cytofix/Cytoperm intracellular staining kit. Numbers on flow cytometry plots indicate frequencies of IFN-γ producing CD8 T cells gated as $CD3\varepsilon^+CD8^+$ lymphocytes. Data are representative from three mice per group. **(B)** Absolute numbers of IFN-γ^+ CD8 T cells (mean ± SEM, $n = 3$ mice per group) gated as shown in panel **(A)**. **(C)** Frequencies and **(D)** absolute numbers (mean ± SEM, $n = 3$ mice per group) of OVA-specific memory CD8 T cells detected 9 months after immunization. OVA immunization and adoptive transfer of unpulsed- or RK-pulsed DCs were done as described in panel **(A)**. Numbers on flow cytometry plots show percentages of OVA tetramer$^+$CD8$^+$ memory T cells among $CD3\varepsilon^+CD8^+$ gated splenocytes. **(E)** Frequencies and **(F)** numbers (mean ± SEM, $n = 3$ mice per group) of $CD44^+CD62L^+$ central memory and $CD44^+CD62L^-$ effector memory antigen-specific CD8 T cells among gated OVA tetramer$^+$CD8$^+$ T cells analyzed 9 months after immunization as shown in panel **(C)**. All experiments were repeated two times with similar results. $^*P < 0.05$; $^{**}P < 0.01$, unpaired Student's *t*-test.

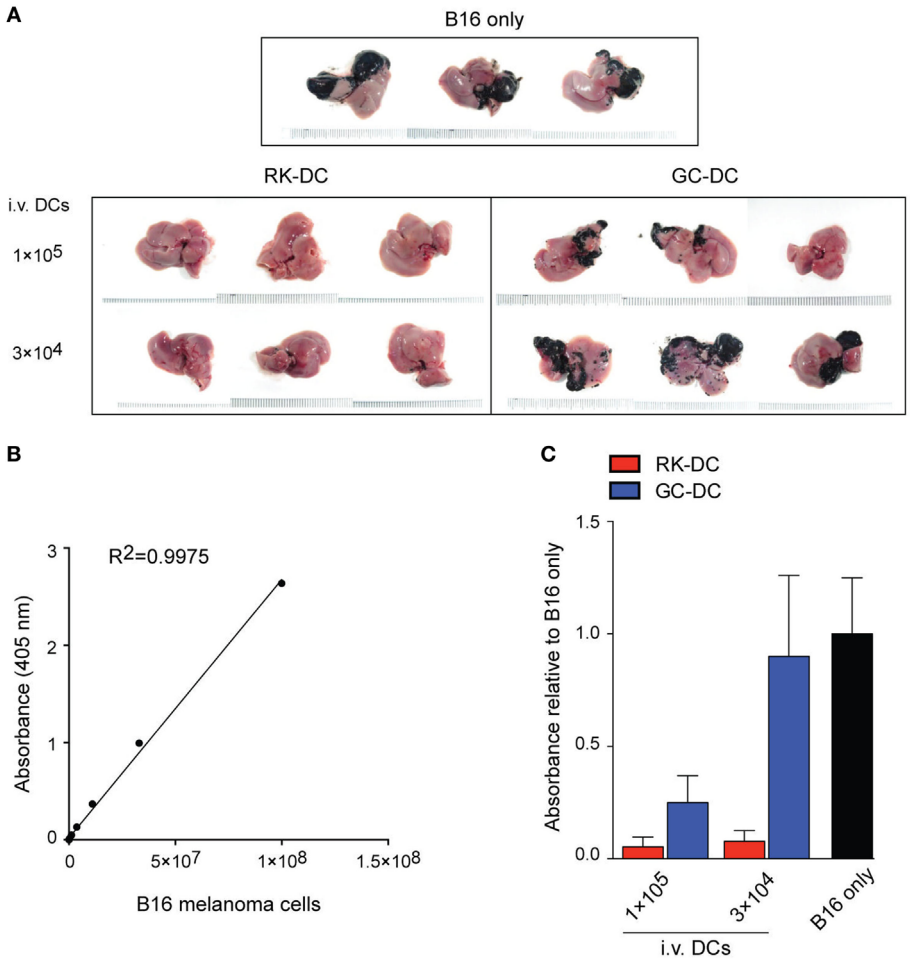

FIGURE 6 | Antitumor activity of RK-pulsed dendritic cells (DCs). **(A)** Inhibition of B16 melanoma metastasis by specific activation of natural killer T cells with RK-DCs. The antitumor effect of RK-DCs was assessed using the B16 melanoma liver metastasis model. Tumor cells were inoculated into B6 mice on day 0, and the indicated numbers of RK- or galactosylceramide (GC)-pulsed DCs were injected intravenously on day 4. Untreated control mice were inoculated only with the B16 melanoma. Individual liver tissue images obtained from three mice per experimental group on day 14 post-injection are shown. **(B)** Standard curve generated by melanin measurement using visible light spectroscopy at 405 nm, obtained from serially diluted B16 melanoma cells. **(C)** Quantitative analysis of B16 melanoma burden in the liver tissues shown in panel **(A)**. Values represent calculated ratios of melanin concentrations assessed by visible light spectroscopy relative to the untreated animals inoculated only with the B16 melanoma (mean ± SEM, $n = 5$ mice per group). All experiments were repeated three times with similar results.

There are several possible mechanisms that could explain this superior antitumor activity resulting from NKT cell activation with RK. One interpretation is that RK has a much stronger ability to induce IFN-γ release from both mouse and human NKT cells compared with GC when presented by professional APCs such as DCs (as shown in **Figure 1B**). In agreement with previous studies suggesting that the strong binding affinity of the TCR with the glycolipid/CD1d complex may lead to the T_H1 skewed cytokine release (27, 36), the staining intensities of RK-loaded human or mouse CD1d molecules showed significantly higher MFI than those of GC-loaded CD1d. This could partly explain the augmented IFN-γ release from NKT cells, which may have resulted from higher binding affinity of the TCR/RK/CD1d triple complex. We also found that adoptive transfer of RK-pulsed DCs strongly induced IL-12 release, presumably induced upon interaction of transferred RK-pulsed DCs with endogenous NKT cells through a CD40/CD40L interaction, which further enhances IFN-γ secretion through a positive feedback mechanism.

We have also made an interesting observation that adoptive transfer of RK-pulsed DCs mediated strong adjuvant effects before the cells were eliminated from the recipient within 72 h, yet they could still activate various downstream effector cell types such as NK and antigen-specific CD8 T cells. This feature of RK-pulsed DCs to activate both NK and CD8 T effector cells and other antitumor effector cells is particularly important for the immunotherapy approach, because for an optimal anticancer therapy both MHC-positive and MHC-negative tumor cells should be eliminated simultaneously to avoid tumor relapse and metastasis. In other words, this dichotomy represents the main threat in anticancer therapy because tumors in general contain both cell types and often undergo mutational changes that allow immune evasion. Furthermore, the adjuvant activity of

RK-pulsed DCs has important implications because tumor cells, unlike pathogens, are usually poorly immunogenic as they do not contain any endogenous adjuvant materials. It is this property of tumor cells that makes it difficult for the immune system to mount efficient antitumor immune responses in patients without use of adjuvants.

Another important finding was that RK-pulsed DCs have the capacity to establish long-term memory responses, where we could still detect ovalbumin-specific $CD44^+CD62L^+$ central memory CD8 T cells even after 9 months in OVA-primed mice that had received only a single injection of RK-pulsed DCs. The establishment of long-term T cell-based memory is particularly important in cancer patients to prevent tumor recurrence by conferring long-lasting tumor protection.

In summary, RK-pulsed DCs strongly activate NKT cells *in vivo,* and through their ability to secrete IL-12, which is induced upon interaction with endogenous NKT cells of the host *in situ*, augment IFN-γ release that results in strong adjuvant activity for both innate and acquired effector systems. The endpoint result of these cellular interactions is the activation of both cytotoxic CD8 T cells and NK cells that eliminate MHC-positive and MHC-negative tumors, respectively, resulting in the tumor eradication without relapse.

Our results warrant further human translational studies using the newly discovered agonist antigen RK. Moreover, RK-pulsed DCs are promising targets for future clinical application because of their potent adjuvant activities on both cytotoxic CD8 T cells and NK cells to eliminate MHC-positive and MHC-negative tumor cells, respectively, and their potential to establish long-term effector memory. Currently, we have started translational research (AMED project) on the establishment of an immunotherapy method using the novel glycolipid ligand RK.

ETHICS STATEMENT

All experiments were performed in accordance with the institutional guidelines and with the approval of RIKEN committee. All patients have provided informed consent according to the institutional regulations.

AUTHOR CONTRIBUTIONS

ND, TS, and MA performed experiments; TT synthesized RK; ND, TC, KH, HS, AK, and MT analyzed and interpreted the data; ND, TC, HO, KH, and MT designed the experiments; ND and MT wrote the manuscript; MT supervised the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01206/full#supplementary-material.

REFERENCES

1. Blattman JN, Greenberg PD. Cancer immunotherapy: a treatment for the masses. *Science* (2004) 305(5681):200–5. doi:10.1126/science.1100369
2. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12(4):252–64. doi:10.1038/nrc3239
3. Fujii S, Goto A, Shimizu K. Antigen mRNA-transfected, allogeneic fibroblasts loaded with NKT-cell ligand confer antitumor immunity. *Blood* (2009) 113(18):4262–72. doi:10.1182/blood-2008-08-176446
4. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* (2003) 21:483–513. doi:10.1146/annurev.immunol.21.120601.141057
5. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol* (2005) 23:877–900. doi:10.1146/annurev.immunol.23.021704.115742
6. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi:10.1146/annurev.immunol.25.022106.141711
7. Fujii S, Shimizu K, Okamoto Y, Kunii N, Nakayama T, Motohashi S, et al. NKT cells as an ideal anti-tumor immunotherapeutic. *Front Immunol* (2013) 4:409. doi:10.3389/fimmu.2013.00409
8. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S. Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *J Exp Med* (2007) 204(11):2641–53. doi:10.1084/jem.20070458
9. Barral DC, Brenner MB. CD1 antigen presentation: how it works. *Nat Rev Immunol* (2007) 7(12):929–41. doi:10.1038/nri2191
10. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* (1997) 278(5343):1626–9. doi:10.1126/science.278.5343.1626
11. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* (1997) 278(5343):1623–6. doi:10.1126/science.278.5343.1623
12. Crowe NY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med* (2002) 196(1):119–27. doi:10.1084/jem.20020092
13. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* (2000) 191(4):661–8. doi:10.1084/jem.191.4.661
14. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* (1999) 163(5):2387–91.
15. Taniguchi M, Harada M, Dashtsoodol N, Kojo S. Discovery of NKT cells and development of NKT cell-targeted anti-tumor immunotherapy. *Proc Jpn Acad Ser B Phys Biol Sci* (2015) 91(7):292–304. doi:10.2183/pjab.91.292
16. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* (2009) 182(4):2492–501. doi:10.4049/jimmunol.0800126
17. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med* (2015) 373(17):1627–39. doi:10.1056/NEJMoa1507643
18. Carreno LJ, Kharkwal SS, Porcelli SA. Optimizing NKT cell ligands as vaccine adjuvants. *Immunotherapy* (2014) 6(3):309–20. doi:10.2217/imt.13.175

19. Robertson FC, Berzofsky JA, Terabe M. NKT cell networks in the regulation of tumor immunity. *Front Immunol* (2014) 5:543. doi:10.3389/fimmu.2014.00543
20. Dashtsoodol N, Shigeura T, Ozawa R, Harada M, Kojo S, Watanabe T, et al. Generation of novel Traj18-deficient mice lacking Valpha14 natural killer T cells with an undisturbed T cell receptor alpha-chain repertoire. *PLoS One* (2016) 11(4):e0153347. doi:10.1371/journal.pone.0153347
21. Tashiro T, Mori K, Shiozaki M, Taniguchi M, Watarai H. *New Carbamate Glycolipid and Use Thereof*. World Intellectual Property Organization. WO Patent No WO2013/162016 A1 (2013).
22. Pauwels N, Aspeslagh S, Vanhoenacker G, Sandra K, Yu ED, Zajonc DM, et al. Divergent synthetic approach to 6''-modified alpha-GalCer analogues. *Org Biomol Chem* (2011) 9(24):8413–21. doi:10.1039/c1ob06235b
23. Watarai H, Nakagawa R, Omori-Miyake M, Dashtsoodol N, Taniguchi M. Methods for detection, isolation and culture of mouse and human invariant NKT cells. *Nat Protoc* (2008) 3(1):70–8. doi:10.1038/nprot.2007.515
24. Moro K, Ealey KN, Kabata H, Koyasu S. Isolation and analysis of group 2 innate lymphoid cells in mice. *Nat Protoc* (2015) 10(5):792–806. doi:10.1038/nprot.2015.047
25. Nyambayar D, Iwabuchi K, Hedlund E, Murakawa S, Shirai K, Iwabuchi C, et al. Characterization of NKT-cell hybridomas expressing invariant T-cell antigen receptors. *J Clin Exp Hematop* (2007) 47(1):1–8. doi:10.3960/jslrt.47.1
26. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* (1992) 176(6):1693–702. doi:10.1084/jem.176.6.1693
27. Tashiro T, Sekine-Kondo E, Shigeura T, Nakagawa R, Inoue S, Omori-Miyake M, et al. Induction of Th1-biased cytokine production by alpha-carba-GalCer, a neoglycolipid ligand for NKT cells. *Int Immunol* (2010) 22(4):319–28. doi:10.1093/intimm/dxq012
28. Watarai H, Rybouchkin A, Hongo N, Nagata Y, Sakata S, Sekine E, et al. Generation of functional NKT cells in vitro from embryonic stem cells bearing rearranged invariant Valpha14-Jalpha18 TCRalpha gene. *Blood* (2010) 115(2):230–7. doi:10.1182/blood-2009-04-217729
29. Yamada D, Iyoda T, Vizcardo R, Shimizu K, Sato Y, Endo TA, et al. Efficient regeneration of human Valpha24+ invariant natural killer T cells and their anti-tumor activity in vivo. *Stem Cells* (2016) 34(12):2852–60. doi:10.1002/stem.2465
30. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* (2002) 99(4):1259–66. doi:10.1182/blood.V99.4.1259
31. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13(2):101–17. doi:10.1038/nri3369
32. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, et al. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* (1999) 189(7):1121–8. doi:10.1084/jem.189.7.1121
33. Tomura M, Yu WG, Ahn HJ, Yamashita M, Yang YF, Ono S, et al. A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J Immunol* (1999) 163(1):93–101.
34. Hayakawa Y, Takeda K, Yagita H, Van Kaer L, Saiki I, Okumura K. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J Immunol* (2001) 166(10):6012–8. doi:10.4049/jimmunol.166.10.6012
35. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* (2004) 199(12):1607–18. doi:10.1084/jem.20040317
36. Oki S, Chiba A, Yamamura T, Miyake S. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J Clin Invest* (2004) 113(11):1631–40. doi:10.1172/JCI20862

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Invariant Natural Killer T Cells in Immune Regulation of Blood Cancers: Harnessing Their Potential in Immunotherapies

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Invariant natural killer T (iNKT) cells are a unique innate T lymphocyte population that possess cytolytic properties and profound immunoregulatory activities. iNKT cells play an important role in the immune surveillance of blood cancers. They predominantly recognize glycolipid antigens presented on CD1d, but their activation and cytolytic activities are not confined to CD1d expressing cells. iNKT cell stimulation and subsequent production of immunomodulatory cytokines serve to enhance the overall antitumor immune response. Crucially, the activation of iNKT cells in cancer often precedes the activation and priming of other immune effector cells, such as NK cells and T cells, thereby influencing the generation and outcome of the antitumor immune response. Blood cancers can evade or dampen iNKT cell responses by downregulating expression of recognition receptors or by actively suppressing or diverting iNKT cell functions. This review will discuss literature on iNKT cell activity and associated dysregulation in blood cancers as well as highlight some of the strategies designed to harness and enhance iNKT cell functions against blood cancers.

Keywords: invariant natural killer T, natural killer T cells, blood cancer, immunosurveillance, immunotherapy, tumor immune evasion

INTRODUCTION

Blood cancers are a heterogeneous group of malignancies broadly encompassing leukemia, myeloma, and lymphoma. As these cancers develop largely in lymphoid tissues, immune surveillance mechanisms are engaged, but inevitably fail due to changes in the microenvironment which are permissive to tumor growth but impede the development of antitumor immunity. Invariant natural killer T (iNKT) cells, an innate-like lymphocyte population defined by their semi-invariant T cell receptor (TCR)—Vα14Jα18 in mice and Vα24Jα18 in humans, have important roles in helping to regulate antitumor responses to cancer (1). These cells share similar properties to that of NK and T cells. The discovery of a potent prototypical NKT cell-activating glycolipid ligand known as α-galactosylceremide (αGalCer) (2, 3) prompted extensive attempts to manipulate this population to enhance antitumor immunity, both in solid and blood cancers. This review focuses on the activities of iNKT cells in blood malignancies and discusses the potential avenues for therapeutic targeting of iNKT cells in humans based on preclinical evidence (**Table 1**).

TABLE 1 | Evidence for the involvement and effective targeting of iNKT cells for blood cancer control in mice and humans.

Blood cancer type	Mouse	Human
Lymphoma	• CD1d$^+$ tumors can be recognized by NKT cells *in vitro* (4) • Altered glycosphingolipids secreted by T lymphoma cell line shield iNKT cell recognition (5) • αGalCer-pulsed tumor cells ± checkpoint agonist provide protection (6, 7) • Pulsing of DCs with αGalCer and tumor antigen provides protection (ATOO) (8) • Adoptive transfer of *ex vivo* activated iNKT cells provides protection (ALC) (9) • NKT cells transduced with CD62L CAR persist *in vivo* and prevents tumor growth (10) • DC-targeted nanoparticle provides prophylactic and therapeutic protection (11)	• Frequency of iNKT cells varies between loci of disease, disease stage, and subtypes (12, 13) • CIK cells activated and expanded *ex vivo* show partial clinical efficacy against advanced lymphoma [reviewed (14, 15)]
Acute/chronic myeloid leukemia	• αGalCer-pulsed tumor cells provide protection (7)	• Low expression of CD1d correlate with poorer prognosis (16) • Functional defects in NKT cells and CD1d downregulation induced by oncogene expression (17, 18) • Tyrosine kinase inhibitor can restore iNKT cell functions (17) • Activated iNKT cells is cytotoxic against CD1d$^+$ tumor cells *in vitro* (19, 20)
Acute lymphocytic leukemia	• αGalCer-pulsed tumor cells provide protection prophylactically. Therapeutic vaccine combined with chemotherapy is protective (C1498) (21) • NKT-like cells transduced with CD19-directed CAR is protective and promotes long term survival (22)	• Low expression of CD1d may contribute to progression (16), yet CD1d$^+$ leukemia can also associate with poor prognosis (23) • CIK cells transduced with CD19-directed CAR kill tumor cells *in vitro* (22)
Chronic lymphocytic leukemia	• CD1d-deficiency shortens survival (TCL1) (24) • NKT cells delay disease onset but become functionally impaired	• Reduced frequency, function and expression of CD1d on tumors is associated with progression of disease (13, 24–28) • Higher CD1d expression can also be associated with poor prognosis (27, 29) • Higher presentation of tumor-associated lipids on CD1d can lead to impairment of CD3ζ signaling and poorer prognosis (29) • Cultured iNKT-like/CIK cells are cytotoxic against tumor *in vitro* (30–33)
Multiple myeloma	• αGalCer-pulsed DCs improves survival outcome of mice (5T33MM) (34) • αGalCer-pulsed tumor cells provides protection (Vk*myc, MOPC315.BM) (7, 35)	• Reduced frequency and function of iNKT cells correlates with disease progression (36) • Inflammation associated lipids skew Th2 responses in iNKT cells (36, 37) • Cultured expanded NKT cells are cytotoxic against CD1d$^+$ myeloma cells *in vitro* (20, 36) • αGalCer-pulsed DCs ± lenalidomide induce NKT cell expansion (38, 39)

IMMUNOREGULATORY AND DIRECT CYTOTOXIC ACTIVITIES OF iNKT CELLS IN BLOOD CANCERS

Invariant natural killer T cells recognize glycolipid antigens presented on the MHC Class I-like molecule CD1d, which are expressed on many cell types, but most highly expressed on antigen-presenting cells (APCs) (40, 41). Both human and murine iNKT cells were found to recognize glycolipid antigens derived from components of bacteria (42, 43), as well as the synthetic molecule, αGalCer (44). However, iNKT cells have also been shown to recognize and respond to a variety of endogenous lipids including lysosomal glycosphingolipids such as isoglobotrihexosylceramide (iGb3) (45–48). iNKT cells were shown to directly recognize and kill various human tumor cell lines *in vitro* and murine tumors *in vitro* and *in vivo* through the recognition of endogenous lipids expressed on CD1d (36, 49, 50). The identities of these tumor-associated lipid antigens are mostly unknown. However, the tumor-associated ganglioside GD3 can be presented on CD1d for the activation of iNKT cells *in vivo* (45).

Early preclinical studies demonstrated that engagement of lipid antigen-CD1d complexes *via* the iNKT TCR results in the production of a diverse range of Th1/Th2 cytokines and chemokines (51–53), which can subsequently modulate both innate and adaptive immune cells. Notably, activation of iNKT cells leads to the downstream activation of NK cells and enhanced IFNγ production (54, 55), dendritic cell (DC) maturation and IL-12 production, and the induction of CD4 and CD8 T cell responses (56–59). Consequently, this cascade of events constitutes the indirect antitumor immunity imparted by activated iNKT cells (transactivation). Indeed, mice lacking iNKT cells (CD1d$^{-/-}$ and Jα18$^{-/-}$ mice) are more susceptible to tumor development in several spontaneous, oncogenic and carcinogenic models (60–63). In recent years, several studies have established the direct and spontaneous role of iNKT cells in the initiation of innate immune

responses against blood cancers such as B/T cell lymphomas, chronic lymphocytic leukemia (CLL) and multiple myeloma (MM) (25, 36, 64–66). These studies show that iNKT cells have the potential to control or delay the progression of premalignant or early stage disease in a CD1d-dependent manner, as seen using murine models and iNKT cells derived from patients (4, 19, 49, 67–69). In addition, innate immune control of blood cancers was found to correlate to the functional ability of iNKT cells to produce inflammatory cytokines IFNγ, and TNFα and as well as the induction of IL-12 production in APCs (64, 70, 71) (**Figure 1**).

In addition to their immunostimulatory effects, activated iNKT cells possess direct cytotoxic activity against blood cancers through the production of cytolytic molecules such as granzyme B and perforin, and through the interaction of death-inducing receptors such as Fas and TRAIL (19, 49, 72–75). More than half of all iNKT cells also express the NKG2D activating receptor enabling direct cytotoxicity against tumors expressing NKG2D ligands (76, 77). More broadly, NKG2D expression on immune effector cells is important for protection against hematological malignancy (78) (**Figure 1**). This was supported by two recent studies performed in NKG2D-deficient mice, which developed spontaneous lymphomas significantly faster than NKG2D-competent mice (79, 80). Similarly, the success of various inhibitors administered in mice that prevent the shedding of NKG2D ligands (NKG2D-L) or induce NKG2D-L expression on leukemic cells, and thereby enhancing cytotoxic killing, further demonstrates the significant role of NKG2D expression in immune surveillance of blood cancers (81, 82). In contrast, the functional role of NKG2D on human iNKT cells against tumors is less well defined. It has, however been demonstrated that human $CD3^+CD56^+$ NKT-like cells derived from the blood of healthy individuals are sensitive towards NKG2D-L-expressing cell lines including monocytic lymphoma (U937) and Burkitt's lymphoma cell lines (Raji) (77, 83). More studies are required to understand the extent to which NKG2D expression on human iNKT cells is effective against blood cancers.

Invariant natural killer T cells have also been identified in the control of host response against allogenic donor cell

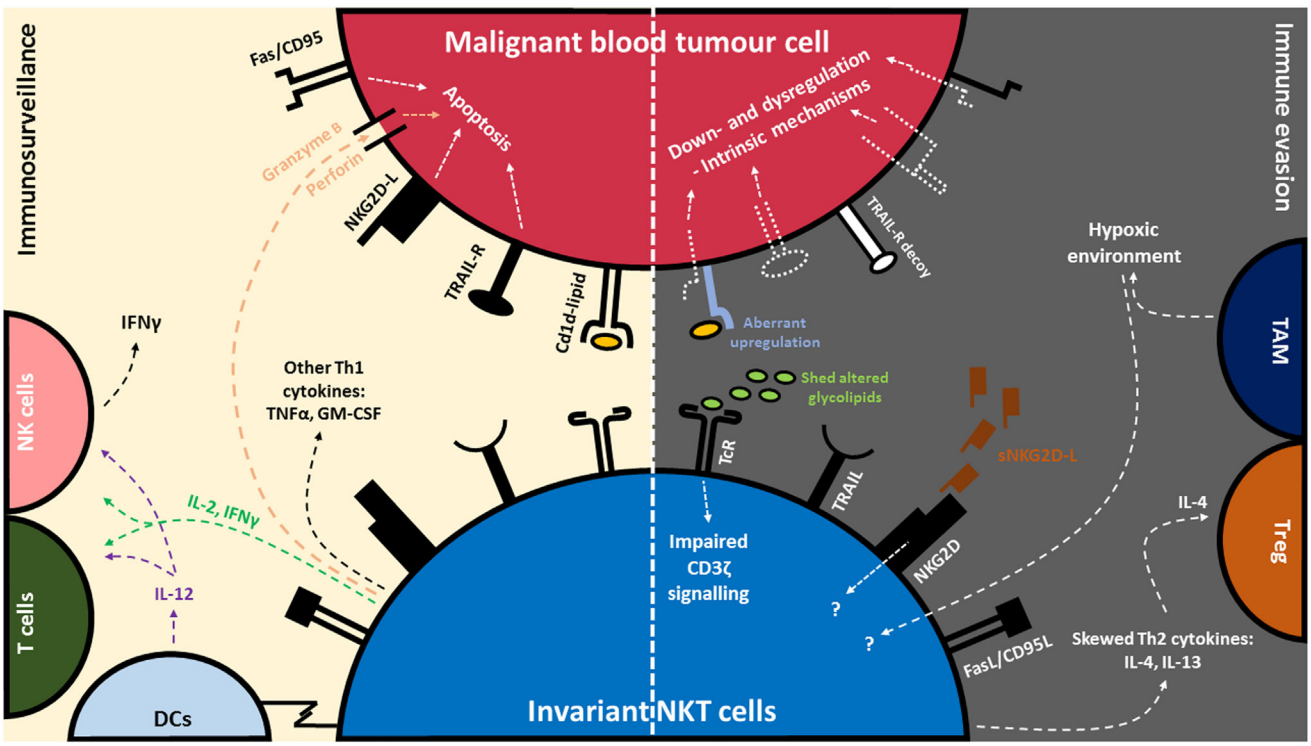

FIGURE 1 | Invariant natural killer T (iNKT) cell-mediated immune surveillance of blood cancer and counteractive evasion strategies utilized by blood cancer cells. (Left) iNKT cells recognize glycolipid antigens presented on CD1d, commonly expressed by blood tumor cells. Recognition of glycolipid:CD1d complex *via* the invariant T cell receptor (TcR) leads to a cascade of events: the production of immunomodulatory cytokines such as interleukin-2 (IL-2), interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF), release of cytolytic mediators such as perforin/granzyme, activation of antigen-presenting cells (APCs) such as dendritic cells (DCs) and IL-12 production, as well as the rapid transactivation of NK cells and T cells. iNKT cells can also recognize tumor and degranulate in a CD1d-independent manner *via* Natural Killer Group 2D **(**NKG2D) receptors. (Right) In turn, tumor cells can evade recognition and killing by downregulating CD1d, NKG2D-L, TNF-related apoptosis-inducing ligand (TRAIL-L) and FAS/CD95. In addition, certain blood tumors can disrupt death signaling pathways to avoid killing. Some blood tumors express aberrant levels of glycolipids or shed soluble glycolipids and NKG2D-L which in turn dysregulate normal signaling pathway in iNKT cells. Blood tumors cells can also skew the production of Th2 cytokines (IL-4 and IL-13) in iNKT cells. IL-4 is associated with the activation of regulatory T cells (Treg) which are involved in dampening of antitumor responses. Dysfunction of iNKT cells have also been associated with tumor-associated macrophages (TAMs) and their ability to induce hypoxia in the tumor microenvironment.

rejection in leukemic patients receiving allogeneic HSCT. The suppression of graft-versus-host-disease (GvHD), while maintaining graft-versus-tumor effect has been shown to be highly dependent on the engraftment of donor iNKT cells, as failure to reconstitute iNKT cells after transplantation strongly correlated with disease relapse (84–87). Studies into the mechanisms of GvHD suppression show that iNKT cells modulate the overall immune response through production of Th2 cytokines such as IL-4, which in turn dampen inflammatory donor T cells, and promote Treg proliferation against both acute and chronic GvHD (88–91). These studies therefore highlight an importance function of the Th2 arm of activated iNKT cells in the facilitation of engraftment of allogenic donor cells against recurrence of leukemia.

iNKT CELL DYSFUNCTION AND EVASION OF iNKT CELL RECOGNITION IN BLOOD CANCERS

Tumor Cell Evasion of iNKT Cell Recognition and Killing

Blood tumor cells possess intricate methods of evading detection and elimination by the immune system (92–94). The downregulation of CD1d on malignant cells is one of the major contributing factors to the evasion of iNKT cell immunosurveillance in blood cancers (34, 95). In fact, lower expression levels of CD1d on a variety of blood cancers is associated with progressive and advanced stages of disease in both murine models and in humans (16, 25, 26, 64, 96). Various mechanisms have been associated with downregulation of CD1d expression in blood cancers. For example, surface CD1d downregulation in Epstein–Barr virus-transformed B cells is thought to be attributed to posttranscriptional mechanisms commonly employed by herpes viruses (97, 98). Downregulation of CD1d expression on CLL B cells is believed to be associated with the elevated levels of a transcriptional protein called lymphoid enhancer-binding factor-1 (26).

Aside from regulation of CD1d expression, blood cancers may also be able to evade recognition by NKG2D on iNKT cells. This assumption is derived from previous observations in solid tumors. In one particular study, serum samples taken from patients with ovarian and prostate cancer had elevated levels of tumor-derived soluble NKG2D ligands, namely MHC class I chain-related (MIC) proteins. When cocultured with freshly isolated iNKT-like $CD3^+CD56^+$ cells *in vitro*, the cytotoxic activity of these cells was compromised and NKG2D expression was downregulated (83). In a more recent study, Lu et al. (99) demonstrated that antibody blockade of soluble MIC in a model of adenocarcinoma could potentiate IFNγ production upon stimulation (100) As elevated levels of soluble NKG2D ligands in the plasma of patients with MM, acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), Hodgkin's lymphoma (HL), and non-HL have been observed (101–105), it is predicted that NKG2D-expressing iNKT cells will be dysregulated in these tumor microenvironments. With evidence showing the capacity for iNKT cells to utilize TRAIL to kill leukemic cells *in vitro* (19), it is anticipated that blood tumors would be able to evade recognition by iNKT cells by altering TRAIL receptor expression. Indeed, myeloma and B cell lymphomas have been reported to resist TRAIL-induced killing (106), by downregulating TRAIL receptors—death receptor 4 (DR4) and DR5 (107, 108), or by dysregulating receptor signaling to evade killing (109, 110). Likewise, AML tumors have been observed to utilize decoy TRAIL receptors to resist apoptosis (111, 112).

Immunosuppressive Effects of Tumors on iNKT Cells

Blood cancer disease progression in humans is associated with a profound decrease in the frequency and function of circulating iNKT cells (12, 113–119). Although iNKT cell numbers have been shown to vary between subtypes and grade of B cell neoplasms in humans (13), this parameter has been used as an independent factor for predicting disease stage and progression in blood cancer patients (25, 36, 118). It is currently unclear how disease progression causes these defects in iNKT cells. Several studies have suggested that iNKT cell dysfunction caused by tumors are indirect, as iNKT cell function and expansion can be rescued upon administration of αGalCer-based treatments (36, 67, 120, 121), or lenalidomide treatment (122, 123). In studies in CML patients, aberrant tyrosine kinase expression and dysfunctional Rho-associated protein kinase (ROCK) expression have been suggested to exert suppressive effects on iNKT cells by regulating the transcription factor PLZF, expression of CD95L and perforin (17) as well as altering CD1d expression on myeloid DCs (mDCs) (18). Indeed, in CML patients who had undergone treatment using a tyrosine kinase inhibitor, iNKT cell functions could be restored (17). Likewise, *in vitro* treatment of CML mDCs with ROCK inhibitors was found to partially restore CD1d expression (18). iNKT cell dysfunction has also been associated with tumor-associated lipid antigen production, such as altered glycosphingolipids secreted by a murine T cell lymphoma cell line. The shedding of these lipid antigens were suggested to shield from iNKT cell recognition, as inhibition of the release of these lipid antigens could rescue iNKT cell functions (5). Interestingly, in certain patients with leukemia, higher CD1d levels have been detected on malignant cells that correlated with poorer prognosis and lower iNKT cell numbers (23, 27, 29). In this instance, higher presentation of tumor-associated lipids on CD1d by leukemic cells was suggested to cause iNKT cell hyporesponsiveness attributed to an impairment of CD3ζ signaling (29). In MM patients, inflammation-associated lysophospholipids and other glycolipids found to be elevated in the plasma were shown to induce iNKT cells to produce the Th2 cytokine IL-13 (36, 37), an anti-inflammatory cytokine associated with downregulation of tumor immunosurveillance (124). iNKT cell dysfunction has also been linked to hypoxia and tumor-associated macrophages (125), as well as interruptions in metabolic signaling caused by acidity of the tumor microenvironment (126) (**Figure 1**). These conditions have been implied to promote lymphoma tumor progression (127, 128). Better understanding of these immunosuppressive strategies of blood cancers will help with designing strategies that better harness the antitumor effects of iNKT cells.

STRATEGIES TO MODULATE iNKT CELL ACTIVITY IN BLOOD CANCERS

Early Use of iNKT Cell Adjuvants

Over the past couple of decades, strategies to exploit iNKT cells have been explored to treat various types of cancer, including blood cancers. Early studies in preclinical models showed that direct injection of αGalCer or its derivatives could induce potent iNKT cell activation and subsequent innate and adaptive immune suppression of tumors, but was also associated with significant liver toxicity (63, 71, 129, 130). Unfortunately however, this antitumor effect was not recapitulated when tested against human cancers. A phase I clinical trial using αGalCer instead found limited value as a direct immunotherapeutic agent against advanced solid cancers, despite a relatively safe toxicity profile tested in dose-escalating studies (131, 132). Patients with a higher frequency of circulating iNKTs did however respond better to treatment and produce enhanced immunological responses (133). Yet, the induction of immunological activity in these patients did not result in any partial or complete responses, and only disease stabilization in some patients could be achieved (131, 132).

DC Vaccines

Subsequently, it was revealed that free-form αGalCer causes profound and enduring hyporesponsiveness in iNKT cells (134, 135). To overcome this treatment-induced anergy, various other delivery strategies have been designed, including the *ex vivo* stimulation and loading of autologous DCs with αGalCer. Initial studies in solid tumor preclinical models showed that administration of αGalCer-pulsed DCs could enhance the frequency of iNKT cells and circulating IFNγ-producing cells, as well as Th1 antitumor responses when compared to free-form αGalCer (38, 136, 137). In addition, αGalCer-pulsed DCs can also efficiently promote the infiltration of lymphocytes including iNKT cells into tumors, enhance circulating levels of IFNγ (138, 139), and promote iNKT cell-induced immune memory upon secondary administration (140). These properties are believed to contribute in part to the long-term survival of tumor-bearing mice receiving DC therapy. For example, αGalCer-pulsed DCs has been shown to improve overall survival of mice with MM (5T33MM model) (34). When tested in patients with advanced MM, administration of αGalCer-pulsed DCs was found to sufficiently induce iNKT cell expansion and persistence in the blood (38). However, this study did not observe any overall clinical improvement in these patients. In a Phase I/II study in six patients with asymptomatic myeloma, the combination therapy of αGalCer-pulsed monocyte-derived DCs with low-dose lenalidomide, resulted in improved modulation of both iNKT and NK cell responses, including the increased surface expression of NKG2D on NK cells. The addition of lenalidomide was intended to augment the effects of DC vaccination (39), as lenalidomide have been previously suggested to skew iNKT cell and cytokine induced killer (CIK) cell responses toward a protective Th1 profile in MM patients (123, 141, 142). Similarly, coloading of DCs with αGalCer and irradiated tumor cells has also been shown to be highly protective against B cell lymphoma in mice (4TOO model) (8). In this instance, the pulsing of DCs with tumor cells served to provide a source of undefined tumor antigens to initiate tumor-specific immune responses enhanced by the adjuvanting effects of αGalCer.

Tumor Cell-Based Vaccines

We and others have previously attempted to use autologous tumor cells as vaccine vehicles for αGalCer delivery in mice. Single administration of an αGalCer-loaded tumor cell vaccine could induce potent antitumor immunity and prolong overall survival in mice with various blood cancers, including B lymphoma (Eμ-myc), acute myeloid leukemia (AML-ETO9a), and myeloma (Vk**myc*) (6, 7, 130, 143, 144). In addition, therapeutic effect of this vaccine approach was significantly enhanced when used in combination with immune checkpoint agonists, such as anti-4-1BB mAb (6). In other studies, the use of αGalCer-loaded tumor vaccines was also demonstrated to induce potent therapeutic responses against a murine model of MM (MOPC315.BM model) and found to generate long-term protection against tumor rechallenge (35). Interestingly, in a murine model of acute leukemia (C1498), the administration of αGalCer-loaded leukemic cells alone was found to be effective as a prophylactic vaccine but ineffective against established leukemia. The study found that while iNKT cells could be effectively activated, the downstream leukemia-specific T cell responses were suppressed. Instead, the benefit of vaccination became apparent following chemotherapy treatment, to prevent relapse of leukemia, and protect against rechallenge (21).

Adoptive Transfer of iNKT Cells and CIK Cells

While the use of autologous cell-based vaccines has proven to be effective in animal models, a potential limitation in human patients is the high variability of iNKT cell frequency. Also, the functionality of iNKT cells often diminishes with tumor progression. Therefore, to circumvent this issue, adoptive transfer of activated and expanded iNKT cells derived from patient peripheral blood mononuclear cells (PBMCs) have been explored. Notably, $CD3^+CD56^+$ CIK cells, which represent a mixture of NK cell-like T cells, and incorporate an iNKT population, possess non-MHC-dependent tumor activity mediated through perforin and NKG2D expression (14, 15). By culturing autologous PBMCs under various conditions (e.g., αGalCer in the presence of GM-CSF and/or IL-2, or with a combination of cytokines such as IFNγ, OKT3, IL-2, and IL-15), *ex vivo* expansion of autologous activated iNKT/CIK cells from patients can be achieved (20, 30, 145). Successful expansion of functional iNKT cells from adult hematopoietic stem-progenitor cells using artificial APCs coated with CD1d-immunoglobulin (146, 147) as well as iNKT cell generation from induced pluripotent stem cells have also been explored (148). Adoptive transfer of *ex vivo* expanded iNKT cells in conjunction with αGalCer administration is an effective treatment against $CD1d^+$ leukemic cells implanted in immunodeficient NOD/SCID mice (67). Similarly, adoptive transfer of iNKT cells activated *ex vivo* with

IL-12 and IL-18 could initiate protection against lymphoma (ALC model) in mice (9). In humans, cultured iNKT/CIK cells are able recognize autologous or allogenic blood tumor cells *in vitro* (20, 30–32, 149). However, therapeutic use of *in vitro* expanded iNKT cells against blood cancers in humans is limited. Thus far, three phase I trials and a phase II trial have looked into the safety profile and efficacy of expanded activated autologous iNKT cells in patients with solid tumors (150–153). All of these studies demonstrated safety and feasibility of treatment as well as induction of IFNγ in circulating iNKT in patients. In the phase II study, αGalCer-loaded APCs administered alongside activated iNKT cells led to iNKT cell accumulation at tumor sites and some clinical efficacy in 50% of patients enrolled (153).

Notably, the use of expanded CIK cells in association with other treatments has led to complete cancer remissions in patients with hematological malignancies [reviewed in Refs. (14, 15)]. CIK cells have also been used in combination with HSCT in a bid to potentiate the overall inhibitory effects of GvHD in blood cancer patients receiving transplants (154). In a phase I study published by Luo et al. (154), patients enrolled were refractory to chemotherapy or had relapsed after early allogenic HSCT treatment. While some patients displayed a response to engraftment of donor cells, and infusion of CIK cells appeared to contribute to the prolonged survival in these patients, the overall efficacy of the combination treatment remains limited for this small cohort of patients with highly aggressive hematological malignancies (154). The extent to which these responses can be attributed to iNKT-like cells specifically, is unknown.

Chimeric Antigen Receptor (CAR) Modified iNKT Cells and CIK Cells

Most recently, several studies have explored CAR engineering of iNKT/CIK cells (10, 22, 155). A summary of the proof of concept findings to date indicate that both CAR-NKT cells and CAR-CIK cells possess greater antitumor activity than their iNKT and CIK cell counterparts [recently reviewed in Ref. (156)]. In one example, donor $CD62L^+$ iNKT cells that were identified to be highly proliferative *in vitro* were transduced with a CD19-specific CAR and tested for therapeutic activity against humanized mouse models of lymphoma and neuroblastoma. These $CD62L^+$ CAR-NKT cells were demonstrated to persist long-term *in vivo* and were also highly effective at inhibiting tumor growth (10). The use of CAR-NKT cells was demonstrated to be safe and did not induce graft-versus-host disease (GvHD) in mice with neuroblastoma (155). In addition, the antitumor effects of CIK cells generated from donor PBMCs could also be further enhanced when transduced with CAR specific for CD19 and the CD28-CD3ζ signaling domain (22). These CAR-CIK cells were found to be highly effective against B-cell ALL (B-ALL) *in vitro*, including against CIK-resistant tumor cells. When tested *in vivo*, CAR-CIK cells were described to be more effective than non-CAR CIK cells in eliminating B-ALL tumors and promoting long-term survival in mice (22). We foresee that these studies will serve to accelerate research into modifying donor iNKT cells for adoptive therapies for blood cancers to complement other CAR-T cell-based therapies (157).

Nanoparticle-Based Delivery Systems for iNKT Cell Adjuvants

To overcome some of the limitations associated with adoptive NKT cell-based approaches and to provide less costly and time-consuming alternatives for NKT cell-targeting immunotherapy, research into the use of nanoparticle-based systems are emerging [reviewed in Ref. (158)]. Briefly, nanoparticle vectors are delivery vehicles less than 1 μM in size and have wide applications in various diagnostic and treatment settings, including tumor immunotherapy (159). Delivery of glycolipid adjuvants in suitable nanoparticles presents several advantages over delivery in soluble form, such as reduced toxicity profile (owing to the reduced amount required to elicit a biological response), the ability to overcome iNKT cell anergy (160) and the preferential targeted delivery to APCs *in vivo* (158). To date, there exists various published studies in preclinical models of solid cancers on the nanoparticulate delivery of αGalCer alone or co-delivered with tumor-associated antigens (11, 161–164). By comparison, few therapeutic applications of nanoparticle delivery of glycolipid adjuvants have been reported for blood cancers. One such study utilized a targeted PLGA nanoparticle to codeliver a model tumor antigen ovalbumin (OVA) and αGalCer to $DEC205^+$ $CD8\alpha^+$ DCs. iNKT cells were rapidly activated using this approach and could drive the induction of cytolytic tumor-specific CD8 T cells. When assessed in prophylactic and therapeutic settings against a model of thymoma, administration of targeted nanoparticles could significantly suppress early tumor growth (11). Recently, a liposomal form of αGalCer (RGI-2001) has been designed to circumvent GvHD after HSCT. Initial preclinical studies show that RGI-2001 could aid in graft-versus-leukemia effect and significantly prevented acute GvHD in lethally irradiated leukemia-bearing mice given allele-mismatched donor bone marrow cells or spleen cells. This effect was believed to be largely due to the enhanced expansion of donor-derived $CD4^+$ regulatory T (Treg) cells that could exert its effects in an antigen-specific manner (165). Although RGI-2001 was demonstrated to induce expansion of NKT cells as well as higher IL-4 levels early after treatment, the correlation between NKT cell expansion and Treg induction was not clearly demonstrated. In a Phase II study in blood cancer patients, RGI-2001 was administered as a single dose in combination with HSCT. Similar to findings in mice, this study showed that RGI-2001 was generally tolerable in most patients and suggested that immunosuppressive Treg cells could be efficiently induced *in vivo* in a small proportion of patients. However, due to limited patient recruitment and difficulties in the detection of NKT cells in the blood in this particular study, the extent to which NKT cells contributed to overall GvL response remained inconclusive (89).

CONCLUDING REMARKS

Increasing knowledge of how different blood cancers modulate their environment to avoid or suppress antitumor immunity

has advanced the development of counteractive measures with immunotherapies. The fortuitous discovery of the potent NKT cell-stimulatory properties of αGalCer has enabled us to better understand how iNKT cells function to transactivate both the innate and adaptive immune system, and importantly, their unique role in antitumor immunity. However, encouraging findings in preclinical studies have not yet convincingly translated to similar outcomes in human cancers. In fact, the number of human trials testing the therapeutic use of various glycolipid compounds against cancer is limited, perhaps not only due to interindividual variability between patients but also due to the lack of understanding on the effects of tumors on decreasing iNKT frequencies and function. This is also true in harnessing the functions of NKT cells against GvHD after HSCT. In general, there still exists an uncertainty on the proper manipulation of iNKT cells and their different responses to a variety of glycolipids. We should continue to fully utilize preclinical models to understand how to best influence the functions of iNKT cells through synthetic glycolipid ligands, but also place more emphasis on the translation of these findings into the clinical setting, with the goal to rescue or enhance iNKT cell functions in different human blood cancer settings.

AUTHOR CONTRIBUTIONS

PL undertook critical review of the literature, wrote the manuscript, and designed the figure. MN contributed to the writing and editing of the manuscript. SM designed the scope of the manuscript and assisted with writing and editing of the manuscript.

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REFERENCES

1. McEwen-Smith RM, Salio M, Cerundolo V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res* (2015) 3(5): 425–35. doi:10.1158/2326-6066.CIR-15-0062
2. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of Valpha14 NKT cells by glycosylceramides. *Science* (1997) 278(5343):1626–9. doi:10.1126/science.278.5343.1626
3. Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. *Nat Rev Immunol* (2002) 2(8):557–68. doi:10.1038/nri854
4. Haraguchi K, Takahashi T, Nakahara F, Matsumoto A, Kurokawa M, Ogawa S, et al. CD1d expression level in tumor cells is an important determinant for anti-tumor immunity by natural killer T cells. *Leuk Lymphoma* (2006) 47(10):2218–23. doi:10.1080/10428190600682688
5. Sriram V, Cho S, Li P, O'Donnell PW, Dunn C, Hayakawa K, et al. Inhibition of glycolipid shedding rescues recognition of a CD1+ T cell lymphoma by natural killer T (NKT) cells. *Proc Natl Acad Sci U S A* (2002) 99(12):8197–202. doi:10.1073/pnas.122636199
6. Kobayashi T, Doff BL, Rearden RC, Leggatt GR, Mattarollo SR. NKT cell-targeted vaccination plus anti-4-1BB antibody generates persistent CD8 T cell immunity against B cell lymphoma. *Oncoimmunology* (2015) 4(3):e990793. doi:10.4161/2162402X.2014.990793
7. Mattarollo SR, West AC, Steegh K, Duret H, Paget C, Martin B, et al. NKT cell adjuvant-based tumor vaccine for treatment of myc oncogene-driven mouse B-cell lymphoma. *Blood* (2012) 120(15):3019–29. doi:10.1182/blood-2012-04-426643
8. Escribà-Garcia L, Alvarez-Fernández C, Tellez-Gabriel M, Sierra J, Briones J. Dendritic cells combined with tumor cells and α-galactosylceramide induce a potent, therapeutic and NK-cell dependent antitumor immunity in B cell lymphoma. *J Transl Med* (2017) 15(1):115. doi:10.1186/s12967-017-1219-3
9. Baxevanis CN, Gritzapis AD, Papamichail M. In vivo antitumor activity of NKT cells activated by the combination of IL-12 and IL-18. *J Immunol* (2003) 171(6):2953–9. doi:10.4049/jimmunol.171.6.2953
10. Tian G, Courtney AN, Jena B, Heczey A, Liu D, Marinova E, et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. *J Clin Invest* (2016) 126(6):2341–55. doi:10.1172/JCI83476
11. Macho-Fernandez E, Cruz LJ, Ghinnagow R, Fontaine J, Bialecki E, Frisch B, et al. Targeted delivery of alpha-galactosylceramide to CD8alpha+ dendritic cells optimizes type I NKT cell-based antitumor responses. *J Immunol* (2014) 193(2):961–9. doi:10.4049/jimmunol.1303029
12. Hus I, Staroslawska E, Bojarska-Junak A, Dobrzynska-Rutkowska A, Surdacka A, Wdowiak P, et al. CD3+/CD16+CD56+ cell numbers in peripheral blood are correlated with higher tumor burden in patients with diffuse large B-cell lymphoma. *Folia Histochem Cytobiol* (2011) 49(1):183–7. doi:10.5603/FHC.2011.0025
13. Gibson SE, Swerdlow SH, Felgar RE. Natural killer cell subsets and natural killer-like T-cell populations in benign and neoplastic B-cell proliferations vary based on clinicopathologic features. *Hum Pathol* (2011) 42(5):679–87. doi:10.1016/j.humpath.2010.07.023
14. Schmeel LC, Schmeel FC, Coch C, Schmidt-Wolf IG. Cytokine-induced killer (CIK) cells in cancer immunotherapy: report of the international registry on CIK cells (IRCC). *J Cancer Res Clin Oncol* (2015) 141(5):839–49. doi:10.1007/s00432-014-1864-3
15. Schmeel FC, Schmeel LC, Gast SM, Schmidt-Wolf IG. Adoptive immunotherapy strategies with cytokine-induced killer (CIK) cells in the treatment of hematological malignancies. *Int J Mol Sci* (2014) 15(8):14632–48. doi:10.3390/ijms150814632
16. Guo W, Dong A, Xing C, Lin X, Pan X, Lin Y, et al. CD1d levels in peripheral blood of patients with acute myeloid leukemia and acute lymphoblastic leukemia. *Oncol Lett* (2014) 8(2):825–30. doi:10.3892/ol.2014.2208
17. Rossignol A, Levescot A, Jacomet F, Robin A, Basbous S, Giraud C, et al. Evidence for BCR-ABL-dependent dysfunctions of iNKT cells from chronic myeloid leukemia patients. *Eur J Immunol* (2012) 42(7):1870–5. doi:10.1002/eji.201142043
18. Basbous S, Levescot A, Piccirilli N, Brizard F, Guilhot F, Roy L, et al. The Rho-ROCK pathway as a new pathological mechanism of innate immune subversion in chronic myeloid leukaemia. *J Pathol* (2016) 240(3):262–8. doi:10.1002/path.4779
19. Metelitsa LS, Weinberg KI, Emanuel PD, Seeger RC. Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells. *Leukemia* (2003) 17(6):1068–77. doi:10.1038/sj.leu.2402943
20. Rogers PR, Emerson M, Nguyen E, Ball E, Bashey A, Kato S. Function and therapeutic potential of in vitro-expanded natural killer T (NKT) cells from patients with hematopoietic malignancies. *Blood* (2007) 110(11): 805a–6a.
21. Gibbins JD, Ancelet LR, Weinkove R, Compton BJ, Painter GF, Petersen TR, et al. An autologous leukemia cell vaccine prevents murine acute leukemia relapse after cytarabine treatment. *Blood* (2014) 124(19):2953–63. doi:10.1182/blood-2014-04-568956
22. Oelsner S, Wagner J, Friede ME, Pfirrmann V, Genssler S, Rettinger E, et al. Chimeric antigen receptor-engineered cytokine-induced killer cells overcome treatment resistance of pre-B-cell acute lymphoblastic leukemia

and enhance survival. *Int J Cancer* (2016) 139(8):1799–809. doi:10.1002/ijc.30217
23. Fais F, Tenca C, Cimino G, Coletti V, Zanardi S, Bagnara D, et al. CD1d expression on B-precursor acute lymphoblastic leukemia subsets with poor prognosis. *Leukemia* (2005) 19(4):551–6. doi:10.1038/sj.leu.2403671
24. Zaborsky N, Gassner FJ, Asslaber D, Reinthaler P, Denk U, Flenady S, et al. CD1d expression on chronic lymphocytic leukemia B cells affects disease progression and induces T cell skewing in CD8 positive and CD4CD8 double negative T cells. *Oncotarget* (2016) 7(31):49459–69. doi:10.18632/oncotarget.10372
25. Gorini F, Azzimonti L, Delfanti G, Scarfo L, Scielzo C, Bertilaccio MT, et al. Invariant NKT cells contribute to chronic lymphocytic leukemia surveillance and prognosis. *Blood* (2017) 129(26):3440–51. doi:10.1182/blood-2016-11-751065
26. Kotsianidis I, Nakou E, Spanoudakis E, Bouchliou I, Moustakidis E, Miltiades P, et al. The diagnostic value of CD1d expression in a large cohort of patients with B-cell chronic lymphoproliferative disorders. *Am J Clin Pathol* (2011) 136(3):400–8. doi:10.1309/AJCP2F2DOXOTXHZA
27. Anastasiadis A, Kotsianidis I, Papadopoulos V, Spanoudakis E, Margaritis D, Christoforidou A, et al. CD1d expression as a prognostic marker for chronic lymphocytic leukemia. *Leuk Lymphoma* (2014) 55(2):320–5. doi:10.3109/10428194.2013.803222
28. Weinkove R, Brooks CR, Carter JM, Hermans IF, Ronchese F. Functional invariant natural killer T-cell and CD1d axis in chronic lymphocytic leukemia: implications for immunotherapy. *Haematologica* (2013) 98(3):376–84. doi:10.3324/haematol.2012.072835
29. Bojarska-Junak A, Hus I, Chocholska S, Tomczak W, Wos J, Czubak P, et al. CD1d expression is higher in chronic lymphocytic leukemia patients with unfavorable prognosis. *Leuk Res* (2014) 38(4):435–42. doi:10.1016/j.leukres.2013.12.015
30. Guven H, Gilljam M, Chambers BJ, Ljunggren HG, Christensson B, Kimby E, et al. Expansion of natural killer (NK) and natural killer-like T (NKT)-cell populations derived from patients with B-chronic lymphocytic leukemia (B-CLL): a potential source for cellular immunotherapy. *Leukemia* (2003) 17(10):1973–80. doi:10.1038/sj.leu.2403083
31. Lefterova P, Schakowski F, Buttgereit P, Scheffold C, Huhn D, Schmidt-Wolf IG. Expansion of CD3+CD56+ cytotoxic cells from patients with chronic lymphocytic leukemia: in vitro efficacy. *Haematologica* (2000) 85(10):1108–9.
32. Linn YC, Lau LC, Hui KM. Generation of cytokine-induced killer cells from leukaemic samples with in vitro cytotoxicity against autologous and allogeneic leukaemic blasts. *Br J Haematol* (2002) 116(1):78–86. doi:10.1046/j.1365-2141.2002.03247.x
33. Linn YC, Hui KM. Cytokine-induced killer cells: NK-like T cells with cytotolytic specificity against leukemia. *Leuk Lymphoma* (2003) 44(9):1457–62. doi:10.3109/10428190309178764
34. Nur H, Fostier K, Aspeslagh S, Renmans W, Bertrand E, Leleu X, et al. Preclinical evaluation of invariant natural killer T cells in the 5T33 multiple myeloma model. *PLoS One* (2013) 8(5):e65075. doi:10.1371/journal.pone.0065075
35. Hong S, Lee H, Jung K, Lee SM, Lee SJ, Jun HJ, et al. Tumor cells loaded with α-galactosylceramide promote therapeutic NKT-dependent anti-tumor immunity in multiple myeloma. *Immunol Lett* (2013) 156(1–2):132–9. doi:10.1016/j.imlet.2013.10.002
36. Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J Exp Med* (2003) 197(12):1667–76. doi:10.1084/jem.20021650
37. Chang DH, Deng H, Matthews P, Krasovsky J, Ragupathi G, Spisek R, et al. Inflammation-associated lysophospholipids as ligands for CD1d-restricted T cells in human cancer. *Blood* (2008) 112(4):1308–16. doi:10.1182/blood-2008-04-149831
38. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* (2005) 201(9):1503–17. doi:10.1084/jem.20042592
39. Richter J, Neparidze N, Zhang L, Nair S, Monesmith T, Sundaram R, et al. Clinical regressions and broad immune activation following combination therapy targeting human NKT cells in myeloma. *Blood* (2013) 121(3):423–30. doi:10.1182/blood-2012-06-435503
40. Dougan SK, Kaser A, Blumberg RS. CD1 expression on antigen-presenting cells. In: Moody DB, editor. *T Cell Activation by CD1 and Lipid Antigens*. Berlin, Heidelberg: Springer (2007). p. 113–41.
41. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol* (2004) 22:817–90. doi:10.1146/annurev.immunol.22.012703.104608
42. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C III, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* (2005) 434(7032):525–9. doi:10.1038/nature03408
43. Zajonc DM, Girardi E. Recognition of microbial glycolipids by natural killer T cells. *Front Immunol* (2015) 6:400. doi:10.3389/fimmu.2015.00400
44. Banchet-Cadeddu A, Henon E, Dauchez M, Renault JH, Monneaux F, Haudrechy A. The stimulating adventure of KRN 7000. *Org Biomol Chem* (2011) 9(9):3080–104. doi:10.1039/c0ob00975j
45. Wu DY, Segal NH, Sidobre S, Kronenberg M, Chapman PB. Cross-presentation of disialoganglioside GD3 to natural killer T cells. *J Exp Med* (2003) 198(1):173–81. doi:10.1084/jem.20030446
46. Dias BR, Rodrigues EG, Nimrichter L, Nakayasu ES, Almeida IC, Travassos LR. Identification of iGb3 and iGb4 in melanoma B16F10-Nex2 cells and the iNKT cell-mediated antitumor effect of dendritic cells primed with iGb3. *Mol Cancer* (2009) 8:116. doi:10.1186/1476-4598-8-116
47. Zhou D, Mattner J, Cantu C III, Schrantz N, Yin N, Gao Y, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science* (2004) 306(5702):1786–9. doi:10.1126/science.1103440
48. Dowds CM, Kornell SC, Blumberg RS, Zeissig S. Lipid antigens in immunity. *Biol Chem* (2014) 395(1):61–81. doi:10.1515/hsz-2013-0220
49. Bassiri H, Das R, Nichols KE. Invariant NKT cells: killers and conspirators against cancer. *Oncoimmunology* (2013) 2(12):e27440. doi:10.4161/onci.27440
50. Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B, et al. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol* (2001) 167(6):3114–22. doi:10.4049/jimmunol.167.6.3114
51. Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J Exp Med* (2005) 202(9):1279–88. doi:10.1084/jem.20050953
52. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci U S A* (2008) 105(32):11287–92. doi:10.1073/pnas.0801631105
53. Smyth MJ, Godfrey DI. NKT cells and tumor immunity – a double-edged sword. *Nat Immunol* (2000) 1(6):459–60. doi:10.1038/82698
54. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, et al. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* (1999) 163(9):4647–50.
55. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* (2002) 99(4):1259–66. doi:10.1182/blood.V99.4.1259
56. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* (1998) 392(6673):245–52. doi:10.1038/32588
57. Fujii S, Shimizu K, Hemmi H, Steinman RM. Innate Valpha14(+) natural killer T cells mature dendritic cells, leading to strong adaptive immunity. *Immunol Rev* (2007) 220:183–98. doi:10.1111/j.1600-065X.2007.00561.x
58. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* (2004) 199(12):1607–18. doi:10.1084/jem.20040317
59. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* (2003) 171(10):5140–7. doi:10.4049/jimmunol.171.10.5140

60. Swann JB, Uldrich AP, van Dommelen S, Sharkey J, Murray WK, Godfrey DI, et al. Type I natural killer T cells suppress tumors caused by p53 loss in mice. *Blood* (2009) 113(25):6382–5. doi:10.1182/blood-2009-01-198564
61. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* (2000) 191(4):661–8. doi:10.1084/jem.191.4.661
62. Crowe NY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med* (2002) 196(1):119–27. doi:10.1084/jem.20020092
63. Hayakawa Y, Rovero S, Forni G, Smyth MJ. Alpha-galactosylceramide (KRN7000) suppression of chemical- and oncogene-dependent carcinogenesis. *Proc Natl Acad Sci U S A* (2003) 100(16):9464–9. doi:10.1073/pnas.1630663100
64. Renukaradhya GJ, Khan MA, Vieira M, Du W, Gervay-Hague J, Brutkiewicz RR. Type I NKT cells protect (and type II NKT cells suppress) the host's innate antitumor immune response to a B-cell lymphoma. *Blood* (2008) 111(12):5637–45. doi:10.1182/blood-2007-05-092866
65. Li J, Sun W, Subrahmanyam PB, Page C, Younger KM, Tiper IV, et al. NKT cell responses to B cell lymphoma. *Med Sci (Basel)* (2014) 2(2):82–97. doi:10.3390/medsci2020082
66. Renukaradhya GJ, Sriram V, Du W, Gervay-Hague J, Van Kaer L, Brutkiewicz RR. Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* (2006) 118(12):3045–53. doi:10.1002/ijc.21764
67. Bagnara D, Ibatici A, Corselli M, Sessarego N, Tenca C, De Santanna A, et al. Adoptive immunotherapy mediated by ex vivo expanded natural killer T cells against CD1d-expressing lymphoid neoplasms. *Haematologica* (2009) 94(7):967–74. doi:10.3324/haematol.2008.001339
68. Xu C, de Vries R, Visser L, Diepstra A, Gadola SD, Poppema S, et al. Expression of CD1d and presence of invariant NKT cells in classical Hodgkin lymphoma. *Am J Hematol* (2010) 85(7):539–41. doi:10.1002/ajh.21743
69. Fais F, Morabito F, Stelitano C, Callea V, Zanardi S, Scudeletti M, et al. CD1d is expressed on B-chronic lymphocytic leukemia cells and mediates alpha-galactosylceramide presentation to natural killer T lymphocytes. *Int J Cancer* (2004) 109(3):402–11. doi:10.1002/ijc.11723
70. Chan AC, Neeson P, Leeansyah E, Tainton K, Quach H, Prince HM, et al. Natural killer T cell defects in multiple myeloma and the impact of lenalidomide therapy. *Clin Exp Immunol* (2013) 175(1):49–58. doi:10.1111/cei.12196
71. Hayakawa Y, Takeda K, Yagita H, Smyth MJ, Van Kaer L, Okumura K, et al. IFN-gamma-mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, alpha-galactosylceramide. *Blood* (2002) 100(5):1728–33.
72. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol* (2010) 185(5):2721–9. doi:10.4049/jimmunol.1001018
73. Nieda M, Nicol A, Koezuka Y, Kikuchi A, Lapteva N, Tanaka Y, et al. TRAIL expression by activated human CD4(+)V alpha 24NKT cells induces in vitro and in vivo apoptosis of human acute myeloid leukemia cells. *Blood* (2001) 97(7):2067–74. doi:10.1182/blood.V97.7.2067
74. Matsuda JL, Zhang Q, Ndonye R, Richardson SK, Howell AR, Gapin L. T-bet concomitantly controls migration, survival, and effector functions during the development of Valpha14i NKT cells. *Blood* (2006) 107(7):2797–805. doi:10.1182/blood-2005-08-3103
75. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, et al. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci U S A* (1998) 95(10):5690–3. doi:10.1073/pnas.95.10.5690
76. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* (2002) 195(5):625–36. doi:10.1084/jem.20011786
77. Kuylenstierna C, Bjorkstrom NK, Andersson SK, Sahlstrom P, Bosnjak L, Paquin-Proulx D, et al. NKG2D performs two functions in invariant NKT cells: direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d. *Eur J Immunol* (2011) 41(7):1913–23. doi:10.1002/eji.200940278
78. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* (2008) 28(4):571–80. doi:10.1016/j.immuni.2008.02.016
79. Belting L, Homberg N, Przewoznik M, Brenner C, Riedel T, Flatley A, et al. Critical role of the NKG2D receptor for NK cell-mediated control and immune escape of B-cell lymphoma. *Eur J Immunol* (2015) 45(9):2593–601. doi:10.1002/eji.201445375
80. Raju S, Kretzmer LZ, Koues OI, Payton JE, Oltz EM, Cashen A, et al. NKG2D-NKG2D ligand interaction inhibits the outgrowth of naturally arising low-grade B cell lymphoma in vivo. *J Immunol* (2016) 196(11):4805–13. doi:10.4049/jimmunol.1501982
81. Poggi A, Catellani S, Garuti A, Pierri I, Gobbi M, Zocchi MR. Effective in vivo induction of NKG2D ligands in acute myeloid leukaemias by all-trans-retinoic acid or sodium valproate. *Leukemia* (2009) 23(4):641–8. doi:10.1038/leu.2008.354
82. Diermayr S, Himmelreich H, Durovic B, Mathys-Schneeberger A, Siegler U, Langenkamp U, et al. NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. *Blood* (2008) 111(3):1428–36. doi:10.1182/blood-2007-07-101311
83. Wang H, Yang D, Xu W, Wang Y, Ruan Z, Zhao T, et al. Tumor-derived soluble MICs impair CD3(+)CD56(+) NKT-like cell cytotoxicity in cancer patients. *Immunol Lett* (2008) 120(1–2):65–71. doi:10.1016/j.imlet.2008.07.001
84. Pillai AB, George TI, Dutt S, Teo P, Strober S. Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol* (2007) 178(10):6242–51. doi:10.4049/jimmunol.178.10.6242
85. Casorati G, de Lalla C, Dellabona P. Invariant natural killer T cells reconstitution and the control of leukemia relapse in pediatric haploidentical hematopoietic stem cell transplantation. *Oncoimmunology* (2012) 1(3):355–7. doi:10.4161/onci.18399
86. Dellabona P, Casorati G, de Lalla C, Montagna D, Locatelli F. On the use of donor-derived iNKT cells for adoptive immunotherapy to prevent leukemia recurrence in pediatric recipients of HLA haploidentical HSCT for hematological malignancies. *Clin Immunol* (2011) 140(2):152–9. doi:10.1016/j.clim.2010.11.015
87. de Lalla C, Rinaldi A, Montagna D, Azzimonti L, Bernardo ME, Sangalli LM, et al. Invariant NKT cell reconstitution in pediatric leukemia patients given HLA-haploidentical stem cell transplantation defines distinct CD4+ and CD4- subset dynamics and correlates with remission state. *J Immunol* (2011) 186(7):4490–9. doi:10.4049/jimmunol.1003748
88. Guan P, Bassiri H, Patel NP, Nichols KE, Das R. Invariant natural killer T cells in hematopoietic stem cell transplantation: killer choice for natural suppression. *Bone Marrow Transplant* (2016) 51(5):629–37. doi:10.1038/bmt.2015.335
89. Chen YB, Efebera YA, Johnston L, Ball ED, Avigan D, Lekakis LJ, et al. Increased Foxp3+helios+ regulatory t cells and decreased acute graft-versus-host disease after allogeneic bone marrow transplantation in patients receiving sirolimus and RGI-2001, an activator of invariant natural killer T cells. *Biol Blood Marrow Transplant* (2017) 23(4):625–34. doi:10.1016/j.bbmt.2017.01.069
90. Hongo D, Tang XB, Dutt S, Nador RG, Strober S. Interactions between NKT cells and Tregs are required for tolerance to combined bone marrow and organ transplants. *Blood* (2012) 119(6):1581–9. doi:10.1182/blood-2011-08-371948
91. Du J, Paz K, Thangavelu G, Schneidawind D, Baker J, Flynn R, et al. Invariant natural killer T cells ameliorate murine chronic GVHD by expanding donor regulatory T cells. *Blood* (2017) 129(23):3121–5. doi:10.1182/blood-2016-11-752444
92. Upadhyay R, Hammerich L, Peng P, Brown B, Merad M, Brody JD. Lymphoma: immune evasion strategies. *Cancers (Basel)* (2015) 7(2):736–62. doi:10.3390/cancers7020736
93. Teague RM, Kline J. Immune evasion in acute myeloid leukemia: current concepts and future directions. *J Immunother Cancer* (2013) 1(13). doi:10.1186/2051-1426-1-13
94. Zheng Z, Venkatapathy S, Rao G, Harrington CA. Expression profiling of B cell chronic lymphocytic leukemia suggests deficient CD1-mediated

immunity, polarized cytokine response, altered adhesion and increased intracellular protein transport and processing of leukemic cells. *Leukemia* (2002) 16(12):2429–37. doi:10.1038/sj.leu.2402711
95. Hix LM, Shi YH, Brutkiewicz RR, Stein PL, Wang CR, Zhang M. CD1d-expressing breast cancer cells modulate NKT cell-mediated antitumor immunity in a murine model of breast cancer metastasis. *PLoS One* (2011) 6(6):e20702. doi:10.1371/journal.pone.0020702
96. Fiedler T, Walter W, Reichert TE, Maeurer MJ. Regulation of CD1d expression by murine tumor cells: escape from immunosurveillance or alternate target molecules? *Int J Cancer* (2002) 98(3):389–97. doi:10.1002/ijc.10141
97. Chaudhry MS, Karadimitris A. Role and regulation of CD1d in normal and pathological B cells. *J Immunol* (2014) 193(10):4761–8. doi:10.4049/jimmunol.1401805
98. Yuan W, Dasgupta A, Cresswell P. Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. *Nat Immunol* (2006) 7(8):835–42. doi:10.1038/ni1364
99. Lu S, Zhang J, Liu D, Li G, Staveley-O'Carroll KF, Li Z, et al. Nonblocking monoclonal antibody targeting soluble MIC revamps endogenous innate and adaptive antitumor responses and eliminates primary and metastatic tumors. *Clin Cancer Res* (2015) 21(21):4819–30. doi:10.1158/1078-0432.CCR-15-0845
100. Bezu L, Gomes-de-Silva LC, Dewitte H, Breckpot K, Fucikova J, Spisek R, et al. Combinatorial strategies for the induction of immunogenic cell death. *Front Immunol* (2015) 6:187. doi:10.3389/fimmu.2015.00187
101. Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. *Blood* (2003) 102(4):1389–96. doi:10.1182/blood-2003-01-0019
102. Nuckel H, Switala M, Sellmann L, Horn PA, Durig J, Duhrsen U, et al. The prognostic significance of soluble NKG2D ligands in B-cell chronic lymphocytic leukemia. *Leukemia* (2010) 24(6):1152–9. doi:10.1038/leu.2010.74
103. Hilpert J, Grosse-Hovest L, Grunebach F, Buechele C, Nuebling T, Raum T, et al. Comprehensive analysis of NKG2D ligand expression and release in leukemia: implications for NKG2D-mediated NK cell responses. *J Immunol* (2012) 189(3):1360–71. doi:10.4049/jimmunol.1200796
104. Zocchi MR, Catellani S, Canevali P, Tavella S, Garuti A, Villaggio B, et al. High ERp5/ADAM10 expression in lymph node microenvironment and impaired NKG2D ligands recognition in Hodgkin lymphomas. *Blood* (2012) 119(6):1479–89. doi:10.1182/blood-2011-07-370841
105. Jinushi M, Vanneman M, Munshi NC, Tai YT, Prabhala RH, Ritz J, et al. MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma. *Proc Natl Acad Sci U S A* (2008) 105(4):1285–90. doi:10.1073/pnas.0711293105
106. Testa U. TRAIL/TRAIL-R in hematologic malignancies. *J Cell Biochem* (2010) 110(1):21–34. doi:10.1002/jcb.22549
107. Kagawa K, Nakano A, Miki H, Oda A, Amou H, Takeuchi K, et al. Inhibition of TACE activity enhances the susceptibility of myeloma cells to TRAIL. *PLoS One* (2012) 7(2):e31594. doi:10.1371/journal.pone.0031594
108. Rubio-Moscardo F, Blesa D, Mestre C, Siebert R, Balasas T, Benito A, et al. Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. *Blood* (2005) 106(9):3214–22. doi:10.1182/blood-2005-05-2013
109. Plumas J, Jacob MC, Chaperot L, Molens JP, Sotto JJ, Bensa JC. Tumor B cells from non-Hodgkin's lymphoma are resistant to CD95 (Fas/Apo-1)-mediated apoptosis. *Blood* (1998) 91(8):2875–85.
110. Mathas S, Lietz A, Anagnostopoulos I, Hummel F, Wiesner B, Janz M, et al. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. *J Exp Med* (2004) 199(8):1041–52. doi:10.1084/jem.20031080
111. Riccioni R, Pasquini L, Mariani G, Saulle E, Rossini A, Diverio D, et al. TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL. *Haematologica* (2005) 90(5):612–24.
112. Chamuleau MED, Ossenkoppele GJ, van Rhenen A, van Dreunen L, Jirka SMG, Zevenbergen A, et al. High TRAIL-R3 expression on leukemic blasts is associated with poor outcome and induces apoptosis-resistance which can be overcome by targeting TRAIL-R2. *Leuk Res* (2011) 35(6):741–9. doi:10.1016/j.leukres.2010.12.032
113. Zeng W, Maciejewski JP, Chen G, Risitano AM, Kirby M, Kajigaya S, et al. Selective reduction of natural killer T cells in the bone marrow of aplastic anaemia. *Br J Haematol* (2002) 119(3):803–9. doi:10.1046/j.1365-2141.2002.03875.x
114. Yoneda K, Morii T, Nieda M, Tsukaguchi N, Amano I, Tanaka H, et al. The peripheral blood Valpha24+ NKT cell numbers decrease in patients with haematopoietic malignancy. *Leuk Res* (2005) 29(2):147–52. doi:10.1016/j.leukres.2004.06.005
115. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, et al. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol* (2001) 167(7):4046–50. doi:10.4049/jimmunol.167.7.4046
116. Jadidi-Niaragh F, Jeddi-Tehrani M, Ansaripour B, Razavi SM, Sharifian RA, Shokri F. Reduced frequency of NKT-like cells in patients with progressive chronic lymphocytic leukemia. *Med Oncol* (2012) 29(5):3561–9. doi:10.1007/s12032-012-0262-4
117. Nowak M, Arredouani MS, Tun-Kyi A, Schmidt-Wolf I, Sanda MG, Balk SP, et al. Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with alpha-galactosylceramide. *PLoS One* (2010) 5(6):e11311. doi:10.1371/journal.pone.0011311
118. Fujii S, Shimizu K, Klimek V, Geller MD, Nimer SD, Dhodapkar MV. Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br J Haematol* (2003) 122(4):617–22. doi:10.1046/j.1365-2141.2003.04465.x
119. Chuc AEN, Cervantes LAM, Retiguin FP, Ojeda JV, Maldonado ER. Low number of invariant NKT cells is associated with poor survival in acute myeloid leukemia. *J Cancer Res Clin Oncol* (2012) 138(8):1427–32. doi:10.1007/s00432-012-1251-x
120. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* (1999) 163(5):2387–91.
121. van der Vliet HJ, Molling JW, Nishi N, Masterson AJ, Kolgen W, Porcelli SA, et al. Polarization of Valpha24+ Vbeta11+ natural killer T cells of healthy volunteers and cancer patients using alpha-galactosylceramide-loaded and environmentally instructed dendritic cells. *Cancer Res* (2003) 63(14):4101–6.
122. Chang DH, Liu N, Klimek V, Hassoun H, Mazumder A, Nimer SD, et al. Enhancement of ligand-dependent activation of human natural killer T cells by lenalidomide: therapeutic implications. *Blood* (2006) 108(2):618–21. doi:10.1182/blood-2005-10-4184
123. Song W, van der Vliet HJ, Tai YT, Prabhala R, Wang R, Podar K, et al. Generation of antitumor invariant natural killer T cell lines in multiple myeloma and promotion of their functions via lenalidomide: a strategy for immunotherapy. *Clin Cancer Res* (2008) 14(21):6955–62. doi:10.1158/1078-0432.CCR-07-5290
124. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* (2000) 1(6):515–20. doi:10.1038/82771
125. Liu D, Song L, Wei J, Courtney AN, Gao X, Marinova E, et al. IL-15 protects NKT cells from inhibition by tumor-associated macrophages and enhances antimetastatic activity. *J Clin Invest* (2012) 122(6):2221–33. doi:10.1172/JCI59535
126. Xie D, Zhu S, Bai L. Lactic acid in tumor microenvironments causes dysfunction of NKT cells by interfering with mTOR signaling. *Sci China Life Sci* (2016) 59(12):1290–6. doi:10.1007/s11427-016-0348-7
127. Vishvakarma NK, Singh SM. Mechanisms of tumor growth retardation by modulation of pH regulation in the tumor-microenvironment of a murine T cell lymphoma. *Biomed Pharmacother* (2011) 65(1):27–39. doi:10.1016/j.biopha.2010.06.012
128. Wein F, Otto T, Lambertz P, Fandrey J, Hansmann ML, Kuppers R. Potential role of hypoxia in early stages of Hodgkin lymphoma pathogenesis. *Haematologica* (2015) 100(10):1320–6. doi:10.3324/haematol.2015.127498
129. Nakagawa R, Motoki K, Ueno H, Iijima R, Nakamura H, Kobayashi E, et al. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an alpha-galactosylceramide, KRN7000. *Cancer Res* (1998) 58(6):1202–7.

130. Fujii S, Shimizu K, Hemmi H, Fukui M, Bonito AJ, Chen G, et al. Glycolipid alpha-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice. *Proc Natl Acad Sci U S A* (2006) 103(30):11252–7. doi:10.1073/pnas.0604812103
131. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* (2002) 8(12): 3702–9.
132. Schneiders FL, Scheper RJ, von Blomberg BM, Woltman AM, Janssen HL, van den Eertwegh AJ, et al. Clinical experience with alpha-galactosylceramide (KRN7000) in patients with advanced cancer and chronic hepatitis B/C infection. *Clin Immunol* (2011) 140(2):130–41. doi:10.1016/j.clim.2010.11.010
133. Schneiders FL, de Bruin RC, van den Eertwegh AJ, Scheper RJ, Leemans CR, Brakenhoff RH, et al. Circulating invariant natural killer T-cell numbers predict outcome in head and neck squamous cell carcinoma: updated analysis with 10-year follow-up. *J Clin Oncol* (2012) 30(5):567–70. doi:10.1200/JCO.2011.38.8819
134. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* (2005) 115(9):2572–83. doi:10.1172/JCI24762
135. Sullivan BA, Kronenberg M. Activation or anergy: NKT cells are stunned by alpha-galactosylceramide. *J Clin Invest* (2005) 115(9):2328–9. doi:10.1172/JCI26297
136. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* (2002) 3(9):867–74. doi:10.1038/ni827
137. Motohashi S, Okamoto Y, Yoshino I, Nakayama T. Anti-tumor immune responses induced by iNKT cell-based immunotherapy for lung cancer and head and neck cancer. *Clin Immunol* (2011) 140(2):167–76. doi:10.1016/j.clim.2011.01.009
138. Nagato K, Motohashi S, Ishibashi F, Okita K, Yamasaki K, Moriya Y, et al. Accumulation of activated invariant natural killer T cells in the tumor microenvironment after alpha-galactosylceramide-pulsed antigen presenting cells. *J Clin Immunol* (2012) 32(5):1071–81. doi:10.1007/s10875-012-9697-9
139. Tatsumi T, Takehara T, Yamaguchi S, Sasakawa A, Sakamori R, Ohkawa K, et al. Intrahepatic delivery of alpha-galactosylceramide-pulsed dendritic cells suppresses liver tumor. *Hepatology* (2007) 45(1):22–30. doi:10.1002/hep.21447
140. Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, et al. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* (2004) 103(2):383–9. doi:10.1182/blood-2003-04-1155
141. Handa H, Saitoh T, Murakami H. [Immunomodulatory effects of lenalidomide]. *Nihon Rinsho* (2015) 73(1):156–61.
142. Handa H, Kamiya A, Nagai K, Saitoh T, Alkebsi L, Mawatari M, et al. Lenalidomide expand CD3+CD56+ T cell cytokine induced killer (CIK) cells in vivo in multiple myeloma patients. *Clin Lymphoma Myeloma Leuk* (2015) 15:e296. doi:10.1016/j.clml.2015.07.603
143. Chung Y, Qin H, Kang CY, Kim S, Kwak LW, Dong C. An NKT-mediated autologous vaccine generates CD4 T-cell dependent potent antilymphoma immunity. *Blood* (2007) 110(6):2013–9. doi:10.1182/blood-2006-12-061309
144. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S. Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *J Exp Med* (2007) 204(11):2641–53. doi:10.1084/jem.20070458
145. Leemhuis T, Wells S, Scheffold C, Edinger M, Negrin RS. A phase I trial of autologous cytokine-induced killer cells for the treatment of relapsed Hodgkin disease and non-Hodgkin lymphoma. *Biol Blood Marrow Transplant* (2005) 11(3):181–7. doi:10.1016/j.bbmt.2004.11.019
146. Webb TJ, Bieler JG, Schneck JP, Oelke M. Ex vivo induction and expansion of natural killer T cells by CD1d1-Ig coated artificial antigen presenting cells. *J Immunol Methods* (2009) 346(1–2):38–44. doi:10.1016/j.jim.2009.05.003
147. Sun W, Wang Y, East JE, Kimball AS, Tkaczuk K, Kesmodel S, et al. Invariant natural killer T cells generated from human adult hematopoietic stem-progenitor cells are poly-functional. *Cytokine* (2015) 72(1):48–57. doi:10.1016/j.cyto.2014.12.009
148. Yamada D, Iyoda T, Vizcardo R, Shimizu K, Sato Y, Endo TA, et al. Efficient regeneration of human Valpha24+ invariant natural killer T cells and their anti-tumor activity in vivo. *Stem Cells* (2016) 34(12):2852–60. doi:10.1002/stem.2465
149. Mattarollo SR, Kenna T, Nieda M, Nicol AJ. Chemotherapy pretreatment sensitizes solid tumor-derived cell lines to Valpha24+ NKT cell-mediated cytotoxicity. *Int J Cancer* (2006) 119(7):1630–7. doi:10.1002/ijc.22019
150. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, et al. Adoptive transfer of invariant NKT cells as immunotherapy for advanced melanoma: a phase I clinical trial. *Clin Cancer Res* (2017) 23(14): 3510–9. doi:10.1158/1078-0432.Ccr-16-0600
151. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* (2006) 12(20 Pt 1):6079–86. doi:10.1158/1078-0432.CCR-06-0114
152. Kunii N, Horiguchi S, Motohashi S, Yamamoto H, Ueno N, Yamamoto S, et al. Combination therapy of in vitro-expanded natural killer T cells and alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* (2009) 100(6):1092–8. doi:10.1111/j.1349-7006.2009.01135.x
153. Yamasaki K, Horiguchi S, Kurosaki M, Kunii N, Nagato K, Hanaoka H, et al. Induction of NKT cell-specific immune responses in cancer tissues after NKT cell-targeted adoptive immunotherapy. *Clin Immunol* (2011) 138(3):255–65. doi:10.1016/j.clim.2010.11.014
154. Luo Y, Zeng HQ, Shen Y, Zhang P, Lou SF, Chen L, et al. Allogeneic hematopoietic stem cell transplantation following donor CIK cell infusion: a phase I study in patients with relapsed/refractory hematologic malignancies. *Leuk Res* (2016) 48:6–10. doi:10.1016/j.leukres.2016.06.006
155. Heczey A, Liu D, Tian G, Courtney AN, Wei J, Marinova E, et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* (2014) 124(18):2824–33. doi:10.1182/blood-2013-11-541235
156. Bollino D, Webb TJ. Chimeric antigen receptor-engineered natural killer and natural killer T cells for cancer immunotherapy. *Transl Res* (2017) 187:32–43. doi:10.1016/j.trsl.2017.06.003
157. Wang Z, Wu Z, Liu Y, Han W. New development in CAR-T cell therapy. *J Hematol Oncol* (2017) 10(1):53. doi:10.1186/s13045-017-0423-1
158. Ghinnagow R, Cruz LJ, Macho-Fernandez E, Faveeuw C, Trottein F. Enhancement of adjuvant functions of natural killer T cells using nanovector delivery systems: application in anticancer immune therapy. *Front Immunol* (2017) 8:879. doi:10.3389/fimmu.2017.00879
159. Baetke SC, Lammers T, Kiessling F. Applications of nanoparticles for diagnosis and therapy of cancer. *Br J Radiol* (2015) 88(1054):20150207. doi:10.1259/bjr.20150207
160. Thapa P, Zhang G, Xia C, Gelbard A, Overwijk WW, Liu C, et al. Nanoparticle formulated alpha-galactosylceramide activates NKT cells without inducing anergy. *Vaccine* (2009) 27(25–26):3484–8. doi:10.1016/j.vaccine.2009.01.047
161. Dolen Y, Kreutz M, Gileadi U, Tel J, Vasaturo A, van Dinther EA, et al. Co-delivery of PLGA encapsulated invariant NKT cell agonist with antigenic protein induce strong T cell-mediated antitumor immune responses. *Oncoimmunology* (2016) 5(1):e1068493. doi:10.1080/2162402X.2015.1068493
162. McKee SJ, Young VL, Clow F, Hayman CM, Baird MA, Hermans IF, et al. Virus-like particles and alpha-galactosylceramide form a self-adjuvanting composite particle that elicits anti-tumor responses. *J Control Release* (2012) 159(3):338–45. doi:10.1016/j.jconrel.2012.02.015
163. Nakamura T, Yamazaki D, Yamauchi J, Harashima H. The nanoparticulation by octaarginine-modified liposome improves alpha-galactosylceramide-

mediated antitumor therapy via systemic administration. *J Control Release* (2013) 171(2):216–24. doi:10.1016/j.jconrel.2013.07.004

164. Gehrmann U, Hiltbrunner S, Georgoudaki AM, Karlsson MC, Naslund TI, Gabrielsson S. Synergistic induction of adaptive antitumor immunity by codelivery of antigen with alpha-galactosylceramide on exosomes. *Cancer Res* (2013) 73(13):3865–76. doi:10.1158/0008-5472.CAN-12-3918
165. Duramad O, Laysang A, Li J, Ishii Y, Namikawa R. Pharmacologic expansion of donor-derived, naturally occurring CD4(+)Foxp3(+) regulatory T cells reduces acute graft-versus-host disease lethality without abrogating the graft-versus-leukemia effect in murine models. *Biol Blood Marrow Transplant* (2011) 17(8):1154–68. doi:10.1016/j.bbmt.2010.11.022

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Alpha-Galactosylceramide/CD1d-Antibody Fusion Proteins Redirect Invariant Natural Killer T Cell Immunity to Solid Tumors and Promote Prolonged Therapeutic Responses

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Major progress in cancer immunotherapies have been obtained by the use of tumor targeting strategies, in particular with the development of bi-functional fusion proteins such as ImmTacs or BiTes, which engage effector T cells for targeted elimination of tumor cells. Given the significance of invariant natural killer T (iNKT) cells in bridging innate and adaptive immunity, we have developed a bi-functional protein composed of the extracellular part of CD1d molecule that was genetically fused to an scFv fragment from high affinity antibodies against HER2 or CEA. Systemic treatments with the CD1d-antitumor fusion proteins loaded with the agonist alpha-galactosylceramide (αGalCer) led to specific iNKT cell activation, resulting in a sustained growth inhibition of established tumors expressing HER2 or CEA, while treatment with the free αGalCer was ineffective. Importantly, we discovered that αGalCer/CD1d-antitumor fusion proteins were able to maintain iNKT cells reactive to multiple re-stimulations in contrast to their anergic state induced after a single injection of free αGalCer. We further demonstrated that the antitumor effects by αGalCer/CD1d-antitumor fusion proteins were largely dependent on the iNKT cell-mediated transactivation of NK cells. Moreover, prolonged antitumor effects could be obtained when combining the CD1d-antitumor fusion protein treatment with a therapeutic peptide/CpG cancer vaccine, which favored the capacity of iNKT cells to transactivate cross-presenting DCs for efficient priming of tumor-specific CD8 T cells. We will also summarize these pre-clinical results with a special focus on the cellular mechanisms underlying iNKT cell unresponsiveness to antigen re-challenge. Finally, we will discuss the perspectives regarding iNKT cell-mediated tumor targeting strategy in cancer immunotherapy.

Keywords: bi-functional fusion protein, CD1d-antitumor scFv, NKT cell, DC activation, innate and adaptive immune response, tumor-associated antigen

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HARNESSING iNKT CELLS FOR CANCER IMMUNOTHERAPY

Invariant natural killer T (iNKT) cells represent a unique T cell subset characterized by an invariant TCR alpha chain paired with a restricted number of TCR beta chains both in mouse and humans (1–3). iNKT cells have the capacity to bridge the innate and adaptive immunity (1, 4–6). First, iNKT cells acquire an effector memory phenotype before birth, which allows their trafficking to the site of inflammation where they exhibit direct cytotoxic capacity by the expression of perforin and granzymes. Second, iNKT cells secrete large amounts of effector cytokines very rapidly after

activation and are potent activators of NK cells through their fast release of IFNγ. Third, activated iNKT cells communicate with DCs *via* the upregulation of CD40L which promotes DCs licensing and maturation, and subsequently effective CD8 T cell responses (7, 8).

The significance of iNKT cells in antitumor immunity has been well studied in both mouse models and clinics (1, 4, 5, 9–12). Mice lacking iNKT cells are more prone to chemical or p53 loss-induced tumor development (13–15). Along the same line, late-stage cancer patients harbor either decreased numbers of iNKT cells or iNKT cells showing certain functional deficiencies (11, 16–19). Also, head and neck squamous cell carcinoma (HNSCC) patients with lower levels of circulating iNKT cells before radiation therapy show poor 3-year survival as compared to patients harboring higher circulating levels of iNKT cells (20). These observations have triggered the development of iNKT-mediated cancer immunotherapy mainly by the use of the CD1d agonist ligand alpha-galactosylceramide (αGalCer), either as a free drug or loaded on DCs before their adoptive transfer, as reviewed by McEwen-Smith et al. (4) and Robertson et al. (21). These approaches have demonstrated potent iNKT cell activation and subsequent NK cell transactivation and CD8 T cell priming. Despite the potent tumor cytotoxicity and transactivating properties of iNKT cells, clinical responses have remained so far limited, resulting on the one hand from the small numbers of iNKT cells, and on the other hand from their short-lived activation followed by long-term unresponsiveness. To address the issue of the small iNKT cell numbers, the adoptive cell transfer (ACT) of *ex vivo* expanded autologous iNKT cells has been tested in HNSCC and melanoma patients with, respectively, some objective clinical responses and Th1 responses, in particular when iNKT cells were inoculated in the vicinity of the tumor in combination with αGalCer-pulsed DCs (22–24). As mentioned above, the powerful initial αGalCer-mediated activation of iNKT cells is followed by long-term unresponsiveness which is another drawback for the therapeutic manipulation of iNKT cells against cancer (9, 25, 26). In this regard, ACT of αGalCer-pulsed DCs was reported to trigger more effective antitumor immunity than administration of free αGalCer in mouse experimental models and cancer patients (25–28).

More recently, ACT of human iNKT cells transduced with a chimeric antigen receptor (CAR) was reported as a novel and safe platform in a humanized mouse tumor model (29). This attractive approach that requires further validation in immunocompetent hosts would combine the ACT of high numbers of tumor-specific iNKT cells which could be co-activated by αGalCer treatment. However, CAR-T cell immunotherapy represents an expensive personalized cancer treatment and alternative cost-effective treatments would be preferred, such as the development of soluble molecules able to activate and redirect endogenous iNKT cells to the tumor site.

TUMOR TARGETING IN CANCER IMMUNOTHERAPIES

Major progress in cancer therapy have been obtained by the development of tumor targeting strategies, which mostly involve monoclonal antibodies (mAbs) specific either of tumor-associated antigens (TAA), or soluble factors released by the tumor or inhibitory and activatory receptors expressed by tumor-infiltrating T cells (TILs). For instance, numerous clinical protocols are now routinely involving tumor targeting antibodies such as anti-CD19, anti-HER2, or anti-EGFR combined with chemotherapy or kinase inhibitors for the treatment of, respectively, B cell lymphoma, breast, gastric, and colon cancers (30, 31). In addition to the use of native mAbs, various antibody formats have been developed, which allowed, for instance, the development of a large array of bi-functional molecules by the genetic fusion of an antibody fragment with an effector molecule, such as another antibody fragment, a toxin, a cytokine, or an antigen-presenting molecule. Yet, even a large array of bi-functional proteins have been tested in pre-clinical studies and some clinical trials, very few have so far entered routine clinical application. Among the few bi-functional molecules that are currently under clinical testing, the most promising are the Bi-specific T cell engagers or BiTes, which directly activate T cells against tumor cells by combining an anti-CD3 scFv fragment with another scFv specific of an antigen over-expressed on tumor cells (32). The second class of bi-functional molecules that are currently tested in metastatic melanoma patients are the so-called ImmTACs for "immune mobilizing monoclonal TCRs against cancer," which combines an optimized TCR specific of HLA-A2/gp100 (IMCgp100) fused to an anti-CD3 scFv (33). While the use of BiTes is restricted to surface-expressed tumor antigens, ImmTACs have the potential to target endogenously processed antigens loaded on MHC I molecules, which greatly increases the possible applications. However, ImmTACs require TCR optimization in the context of defined HLA haplotypes, which represents a personalized and expensive approach. By contrast, BiTes have the advantage to be one drug which fits all patients. Along the same line, we and others have initially developed bi-functional molecules, which combine an MHC I molecule with an antitumor antibody fragment (34–37). We could demonstrate the capacity of these bi-functional molecules to redirect tumor-specific T cells to the tumor site, which led to a significant inhibition of tumor growth (34, 35). More recently, we developed CD1d-antitumor fusion proteins, which offered two main advantages. First, CD1d bi-functional molecules are exploiting a monomorphic antigen-presenting molecule that would fit all patients. Second, when loaded with αGalCer, these CD1d-antitumor fusion proteins will specifically activate Type 1 iNKT cells and redirect both the innate and the adaptive antitumor responses to the tumor site, in view of the transactivating properties of iNKT cells.

CD1d-ANTITUMOR FUSION PROTEINS

In order to redirect iNKT cell immunity at the tumor site, we have developed CD1d molecules genetically fused to an antibody scFv fragment specific of the HER2 or CEA antigens, which are overexpressed in several cancers (9, 10). Briefly, mouse β2-microglobulin coding sequence (β2M) was fused to the soluble part of CD1d followed by the antibody scFv fragment and a 6xHIS-tag for purification. Flexible glycine/serine

linkers were inserted after β2M, CD1d, and scFv to facilitate proper folding. Recombinant proteins were produced in 293-EBNA cells. Strikingly, unlike MHC I/peptide monomer and conventional CD8 T cells, αGalCer/CD1d monomers were able to activate iNKT cells *in vivo* as seen by iNKT TCR down-modulation, as well as iNKT and NK cell proliferation and DC maturation (9). The iNKT cell activation by CD1d monomers may result from the significantly higher binding affinity of the iNKT TCR for αGalCer/CD1d (K_D ~ 0.3 μM) (38), as compared to conventional TCR for MHC/peptide (K_D range 1–50 μM) (39). Moreover, it is possible that *in vivo* aggregation or loose cell binding may also facilitate iNKT cell monomer activation. Nonetheless, significant antitumor activity only occurred when the CD1d protein was targeted to a tumor antigen by its fusion to an antibody scFv fragment. First, we demonstrated that B16-HER2 tumor cells pre-coated with αGalCer/CD1d-anti-HER2 fusion proteins totally abolished their potency to initiate tumors (9). In view of these encouraging results, we tested the therapeutic efficacy of αGalCer/CD1d-anti-HER2 proteins in mice bearing established B16-HER2 lung tumor nodules or subcutaneous tumors. In both models, we could demonstrate a significant inhibition of tumor growth, which was dependent on the presence of iNKT and NK cells as the antitumor effects were abolished in CD1d-deficient mice or upon depletion of NK cells (9). The analysis of peripheral lymphoid organs and tumor tissue revealed (i) localization of CD1d-antitumor proteins at the tumor site, (ii) recruitment of iNKT, NK, and T cells at the tumor, (iii) sustained activation of iNKT cells, and (iv) adjuvant effect on CD8 T cell priming (as depicted in the **Figure 1**).

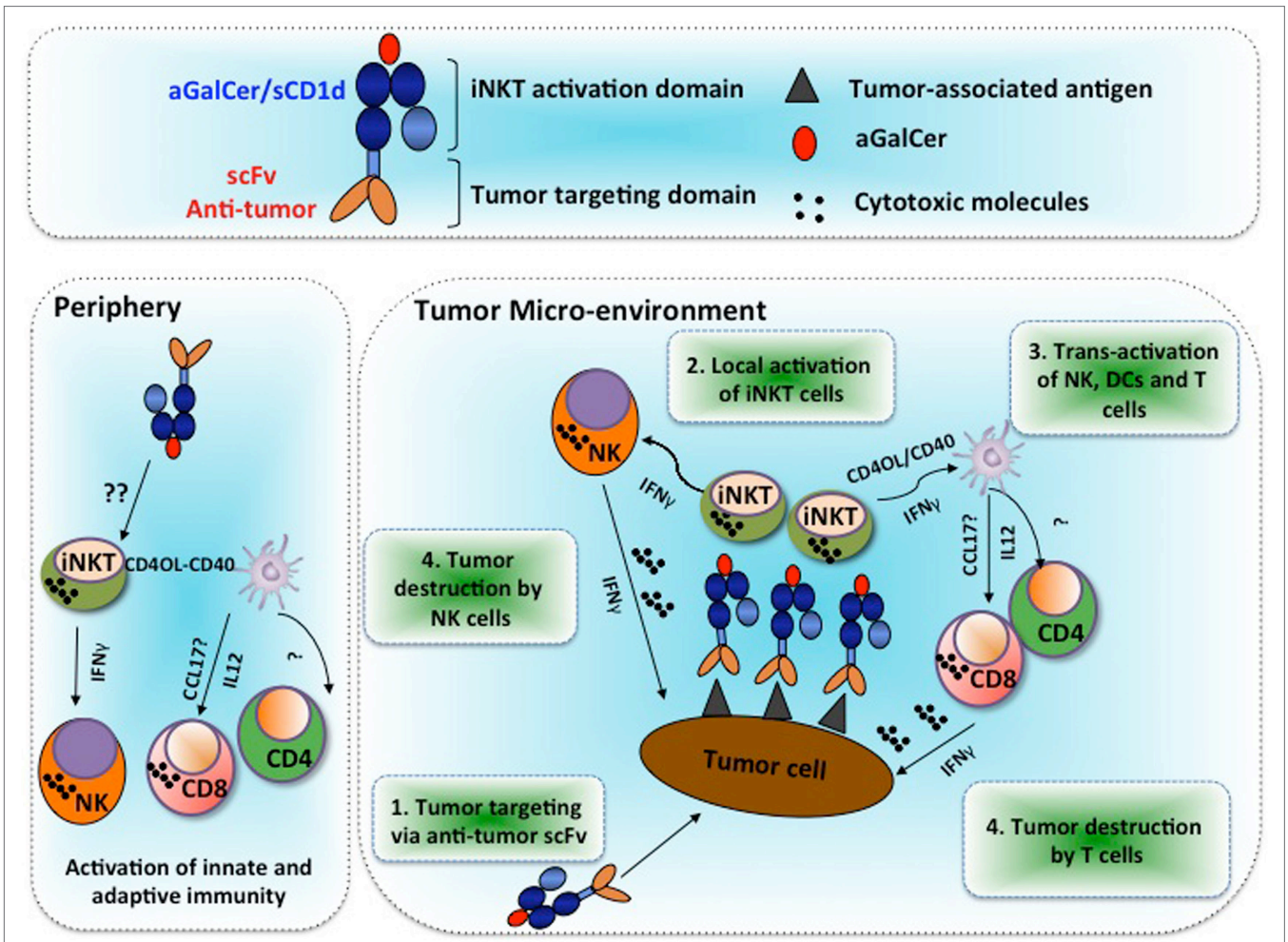

FIGURE 1 | Alpha-galactosylceramide (αGalCer)/CD1d-antibody fusion proteins redirect invariant natural killer T (iNKT), NK, and T cell immunity to solid tumors and promote prolonged therapeutic responses. Tumor microenvironment: Step 1: systemic treatments with the αGalCer/CD1d-antitumor fusion protein lead to its binding to the tumor-associated antigen at the tumor site. Step 2: tumor-bound αGalCer/CD1d-antitumor fusion proteins lead to specific activation of iNKT cells. Step 3: rapid iNKT-mediated transactivation of NK cells and DCs, which then activate and recruit T cells. Step 4: release of IFNγ and cytotoxic molecules from NK and CD8 T cells that mediate targeted tumor destruction. Periphery: soluble αGalCer/CD1d fused or not to an antitumor scFv antibody fragment can also activate iNKT cells in peripheral lymphoid and non-lymphoid organs. The underlying mechanisms for this extra-tumoral activation of iNKT cells remain unclear.

Localization of CD1d-Antitumor Proteins at the Tumor Site

CD1d-antitumor proteins were initially validated *in vitro* for their specific binding to tumor cells expressing the relevant tumor antigen. Next, we investigated whether intravenously injected fusion proteins would reach the tumor site in sufficient amounts to attract iNKT cells. Indeed, when injecting radiolabeled αGalCer/CD1d-anti-HER2 proteins in mice bearing on each flank either HER2-positive or HER2-negative tumors, up to twofold more radioactivity was found in HER2-positive tumors, as compared to HER2-negative tumors, while non-targeted αGalCer/CD1d protein did not localize preferentially to any of the tumors, although it induced systemic iNKT cell activation to some extent (9) (see **Figure 1**).

Recruitment of iNKT, NK, and T Cells at the Tumor Site

When BrdU-positive iNKT cells in lung tumor nodules, there was a fivefold or twofold increase when compared to untreated mice or the ones treated with untargeted αGalCer/CD1d protein, respectively. Most importantly, we observed a sevenfold enrichment of BrdU-positive NK cells and conventional T cells at tumor site, illustrating that the iNKT-mediated transactivation could trigger their increased proliferation capacity. Interestingly, all three lymphocyte populations were instead decreased in the blood and spleen of CD1d-anti-HER2-treated animals as compared to the untargeted CD1d treatment, which might reflect their preferential recruitment to the tumor site upon HER2 targeting.

Sustained Activation of iNKT Cells

Strikingly, iNKT cells remained reactive even after multiple treatments with αGalCer/CD1d-antitumor fusion proteins, in contrast to the hyporesponsive state that typically follows the injection of the free ligand αGalCer. This preservation of iNKT cell responsiveness allowed multiple injections of the fusion proteins, which greatly enhanced antitumor efficacy of tumor-targeted CD1d as compared to αGalCer/CD1d molecules targeted to an irrelevant tumor antigen (10). Although iNKT cells remained substantially reactive to several injections of αGalCer/CD1d fusion proteins, we did observe a progressive loss of iNKT cell activation with reduced cytokine production. It is highly possible that a proportion of loaded glycolipid analog was lost from the fusion protein *in vivo* and, thus, processed by APCs, whereby they progressively induced iNKT cell anergy. In this regard, studies are in progress to assess the activities of CD1d fusion proteins loaded with photo-reactive αGalCer analogs that can be UV-crosslinked to CD1d. Initial studies show that complexes of mCD1d with a covalently bound αGalCer are resistant to dissociation and are potent iNKT cell activators *in vitro* and *in vivo* (personal communication, S. Porcelli, Albert Einstein College of Medicine, NY, USA). The validation of these covalently bound αGalCer on CD1d antitumor fusion proteins is in progress with regard to their antitumor activity and capacity to maintain iNKT cells reactive to multiple stimulations.

The mechanism by which systemic treatments with αGalCer/CD1d fusion proteins activate iNKT cells without inducing anergy, as compared to free αGalCer analogs, remains an area of active exploration. High level of surface PD-1 expression has been well defined in exhausted CD8 T cells during chronic viral infection or tumor exposure, which closely correlated with T cell functional decline (40). Likewise, PD-1 expression was also proposed to regulate iNKT cell anergy induction (41, 42). Indeed, upregulation of PD-1 was observed shortly after αGalCer injections, which could last for at least 1 month (41). Moreover, two studies showed that blocking of the interaction between PD-1 and its ligand PD ligand 1 (PD-L1) or PD-L2 at the time of αGalCer injection could prevent the anergy induction of iNKT cells (41, 42). In addition, injection of αGalCer into PD-1 deficient mice failed to induce iNKT cell anergy (42). In a different context, lymphocyte activation gene 3 (LAG-3), another co-inhibitory molecule, was highly expressed on iNKT and NK cells rather than conventional T cells from chronically HIV-infected patients. Interestingly, LAG-3, but not PD-1, was associated with the reduced IFNγ production from iNKT cells, indicating that distinct mechanisms underlying the anergy induction of iNKT cells are context dependent (43). Yet, other mechanisms were also described (44, 45). For instance, deficiency of tuberous sclerosis 1 (TSC1), the upstream inhibitor of mTORC1 signaling, in iNKT cells results in increased resistance to αGalCer induced anergy, which is correlated with impaired upregulation of Egr2 and Grail (46). Altogether, it appears that PD-1 upregulation alone is not enough to mediate iNKT cell anergy.

Interestingly, a recent report showed that, instead of being anergic, iNKT cells were rather reprogrammed toward a suppressive phenotype with the secretion of IL-10 associated with markedly reduced production of effector cytokines (47). Importantly, the study by Wingender et al. (48) showed that Th2-biased αGalCer analogs, which are less hydrophobic than Th1 analogs, are mostly surface-loaded as monomers on CD1d, resulting in a fast and transient iNKT cell activation which preserved their responsiveness to antigen re-challenge. By contrast, the more hydrophobic so-called Th1 αGalCer analogs characterized by a higher critical micelle concentration (CMC) are mostly loaded as micelles and internalized and processed on CD1d *via* the endosomal pathway, leading to a delayed and prolonged iNKT cell activation followed by long-term unresponsiveness (49, 50).

Therefore, we speculate that the surface loading of Th2 analogs on APCs is similar to the loading of αGalCer on CD1d fusion protein which in both cases triggers the fast and transient kinetic of iNKT cell activation, which might be instrumental for their retained reactivity to antigen re-challenge.

Adjuvant Effect on CD8 T Cell Priming

CD1d-restricted iNKT cells have been shown to promote the transactivation of DCs *via* the CD40L–CD40 interaction (see **Figure 1**), and their adjuvant properties on the adaptive immunity are well reported (51). For instance, DCs receive cognate "licensing" either from helper T cells or iNKT cells. With regard to iNKT cell, their licensing of cross-priming CD8α$^+$ DCs induces them to produce CCL17, which thus attracts CCR4 expressing CD8 T cells (8). However, it remains largely undefined regarding how NK and T cells are recruited upon iNKT cell activation to the

tumor site. In this regard, several laboratories have developed vaccine strategies involving either the development of novel αGalCer analogs (52), αGalCer-loaded DC vaccines (53) or DC-targeted nanoparticles loaded with αGalCer (54). With regard to αGalCer/CD1d proteins, repeated iNKT cell activation by untargeted αGalCer/CD1d monomers efficiently promoted the maturation of pro-inflammatory DCs, while αGalCer as a free drug had only a marginal effect (9). Moreover, when tumor-bearing mice received an OVA peptide/CpG-ODN vaccination combined with systemic treatments of αGalCer/CD1d-antitumor fusion proteins, a synergistic expansion of OVA-specific CD8 T cells and NK cells was obtained, as compared to each regimen alone (12). The optimal adjuvant effect on innate and adaptive immune responses likely resulted from the enhanced production of the pro-inflammatory cytokine IL-12 by mature DCs, which was 10-fold higher with the combined stimuli of CpG-ODN and αGalCer/CD1d-anti-HER2 fusion. Most importantly, the combined treatment resulted in an improved enrichment of tumor antigen-specific CD8 T cells and NK cells at the tumor site, associated with better tumor inhibition against tumors co-expressing HER2 and OVA (12). Interestingly, the antibody-mediated depletion of either NK cells or CD8 T cells, demonstrated an early and transient NK-mediated antitumor activity that was quickly replaced by the CD8 antitumor response. Thus, in this context, the direct antitumor activity of iNKT cells was minimal but was instead instrumental for the development of a tumor-targeted innate and adaptive antitumor responses.

PERSPECTIVES

CD1d-antitumor fusion proteins represent an attractive tool to redirect at the tumor site the immunoregulatory properties of iNKT cells on the innate and adaptive immune responses. Importantly, this strategy holds the advantage to maintain iNKT cells reactive to multiple treatments in contrast to the use of an αGalCer analog as a free drug. Yet, the development of covalently bound αGalCer on CD1d fusion proteins will improve their stability *in vivo* and should greatly increase the sustained activation of iNKT cells and antitumor efficacy. Although, these molecules did not show significant liver toxicity, more in-depth pharmacological studies need to be done. Moreover, imaging techniques will help demonstrate that oligomerization of CD1d-antitumor proteins on the tumor cell likely optimize the formation of an immunological synapse with the iNKT cell. However, in view of the low numbers of iNKT cells in cancer patients, the benefit would primarily result from their adjuvant effects rather than their direct antitumor cytotoxicity, unless iNKT ACT is included. Finally, CD1d is monomorphic and a single fusion protein would fit all patients, in contrast to other approaches such as ImmTacs, which involves individual TCRs.

ETHICS STATEMENT

All animal experiments were conducted under an authorization delivered by the Swiss veterinary department.

AUTHOR CONTRIBUTIONS

AD has initiated the project and led its subsequent developments. LZ has contributed to the project in a later stage. AD and LZ wrote the review together.

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REFERENCES

1. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi:10.1146/annurev.immunol.25.022106.141711
2. Salio M, Silk JD, Jones EY, Cerundolo V. Biology of CD1- and MR1-restricted T cells. *Annu Rev Immunol* (2014) 32:323–66. doi:10.1146/annurev-immunol-032713-120243
3. Zhang L, Tschumi BO, Corgnac S, Ruegg MA, Hall MN, Mach JP, et al. Mammalian target of rapamycin complex 1 orchestrates invariant NKT cell differentiation and effector function. *J Immunol* (2014) 193(4):1759–65. doi:10.4049/jimmunol.1400769
4. McEwen-Smith RM, Salio M, Cerundolo V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res* (2015) 3(5):425–35. doi:10.1158/2326-6066.CIR-15-0062
5. Carreno LJ, Saavedra-Avila NA, Porcelli SA. Synthetic glycolipid activators of natural killer T cells as immunotherapeutic agents. *Clin Transl Immunology* (2016) 5(4):e69. doi:10.1038/cti.2016.14
6. Taniguchi M, Tashiro T, Dashtsoodol N, Hongo N, Watarai H. The specialized iNKT cell system recognizes glycolipid antigens and bridges the innate and acquired immune systems with potential applications for cancer therapy. *Int Immunol* (2010) 22(1):1–6. doi:10.1093/intimm/dxp104
7. Gottschalk C, Mettke E, Kurts C. The role of invariant natural killer T cells in dendritic cell licensing, cross-priming, and memory CD8(+) T cell generation. *Front Immunol* (2015) 6:379. doi:10.3389/fimmu.2015.00379
8. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, et al. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. *Nat Immunol* (2010) 11(4):313–20. doi:10.1038/ni.1848
9. Stirnemann K, Romero JF, Baldi L, Robert B, Cesson V, Wurm F, et al. Sustained activation and tumor targeting of NKT cells using a CD1d-anti-HER2-scFv fusion protein induce antitumor effects in mice. *J Clin Invest* (2008) 118(3):994–1005. doi:10.1172/JCI33249
10. Corgnac S, Perret R, Derre L, Zhang L, Stirnemann K, Zauderer M, et al. CD1d-antibody fusion proteins target iNKT cells to the tumor and trigger long-term therapeutic responses. *Cancer Immunol Immunother* (2013) 62(4):747–60. doi:10.1007/s00262-012-1381-7
11. Fujii S, Shimizu K, Okamoto Y, Kunii N, Nakayama T, Motohashi S, et al. NKT cells as an ideal anti-tumor immunotherapeutic. *Front Immunol* (2013) 4:409. doi:10.3389/fimmu.2013.00409
12. Corgnac S, Perret R, Zhang L, Mach JP, Romero P, Donda A. iNKT/CD1d-antitumor immunotherapy significantly increases the efficacy of therapeutic CpG/peptide-based cancer vaccine. *J Immunother Cancer* (2014) 2(1):39. doi:10.1186/s40425-014-0039-8
13. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* (2000) 191(4):661–8. doi:10.1084/jem.191.4.661
14. Nishikawa H, Kato T, Tawara I, Takemitsu T, Saito K, Wang L, et al. Accelerated chemically induced tumor development mediated by CD4+CD25+ regulatory T cells in wild-type hosts. *Proc Natl Acad Sci U S A* (2005) 102(26):9253–7. doi:10.1073/pnas.0503852102
15. Swann JB, Uldrich AP, van Dommelen S, Sharkey J, Murray WK, Godfrey DI, et al. Type I natural killer T cells suppress tumors caused by

p53 loss in mice. *Blood* (2009) 113(25):6382–5. doi:10.1182/blood-2009-01-198564
16. Metelitsa LS, Wu HW, Wang H, Yang Y, Warsi Z, Asgharzadeh S, et al. Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2. *J Exp Med* (2004) 199(9):1213–21. doi:10.1084/jem.20031462
17. Schneiders FL, de Bruin RC, van den Eertwegh AJ, Scheper RJ, Leemans CR, Brakenhoff RH, et al. Circulating invariant natural killer T-cell numbers predict outcome in head and neck squamous cell carcinoma: updated analysis with 10-year follow-up. *J Clin Oncol* (2012) 30(5):567–70. doi:10.1200/JCO.2011.38.8819
18. Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. *J Immunol* (2002) 168(12):6494–9. doi:10.4049/jimmunol.168.12.6494
19. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: natural killer T cell defects and human disease. *Nat Rev Immunol* (2011) 11(2):131–42. doi:10.1038/nri2904
20. Molling JW, Langius JA, Langendijk JA, Leemans CR, Bontkes HJ, van der Vliet HJ, et al. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. *J Clin Oncol* (2007) 25(7):862–8. doi:10.1200/JCO.2006.08.5787
21. Robertson FC, Berzofsky JA, Terabe M. NKT cell networks in the regulation of tumor immunity. *Front Immunol* (2014) 5:543. doi:10.3389/fimmu.2014.00543
22. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, et al. Adoptive transfer of invariant NKT cells as immunotherapy for advanced melanoma: a phase I clinical trial. *Clin Cancer Res* (2017) 23(14):3510–9. doi:10.1158/1078-0432.CCR-16-0600
23. Kunii N, Horiguchi S, Motohashi S, Yamamoto H, Ueno N, Yamamoto S, et al. Combination therapy of in vitro-expanded natural killer T cells and alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* (2009) 100(6):1092–8. doi:10.1111/j.1349-7006.2009.01135.x
24. Yamasaki K, Horiguchi S, Kurosaki M, Kunii N, Nagato K, Hanaoka H, et al. Induction of NKT cell-specific immune responses in cancer tissues after NKT cell-targeted adoptive immunotherapy. *Clin Immunol* (2011) 138(3):255–65. doi:10.1016/j.clim.2010.11.014
25. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* (2005) 115(9):2572–83. doi:10.1172/JCI24762
26. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* (2002) 3(9):867–74. doi:10.1038/ni827
27. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* (2005) 201(9):1503–17. doi:10.1084/jem.20042592
28. Uchida T, Horiguchi S, Tanaka Y, Yamamoto H, Kunii N, Motohashi S, et al. Phase I study of alpha-galactosylceramide-pulsed antigen presenting cells administration to the nasal submucosa in unresectable or recurrent head and neck cancer. *Cancer Immunol Immunother* (2008) 57(3):337–45. doi:10.1007/s00262-007-0373-5
29. Heczey A, Liu D, Tian G, Courtney AN, Wei J, Marinova E, et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* (2014) 124(18):2824–33. doi:10.1182/blood-2013-11-541235
30. Parakh S, Gan HK, Parslow AC, Burvenich IJG, Burgess AW, Scott AM. Evolution of anti-HER2 therapies for cancer treatment. *Cancer Treat Rev* (2017) 59:1–21. doi:10.1016/j.ctrv.2017.06.005
31. Robak T, Blonski JZ, Robak P. Antibody therapy alone and in combination with targeted drugs in chronic lymphocytic leukemia. *Semin Oncol* (2016) 43(2):280–90. doi:10.1053/j.seminoncol.2016.02.010
32. Klinger M, Benjamin J, Kischel R, Stienen S, Zugmaier G. Harnessing T cells to fight cancer with BiTE(R) antibody constructs – past developments and future directions. *Immunol Rev* (2016) 270(1):193–208. doi:10.1111/imr.12393
33. Liddy N, Bossi G, Adams KJ, Lissina A, Mahon TM, Hassan NJ, et al. Monoclonal TCR-redirected tumor cell killing. *Nat Med* (2012) 18(6):980–7. doi:10.1038/nm.2764
34. Cesson V, Stirnemann K, Robert B, Luescher I, Filleron T, Corradin G, et al. Active antiviral T-lymphocyte response can be redirected against tumor cells by antitumor antibody x MHC/viral peptide conjugates. *Clin Cancer Res* (2006) 12(24):7422–30. doi:10.1158/1078-0432.CCR-06-1862
35. Donda A, Cesson V, Mach JP, Corradin G, Primus FJ, Robert B. In vivo targeting of an anti-tumor antibody coupled to antigenic MHC class I complexes induces specific growth inhibition and regression of established syngeneic tumor grafts. *Cancer Immun* (2003) 3:11.
36. King BC, Hamblin AD, Savage PM, Douglas LR, Hansen TH, French RR, et al. Antibody-peptide-MHC fusion conjugates target non-cognate T cells to kill tumour cells. *Cancer Immunol Immunother* (2013) 62(6):1093–105. doi:10.1007/s00262-013-1408-8
37. Lev A, Noy R, Oved K, Novak H, Segal D, Walden P, et al. Tumor-specific Ab-mediated targeting of MHC-peptide complexes induces regression of human tumor xenografts in vivo. *Proc Natl Acad Sci U S A* (2004) 101(24):9051–6. doi:10.1073/pnas.0403222101
38. Patel O, Cameron G, Pellicci DG, Liu Z, Byun HS, Beddoe T, et al. NKT TCR recognition of CD1d-alpha-C-galactosylceramide. *J Immunol* (2011) 187(9):4705–13. doi:10.4049/jimmunol.1100794
39. Davis MM, Boniface JJ, Reich Z, Lyons D, Hampl J, Arden B, et al. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* (1998) 16:523–44. doi:10.1146/annurev.immunol.16.1.523
40. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* (2015) 15(8):486–99. doi:10.1038/nri3862
41. Chang WS, Kim JY, Kim YJ, Kim YS, Lee JM, Azuma M, et al. Cutting edge: programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. *J Immunol* (2008) 181(10):6707–10. doi:10.4049/jimmunol.181.10.6707
42. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* (2009) 182(5):2816–26. doi:10.4049/jimmunol.0803648
43. Juno JA, Stalker AT, Waruk JL, Oyugi J, Kimani M, Plummer FA, et al. Elevated expression of LAG-3, but not PD-1, is associated with impaired iNKT cytokine production during chronic HIV-1 infection and treatment. *Retrovirology* (2015) 12:17. doi:10.1186/s12977-015-0142-z
44. Iyoda T, Ushida M, Kimura Y, Minamino K, Hayuka A, Yokohata S, et al. Invariant NKT cell anergy is induced by a strong TCR-mediated signal plus co-stimulation. *Int Immunol* (2010) 22(11):905–13. doi:10.1093/intimm/dxq444
45. Kojo S, Elly C, Harada Y, Langdon WY, Kronenberg M, Liu YC. Mechanisms of NKT cell anergy induction involve Cbl-b-promoted monoubiquitination of CARMA1. *Proc Natl Acad Sci U S A* (2009) 106(42):17847–51. doi:10.1073/pnas.0904078106
46. Wu J, Shin J, Xie D, Wang H, Gao J, Zhong XP. Tuberous sclerosis 1 promotes invariant NKT cell anergy and inhibits invariant NKT cell-mediated antitumor immunity. *J Immunol* (2014) 192(6):2643–50. doi:10.4049/jimmunol.1302076
47. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124(9):3725–40. doi:10.1172/JCI72308
48. Wingender G, Birkholz AM, Sag D, Farber E, Chitale S, Howell AR, et al. Selective conditions are required for the induction of invariant NKT cell hyporesponsiveness by antigenic stimulation. *J Immunol* (2015) 195(8):3838–48. doi:10.4049/jimmunol.1500203
49. Arora P, Venkataswamy MM, Baena A, Bricard G, Li Q, Veerapen N, et al. A rapid fluorescence-based assay for classification of iNKT cell activating glycolipids. *J Am Chem Soc* (2011) 133(14):5198–201. doi:10.1021/ja200070u
50. Im JS, Arora P, Bricard G, Molano A, Venkataswamy MM, Baine I, et al. Kinetics and cellular site of glycolipid loading control the outcome of natural killer T cell activation. *Immunity* (2009) 30(6):888–98. doi:10.1016/j.immuni.2009.03.022
51. Cerundolo V, Silk JD, Masri SH, Salio M. Harnessing invariant NKT cells in vaccination strategies. *Nat Rev Immunol* (2009) 9(1):28–38. doi:10.1038/nri2451

52. Li X, Huang J, Kaneko I, Zhang M, Iwanaga S, Yuda M, et al. A potent adjuvant effect of a CD1d-binding NKT cell ligand in human immune system mice. *Expert Rev Vaccines* (2017) 16(1):73–80. doi:10.1080/14760584.2017.1256208
53. Petersen TR, Sika-Paotonu D, Knight DA, Simkins HM, Hermans IF. Exploiting the role of endogenous lymphoid-resident dendritic cells in the priming of NKT cells and CD8+ T cells to dendritic cell-based vaccines. *PLoS One* (2011) 6(3):e17657. doi:10.1371/journal.pone.0017657
54. Macho-Fernandez E, Cruz LJ, Ghinnagow R, Fontaine J, Bialecki E, Frisch B, et al. Targeted delivery of alpha-galactosylceramide to CD8alpha+ dendritic cells optimizes type I NKT cell-based antitumor responses. *J Immunol* (2014) 193(2):961–9. doi:10.4049/jimmunol.1303029

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REVIEW
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Mixed Signals: Co-Stimulation in Invariant Natural Killer T Cell-Mediated Cancer Immunotherapy

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Invariant natural killer T (iNKT) cells are an integral component of the immune system and play an important role in antitumor immunity. Upon activation, iNKT cells can directly kill malignant cells as well as rapidly produce cytokines that stimulate other immune cells, making them a front line defense against tumorigenesis. Unfortunately, iNKT cell number and activity are reduced in multiple cancer types. This anergy is often associated with upregulation of co-inhibitory markers such as programmed death-1. Similar to conventional T cells, iNKT cells are influenced by the conditions of their activation. Conventional T cells receive signals through the following three types of receptors: (1) T cell receptor (TCR), (2) co-stimulation molecules, and (3) cytokine receptors. Unlike conventional T cells, which recognize peptide antigen presented by MHC class I or II, the TCRs of iNKT cells recognize lipid antigen in the context of the antigen presentation molecule CD1d (Signal 1). Co-stimulatory molecules can positively and negatively influence iNKT cell activation and function and skew the immune response (Signal 2). This study will review the background of iNKT cells and their co-stimulatory requirements for general function and in antitumor immunity. We will explore the impact of monoclonal antibody administration for both blocking inhibitory pathways and engaging stimulatory pathways on iNKT cell-mediated antitumor immunity. This review will highlight the incorporation of co-stimulatory molecules in antitumor dendritic cell vaccine strategies. The use of co-stimulatory intracellular signaling domains in chimeric antigen receptor-iNKT therapy will be assessed. Finally, we will explore the influence of innate-like receptors and modification of immunosuppressive cytokines (Signal 3) on cancer immunotherapy.

Keywords: invariant natural killer T, co-stimulation, cancer, immunotherapy, chimeric antigen receptor, checkpoint, natural killer T

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INVARIANT NATURAL KILLER T (iNKT) CELLS AND CO-STIMULATION

Natural killer T (NKT) cells exhibit similar traits to their namesakes. They express cell surface markers similar to natural killer (NK) cells such as CD161, CD56, and CD16. As a subset of T cells, NKT cells develop in the thymus and possess a T cell receptor (TCR) (1). Unlike conventional T cells, the NKT TCR recognizes lipid antigens in the context of the MHC class Ib molecule, CD1d (2). CD1d is expressed on many types of epithelial and endothelial cells and antigen-presenting cells (APCs), such

as B cells and dendritic cells (DCs) (3, 4). There are two subsets of NKT cells that are differentiated by their TCRs. Type I NKT (iNKT) cells have an invariant TCR whereas type II have diverse TCRs (5–8). The TCR of iNKT cells is composed of a single α chain (Vα14Jα18 in mice and Vα24Jα18 in humans) paired with β chains of limited diversity (Vβ8.2, 7 or 2 in mice and Vβ11 in humans) (9). While endogenous activating and suppressive antigens remain contested, iNKT cells respond to the exogenous glycolipid antigen, α-galactosylceramide (α-GalCer), while type II NKT cells do not (10). Upon antigenic stimulation, iNKT cells once again mirror NK and T cells. As innate-like lymphocytes, they respond to antigenic stimulation within a few hours by producing large amounts of Th1, Th2, and Th17 cytokines (11). This rapid response can be attributed to their storage of cytokine mRNA before activation (12). Like both NK cells and cytotoxic T lymphocytes (CTLs), iNKT cells can be directly cytotoxic (13). This combination of effector functions allows them to address stimuli directly and incite the immune system at large to mount an effective immune response against various assaults.

Naïve T cells conventionally require three signals for effective activation. Signal 1 is TCR: antigen–MHC engagement, Signal 2 is co-stimulatory molecules, and Signal 3 is cytokine stimulation. Signals 1 and 2 are regarded as mandatory for activation whereas Signal 3 is thought to direct the immune response (14, 15). This review focuses on the importance of Signal 2 for type I iNKT cell activation and function in antitumor immunity. Because the majority of the references presented herein refer to mouse iNKT cells, it will be explicitly stated when the data refer to human iNKT cells. Co-stimulation receptors can provide multiple types of signals, including positive/stimulatory and negative/inhibitory, and influence the type of response. There are two primary families of co-receptors: the CD28/B7 family and the TNF receptor superfamily (TNFRSF). The CD28/B7 family members are composed of immunoglobulin domains whereas the TNFRSF members have cysteine-rich extracellular domains (15). First, we will review the literature that addresses the effects of co-stimulatory receptors on iNKT cell biology, which are summarized in **Figure 1**.

CD28 is the canonical co-stimulatory molecule referred to during T cell activation. It is known to compete with CTLA-4, an inhibitory signaler, to interact with CD80/86 (also known as B7-1/2). In iNKT cells, CD28 is important for expansion during thymic development and after stimulation in the periphery (16–19). CD28 is constitutively expressed on iNKT cells, but its expression is downregulated during anergy or exhaustion (20). Blockade of CD80/86 suppresses production of both Th1 and Th2 cytokines and immune responses (21). In an experimental autoimmune encephalomyelitis model, blockade of CD86 during α-GalCer activation resulted in a Th2 bias (22). The literature available clearly shows a stimulatory role for CD28 signaling in iNKT cells.

Inducible T cell costimulator (ICOS or CD278), another member of the CD28 family, is constitutively expressed on iNKT cells, and its expression is increased after activation (23). The ICOS:ICOSL pathway is important for homeostatic proliferation and Th1 and Th2 immune responses (23, 24). During stimulation by marginal zone B cells, ICOS:ICOSL interactions are necessary to produce Th2 cytokines (25). While ICOS engagement is distinctly positive, its influence over the immune response is uncertain.

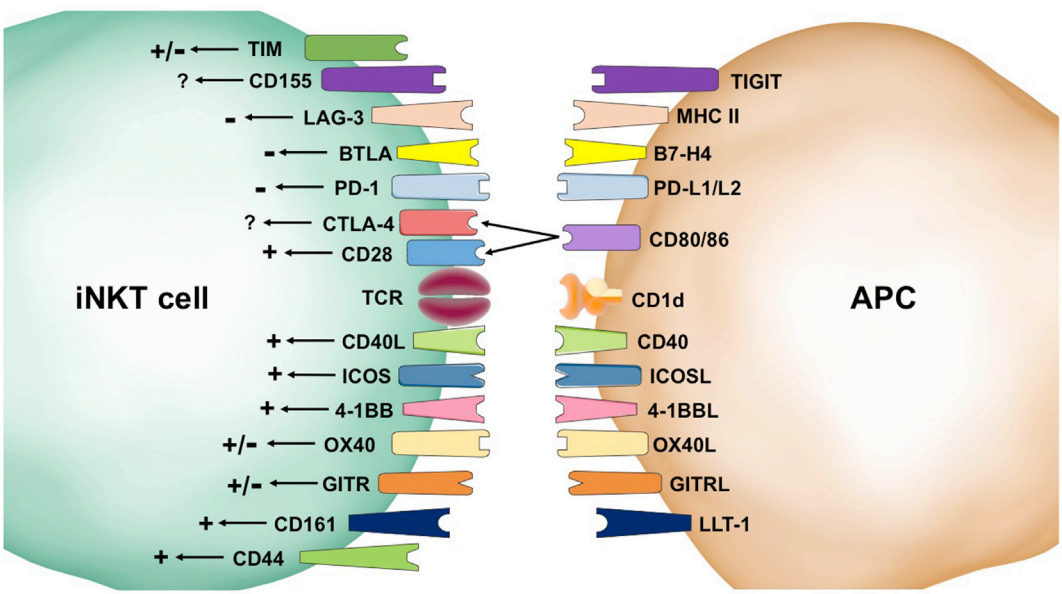

FIGURE 1 | Second signals provided by co-receptors influence invariant natural killer T (iNKT) cell biology. Stimulatory (+) pathways result in homeostatic survival and enhanced activation, cytokine production, expansion, and cytotoxicity. These positive signals come from CD44, CD161, OX40, 4-1BB, ICOS, CD40L, and CD28. Inhibitory (–) signaling can result in cell death and inhibition of iNKT cell activation. Receptors that have shown negative signaling effects include programmed death (PD)-1, B and T lymphocyte attenuator (BTLA), and lymphocyte activation gene (LAG-3). The impacts of co-receptors, such as T cell immunoglobulin mucin (TIM), CD155, CTLA-4, OX40, and GITR, are not settled in the literature and are indicated by a +/– symbol. Some co-receptors, such as CD40L, selectively skew the immune response.

CD40L, a member of the TNFRSF that interacts with CD40 on APCs, has both positive co-stimulatory abilities as well as influence over the type of immune response generated. iNKT cells have been shown to provide cognate help to B cells that is independent of CD40:CD40L interactions (26) and non-cognate help in a method dependent on CD40:CD40L interactions (27). CD40 is upregulated after activation and necessary for production of a Th1 inflammatory response in intracellular infections (28) and antitumor immunity (29, 30). Th2 cytokine production decreases in NKT cells activated by APCs that had been treated with an agonistic anti-CD40 antibody (22). Blockade of CD40L results in decreased Th1 responses (21) and increased Th2 responses (31) making this pathway a likely target for enhancing transplant tolerance. CD40L signaling is distinctly positive and demonstrates importance for Th1 immune responses.

4-1BB (CD137), another member of the TNFRSF, is expressed on iNKT cells after activation. 4-1BB stimulation during or after activation results in increased cytokine production (32) and enhanced iNKT cell proliferation in mice and humans (33). Under resting conditions, 4-1BB:4-1BBL interactions between iNKT cells and monocytes in the lungs provide homeostatic survival signals for both cell types in both human and mouse models (33). Blockade of 4-1BB results in decreased immune responses, including Th1 and Th2 responses (34). 4-1BB signaling is an important stimulatory pathway for iNKT cell function.

Although CD44 is expressed on all T cells, its function differs in iNKT cells. Unlike in conventional T cells, iNKT cell CD44 can bind hyaluronic acid and induce activation. Crosslinking CD44 results in iNKT cell activation and increased cytokine production as well as protection from activation-induced cell death (35). Stimulation of iNKT cells with artificial antigen-presenting cells that only possess CD1d and anti-CD44 on their surface results in potent iNKT cell cytokine production (36). Human iNKT cells also express CD161, which is a C-type lectin receptor that interacts with lectin-like transcript-1. While CD161 crosslinking by itself does not induce activation, CD161 blockade decreases cytokine production and proliferation. iNKT cell mediated cytotoxicity is independent of CD161 (37). CD44 and CD161 exert a positive influence over iNKT cell activation.

Glucocorticoid-induced TNFR-related (GITR or CD357), a TNFRSF member, is constitutively expressed on iNKT cells and is upregulated after activation. The effects of GITR signaling on iNKT cells is somewhat contested. A paper by Chen et al. shows that GITR has a co-inhibitory role in iNKT cell activation as demonstrated by decreased proliferation and cytokine production in WT mice compared with GITR-KO mice (38). However, GITR:GITRL interactions are necessary for Th1 and Th17 cytokine production by iNKT cells after stimulation by conventional DCs (25) and GITR stimulation using an agonistic monoclonal antibody enhances overall cytokine production by iNKT hybridomas *in vitro* (39). Further studies are needed to address these disparities found in the literature to determine the effects of GITR on iNKT cell activation.

OX40 (CD134), a TNFRSF member, is expressed on iNKT cells and interacts with OX40L on APCs but the outcome of this interaction is debated. In the pancreas, the OX40:OX40L interaction between iNKT cells and plasmacytoid DCs during LCMV infection, tested using neutralizing antibodies, induces IFN-α/β production by the DCs and dampens the adaptive immune response to avoid tissue damage (40). By contrast, stimulation of OX40 with an agonistic monoclonal antibody on liver-resident iNKT cells results in caspase-1-dependent pyroptosis and release of inflammatory cytokines that cause tissue injury (41). In a tumor model, iNKT cell expansion and IFN-γ production are enhanced by upregulation of OX40L on DCs (42). OX40 is stereotypically thought of as a stimulatory co-receptor, but its role in iNKT cell responses is unclear and may be tissue specific.

CD155, a member of the immunoglobulin superfamily, is expressed on iNKT cells and interacts with CD226, CD96, and TIGIT. CD155 blockade or knockout increases NKT1 cells and decreases both NKT2 and NKT17 cell generation during development in Balb/c and C57BL/6 mice (43). Its effect on iNKT cell activation and cytokine production has not been published.

There are three different T cell immunoglobulin mucin (TIM) receptors expressed by iNKT cells (TIM-1, 3, and 4), and they have differing effects on iNKT cell activation. TIM-1 engagement on iNKT cells by monoclonal antibodies suppresses Th1 responses but enhances Th2 responses in both *in vitro* and *in vivo* models (44). Conversely, TIM-1 engagement by phosphatidylserine—a marker of apoptosis—enhances iNKT cell activation, proliferation, and cytokine production (45). In a nonalcoholic fatty liver disease model, TIM-3 is shown to control liver-resident iNKT cell homeostasis with direct TIM-3 signaling inducing apoptosis and indirect signaling from IL-15, produced by TIM-3 stimulated Kupffer cells, promoting iNKT cell proliferation (46). TIM-3 is classically an inhibitory receptor and is upregulated on human iNKT cells in chronic viral infections (47). TIM-4 is expressed but does not have an effect on iNKT cell development or function (48). The effects of TIM-1 and TIM-3 need to be further assessed in iNKT cell biology.

B and T lymphocyte attenuator (BTLA), a member of the CD28 family that interacts with B7-H4, is an inhibitory co-receptor that is expressed on iNKT cells. Thus far, it has only been examined in ConA-induced hepatitis with both studies demonstrating that BTLA knockout increases iNKT cell cytokine production and exacerbates hepatitis (49, 50), indicating an inhibitory role of BTLA in iNKT cell function. Although these results align with the role of BTLA in conventional T cells, more research is needed to assess the role of BTLA in other immune models.

Lymphocyte activation gene (LAG)-3, a member of the immunoglobulin superfamily that interacts with MHC class II, is induced on iNKT cells after activation. It has an inhibitory affect with overexpression resulting in inhibition of proliferation due to cell cycle arrest (51). LAG-3 is upregulated on human iNKT cells in chronic viral infection and is associated with decreased cytokine production (52). These inhibitory effects are consistent with the effects of LAG-3 in conventional T cells.

Programmed death (PD)-1, a member of the CD28 family, is constitutively expressed on iNKT cells at low levels, rapidly upregulated after activation, and thought to play a role in iNKT cell anergy (53–55). Blockade of PD-1 signaling during iNKT cell activation enhances Th1 immunity (56). PD-1 interacts with both PD-L1 and PD-L2, with PD-L1 also being expressed on iNKT cells. Blockade of PD-L1 increases IFN-γ production in

mice and humans whereas blockade of PD-L2 increases IL-4 and IL-13 production (57–59). In chronic viral infections and tumor models, human iNKT cells are dysfunctional—failing to proliferate or produce cytokines after activation—and have upregulated PD-1 (47, 60). Blockade of PD-1 signaling after iNKT cell activation and upregulation of PD-1 is debated with one paper showing ability to rescue anergy (55) and two others showing inability to rescue anergy (54, 61). iNKT cells require CD28 signaling to produce cytokines in the presence of PD-1:PD-L1 signaling (53). The role of the PD-1 pathway in iNKT cell function is distinctly inhibitory.

Due to the innate-like qualities of iNKT cells, it was at one time contested how co-receptors affected iNKT cell activation. The research summarized above demonstrates that iNKT cells are sensitive to both positive and negative signaling. Indeed, the context of these signals can have dramatic effects on the type of immune response generated. The next sections will explore the effects of co-stimulatory molecules on the ability of iNKT cells to mount an effective antitumor immune response.

iNKT CELLS IN CANCER

The antitumor capabilities of iNKT cells were demonstrated soon after their discovery in 1987 (1). In fact, the 1993 discovery of their exogenous activating ligand, α-GalCer, was tested using a B16 melanoma model (62). Multiple papers noted the potent bioactivity of α-GalCer—including inducing lymphocyte proliferation, NK cell activation, fewer metastases, and prolonged lifespan of tumor-bearing mice. Increased survival was correlated with IL-2 and IFN-γ production, APC activation, NK cell activation, and tumor-specific CTL production (63–66). However, it was not until 1997 that the proliferative effects of α-GalCer were shown to be dependent on CD1d, Vβ8, and co-stimulatory molecules (CD40/CD40L, B7/CTLA-4/CD28) (10), linking iNKT cells to α-GalCer. A key piece of evidence was provided when surface plasmon resonance was used to prove that glycolipids such as α- and β-GalCer can bind both mouse CD1 and human CD1d (67). In 2000, the importance of iNKT cells in tumor immunosurveillance and initiation of the antitumor immune response was demonstrated using a carcinogen-induced tumor model in mice that had various lymphocyte subsets knocked out by gene targeting or depletion (68).

Both CD28 and CD40 are needed to spur an effective antitumor immune response after α-GalCer injection (29, 30, 69). α-GalCer presentation to iNKT cells results in the production of IFN-γ and TNF-α. The CD40:CD40L interaction induces production of IL-12 by the DCs and upregulation of the IL-12Rα on iNKT cells. Activated iNKT cells can directly kill tumor cells *via* perforin/granzyme and Fas:FasL interactions (70, 71). Coadministration of α-GalCer and IL-12 works synergistically for iNKT activation, cytokine production, and cytotoxicity (29). IFN-γ release by iNKT cells activates NK cells to produce IFN-γ and directly kill tumor cells (72). IFN-γ and TNF-α upregulate CD80/86 on DCs whereas IL-12 induces a Th1/CTL immune response—promoting effective antitumor T cell immunity (73). Thus, malignant cells are killed directly by iNKT cells as well as indirectly *via* the activation of cytotoxic NK and CD8+ T cells. The potent effects of iNKT cells in antitumor immunity are summarized in **Figure 2**.

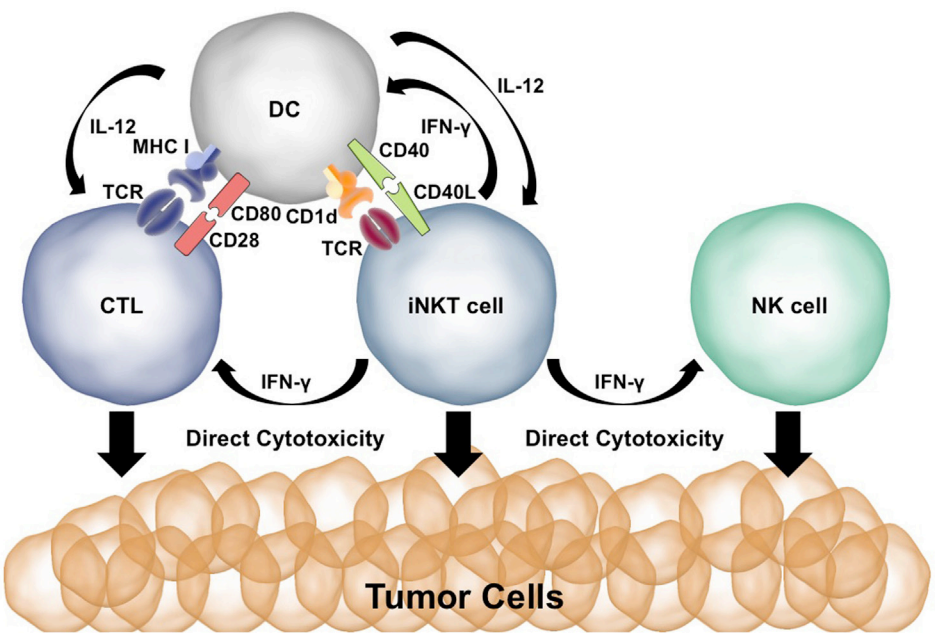

FIGURE 2 | Co-stimulation plays a key role in the ability of invariant natural killer T (iNKT) cells to initiate antitumor immune responses. Presentation of α-GalCer to iNKT cells results in IFN-γ production. IFN-γ stimulates dendritic cells (DCs) to upregulate CD80/86 and activates natural killer (NK) cells. CD40L:CD40 interactions between the DC and iNKT cell activate the DC and result in IL-12 production. Cytotoxic T lymphocytes (CTLs) are activated by MHC:T cell receptor (TCR) interactions, CD80:CD28 co-stimulation, and IFN-γ and IL-12 signaling. iNKT cells, NK cells, and CTLs are able to directly kill tumor cells using perforin/granzyme and Fas:FasL.

iNKT CELLS AND MONOCLONAL ANTIBODY THERAPIES

Progression of cancer to the stage of diagnosis indicates that the cancer has already undergone extensive immunoediting and has proceeded to the immune escape phase of immunosurveillance. In other words, cancers evolve to suppress and subvert the immune response (74). As reviewed by Joyce et al., the tumor microenvironment (TME) employs hypoxia, reactive nitrogen species, immunosuppressive chemokines and cytokines, dense extracellular matrix, and Th1-suppressive immune cells, such as regulatory T cells, myeloid-derived suppressor cells (MDSC), and tumor-associated macrophages, to suppress antitumor immunity (75). In fact, iNKT cells are frequently suppressed in cancer patients—displaying decreased cytokine production, cytolytic activity, and proliferation (76). Inhibitory co-receptor molecules are meant to stop aberrant immune responses such as autoimmunity (77). This system is termed a "checkpoint," but tumors have hijacked expression of molecules such as PD-L1 to suppress and evade the antitumor immune response in humans and mice (78). A key branch of cancer immunotherapy research is on the use of monoclonal antibodies that block inhibitory co-receptor pathways (checkpoint inhibitors) and antibodies that engage co-stimulatory pathways to enhance antitumor immunity (79–85).

Two checkpoint inhibitor pathways have been extensively explored: CTLA-4 and PD-1. CTLA-4 is an inhibitory co-receptor that competes with CD28 for interaction with CD80/86. PD-1 and PD-L1 are both expressed on iNKT cells, but PD-1 engagement with PD-L1/L2 is inhibitory to iNKT cell function. Despite the popularity of CTLA-4 checkpoint inhibitors, research on the effects of α-CTLA-4 on iNKT cell activation is extremely limited. A set of papers from Pilones et al. came out examining the effects of iNKT cells on an immunotherapy regimen of radiation treatment and CTLA-4 blockade in a BALB/c breast cancer model (86, 87). This immunotherapy regimen is more successful in the absence of iNKT cells due to an increased influx of cross-presenting DCs in the tumor draining lymph node, but it is important to note that iNKT cell activation *via* α-GalCer administration is not included in the regimen. There has been slightly more research into PD-1/PD-L1 checkpoint inhibitors in tumor models. Checkpoint blockade of PD-1 or PD-L1, but not PD-L2, at the time of iNKT cell activation (by α-GalCer) increases cytokine production and cytotoxicity *in vitro* and *in vivo*, and decreases iNKT cell anergy, B16 melanoma tumor size, and metastatic lesions (54–56). It is still contested whether PD-1 blockade post-α-GalCer activation can rescue iNKT cells from anergy.

While checkpoint inhibitors have side effects such as autoimmunity, agonistic monoclonal antibodies against stimulatory co-receptors can cause rampant, destructive immune activation—making researchers more cautious with their use. Two such agonists, against members of the TNFRSF, have been explored in conjunction with iNKT cell immunotherapy: 4-1BB and GITR. In a mouse model of B cell lymphoma, treatment with α-GalCer-loaded, irradiated tumor cells and α-4-1BB increases overall survival and tumor-free survival dependent on IFN-γ and KLRG1+ CTLs (88). This immunotherapy also generates a memory immune response. Another group designed a therapy called NKTMab that includes α-4-1BB, α-DR5, and α-GalCer or α-C-GalCer (a modified version of α-GalCer known to skew the iNKT cell response to Th1). This combination immunotherapy causes effective rejection of 4T1 breast cancer tumors in Balb/c mice that is dependent on CD4+ T cells, CTLs, iNKT cells, and IFN-γ, and they found that α-C-GalCer was more effective in a wider range of concentrations (89). The role of GITR in iNKT cell mediated antitumor immune responses is not fully elucidated. In one paper using a C57Bl/6 T cell lymphoma model, iNKT cells in GITR-KO mice exhibit increased survival compared with WT mice (38). In a B16 melanoma model, mice treated with an agonistic mAb against GITR (DTA-1) exhibit increased survival that was dependent on NK1.1+ cells and T cells (90).

Checkpoint inhibitors have excelled in the clinic, but research into their effects on iNKT cells is lacking. Treatment regimens that combine iNKT cell activation and checkpoint blockade or agonistic antibody treatments hold promise for the future.

MODIFIED APCs

Antibody treatments can be harsh due to off-target effects. One method of co-stimulatory delivery is DC vaccines. DC vaccines have been researched and improved upon for decades, with the first cancer vaccine approved by the FDA in 2010. DCs provide co-stimulatory molecules in a more natural context—thus limiting off-target effects. Loading DCs with α-GalCer before vaccination enhances iNKT cell IFN-γ production and decreases tumor metastasis in B16 melanoma and Lewis lung carcinoma models (91, 92). In cancer patients, administration of α-GalCer-loaded DCs results in sustained iNKT cell expansion and enhanced antigen-specific T cell responses (93). Coadministration of irradiated tumor cells with α-GalCer or injection of α-GalCer-loaded, irradiated tumor cells enhances iNKT cell-mediated antitumor immune response *via* DC cross-presentation in plasmacytoma, lymphoma, and B16 melanoma models (94, 95). One vaccination strategy injects α-GalCer-loaded MDSCs—immunosuppressive immune cells created by the tumor—and demonstrates enhanced survival dependent on CTLs, NK cells, and iNKT cells. This enhanced immunity is due to increased positive co-stimulatory molecule (CD40, CD80/86) expression on the MDSC cell surface after iNKT cell interaction (96). Pretreatment of DCs with the Th1, pro-inflammatory cytokine TNF-α enhances positive co-stimulatory molecule expression such as CD80, CD86, 4-1BBL, and OX40L. OX40L expression drastically enhances antitumor immunity by enhancing iNKT cell activation, cytokine production, expansion, and stimulation of antitumor CTL responses (42). These papers demonstrate the impact of APC modification and how this influences iNKT cell mediated antitumor immunity.

CHIMERIC ANTIGEN RECEPTORS (CARs) IN iNKT CELLS

In addition to checkpoint inhibition and modified APCs, another unique approach that takes advantage of the antitumor capabilities of iNKT cells involves the use of CARs. A CAR is an artificially engineered receptor containing an extracellular

antigen recognition domain attached to an intracellular T cell activation domain. Traditionally, in cancer immunotherapy, CARs are placed in conventional T cells and contain an extracellular domain that recognizes a tumor antigen along with intracellular CD3ζ and co-stimulatory domains that provide the appropriate signals needed to fully activate the T cell against the tumor. First-generation CARs were composed of an extracellular single-chain variable fragment (scFv) and a CD3ζ, which meant they required endogenous co-stimulation for activation. Second and third generation CARs included one or two co-stimulatory signaling domains, respectively, in addition to the CD3ζ chain, which eliminated the need for endogenous co-stimulation (97).

However, there are several issues with using conventional T cells in CAR based cancer immunotherapy that may be overcome by expressing CARs in iNKT cells. One major complication is graft-versus-host disease (GVHD). Conventional TCRs are restricted to the polymorphic MHC (98), which can result in an allogenic anti-host response by donor T cells. By contrast, iNKT cells are restricted to the monomorphic CD1d molecule. Since CD1d is monomorphic, meaning it is conserved across individuals, iNKT cells can be adoptively transferred without concern for HLA matching (3, 4, 10). Another advantage iNKT cells have over conventional T cells is their ability to regulate off tumor effects. Several studies have reported that GVHD is exacerbated in CD1d−/− or Jα18−/− mice and that stimulation of iNKT cells can increase antileukemia responses while simultaneously mitigating the severity of GVHD (99, 100).

Human and mouse iNKT cells have the unique ability to secrete both Th1 and Th2 type cytokines, which may partly explain how they can simultaneously regulate GVHD and promote antitumor immunity (101, 102). Lee et al. showed that in humans, CD4+ iNKT cells were able to secrete the Th2 cytokines IL-4 and IL-13 whereas DN iNKT cells were able to secrete Th1 cytokines (103). They proposed that this may explain the ability of iNKT cells to facilitate both Th1 and Th2 type responses. A study later conducted by Tian et al. showed that stimulation with the combination of CD1d, CD86, 4-1BBL, and OX40L resulted in the greatest production of Th1 type cytokines by human CD19-specific CAR-iNKT cells containing a 4-1BB co-stimulatory domain (104). A future generation CAR containing the signaling domains of all these co-stimulatory molecules could be more effective at generating antitumor Th1 type responses.

Despite all the promising reasons to the use CAR-iNKT cells in cancer immunotherapy, there have been relatively few studies completed (105). However, the few studies that do exist have yielded promising results. In 2014, Heczey et al. generated a human anti-GD2 CAR-iNKT cell to target GD2+ neuroblastoma and found that these cells were able to localize to the tumor and initiate antitumor responses to neuroblastoma *in vivo* with no indication of the development of GVHD (106). Two years later, the same group generated anti-CD19 CAR-iNKT cells (104). CD19 is expressed on B cells and is being actively explored as a therapeutic target to treat various types of lymphoma derived from B cells. This study showed that anti-CD19 CAR-iNKT cells were able to selectively target CD19+ cells both *in vitro* and *in vivo*. In addition, they identified CD62L+ as a marker of the most effective CAR-iNKT cells due to greater proliferative potential and enhanced tumor reduction when compared with their CD62L− counterparts (104).

CAR-T cells are emerging as a powerful tool in the field of cancer immunotherapy. Given the current evidence suggesting that using iNKT cells may be able to overcome some of the problems associated with CAR-T cell therapy (highlighted in **Figure 3**), further study of CAR-iNKT cells, especially revolving around the use of various co-stimulatory domains to take advantage of their poly-functional cytokine secretion profiles, should prove rewarding. In addition, there have been other recent advances in the ability to isolate and expand human and mouse iNKT cells *ex vivo* for adoptive transfer that is beyond the scope of this review but will further facilitate the therapeutic use of these cells (36, 107, 108).

FUTURE DIRECTIONS

The development of CARs has followed a predictable pattern of continuously trying to add more and more co-stimulatory domains to the receptor to enhance activation. To make these receptors as efficient as possible, it is worth exploring new possible co-stimulatory domains not traditionally included in CARs. A recent study conducted by Baglaenko et al. found a new role for Ly108 in iNKT cells (109). Ly108 has been previously established to play a role in iNKT cell development; however, this recent study found that peripheral trans-Ly108 interactions between APCs and iNKT cells enhanced the ability of iNKT cells to secrete cytokines and that loss of Ly108 expression resulted in defective iNKT cell homeostasis in mice. They went on to find that Ly108 activation in human iNKT cells led to increased secretion of the Th1 cytokines IFN-γ and TNF-α whereas levels of Th2 or regulatory cytokines, including IL-4 or IL-10, were unchanged. In addition to Ly108, there are several other innate-like co-stimulatory molecules such as TLR3, TLR9, and NKG2D that are known to be expressed on iNKT cells and be involved in immune surveillance. TLR3 and TLR9 agonists have been shown to enhance iNKT cell's ability to mature DCs (110), whereas tumor cells are thought to shed NKG2D ligands in exosomes to block the receptor from recognizing the tumor cell (111). The unique signaling cascades and the involvement of adaptor proteins could complicate the use of these signaling domains in CARs. However, they enhance iNKT cell-mediated antitumor immunity, thus their potential may outweigh the costs.

We have primarily focused on co-stimulatory domains; however, it is also important to note the inhibitory domains and how they might be taken advantage of to enhance iNKT cell mediated antitumor immunity. Tumor cells will upregulate inhibitory molecules in response to inflammatory cytokines, which serve to inhibit any local antitumor immune response. One of the most well-known co-inhibitory molecules upregulated by many tumor types is PD-L1, which binds to PD-1 on activated immune cells to inhibit their function. Cherkassky et al. found that human CAR-T cells became exhausted due to tumor cell expression of PD-L1. They also found that CAR-T cell function could be rescued by anti-PD-1 therapy or by overexpression of a dominant negative PD-1 receptor. The dominant negative receptor consisted

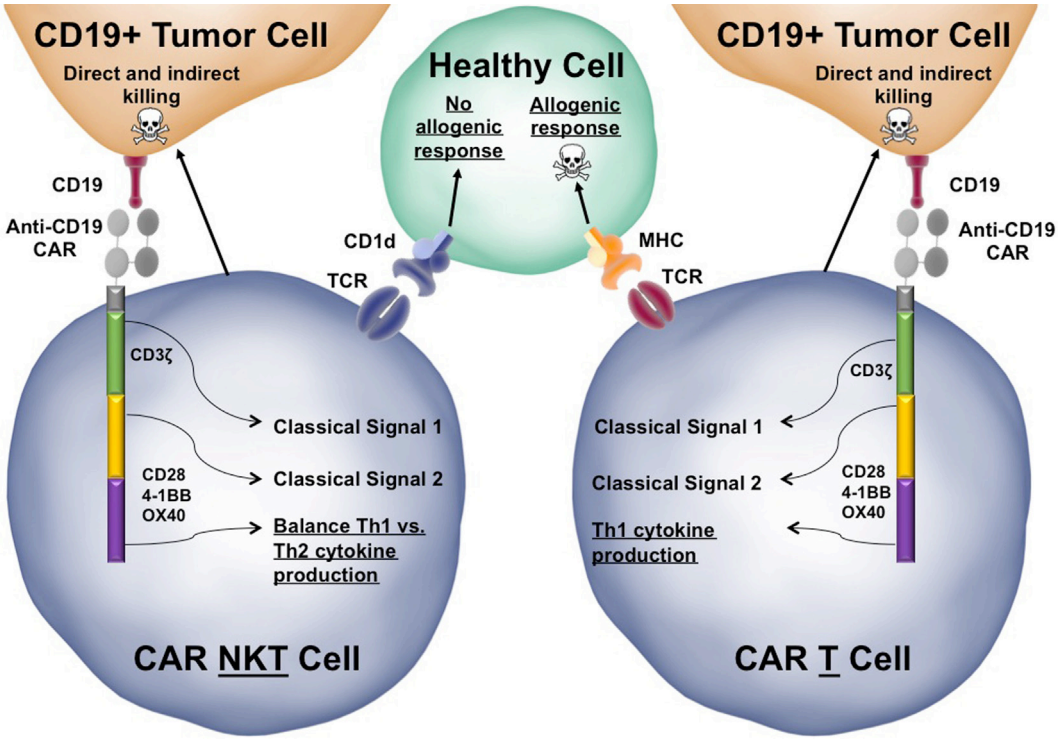

FIGURE 3 | Functional advantages of chimeric antigen receptor (CAR)-invariant natural killer T (iNKT) cells over conventional CAR-T cells. The structure of a third-generation anti-CD19 CAR-iNKT cell is depicted interacting with both a target CD19+ tumor cells and a healthy bystander cell. Third generation CARs have three endodomains that can be modified to tune the response of the iNKT cell when activated. Similar to conventional iNKT cells, CAR-iNKT cells would be able to directly kill tumor cells using Fas:FasL interactions and secretion of perforin/granzyme. CAR-iNKT cells also secrete stimulatory cytokines, such as IFN-γ, that can license dendritic cells as well as indirectly activate cytotoxic T lymphocytes and natural killer cells to kill tumor cells (not depicted). The CAR-iNKT cells is juxtaposed to a similar CAR-T cell to highlight a few key differences between CARs in iNKT cells and CARs in conventional T cells (differences are underlined). Skull and crossbones indicate cell killing.

of the PD-1 extracellular ligand binding domain without any intracellular signaling domain (112).

Along the same lines as co-inhibitory molecules, blocking inhibitory cytokines commonly present in the TME, such as TGF-β, is being actively explored and has shown some promise (113). Terabe et al. found that CD11b+ or Gr1+ myeloid cells secreted TGF-β in a CD1d and IL-13 dependent manner and that removal of these cells prevented tumor recurrence, suggesting iNKT cells were actually playing a role in promoting an immunosuppressive environment (114). Directing the CAR against TGF-β could augment the ability of CAR-iNKT cells by simultaneously enhancing antitumor immunity while inhibiting immunosuppressive functions. Considering that there are already multiple known co-inhibitory receptors and cytokines, the possibility of including dominant negative receptors for each or even attaching CARs to co-inhibitory ligand binding domains provides new avenues of exploration to enhance iNKT cell-mediated antitumor immunity.

AUTHOR CONTRIBUTIONS

SS and ML equally contributed to the literature search and writing of this study. TW provided guidance for and editing of the manuscript. All the authors approved the final manuscript.

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REFERENCES

1. Fowlkes BJ, Kruisbeek AM, Ton-That H, Weston MA, Coligan JE, Schwartz RH, et al. A novel population of T-cell receptor αβ-bearing thymocytes which predominantly expresses a single V β gene family. *Nature* (1987) 329:251–4. doi:10.1038/329251a0
2. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science* (1995) 268:863–5. doi:10.1126/science.7538697
3. Canchis PW, Bhan AK, Landau SB, Yang L, Balk SP, Blumberg RS. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. *Immunology* (1993) 80:561–5.

4. Blumberg RS, Terhorst COX, Bleicher P, Mcdermott FV, Allan CH, Landau SB, et al. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. *J Immunol* (1991) 147:2518–24.
5. Lantz O, Bendelac A. An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med* (1994) 180:1097–106. doi:10.1084/jem.180.3.1097
6. Behar SM, Podrebarac TA, Roy CJ, Wang CR, Brenner MB. Diverse TCRs recognize murine CD1. *J Immunol* (1999) 162:161–7.
7. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- α/β T cells demonstrates preferential use of several V β genes and an invariant TCR α chain. *J Exp Med* (1993) 178:1–16. doi:10.1084/jem.178.1.1
8. Dellabona P, Padovan E, Casorati G, Brockhaus M, Lanzavecchia A. An invariant Vα24-JαQ/Vβ11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med* (1994) 180:1171–6. doi:10.1084/jem.180.3.1171
9. Koseki H, Imai K, Nakayama F, Sado T, Moriwaki K, Taniguchi M. Homogenous junctional sequence of the V14+ T-cell antigen receptor α chain expanded in unprimed mice. *Proc Natl Acad Sci U S A* (1990) 87:5248–52. doi:10.1073/pnas.87.14.5248
10. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* (1997) 278:1626–9. doi:10.1126/science.278.5343.1626
11. Niemeyer M, Darmoise A, Mollenkopf H-J, Hahnke K, Hurwitz R, Besra GS, et al. Natural killer T-cell characterization through gene expression profiling: an account of versatility bridging T helper type 1 (Th1), Th2 and Th17 immune responses. *Immunology* (2007) 123:45–56. doi:10.1111/j.1365-2567.2007.02701.x
12. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang Z-E, Gapin L, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* (2003) 198:1069–76. doi:10.1084/jem.20030630
13. Arase H, Arase N, Kobayashi Y, Nishimura Y, Yonehara S, Onoe K. Cytotoxicity of fresh NKI.1+ T cell receptor α/β+ thymocytes against a CD4+8+ thymocyte population associated with intact fas antigen expression on the target. *J Exp Med* (1994) 180:423–32. doi:10.1084/jem.180.2.423
14. Gutcher I, Becher B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest* (2007) 117:1119–27. doi:10.1172/JCI31720
15. Beier KC, Kallinich T, Hamelmann E. Master switches of T-cell activation and differentiation. *Eur Respir J* (2007) 29:804–12. doi:10.1183/09031936.00094506
16. Zheng X, Zhang H, Yin L, Wang CR, Liu Y, Zheng P. Modulation of NKT cell development by B7-CD28 interaction: an expanding horizon for costimulation. *PLoS One* (2008) 3:e2703. doi:10.1371/journal.pone.0002703
17. Williams JA, Lumsden JM, Yu X, Feigenbaum L, Zhang J, Steinberg SM, et al. Regulation of thymic NKT cell development by the B7-CD28 costimulatory pathway. *J Immunol* (2008) 181:907–17. doi:10.1055/s-0029-1237430. Imprinting
18. Uldrich AP, Crowe NY, Kyparissoudis K, Pellicci DG, Zhan Y, Lew AM, et al. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J Immunol* (2005) 175:3092–101. doi:10.4049/jimmunol.175.5.3092
19. Chung Y, Nurieva R, Esashi E, Wang Y-H, Zhou D, Gapin L, et al. A critical role of costimulation during intrathymic development of invariant NK T cells. *J Immunol* (2008) 180:2276–83. doi:10.4049/jimmunol.180.4.2276
20. Wang XF, Lei Y, Chen M, Chen CB, Ren H, Shi TD. PD-1/PDL1 and CD28/CD80 pathways modulate natural killer T cell function to inhibit hepatitis B virus replication. *J Viral Hepat* (2013) 20:27–39. doi:10.1111/jvh.12061
21. Hayakawa Y, Takeda K, Yagita H, Van Kaer L, Saiki I, Okumura K. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J Immunol* (2001) 166:6012–8. doi:10.4049/jimmunol.166.10.6012
22. Pál E, Tabira T, Kawano T, Taniguchi M, Miyake S, Yamamura T. Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of Vα14 NK T cells 1. *J Immunol* (2001) 166:662–8. doi:10.4049/jimmunol.166.1.662
23. Kaneda H, Takeda K, Ota T, Kaduka Y, Akiba H, Ikarashi Y, et al. ICOS costimulates invariant NKT cell activation. *Biochem Biophys Res Commun* (2005) 327:201–7. doi:10.1016/j.bbrc.2004.12.004
24. Akbari O, Stock P, Meyer EH, Freeman GJ, Sharpe AH, Umetsu DT, et al. ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. *J Immunol* (2008) 180:5448–56. doi:10.4049/jimmunol.180.8.5448
25. Zietara N, Łyszkiewicz M, Krueger A, Weiss S. ICOS-dependent stimulation of NKT cells by marginal zone B cells. *Eur J Immunol* (2011) 41:3125–34. doi:10.1002/eji.201041092
26. Shah HB, Joshi SK, Lang ML. CD40L-null NKT cells provide B cell help for specific antibody responses. *Vaccine* (2011) 29:9132–6. doi:10.1016/j.vaccine.2011.09.060
27. Tonti E, Galli G, Malzone C, Abrignani S, Casorati G, Dellabona P. NKT-cell help to B lymphocytes can occur independently of cognate interaction. *Blood* (2009) 113:370–6. doi:10.1182/blood-2008-06-166249
28. Joyee AG, Qiu H, Fan Y, Wang S, Yang X. Natural killer T cells are critical for dendritic cells to induce immunity in chlamydial pneumonia. *Am J Respir Crit Care Med* (2008) 178:745–56. doi:10.1164/rccm.200804-517OC
29. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, et al. The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* (1999) 189:1121–7. doi:10.1084/jem.189.7.1121
30. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* (2004) 199:1607–18. doi:10.1084/jem.20040317
31. Hirai T, Ishii Y, Ikemiyagi M, Fukuda E, Omoto K, Namiki M, et al. A novel approach inducing transplant tolerance by activated invariant natural killer T cells with costimulatory blockade. *Am J Transplant* (2014) 14:554–67. doi:10.1111/ajt.12606
32. Kim D-H, Chang W-S, Lee Y-S, Lee K-A, Kim Y-K, Kwon BS, et al. 4-1BB engagement costimulates NKT cell activation and exacerbates NKT cell ligand-induced airway hyperresponsiveness and inflammation. *J Immunol* (2008) 180:2062–8. doi:10.4049/jimmunol.180.4.2062
33. Cole SL, Benam KH, McMichael AJ, Ho L-P. Involvement of the 4-1BB/4-1BBL pathway in control of monocyte numbers by invariant NKT cells. *J Immunol* (2014) 192:3898–907. doi:10.4049/jimmunol.1302385
34. Vinay DS, Choi BK, Bae JS, Kim WY, Gebhardt BM, Kwon BS. CD137-deficient mice have reduced NK/NKT cell numbers and function, are resistant to lipopolysaccharide-induced shock syndromes, and have lower IL-4 responses. *J Immunol* (2004) 173:4218–29. doi:10.4049/jimmunol.173.6.4218
35. Larkin J, Renukaradhya GJ, Sriram V, Du W, Gervay-Hague J, Brutkiewicz RR. CD44 differentially activates mouse NK T cells and conventional T cells. *J Immunol* (2006) 177:268–79. doi:10.4049/jimmunol.177.1.268
36. Webb TJ, Bieler JG, Schneck JP, Oelke M. Ex vivo induction and expansion of natural killer T cells by CD1d1-Ig coated artificial antigen presenting cells. *J Immunol Methods* (2009) 346:38–44. doi:10.1016/j.jim.2009.05.003
37. Exley M, Pocelli S, Furman M, Garcia J, Balk S. CD161 (NKR-P1A) costimulation of CD1d-dependent activation of human T cells expressing invariant Vα24JαQ T cell receptor α chains. *J Exp Med* (1998) 188:867–76. doi:10.1084/jem.188.5.867
38. Chen S, Ndhlovu LC, Takahashi T, Takeda K, Ikarashi Y, Kikuchi T, et al. Co-inhibitory roles for glucocorticoid-induced TNF receptor in CD1d-dependent natural killer T cells. *Eur J Immunol* (2008) 38:2229–40. doi:10.1002/eji.200838167
39. Kim HJ, Kim HY, Kim BK, Kim S, Chung DH. Engagement of glucocorticoid-induced TNF receptor costimulates NKT cell activation in vitro and in vivo. *J Immunol* (2006) 176:3507–15. doi:10.4049/jimmunol.176.6.3507
40. Diana J, Griseri T, Lagaye S, Beaudoin L, Autrusseau E, Gautron AS, et al. NKT cell-plasmacytoid dendritic cell cooperation via OX40 controls viral infection in a tissue-specific manner. *Immunity* (2009) 30:289–99. doi:10.1016/j.immuni.2008.12.017
41. Lan P, Fan Y, Zhao Y, Lou X, Monsour HP, Zhang X, et al. TNF superfamily receptor OX40 triggers invariant NKT cell pyroptosis and liver injury. *J Clin Invest* (2017) 127:2222–34. doi:10.1172/JCI91075
42. Zaini J, Andarini S, Tahara M, Saijo Y, Ishii N, Kawakami K, et al. OX40 ligand expressed by DCs costimulates NKT and CD4+ Th cell antitumor immunity in mice. *J Clin Invest* (2007) 117:3330–8. doi:10.1172/JCI32693

43. Georgiev H, Ravens I, Shibuya A, Förster R, Bernhardt G. CD155/CD226-interaction impacts on the generation of innate CD8+ thymocytes by regulating iNKT-cell differentiation. *Eur J Immunol* (2016) 46:993–1003. doi:10.1002/eji.201546073
44. Kim HS, Kim HS, Lee CW, Chung DH. T cell Ig domain and mucin domain 1 engagement on invariant NKT cells in the presence of TCR stimulation enhances IL-4 production but inhibits IFN-γ production. *J Immunol* (2010) 184:4095–106. doi:10.4049/jimmunol.0901991
45. Lee H-H, Meyer EH, Goya S, Pichavant M, Kim HY, Bu X, et al. Apoptotic cells activate NKT cells through T cell Ig-like mucin-like-1 resulting in airway hyperreactivity. *J Immunol* (2010) 185:5225–35. doi:10.4049/jimmunol.1001116
46. Tang Z-H, Liang S, Potter J, Jiang X, Mao H-Q, Li Z. Tim-3/galectin-9 regulate the homeostasis of hepatic NKT cells in a murine model of non-alcoholic fatty liver disease. *J Immunol* (2013) 190:1788–96. doi:10.4049/jimmunol.1202814
47. Yang Z, Lei Y, Chen C, Ren H, Shi T. Roles of the programmed cell death 1, T cell immunoglobulin mucin-3, and cluster of differentiation 288 pathways in the low reactivity of invariant natural killer T cells after chronic hepatitis B virus infection. *Arch Virol* (2015) 160:2535–45. doi:10.1007/s00705-015-2539-3
48. Zhang X, Gu J, Zhou L, Mi Q. TIM-4 is expressed on invariant NKT cells but dispensable for their development and function. *Oncotarget* (2016) 7:71099–111. doi:10.18632/oncotarget.12153
49. Iwata A, Watanabe N, Oya Y, Owada T, Ikeda K, Suto A, et al. Protective roles of B and T lymphocyte attenuator in NKT cell-mediated experimental hepatitis. *J Immunol* (2010) 184:127–33. doi:10.4049/jimmunol.0900389
50. Miller ML, Sun Y, Fu Y-X. Cutting edge: B and T lymphocyte attenuator signaling on NKT cells inhibits cytokine release and tissue injury in early immune responses. *J Immunol* (2009) 183:32–6. doi:10.4049/jimmunol.0900690
51. Byun H-J, Jung W-W, Lee D-S, Kim S, Kim SJ, Park C-G, et al. Proliferation of activated CD1d-restricted NKT cells is down-modulated by lymphocyte activation gene-3 signaling via cell cycle arrest in S phase. *Cell Biol Int* (2007) 31:257–62. doi:10.1016/j.cellbi.2006.11.002
52. Juno JA, Stalker AT, Waruk JL, Oyugi J, Kimani M, Plummer FA, et al. Elevated expression of LAG-3, but not PD-1, is associated with impaired iNKT cytokine production during chronic HIV-1 infection and treatment. *Retrovirology* (2015) 12:1–17. doi:10.1186/s12977-015-0142-z
53. Wang J, Cheng L, Wondimu Z, Swain M, Santamaria P, Yang Y. Cutting edge: CD28 engagement releases antigen-activated invariant NKT cells from the inhibitory effects of PD-1. *J Immunol* (2009) 182:6644–7. doi:10.4049/jimmunol.0804050
54. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* (2009) 182:2816–26. doi:10.4049/jimmunol.0803648
55. Chang W, Kim J-Y, Kim Y-J, Kim Y, Lee J, Azuma M, et al. Cutting edge: programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. *J Immunol* (2008) 181:6707–10. doi:10.4049/jimmunol.181.10.6707
56. Durgan K, Ali M, Warner P, Latchman YE. Targeting NKT cells and PD-L1 pathway results in augmented anti-tumor responses in a melanoma model. *Cancer Immunol Immunother* (2011) 60:547–58. doi:10.1007/s00262-010-0963-5
57. Maazi H, Singh AK, Speak AO, Lombardi V, Lam J, Khoo B, et al. Lack of PD-L1 expression by iNKT cells improves the course of influenza A infection. *PLoS One* (2013) 8:e59599. doi:10.1371/journal.pone.0059599
58. Akbari O, Stock P, Singh AK, Lombardi V, Lee W-L, Freeman GJ, et al. PD-L1 and PD-L2 modulate airway inflammation and iNKT-cell-dependent airway hyperreactivity in opposing directions. *Mucosal Immunol* (2010) 3:81–91. doi:10.1038/mi.2009.112
59. Kamata T, Suzuki A, Mise N, Ihara F, Takami M, Makita Y, et al. Blockade of programmed death-1/programmed death ligand pathway enhances the antitumor immunity of human invariant natural killer T cells. *Cancer Immunol Immunother* (2016) 65:1477–89. doi:10.1007/s00262-016-1901-y
60. Moll M, Kuylenstierna C, Gonzalez VD, Anderson SK, Bosnjak L, Sönnerborg A, et al. Severe functional impairment and elevated PD-1 expression in CD1d-restricted NKT cells retained during chronic HIV-1 infection. *Eur J Immunol* (2009) 39:902–11. doi:10.1002/eji.200838780
61. Iyoda T, Ushida M, Kimura Y, Minamino K, Hayuka A, Yokohata S, et al. Invariant NKT cell anergy is induced by a strong TCR-mediated signal plus co-stimulation. *Int Immunol* (2010) 22:905–13. doi:10.1093/intimm/dxq444
62. Natori T, Morita M, Akimoto K, Koezuka Y. Agelasphins, novel antitumor and immunostimulatory cerebrosides from the marine sponge *Agelas mauritianus*. *Tetrahedron* (1994) 50:2771–84. doi:10.1016/S0040-4020(01)86991-X
63. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, et al. Structure-activity relationship of α-galactosylceramides against B16-bearing mice. *J Med Chem* (1995) 38:2176–87. doi:10.1021/jm00012a018
64. Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* (1995) 7:529–34.
65. Motoki K, Maeda K, Ueno H, Kobayashi E, Uchida T, Fukushima H, et al. Antitumor activities of combined treatment with a novel immunomodulator, (2S,3S,4R)-1-O-(a-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000), and radiotherapy in tumor-bearing mice. *Oncol Res* (1996) 8:155–62.
66. Yamaguchi Y, Motoki K, Ueno H, Maeda K, Kobayashi E, Inoue H, et al. Enhancing effects of (2S,3S,4R)-1-O-(a-Galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000) on antigen-presenting function of antigen-presenting cells and antimetastatic activity of KRN7000-pretreated antigen-presenting cells. *Oncol Res* (1996) 8:399–407.
67. Sakai T, Naidenko OV, Iijima H, Kronenberg M, Koezuka Y. Syntheses of biotinylated α-galactosylceramides and their effects on the immune system and CD1 molecules. *J Med Chem* (1999) 42:1836–41. doi:10.1021/jm990054n
68. Smyth MJ, Thia KYT, Street SEA, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* (2000) 191:661–8. doi:10.1084/jem.191.4.661
69. Cui J, Shin T, Kawano T, Sato H, Kondo E, Kanno M, et al. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science* (1997) 278:1623–6. doi:10.1126/science.278.5343.1623
70. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, et al. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci U S A* (1998) 95:5690–3. doi:10.1073/pnas.95.10.5690
71. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol* (2010) 185:2721–9. doi:10.4049/jimmunol.1001018
72. Carnaud C, Lee D, Donnars O, Park S-H, Beavis A, Koezuka Y, et al. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* (1999) 163:4647–50.
73. Nakagawa R, Nagafune I, Tazunoki Y, Ehara H, Tomura H, Iijima R, et al. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by α-galactosylceramide in mice. *J Immunol* (2001) 166:6578–84. doi:10.4049/jimmunol.166.11.6578
74. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* (2004) 21:137–48. doi:10.1016/j.immuni.2004.07.017
75. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. *Science* (2015) 348:74–80. doi:10.1126/science.aaa6204
76. Sun W, Wang Y, East JE, Kimball AS, Tkaczuk K, Kesmodel S, et al. Invariant natural killer T cells generated from human adult hematopoietic stem-progenitor cells are poly-functional. *Cytokine* (2015) 72:48–57. doi:10.1016/j.cyto.2014.12.009
77. Buchbinder EI, Desai A. CTLA-4 and PD-1 pathways: similarities, differences, and implications of their inhibition. *Am J Clin Oncol* (2016) 39:98–106. doi:10.1097/COC.0000000000000239
78. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* (2002) 8:793–800. doi:10.1038/nm730
79. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U S A* (2010) 107:4275–80. doi:10.1073/pnas.0915174107
80. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* (1996) 271:1734–6. doi:10.1126/science.271.5256.1734

81. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* (2002) 99:12293–7. doi:10.1073/pnas.192461099
82. Gough M, Crittenden M, Sarff M, Pang P, Seung S, Vetto J, et al. Adjuvant therapy with agonistic antibodies to CD134 (OX40) increases local control following surgical or radiation therapy of cancer in mice. *J Immunother* (2010) 33:798–809. doi:10.1097/CJI.0b013e3181ee7095
83. Wilcox RA, Flies DB, Zhu G, Johnson AJ, Tamada K, Chapoval AI, et al. Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. *J Clin Invest* (2002) 109:651–9. doi:10.1172/JCI14184
84. Ko K, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, et al. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3+ CD25+ CD4+ regulatory T cells. *J Exp Med* (2005) 202:885–91. doi:10.1084/jem.20050940
85. Todryk SM, Tutt AL, Green MHA, Smallwood JA, Halanek N, Dalgleish AG, et al. CD40 ligation for immunotherapy of solid tumours. *J Immunol Methods* (2001) 248:139–47. doi:10.1016/S0022-1759(00)00349-5
86. Pilones KA, Kawashima N, Yang AM, Babb JS, Formenti SC, Demaria S. Invariant natural killer T cells regulate breast cancer response to radiation and CTLA-4 blockade. *Clin Cancer Res* (2009) 15:597–606. doi:10.1158/1078-0432.CCR-08-1277
87. Pilones KA, Aryankalayil J, Babb JS, Demaria S. Invariant natural killer T cells regulate anti-tumor immunity by controlling the population of dendritic cells in tumor and draining lymph nodes. *J Immunother Cancer* (2014) 2:1–13. doi:10.1186/s40425-014-0037-x
88. Kobayashi T, Doff BL, Rearden RC, Leggatt GR, Mattarollo SR. NKT cell-targeted vaccination plus anti-4-1BB antibody generates persistent CD8 T cell immunity against B cell lymphoma. *Oncoimmunology* (2015) 4:e990793. doi :10.4161/2162402X.2014.990793
89. Teng MWL, Westwood JA, Darcy PK, Sharkey J, Tsuji M, Franck RW, et al. Combined natural killer T-cell-based immunotherapy eradicates established tumors in mice. *Cancer Res* (2007) 67:7495–504. doi:10.1158/0008-5472.CAN-07-0941
90. Ramirez-Montagut T, Chow A, Hirschhorn-cymerman D, Terwey TH, Kochman AA, Lu S, et al. Glucocorticoid-induced TNF receptor family related gene activation overcomes tolerance/ignorance to melanoma differentiation antigens and enhances antitumor immunity. *J Immunol* (2006) 176:6434–42. doi:10.4049/jimmunol.176.11.6434
91. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-γ-producing NKT response induced with α-galactosylceramide-loaded DCs. *Nat Immunol* (2002) 3:867–74. doi:10.1038/ni827
92. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with α-galactosylceramide. *J Immunol* (1999) 163:2387–91.
93. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of α-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* (2005) 201:1503–17. doi:10.1084/jem.20042592
94. Liu K, Idoyaga J, Charalambous A, Fujii S, Bonito A, Mordoh J, et al. Innate NKT lymphocytes confer superior adaptive immunity via tumor-capturing dendritic cells. *J Exp Med* (2005) 202:1507–16. doi:10.1084/jem.20050956
95. Shimizu K, Goto A, Fukui M, Taniguchi M, Fujii S. Tumor cells loaded with α-galactosylceramide induce innate NKT and NK cell-dependent resistance to tumor implantation in mice. *J Immunol* (2007) 178:2853–61. doi:10.4049/jimmunol.178.5.2853
96. Ko H-J, Lee J-M, Kim Y-J, Kim Y-S, Lee K-A, Kang C-Y. Immunosuppressive myeloid-derived suppressor cells can be converted into immunogenic APCs with the help of activated NKT cells: an alternative cell-based antitumor vaccine. *J Immunol* (2009) 182:1818–28. doi:10.4049/jimmunol.0802430
97. Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Blood* (2015) 125:4017–23. doi:10.1182/blood-2014
98. Mungall AJ, Palmer SA, Sims SK, Edwards CA, Ashurst JL, Wilming L, et al. The DNA sequence and analysis of human chromosome 6. *Nature* (2003) 425:805–11. doi:10.1038/nature02055
99. Morris ES, MacDonald KPA, Rowe V, Banovic T, Kuns RD, Don ALJ, et al. NKT cell-dependent leukemia eradication following stem cell mobilization with potent G-CSF analogs. *J Clin Invest* (2005) 115:3093–103. doi:10.1172/JCI25249
100. Pillai AB, George TI, Dutt S, Teo P, Strober S. Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol* (2007) 178:6242–51. doi:10.4049/jimmunol.178.10.6242
101. Hammond KJ, Poulton LD, Palmisano LJ, Silveira PA, Godfrey DI, Baxter AG. alpha/beta-T cell receptor (TCR)+CD4-CD8- (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. *J Exp Med* (1998) 187:1047–56. doi:10.1084/jem.187.7.1047
102. Wilson SB, Kent SC, Patton KT, Orban T, Jackson RA, Exley M, et al. Extreme Th1 bias of invariant Vα24JαQ T cells in type 1 diabetes. *Nature* (1998) 391:177–81. doi:10.1038/34419
103. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med* (2002) 195:637–41. doi:10.1084/jem.20011908
104. Tian G, Courtney AN, Jena B, Heczey A, Liu D, Marinova E, et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. *J Clin Invest* (2016) 126:2341–55. doi:10.1172/JCI83476
105. Bollino D, Webb TJ. Chimeric antigen receptor-engineered natural killer and natural killer T cells for cancer immunotherapy. *Transl Res* (2017) 187:32–43. doi:10.1016/j.trsl.2017.06.003
106. Heczey A, Liu D, Tian G, Courtney AN, Wei J, Marinova E, et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* (2014) 124:2824–34. doi:10.1182/blood-2013-11-541235
107. Rissiek B, Danquah W, Haag F, Koch-Nolte F. Technical advance: a new cell preparation strategy that greatly improves the yield of vital and functional Tregs and NKT cells. *J Leukoc Biol* (2014) 95:543–9. doi:10.1189/jlb.0713407
108. Yamada D, Iyoda T, Vizcardo R, Shimizu K, Sato Y, Endo TA, et al. Efficient regeneration of human Vα24+ invariant natural killer T cells and their anti-tumor activity in vivo. *Stem Cells* (2016) 34:2852–60. doi:10.1002/stem.2465
109. Baglaenko Y, Cruz Tleugabulova M, Gracey E, Talaei N, Manion KP, Chang N-H, et al. Invariant NKT cell activation is potentiated by homotypic trans-Ly108 interactions. *J Immunol* (2017) 198:3949–62. doi:10.4049/jimmunol.1601369
110. Kulkarni RR, Villanueva AI, Elawadli I, Jayanth P, Read LR, Haeryfar SM, et al. Costimulatory activation of murine invariant natural killer T cells by toll-like receptor agonists. *Cell Immunol* (2012) 277:33–43. doi:10.1016/j.cellimm.2012.06.002
111. Mincheva-Nilsson L, Baranov V. Cancer exosomes and NKG2D receptor-ligand interactions: impairing NKG2D-mediated cytotoxicity and anti-tumour immune surveillance. *Semin Cancer Biol* (2014) 28:24–30. doi:10.1016/j.semcancer.2014.02.010
112. Cherkassky L, Morello A, Villena-Vargas J, Feng Y, Dimitrov DS, Jones DR, et al. Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. *J Clin Invest* (2016) 126:3130–44. doi:10.1172/JCI83092
113. Takaku S, Terabe M, Ambrosino E, Peng J, Lonning S, McPherson JM, et al. Blockade of TGF-β enhances tumor vaccine efficacy mediated by CD8+ T cells. *Int J Cancer* (2010) 126:1–19. doi:10.1002/ijc.24961
114. Terabe M, Matsui S, Park J-M, Mamura M, Noben-Trauth N, Donaldson DD, et al. Transforming growth factor-β production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* (2003) 198:1741–52. doi:10.1084/jem.20022227

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New Directions for Natural Killer T Cells in the Immunotherapy of Cancer

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Natural killer T (NKT) cells have been placed at the interface between innate and adaptive immunity by a long series of experiments that convincingly showed that beyond cytokine secretion and NK cell recruitment, NKT cells were coordinating dendritic cell and B cell maturation through direct membrane contacts and initiate productive responses. As such, NKT cells are the cellular adjuvant of many immune reactions and have functions that go much beyond what their name encapsulates. In addition, the initial discovery of the ligands of NKT cells is deeply linked to cancer biology and therapy. However, for a host of reasons, animal models in which agonists of NKT cells were used did not translate well to human cancers. A systematic reassessment of NKT cells role in tumorigenesis, especially spontaneous one, is now accessible using single cell analysis technologies both in mouse and man, and should be taken advantage of. Similarly, the migration, localization, phenotype of NKT cells following induced expansion after injection of an agonist can be examined at the single cell level. This technological revolution will help evaluate where and how NKT cells can be used in cancer.

Keywords: natural killer T cells, endogenous ligands, single cell analysis, anti-PD1 treatment, vaccines

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INTRODUCTION

Natural killer T (NKT) cells were discovered more than 30 years ago as a small population of double negative or CD4+ T cells in the mouse and characterized by the usage of a unique T cell receptor (TCR) α chain with an invariant CDR3 segment. The same population was subsequently isolated from human blood and shown to express the same TCRα chain as the mouse, instantly raising the attention of researchers around the world as conservation of T cell population across distant species usually correlates to essential functions (1). In addition, it was also reasonable to assume that a semi-invariant TCR would recognize a limited set of antigens. The discovery of the first of these antigens took 10 years and serendipity, and linked forever NKT cells with cancer biology (2). Indeed, α-galactosylceramide (αGalcer) was isolated by a team of researchers at Kirin Pharmaceuticals who were seeking antitumor properties in natural compounds. The extracts from Agelas Mauritanus proved great antitumor effects in a melanoma model that they used for screening. The chemical nature of the natural product responsible for the activity was surprising and related to species that were isolated in 1989 by Mangoni and collaborators, from *Amphimedon viridis*, a marine sponge, and were identified as α-glucosamine ceramides (3). The connection of this new glycolipid, αGalcer, with NKT cell was also very circumstantial as in the same year, the crystal structure of CD1d was elucidated and revealed the lipid binding properties of this class of MHC-like molecules (4). With the rapid development of CD1d- αGalcer tetramers, a field was born as NKT cells were now accessible in

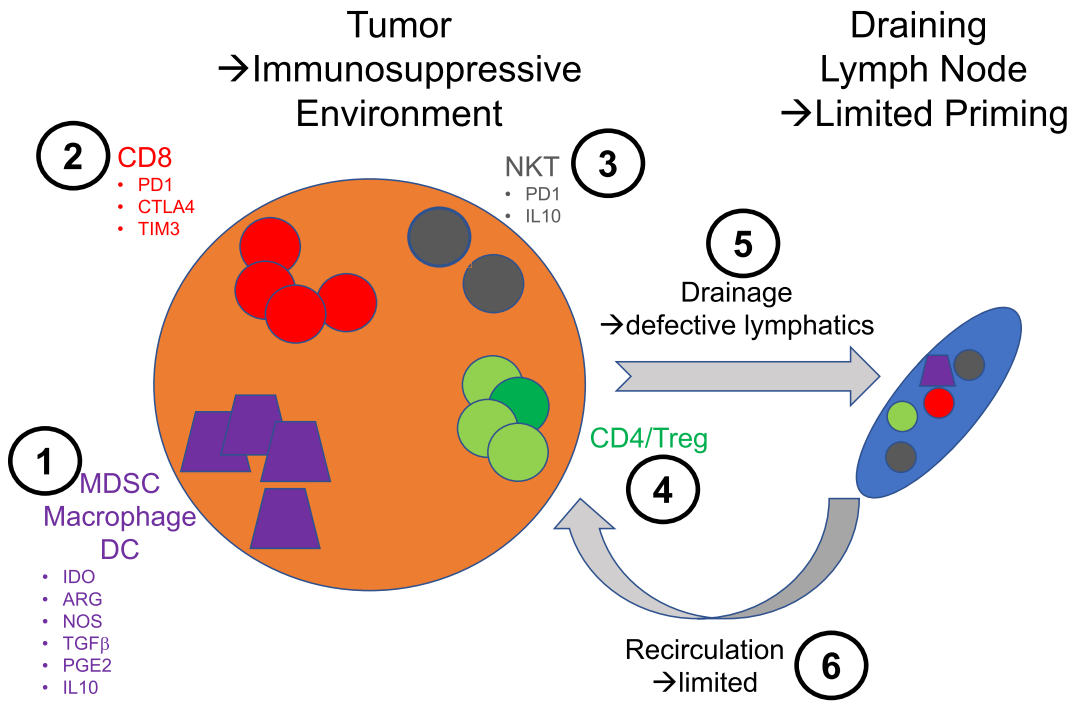

FIGURE 1 | Schematic illustration of the various therapeutic intervention nodes that are relevant to the use of natural killer T (NKT) cells in immunotherapy. **1**: Monocyte lineage—these cells are essential to sample tumors and traffic to the lymph node to prime immune responses. The secretion of indoleamine 2,3-dioxygenase by the tumor cells promotes the emergence of a suppressive phenotype linked to the downstream expression of immunosuppressive mediators such as arginase (ARG), nitric oxide synthase (NOS), transforming growth factor β (TGFβ), prostaglandin E2 (PGE2), and IL10. **2**: CD8 T cells—the exhausted phenotype of these cells in tumor is directly exploited for intervention with checkpoint inhibitors. **3**: NKT cells—in very much the same way CD8 T cell responses are improved by checkpoint blockade, NKT cells could benefit from the same treatment. In addition, the blockade of some of the immunosuppressive factors they may produce could be beneficial. **4**: CD4 T cells—there is not to this day therapies intervening directly on this population of T cells to enhance their effector function or block their regulatory activities. **5**: Lymphatics—the lymphatic architecture of tumors is notably compromised and likely contributes to poor priming against tumors. **6**: Blood vessels—the normal circulation is similarly aberrant and most likely compromises the access of effector cells to tumors.

and *ex vivo* for studies. The discovery that CD1d was presenting other lipids and glycolipids to non-semi-invariant T cells was in the shadow of the semi-invariant NKT cells for years and led to a rather incongruous and inappropriate split in terminology of lipid-specific T cells with type 1 and type 2 NKT cells for the semi-invariant and non-semi-invariant cells, respectively. The NKT name is related to the expression of NK markers by NKT cells but this name brings numerous confusions between the two cell types, while type 2 NKT cells appear to be mainstream T cells (5). In any case, we will only discuss the classic type 1 NKT cells in this communication.

As mentioned, the discovery of αGalcer in the context of a cancer program focused the attention of many on the potential linkage of NKT cells with the biology of cancer. The demonstration of a potent antimelanoma activity in mice and the analysis of some of the mechanisms that led to this response such as the ability of some NKT cells to lyse target cells, enticed clinicians to trying αGalcer as monotherapy in advanced cancers (6). In these trials, if the bioactivity of the compound could be demonstrated based on the expansion of the blood NKT cell population, no clinical benefit could be shown (7–9). As often in the field of immunotherapy, initial failures greatly diminish the appetite of companies and clinicians to use a particular compound, and the consideration of αGalcer for cancer treatment has greatly diminished if not vanished. However, the biology of NKT cells, the identification of their natural ligands, the behavior of their agonists *in vivo*, and the entire field of immunotherapy have made huge progress over the past decade and new approaches that would put NKT cells at the center of the treatment of cancer are emerging (**Figure 1**). This is precisely what we will discuss and place in the context of understanding the particular role of NKT cells in tumorigenesis in both mouse and man.

DEFINITION OF THE NKT CELL POPULATIONS IN MOUSE AND MAN, HEALTH AND DISEASES

As a preamble, it is important to mention that a substantial part of the NKT cell-cancer field has been shrouded in its early years by discrepancies and controversies that have to do with the relationship between cancer and the presence of NKT cells. In mouse models where NKT cells can be removed by either deletion of CD1d, the antigen-presenting molecule, or Jα18, the junctional segment of NKT cells, some have found an increase in MCA-induced tumors in the absence of NKT cells (10), whereas other

have not (11). However, the dramatic effect of NKT cell activation on the fate of metastatic melanoma in mice kept the interest high enough for clinical trials to be carried out with the outcome that we have just mentioned. This discrepancy between mouse studies and clinical trials could be used for the sake of making the argument that mouse models are poorly predictive or human studies, but is this true? The mouse models offer the advantage of speed and the luxury of accessing every organ and lymph node during the course of a disease that we initiate. We often overlook the fact that the B16 melanoma model in its subcutaneous solid tumor or metastatic versions are acute cancers, a situation exceptionally seen in human. So, in this case, it is not the mouse biology that trumps us but the model we decide to use for practical and financial reasons. Spontaneous tumors would and should offer a much better model to evaluate NKT cells in the control of tumorigenesis and the NKT cell-based therapies but they take a long time. However, even in the case of the B16 melanoma model, most of the studies are shortcoming in understanding the role of NKT cells. We live on a model in which the secretion of IFNγ by NKT cells explains all the therapeutic effects of αGalcer by allowing not only the maturation of dendritic cells but a large recruitment of NK cells and cytotoxic activities (12). However, little is known about where NKT cells are localized and what their function is before αGalcer is injected. Why cannot they control tumor seeding and development? Are NKT cells exclusively in the lymph node or also in the tumor? What gene expression profiles have those NKT cells? Are they tissue- or blood-resident? Are some of them negative regulators of immune responses? Why do not they initiate a spontaneous immune response and tumor rejection? In all cases, the difficulty to study these functions and mechanisms is the paucity of the NKT cell population even in mice. In human, it is often brought up that NKT cell numbers vary greatly in the general population, spanning at least three orders of magnitude. However, this observation pertains only to peripheral blood NKT cells and variability in other tissue-resident NKT cells has never been systematically documented. The issue that is often overlooked is that NKT cells are tissue-resident and organ-specific (13), and that circulating cells are simply resident of the circulatory system, not merely circulating to the next organ. The molecular basis of this tissue residency is mostly unknown at the exception of the relationship between CXCR6 expression and liver NKT cells (14). What makes an NKT cell resident of a lymph node at a particular location is a field to be explored. In any instance, these considerations are key to understanding what NKT cells do in the vicinity of a tumor, e.g., the lymph node, or within the tumor environment when they are found locally.

SINGLE CELL STUDIES

An exhaustive description of NKT cell subsets has been done over the past decade: NKT1, NKT2, NKT17, NKTreg, double negative, CD4+ (1). This diversity demonstrates the plasticity of T cells and the ability of specifying function in various environments. In relationship to cancer, no clear phenotyping has emerged for NKT cells that are inside the tumor and in draining lymph nodes but unexplained observations have been noted in relationship to phenotype; for instance, it appeared that only DN NKT cells from the liver could protect against MCA tumor development, while spleen and thymic NKT cells could not (15). Therefore, it seems obvious that a detailed description of the various NKT cells in the local environment of tumors must be carried out to understand the fundamental role of NKT cells and tumorigenesis. Because NKT cells are so few, classical approaches cannot be used to address this question. However, we have stepped into the era of single cell analysis and pathway mapping, and the topic "NKT cells and cancer" is ideally suited for applying targeted single cell (SC) gene expression profiling by Q-PCR and systemic gene expression profiling by SC RNA-seq techniques. Microfluidics have made these techniques amenable to both mouse and human studies (16, 17), and in the case of NKT cells, the exceptional quality of the CD1d tetramers make the step of single cell isolation relatively trivial. In addition to pure phenotypic assignment to one of the subsets that we have just cited, RNAseq allows to map pathways that are up- or downregulated and gives a deep understanding of the biology that the isolated cells undergo at the time of sampling. It might not be surprising if these studies uncover an anergic state or a suppressive phenotype for intra-tumoral and draining lymph nodes NKT cells. For instance, the increased expression of PD1 could explain anergy and would beneficiate from anti-PD1 interventions (see below). Such studies and the head to head comparison between mouse and man would allow the best possible translational research in the field. In addition, the same techniques could be used to follow therapeutic administration of NKT cell agonists and examine circulating as well as NKT cells recovered from biopsies.

OVERCOMING ANERGY AND ACTIVATION INDUCED CELL DEATH

The systemic delivery of agonists of NKT cells has been shown to lead to a depletion in numbers and function of NKT cells, both in the mouse and man (18, 19). One obvious reason for dwindling numbers post-αGalcer administration is activation induced cell death by overstimulation, αGalcer being a very potent ligand *in vitro* and *in vivo*. In human, in the course of a phase 1 clinical trial for hepatitis B, our group discovered that maximal effects were obtained at 2 μg IM and followed by a long-term depletion of peripheral NKT cells (18). Concurrently, it has been shown that higher dosage was accompanied by systemic adverse effects related to liver delivery and IFNγ release. It is obvious that the stunning of the NKT cell compartment by a single injection would be detrimental to therapeutic schemes in which multiple rounds of administration of the therapy, e.g., NKT cell agonist or NKT cell agonist + antigen, would be beneficial. As such, it is highly unlikely that there is a future for therapies that would use αGalcer or similar agonists by itself or simply mixed with an antigen in the formulation of a therapeutic vaccine.

A second issue that might be pertinent to the relationship of NKT cells with cancer, is that there is a possibility that tumorigenesis is accompanied by a chronic low level stimulation of NKT cells and their exhaustion (20). A similar phenomenon has been noted in chronic infections such as HIV and hepatitis B (21), and correlated to an increase PD1 expression. Surprisingly,

the NKT cell PD1 expression levels have not been examined yet in cancer patients. The broad use of anti-PD1 therapies creates the perfect opportunity to examine the status of NKT cells before and during the course of treatment. Would PD-1 be increased on NKT cells during malignancies, it might indicate that the manipulation of NKT cells during anti-PD1 antibody treatment might contribute to increasing the effects of this type of immunotherapy. Indeed, it would seem logical that NKT cells engagement would improve antigen presentation and T cell recruitment, therefore enhancing the expansion of anti-tumor T cells.

CLINICAL MONITORING. BEWARE OF THE BLOOD NUMBERS

As mentioned above, both in mouse and man, we have used circulating NKT cell numbers as a surrogate for local effects but no study has systematically examined the correlation between peripheral and local expansion of NKT cells. The possibility of discrepancy between compartments was documented in the mouse when we studied glycolipid transport. Indeed, in the absence of FAAH, a αGalcer lipid binding protein, systemic transport of αGalcer is greatly impaired but local effects are increased (22). Given the alterations of vascularization of tumors and their vicinity, it is difficult to assume that NKT cells agonists will be delivered locally with the same pharmacological parameters than in normal tissues. This possible dissociation of effects between organs should be kept in mind when trying to understand low circulating NKT cell numbers and cancer, even though in the case of neck and head malignancies it appears that low numbers and poor prognosis are correlated (23). Answering this concern is not trivial even in animal models as the recovery of NKT cells from various tissues is illustrious for its inconsistency and variability. Once again, SC analysis should be helpful to investigate some of these issues.

USE ENDOGENOUS NKT CELL LIGANDS

In natural circumstances, NKT cells initiate activation when their TCR encounters endogenous ligands and then sustain activity on cytokine-mediated signaling. The critical TCR engagement step is mediated by a strong agonist, and is brief as one would expect to avoid overstimulation and cell death (24). The stunning or disappearance of NKT cells mediated by the production of endogenous ligands such as after TLR engagement, have never been noted. It appears that endogenous NKT cell activation is mainly controlled by the availability of ligands and that degradation of stimulatory α-linked glysosylceramides is key to this tight regulation (24). Three enzymes are critical to this catabolism, acid ceramidase, ASAH1, responsible for the first step of degradation, acid α-galactosidase, GLA, and acid α-glucosidase, GAA, both responsible for the removal of the head glycan and the production of free sphingosine. It is notable that during TLR-mediated activation of dendritic cells, all three enzymes are briefly down-regulated before returning to normal levels after about 2 h (25). In addition, we have shown that the chemical blockade of these enzymes increased the stimulatory capacity of dendritic cells and more importantly was efficient *in vivo* at expanding NKT cell populations over prolonged periods of time (Luc Teyton, unpublished). It is also of interest to note that ASAH1 inhibitors have been developed in the context of cancer therapy as chemosensitizing agents based on the hypothesis that ceramides were promoting cell death by apoptosis (26). We would argue that some of the effects that have been observed using these inhibitors are directly linked to the effects on NKT cells. This hypothesis will be interesting to examine in patients receiving anti-ASAH1 inhibitors. It will also be of interest of testing the *in vivo* effects of GLA and GAA inhibitors alone and in combination with ASAH1 inhibitors with respect to expanding NKT cell populations without inducing cell death. Some of these inhibitors have been developed as folding-inducers in some of the forms of GLA and GAA deficiencies and appear safe for use in human. This concept of manipulating a cell population by tuning the availability of it endogenous ligand has never applied in any form of immunotherapy so far; the NKT cell system might offer the first opportunity. Finally, it would also be of great interest to appreciate whether some tumors, in addition to expressing CD1d, might express NKT cell ligands and in what amount. Local overproduction could result in the disappearance or functional anergy of local NKT cells.

OTHER POTENTIAL AVENUES OF USING NKT CELLS IN IMMUNOTHERAPY

Conceptually, and before we really understand the position of NKT cells in cancer and tumorigenesis, a large number of potential therapeutic avenues focused on NKT cells could be explored (**Figure 1**). The *in vitro* expansion of autologous NKT cells and reinfusion are practically achievable, but one could argue that in the absence of understanding the key mechanisms to NKT cell organ residency, the exercise will be futile, cumbersome and expensive. Similarly, it could be interesting to explore the potential of CAR T cells made with NKT cell receptors or the Fab of an anti-CD1d-αGalcer antibody, to hardwire recognition and killing; however, this approach could only be tested for tumors expressing CD1d. The usage of αGalcer and related compounds in the context of therapeutic vaccines is more mainstream and would simply take advantage of the remarkable adjuvant properties of NKT cells. The administration of autologous dendritic cells loaded with antigen and αGalcer has been shown effective in mouse models (27) and could ideally be tested to potentiate vaccines such as Provenge™ (7). In addition, this mode of delivery of αGalcer bypasses systemic and liver delivery and as such should be devoid of side effects. On the other hand, direct administration of an antigen mixed with αGalcer would expose the patient to this undesirable stimulation of all NKT cells in his/her body; it cannot be conceived without controlling local delivery and limiting or eliminating systemic transport of αGalcer. With this in mind, direct coupling to the antigen or to particles, or unique routes of administration such as intradermal

or intranasal, have been explored with some success to control this issue.

CONCLUSION

As often in translational research, the urgency of obtaining results combined with the infatuation of each researcher with his/her favorite cell type, leads to disappointing results. Taking a step back is often granted and querying additional basic knowledge never ill-advised. With the arising of SC technologies, we are today in a unique position of understanding what NKT cells do in the course of tumorigenesis and what role they play together with adaptive immunity to reject cancer cells. This knowledge will be essential to manipulate and utilize NKT cells in the arsenal of immunotherapy.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REFERENCES

1. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi:10.1146/annurev.immunol.25.022106.141711
2. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* (1997) 278:1626–9. doi:10.1126/science.278.5343.1626
3. Costantino V, Fattorusso E, Imperatore C, Mangoni A, Freigang S, Teyton L. Corrugoside, a new immunostimulatory alpha-galactoglycosphingolipid from the marine sponge *Axinella corrugata*. *Bioorg Med Chem* (2008) 16:2077–85. doi:10.1016/j.bmc.2007.10.098
4. Zeng Z, Castano AR, Segelke BW, Stura EA, Peterson PA, Wilson IA. Crystal structure of mouse CD1: an MHC-like fold with a large hydrophobic binding groove. *Science* (1997) 277:339–45. doi:10.1126/science.277.5324.339
5. Ambrosino E, Terabe M, Halder RC, Peng J, Takaku S, Miyake S, et al. Cross-regulation between type I and type II NKT cells in regulating tumor immunity: a new immunoregulatory axis. *J Immunol* (2007) 179:5126–36. doi:10.4049/jimmunol.179.8.5126
6. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S. Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *J Exp Med* (2007) 204:2641–53. doi:10.1084/jem.20070458
7. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* (2005) 201:1503–17. doi:10.1084/jem.20042592
8. Schneiders FL, Scheper RJ, von Blomberg BM, Woltman AM, Janssen HL, van den Eertwegh AJ, et al. Clinical experience with alpha-galactosylceramide (KRN7000) in patients with advanced cancer and chronic hepatitis B/C infection. *Clin Immunol* (2011) 140:130–41. doi:10.1016/j.clim.2010.11.010
9. Wilson MT, Singh AK, Van Kaer L. Immunotherapy with ligands of natural killer T cells. *Trends Mol Med* (2002) 8:225–31. doi:10.1016/S1471-4914(02)02325-0
10. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* (2000) 191:661–8. doi:10.1084/jem.191.4.661
11. Kammertoens T, Qin Z, Briesemeister D, Bendelac A, Blankenstein T. B-cells and IL-4 promote methylcholanthrene-induced carcinogenesis but there is no evidence for a role of T/NKT-cells and their effector molecules (Fas-ligand, TNF-alpha, perforin). *Int J Cancer* (2012) 131:1499–508. doi:10.1002/ijc.27411
12. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* (2002) 3:867–74. doi:10.1038/ni827
13. Thomas SY, Scanlon ST, Griewank KG, Constantinides MG, Savage AK, Barr KA, et al. PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. *J Exp Med* (2011) 208:1179–88. doi:10.1084/jem.20102630
14. Geissmann F, Cameron TO, Sidobre S, Manlongat N, Kronenberg M, Briskin MJ, et al. Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol* (2005) 3:e113. doi:10.1371/journal.pbio.0030113
15. Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J Exp Med* (2005) 202:1279–88. doi:10.1084/jem.20050953
16. Chen X, Love JC, Navin NE, Pachter L, Stubbington MJ, Svensson V, et al. Single-cell analysis at the threshold. *Nat Biotechnol* (2016) 34:1111–8. doi:10.1038/nbt.3721
17. Vieira Braga FA, Teichmann SA, Chen X. Genetics and immunity in the era of single-cell genomics. *Hum Mol Genet* (2016) 25:R141–8. doi:10.1093/hmg/ddw192
18. Tefit JN, Crabe S, Orlandini B, Nell H, Bendelac A, Deng S, et al. Efficacy of ABX196, a new NKT agonist, in prophylactic human vaccination. *Vaccine* (2014) 32:6138–45. doi:10.1016/j.vaccine.2014.08.070
19. Wilson MT, Johansson C, Olivares-Villagomez D, Singh AK, Stanic AK, Wang CR, et al. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. *Proc Natl Acad Sci U S A* (2003) 100:10913–8. doi:10.1073/pnas.1833166100
20. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* (2009) 182:2816–26. doi:10.4049/jimmunol.0803648
21. Juno JA, Keynan Y, Fowke KR. Invariant NKT cells: regulation and function during viral infection. *PLoS Pathog* (2012) 8:e1002838. doi:10.1371/journal.ppat.1002838
22. Freigang S, Zadorozhny V, McKinney MK, Krebs P, Herro R, Pawlak J, et al. Fatty acid amide hydrolase shapes NKT cell responses by influencing the serum transport of lipid antigen in mice. *J Clin Invest* (2010) 120:1873–84. doi:10.1172/JCI40451
23. Berzins SP, Ritchie DS. Natural killer T cells: drivers or passengers in preventing human disease? *Nat Rev Immunol* (2014) 14:640–6. doi:10.1038/nri3725
24. Kain L, Webb B, Anderson BL, Deng S, Holt M, Costanzo A, et al. The identification of the endogenous ligands of natural killer T cells reveals the presence of mammalian alpha-linked glycosylceramides. *Immunity* (2014) 41:543–54. doi:10.1016/j.immuni.2014.08.017
25. Meissner F, Scheltema RA, Mollenkopf HJ, Mann M. Direct proteomic quantification of the secretome of activated immune cells. *Science* (2013) 340:475–8. doi:10.1126/science.1232578
26. Realini N, Solorzano C, Pagliuca C, Pizzirani D, Armirotti A, Luciani R, et al. Discovery of highly potent acid ceramidase inhibitors with in vitro tumor chemosensitizing activity. *Sci Rep* (2013) 3:1035. doi:10.1038/srep01035
27. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* (2003) 198:267–79. doi:10.1084/jem.20030324

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REVIEW
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Harnessing the Power of Invariant Natural Killer T Cells in Cancer Immunotherapy

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Invariant natural killer T (iNKT) cells are a distinct subset of innate-like lymphocytes bearing an invariant T-cell receptor, through which they recognize lipid antigens presented by monomorphic CD1d molecules. Upon activation, iNKT cells are capable of not only having a direct effector function but also transactivating NK cells, maturing dendritic cells, and activating B cells, through secretion of several cytokines and cognate TCR-CD1d interaction. Endowed with the ability to orchestrate an all-encompassing immune response, iNKT cells are critical in shaping immune responses against pathogens and cancer cells. In this review, we examine the critical role of iNKT cells in antitumor responses from two perspectives: (i) how iNKT cells potentiate antitumor immunity and (ii) how CD1d$^+$ tumor cells may modulate their own expression of CD1d molecules. We further explore hypotheses to explain iNKT cell activation in the context of cancer and how the antitumor effects of iNKT cells can be exploited in different forms of cancer immunotherapy, including their role in the development of cancer vaccines.

Keywords: invariant natural killer T cells, CD1d molecules, tumor immunology, innate immune response, lipid antigens

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INTRODUCTION

The evidence that peptide-specific T cells play an important role in the immune defense against pathogens and cancer progression is compelling (1–3). In the last two decades, it has emerged that in addition to peptide-specific T cells, hereafter referred to as conventional T cells, α/β T cells can also recognize lipids and metabolites of vitamin B2 in the context of monomorphic MHC class I-like molecules (4, 5). Such T cells, hereafter referred to as unconventional T cells, can orchestrate an immune response against pathogens and cancer (4). This review will focus on the description of one of these unconventional T cell populations—invariant natural killer T (iNKT) cells. We will also highlight potential mechanisms of iNKT cell activation in cancer and how these cells can be manipulated for the purpose of cancer immunotherapy.

Development and Function

Invariant natural killer T cells originate from bone marrow-derived progenitors that, like conventional T cells, migrate to the thymus. However, unlike conventional T cells, which are selected by self-peptides presented by MHC class I and II on thymic epithelial cells, iNKT cells are positively selected by CD1d molecules expressed by double-positive (CD4$^+$CD8$^+$) thymocytes (6, 7). Such CD1d molecules present self-lipid ligand(s), not yet fully characterized, and upon expression of the transcription factor PLZF, the thymocytes acquire the iNKT cell effector program (8). iNKT cells

subsequently migrate out of the thymus and reach maturity in the periphery (8). Unlike naïve conventional T cells, iNKT cell numbers in humans are high, particularly in the spleen and in the liver, reaching about 1% of total lymphocytes in the latter tissue (9).

Invariant natural killer T cells are considered innate-like lymphocytes as they exhibit characteristics of both innate and adaptive immune cells. Their activation is driven by antigen recognition, a characteristic of conventional adaptive immune cells. However, unlike conventional T cells, iNKT cells bear a semi-invariant TCR that recognizes different lipid antigens presented on monomorphic CD1d molecules. This recognition manner has been likened to a pattern recognition mode (10). iNKT cells further deviate from conventional T cells by their ability to rapidly secrete copious amounts of cytokines, mainly IFN-γ and IL-4, shortly upon activation—a characteristic reminiscent of innate immune responses, and which is imparted at the epigenetic level by their unique developmental program (11–14).

Since iNKT cells are uniquely placed at the interface between innate and adaptive immunity, they have a tremendous influence in shaping immune responses. Cytokine stimulation and cognate interaction between iNKT cells and dendritic cells (DCs), B cells, neutrophils, and macrophages often polarizes these cells toward a pro-inflammatory phenotype (15–25). Similarly, activated iNKT cells can transactivate natural killer cells (26) and enhance stimulation of conventional T cells through their ability to secrete cytokines and mature DCs (16, 18). Although the frequency of iNKT cells in humans ranges from 0.01 to 0.1% in peripheral blood (lower than in mice), this frequency is still orders of magnitude higher than that of naïve peptide-specific T cells (9, 27). In addition, their constitutive expression of CD40L and ability to rapidly secrete cytokines make iNKT cells critical players in immunity, by orchestrating all-encompassing immune responses (9).

Means of Activation

There are two primary means of iNKT cell activation: CD1d-dependent and cytokine-driven activation. CD1d molecules are transmembrane proteins that, similar to MHC class I molecules, bind non-covalently to β_2-microglobulin. The surface-exposed antigen-binding groove consists of two deep hydrophobic channels that bind the fatty acid tails of lipid antigen, while the head moiety is exposed for recognition by the iNKT-TCR (28, 29). Ceramide-based glycolipids (glycosphingolipids) and glycerol-based lipids (such as membrane phospholipids) are the two main types of iNKT-activating lipids bound to CD1d molecules (30–34). While the most potent iNKT-activating lipid agonists described to date is threitol-6-ceramide (35), the classical iNKT-activating lipid agonist most frequently used in the literature is α-galactosylceramide (αGC), which is derived from a bacterium on the *Agelas mauritianus* marine sponge (23, 36–38). Analysis of the crystal structure of CD1d monomers with or without αGC, which exploits the full binding capacity of CD1d, allowed for the identification of the hydrogen bonds required to hold the polar head of iNKT cell agonists (29). The presence of both a lipid binding and non-lipid binding molecule in the asymmetric unit of the CD1d crystals has enabled the identification of two different conformations of the antigen-binding groove (29). Using planar lipid bilayers and surface plasmon resonance, the contribution of the length and saturation of the alkyl chains occupying the A′ and F′ channel of human CD1d molecules to the stability of CD1d-lipid complexes and to the affinity of iNKT-TCR binding was further analyzed (39). These results led to the description of a general mechanism by which the length of the lipid chain occupying the F′ channel plays a role in controlling the affinity of lipid-specific CD1d-restricted T cells (39). This concept can be more generally extend to other CD1-restricted cells (40).

In a more physiological context, iNKT cells become activated by microbial or self-lipid antigens bound to CD1d molecules. For example, isoglobotriosylceramide (iGB3), a neutral glycosphingolipid, has been identified as a weak self-lipid antigen for human and murine iNKT cells (41–43), although its role as the only positive-selecting self-lipid in the thymus remains controversial, given that mice lacking the required synthases for iGB3 production maintain an intact iNKT cell repertoire (44, 45). Lysophospholipids and charged glycosphingolipids have been shown to be self-lipid antigens in different contexts (46–48). Self-lipid antigens are weakly immunogenic and iNKT cell activation in this case is often largely driven by IL-12 and IL-18. In a model of hepatitis B infection, it has been shown that viral-induced phospholipases generate lysophospholipids that lead to iNKT cell activation (30, 47).

Cytokine-driven activation is common when lipid antigen is weakly immunogenic (47). Although CD1d-activated iNKT cells can undergo further activation *via* cytokines secreted from matured DCs, certain cytokines, namely IL-12 and IL-18, are alone sufficient to activate iNKT cells (49, 50). Avidity might play a more important role in iNKT cell activation than previously considered, especially iNKT cell activation by self-lipid antigen repertoire. Alterations in the actin cytoskeleton are evidenced to create CD1d nanoclusters of higher avidity, increasing basal iNKT autoreactivity (51).

iNKT CELLS IN ANTITUMOR IMMUNITY

The ability of iNKT cells to orchestrate immune responses against cancer is perhaps the most striking example of their role in disease. Work from the laboratory led by Dale Godfrey highlighted the essential role of iNKT cells in tumor immunity by demonstrating that mice lacking iNKT cells were more susceptible to methylcholanthrene-induced sarcomas, consistent with the role of iNKT cells in immunosurveillance (52). This effect was reversed upon iNKT cell reconstitution, an observation that further supports their role in tumor clearance. Although the antitumor effector activity of iNKT cells upon αGC injection was recently confirmed using newly generated Jα18-deficient mice, which bear an otherwise normal T cell repertoire (53), the role of iNKT cells in immunosurveillance of methylcholanthrene-induced sarcomas was called into question in a separate study (54).

Invariant natural killer T cells' ability to modulate various immune subsets is key to their role in antitumor immune responses. iNKT cells can mature DCs, activate $CD4^+$ T cells, $CD8^+$ T cells, and B cells, and transactivate NK cells (19, 23). In

murine models of lung and liver cancers, the antitumor effect of αGC administration was attributed to IFN-γ secretion from iNKT cells and transactivated NK cells, which culminated in NK perforin-mediated cytolysis of tumor cells (23). iNKT cell-derived IFN-γ is also responsible for enhanced activation of tumor antigen-specific $CD8^+$ T cells (19, 55, 56). Additionally IL-12 derived from iNKT cell-matured DCs helped priming of tumor antigen-specific T cells (19, 57).

Invariant natural killer T cells can also augment an antitumor response by diminishing the immunosuppressive activities of immune subsets that promote tumorigenesis. It has been shown that iNKT cells can have a profound effect on the number and function of pro-tumorigenic myeloid populations (22, 58, 59). Tumor-associated macrophages (TAMs), which secrete immunosuppressive molecules such as IL-6 and TGF-β that dampen T-cell responses to MHC-presented tumor antigen, are found in the tumor microenvironment of a variety of cancers, including renal cell carcinomas and neuroblastoma (59). In primary human neuroblastoma samples, iNKT cells specifically killed the tumor-antigen-loaded TAMs rather than neuroblastoma cells, in part relieving the immunosuppressive tumor microenvironment and limiting metastases (59). iNKT cells are also capable of reducing myeloid-derived suppressor cells (MDSC) numbers and immunosuppressive activity (22, 58). These findings beg the question of how iNKT cells remain unaffected by the immunosuppressive microenvironment. It is reported that in patients with head and neck cancer, iNKT cells, unlike conventional T cells, are resistant to hydrogen peroxide produced by $CD15^+$ MDSCs (60). This observation potentially explains their persistent activation and cytotoxic activity within an immunosuppressive tumor microenvironment.

While iNKT cells are best known to potentiate their antitumor effect through enhancing the immunogenic activities of a variety of immune cell subsets, they are capable of themselves recognizing and killing $CD1d^+$ tumor cells. Such is true for the EL4 T-cell lymphoma model, where both *in vitro* and *in vivo* iNKT cells directly executed perforin-mediated cytolysis of lymphoma cells in a CD1d-dependent manner (61, 62). Furthermore, in a TRAMP murine model of $CD1d^+$ prostate cancer, iNKT cells directly and predominantly reduce tumorigenesis, to a greater extent than cytotoxic T lymphocytes (63). In addition, in naturally expressing $CD1d^+$ human osteosarcoma cell lines, iNKT cells selectively killed the tumor cells through Fas-FasL interaction, while leaving cocultured $CD1d^-$ osteoclasts and $CD1d^+$ mesenchymal stem cells unaffected (64). Glioma and breast cancer cell lines transduced with CD1d are targets of iNKT cell-dependent lysis (65, 66). These results collectively indicate that iNKT cells are capable of directly killing $CD1d^+$ tumors. In the large proportion of cases where solid tumors are $CD1d^-$, tumor-infiltrating $CD1d^+$ myeloid populations might activate iNKT cells either within the tumor or in distal lymphoid tissues enriched in iNKT cells.

Though their activation can contribute greatly to antitumor immune responses, there is a dearth of evidence that iNKT cells are present within the tumor microenvironment, particularly in human solid tumors. This issue might stem from low frequency of iNKT cells in humans, potentially making them difficult to detect by immunohistochemical techniques. While iNKT cell-specific antibodies, including the 6B11 antibody (67), do exist, there are few reports of their use in identifying iNKT cell populations within tumor microenvironments.

MODULATION OF CD1d: TUMOR EVASION FROM iNKT CELL IMMUNOSURVEILLANCE

Tumor cells use a variety of mechanisms to escape detection and elimination by immune cells. These mechanisms include releasing soluble mediators to dampen antitumor immune responses, notably TGF-β into the microenvironment and inducing T cell anergy and exhaustion (68). Another mechanism involves hindering antigen presentation, often by limiting expression of antigen-presenting molecules in infiltrating myeloid cells or on the tumor cells themselves. MHC class I molecules, which present peptide antigens to $CD8^+$ T cells, are well characterized as a target of such escape mechanisms (68). Similarly, a variety of tumors downregulate CD1d molecules, further emphasizing the important role of iNKT cells in antitumor immunity (**Figure 1**).

The correlation between reduced CD1d expression and enhanced tumor progression has been reported in a variety of types of CD1d-transduced solid cancers, including breast, cervical, ovarian, prostate, lung, and melanoma (66, 69–72). This observation holds true for many naturally $CD1d^+$ and transduced $CD1d^+$ liquid tumors, such as mantle cell lymphoma, multiple myeloma, and chronic lymphocytic leukemia (61, 71, 73, 74). However, different tumors engage different mechanisms to reduce

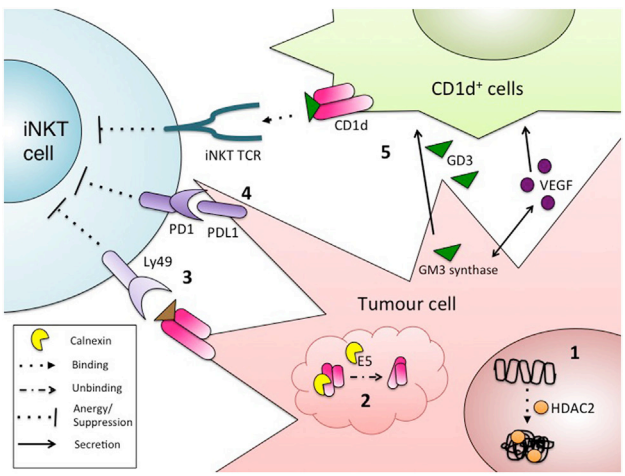

FIGURE 1 | Mechanisms of tumor evasion from invariant natural killer T (iNKT) immunosurveillance. Some tumors cells escape detection by iNKT cells *via* the regulation of surface CD1d, by: **(1)** heterochromatin formation at the CD1d locus by histone deacetylases (71, 72); or **(2)** improper folding and retention of CD1d in the ER (69). Other mechanisms to escape iNKT cell detection include: **(3)** engagement of surface CD1d with the inhibitory NK receptor Ly49, leading to the induction of iNKT cell anergy (70); **(4)** inhibitory signaling through PD1/PDL1 between iNKT cells and tumor cells (139); and **(5)** CD1d-dependent suppression of iNKT cells through presentation of the inhibitory, tumor-derived glycolipid GD3. GD3 production is also driven by the secretion of tumor-derived VEGF (75).

CD1d surface expression. On the RNA level, modulation of CD1d expression is largely driven by epigenetic changes. Treatment of mantle cell lymphoma cell lines with histone deacetylase inhibitors resulted in enhanced iNKT cell activation upon coculture (71). This observation was attributed to the removal of HDAC2 from the CD1d promoter, resulting in increased CD1d expression (71). Another report, which substantiates these findings, demonstrated that treating human and murine lung cancer and melanoma cell lines with HDAC2 inhibitors induce CD1d expression, although the functional relevance was not investigated (72). CD1d assembly in the endoplasmic reticulum (ER) is another potential target for tumor CD1d downregulation. In a model of HPV-driven cervical cancer, early-infected epithelial cells exhibited reduced CD1d expression compared to uninfected cells. In infected cells, the viral protein E5 inhibited calnexin, resulting in improper folding of CD1d, retention of CD1d molecules in the ER, and subsequent proteasomal degradation (69).

Modulation of iNKT cell function, even when CD1d molecules reach the surface of tumor cells, can contribute to evasion of iNKT surveillance. In the TRAMP murine model of prostate cancer, tumor cells express functional CD1d molecules, but lead to aberrant iNKT-cell activation akin to anergy, likely through the inhibitory receptor Ly49 (70). This phenotype could be rescued by simultaneous stimulation with αGC and IL-12, which likely overrides the inhibitory signal.

Tumor-derived factors can also inhibit trans-CD1d-dependent antigen presentation. When murine CD1d$^+$ fibroblasts were treated with human ascites from ovarian cancer patients, CD1d-dependent iNKT cell activation was markedly reduced, suggesting that a soluble factor released from ovarian tumors could affect CD1d-dependent activation (75). VEGF, a pro-angiogenic and pro-tumorigenic soluble factor, and the suppressive glycolipid antigen GD3, were identified as the factors present in the ascitic fluid inhibiting iNKT cell activation (75). Interestingly, the authors also showed that GD3 synthesis was dependent on VEGF-mediated upregulation of GM3 synthase in the ovarian cancer cells (75).

While these findings illustrate the importance of CD1d in mounting iNKT-driven antitumor immune responses, there exists at least one example where increased CD1d expression and tumor progression are positively correlated (76, 77). Through microarrays, immunohistochemistry, and patient statistics, enhanced CD1d expression was associated with increased malignancy and higher relapse rates in a subset of human renal cell carcinoma, clear cell renal carcinoma (76). This result serves as a rare example of enhanced CD1d expression as a predictor of tumor progression. It is possible that CD1d-dependent activation of suppressive type II iNKT cells, to be discussed later, might contribute to this phenotype (78, 79).

Both tumor cells and tumor-infiltrating immune cells are subject to microenvironmental stress due to nutrient deprivation, hypoxia, or accumulation of toxic products of catabolism (80). This suboptimal environment can lead to upregulation of autophagy, a survival-promoting pathway centered on lysosomal-recycling intracellular material (80). Tumor cells that engage the autophagy pathway become more robust and are able to better persist and metastasize (80). It has been recently shown that in murine bone marrow DCs, deletion of the autophagy regulator protein ATG5 led to increased CD1d-dependent antigen presentation, due to limited CD1d internalization (81). However, it has also been demonstrated that during thymic iNKT cell development, ATG5 is dispensable to CD1d expression (82, 83). More research is required to clarify the role of autophagy in CD1d expression, as perhaps this mechanism is cell and time dependent.

iNKT CELL ACTIVATION IN STERILE INFLAMMATION—CANCER

The modulation of CD1d expression in tumor cells provides strong evidence for the critical role of iNKT cells in mounting antitumor immune responses. However, it remains unclear how iNKT cells become activated in the context of cancer, a form of sterile inflammation. The characterization of a growing number of activating stimuli and pathways, some of which might affect lipid antigen presentation, sheds light on a number of mechanisms that might contribute to iNKT sterile activation in cancer (**Figure 2**).

At the intersection of chemotherapy and immunotherapy lies a class of drugs that provoke a type of cell death in tumor cells that results in the activation of innate immune cells. This type of cell death is termed immunogenic cell death (ICD). When cancer cells die, for example, by necrosis, they surface-expose or release molecules called danger-associated molecular patterns (DAMPs) that are usually contained within the cell, not on the cell surface or in the extracellular milieu (84). These DAMPs can be recognized by receptors on a variety of immune cells, including toll-like receptors (TLRs), and initiate an immune response (84).

One prototypic DAMP is a protein called high mobility group box 1 (HMGB1). HMGB1, ubiquitously expressed in a variety of cell types, typically resides in the nucleus as a chromatin binding protein (85). However, HMGB1 can be released into the extracellular environment where it behaves as a DAMP (85). Its release may be passive or mediated by an active mechanism. In the passive form, HMGB1 is released from cells dying by necrosis or other forms of ICD (86). In the active form, HMGB1 is secreted from myeloid cells, namely DCs and macrophages (87). In the extracellular milieu, HMGB1 can bind and signal through TLRs 2, 4, and the receptor for advanced glycation end products (RAGE) expressed on innate immune cells, triggering an immune response (86). RAGE is reportedly expressed on iNKT cells and can bind HMGB1, resulting in a Th17 activation profile (88). While the presence of TLRs on iNKT cells is disputed, most CD1d$^+$ cells also bear TLRs (46, 89–91). Viral signaling through TLR7 on human DCs has also been implicated in enhanced *de novo* synthesis of CD1d molecules (91). Furthermore, engagement of TLR4 on CD1d$^+$ myeloid cells, both murine and human, enhanced loading of self-lipid antigens onto CD1d molecules leading to iNKT cell activation (46, 90). These findings give rise to the possibility that HMGB1 signaling through TLR4 could induce loading of immunogenic self-lipid antigens onto CD1d, thus providing an explanation for iNKT cell activation in cancer.

Another soluble DAMP involved in ICD in cancer is adenosine triphosphate (ATP) (92, 93). ATP can interact with various immunomodulatory receptors and channels on myeloid cells and

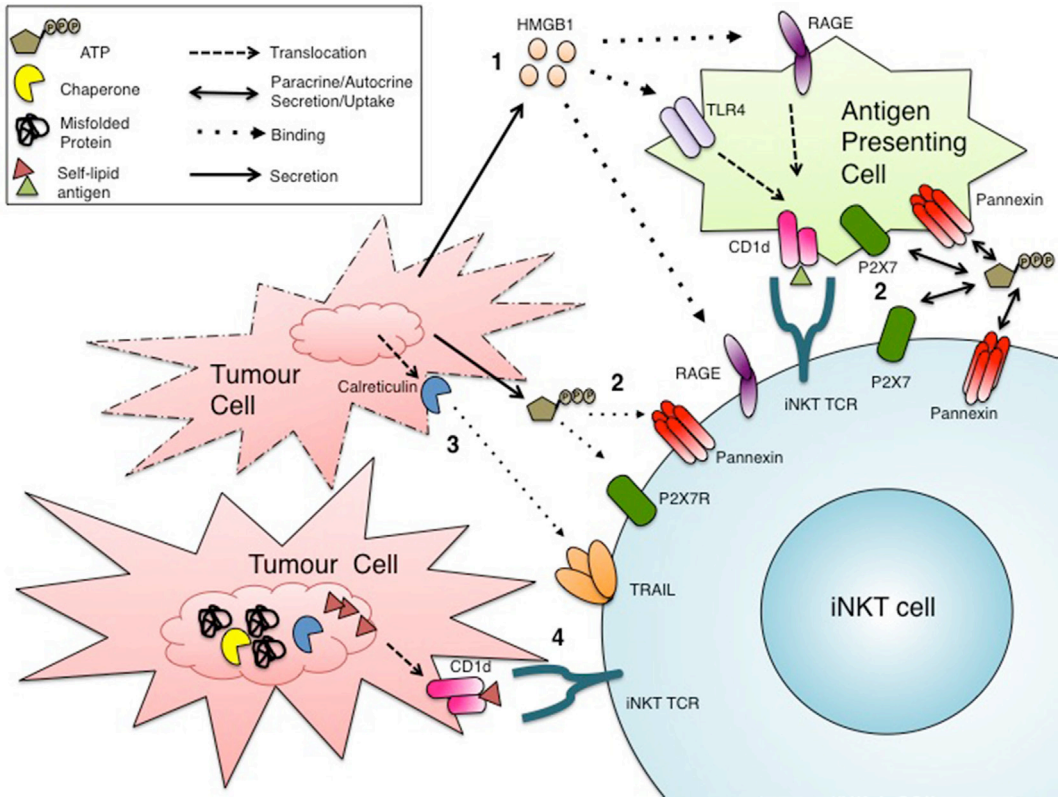

FIGURE 2 | Potential mechanisms of invariant natural killer T (iNKT) cell activation in cancer. Tumor cells subject to drugs or conditions that induce stress might activate iNKT cells through several pathways: **(1)** secretion or passive release of DAMPs, such as HMGB1, that bind RAGE receptors directly on iNKT cells or TLR4/RAGE receptors on CD1d+ antigen-presenting cells (APCs), leading to the presentation of an immunogenic self-lipid antigen (46, 88, 90); **(2)** paracrine (from APCs) or autocrine release of ATP into the extracellular environment for uptake by iNKT cells, ultimately leading to iNKT cell activation (94, 95); **(3)** binding of the surface DAMP calreticulin to TRAIL on iNKT cells (103–110); **(4)** induction of ER stress in CD1d+ cells, due to the suboptimal physiological tumor microenvironment, might trigger the alternate loading immunogenic self-lipid antigen(s), resulting in enhanced iNKT cell activation (111–113).

lymphocytes, a process termed purinergic signaling/regulation (94). Myeloid cells and T cells uptake extracellular ATP through P2X7 or pannexin channels, which can enhance inflammasome activation and amplify TCR-mediated activation, respectively (94). This mode of purinergic signaling might be especially relevant in the context of cancer, where stressed and necrotic tumor cells could release ATP into the tumor microenvironment and in turn augment TCR stimulation in response to weak tumor antigens (94). Like conventional T cells, iNKT cells bear P2XR and pannexins that allow uptake of extracellular ATP, which could provide additional costimulation to CD1d-mediated activation (95). iNKT cells also express two ectonucleotidases, CD39 and CD73 (95). CD39 converts ATP, a pro-inflammatory mediator to ADP, which is in turn converted by CD73 into AMP, an anti-inflammatory mediator (96). This step-wise generation of AMP from ATP is thought to cause a shift toward an immunosuppressive microenvironment, which might be advantageous for tumor progression (96). While the balance between extracellular ATP release and catabolism in the tumor microenvironment is poorly understood, we are gaining insights into mechanisms underlying purinergic signaling in iNKT cells.

The A2 adenosine receptor (A2AR), which binds adenosine to shift immune cells toward an immunosuppressive phenotype, also has a great influence on iNKT cell activation. In a model of concanavalin A-induced hepatitis, which is predominantly iNKT cell dependent, severity of the disease phenotype was dependent on the strength of A2A receptor signaling, with an exaggerated version of the disease seen in $A2AR^{-/-}$ mice, and an abrogation of the disease phenotype in mice treated with an A2AR agonist (97). It was later determined that A2AR exerts control over cytokine secretion in iNKT cells, particularly IL-4 and IL-10 (98).

Adenosine triphosphate is also involved in DC–iNKT cell interactions culminating in the release of inflammatory mediators that promote neutrophil recruitment. In monocyte-derived DC and iNKT cocultures, release of ATP from one or both of these immune cells (the secreting cell type was not identified) induced calcium flux within the DC through P2X7 signaling, which in turn triggered the release of prostaglandin E2 and soluble factors that promote neutrophil recruitment (99, 100). These observations were made in the setting of sterile inflammation, hinting that the influence of ATP in iNKT–DC interactions could hold true in the context of cancer (99). Maturation and stimulation of different immune cell populations by iNKT cells are thought to underlie their ability to induce antitumor immunity. This observation might be explained by the influence of purinergic

signaling leading to the recruitment of neutrophils and perhaps other cell types.

Another potentially relevant soluble factor is heat shock protein 70 (Hsp70) an ER-derived chaperone (101). Similar to HMGB1, Hsp70 is upregulated during a variety of stress conditions, and under extreme stress conditions that induce tissue injury or necrotic cell death, Hsp70 is released in the extracellular environment (101). Hsp70, or more specifically the Hsp70-derived 14-amino acid peptide, in combination with either IL-2 or IL-15, enhanced the expression of NK-activating receptors, including their expression on the surface of iNKT cells (102). It remains to be seen whether this observation drives iNKT cell activation in the context of cancer.

Invariant natural killer T cells upregulate a number of different receptors upon activation, including activation markers such as CD69 and CD25, and cytotoxicity molecules including FASL and TNF-related apoptosis-inducing ligand (TRAIL) (103). TRAIL, best known for inducing apoptosis in cells expressing TRAIL receptors such as DR4 and DR5, shares a high degree of homology with FASL in the extracellular binding motif (104). Although FASL-mediated killing is often indiscriminate due to rather ubiquitous expression of FAS on mammalian tissues, TRAIL-mediated cytotoxicity is more selective toward virally infected cells and tumor cells, making it a potential target in immunotherapy (104). In humans, TRAIL is upregulated on iNKT cells upon activation and is consequently able to induce apoptosis is acute myeloid leukemia (AML) cells (105), which bear TRAIL receptors (103). This finding is substantiated in an AML murine model (103). Upon αGC administration, iNKT-derived IFN-γ upregulated TRAIL expression on activated NK cells, which in turn limited the metastasis of liver and lung tumors (106). While TRAIL has a number of well-recognized receptors, a less characterized interaction is its binding and signaling through calreticulin. Under normal circumstances, calreticulin is retained in the ER where it acts as a chaperone. However, under conditions of extreme stress leading to ICD, as is often the case with tumor cells during chemotherapy, calreticulin can be translocated to the surface of dying cells (107). In fact, calreticulin is a marker of ICD and is considered a DAMP. Soluble TRAIL has been found to interact with calreticulin expressed on A375M melanoma cells (107). Furthermore, calreticulin exposure on malignant AML blasts is correlated with increased frequency of T lymphocytes and improved survival—a finding that complements TRAIL$^+$ iNKT cells' killing of AML cells, although in this cohort of AML patients iNKT cells were not investigated (108). While the link between calreticulin-TRAIL cognate interaction and iNKT-dependent tumor killing requires further corroborating research, it would potentially provide a molecular mechanism for the iNKT cell-mediated antitumor effects in a variety of cancers.

Cells subject to TRAIL-induced killing typically undergo apoptosis, regulated non-inflammatory cell death, or necroptosis, a form of regulated inflammatory cell death due to the release of DAMPs (109). Both apoptosis and necroptosis utilize RIPK1/3 signaling (109). In TRAIL-mediated cytotoxicity, the switch between the two types of death is dictated by the acidity of microenvironment. It is tempting to speculate that in the tumor microenvironment, in which nutrients are in short supply and hypoxia is a hallmark, the consequent acidic surrounding might shift TRAIL-mediated cell death towards necroptosis. Indeed, TRAIL-induced necroptosis can contribute to *in vitro* killing of human HepG2 liver and HT29 colon cancer cell lines. iNKT cells also induce TRAIL-mediated necroptosis in a ConA model of hepatitis (110). Although iNKT cells exert cytolytic functions via necroptotic signaling in target cells, aspects of the necroptotic signaling pathway are also essential in iNKT cells themselves.

RIPK3, a kinase involved in the transduction of the necroptotic signaling pathway, regulates iNKT cell activation independent of necroptosis, as RIPK3 knockdown iNKT cells exhibited impaired cytokine secretion, including IFN-γ, upon αGC stimulation (110). Furthermore, wild-type mice inoculated with B16 melanoma were able to clear the tumor burden upon administration of αGC, but RIPK3$^{-/-}$ mice were unable to do so, suggesting that RIPK3 is essential in iNKT-mediated anti-tumor responses (110). Further dissection of the mechanism involved illustrated that RIK3 signaling can induce the mitochondrial phosphatase PGAM5, which in turn upregulates NFAT translocation into the nucleus and stimulates the mitochondrial GTPase Drp1 (110). These factors appear to regulate TCR- and cytokine-mediated iNKT cell activation (110). These findings lay the foundation for a new pathway that can be manipulated in therapies centered on enhancing iNKT cell activation.

Cancer cells are subject to rapid cell division, leading to a reduction in available nutrients in the microenvironment and accumulation of nascent and/or mutated proteins. These suboptimal conditions, both intrinsic and extrinsic to the cell, compromise ER homeostasis and trigger the unfolded protein response (UPR) (111). Additionally, the UPR is likely triggered in immune cells in the suboptimal tumor microenvironment. UPR activation in malignant and infiltrating immune cells would alter lipid biosynthetic pathways (112). UPR activation might lead to sorting of self-lipid antigens onto CD1d complexes on CD1d$^+$ tumor or surrounding immune cells—which in conjunction with inflammatory cytokines might become immunogenic. It has been shown that the microsomal triglyceride transfer protein (MTTP) lies at the intersection between the UPR and CD1d–lipid complex formation (113). MTTP forms a heterodimer with protein disulfide isomerase (PDI) and transfers different lipid antigens onto assembling CD1d complexes in the ER. Importantly, PDIs are upregulated during UPR activation (112, 113). It is thus possible that ER-stressed CD1d$^+$ cells exhibit altered self-lipid loading, such that immunogenic self-lipid antigens are presented to and activate iNKT cells in the context of cancer and other forms of sterile inflammation (113).

SUPPRESSIVE NKT CELLS—A ROLE IN PROMOTING TUMOR PROGRESSION

While iNKT cells are generally thought to augment antitumor immune responses, there exist subsets of NKT cells that exhibit a regulatory phenotype, which in fact might hinder antitumor responses and promote tumor progression. One of the earliest reports suggesting the presence of regulatory NKT cells identified that IL-13 secreted from NKT cells could signal through the

IL4R-STAT6 pathways in cytotoxic T lymphocytes and consequently hinder their immunosurveillance of colon carcinoma and fibrosarcoma tumors (114). NKT cell-derived IL-13 can further drive impaired tumor immunosurveillance by inducing TGF-β secretion from a population of myeloid cells (115). These findings led to the identification of a Vα14Jα18$^-$ CD1d-restricted NKT population, dubbed type II NKT cells, which regulate and suppress antitumor immunity independent of IL-4, in contrast with the better-characterized Vα14Jα18$^+$ CD1d-restricted iNKT population, or type I iNKT cells, that augment antitumor immunity (79). However, in a murine model of osteosarcoma, CD1d-restricted NKT cells activated an immunoregulatory pathway independent of IL-13, IL4R-STAT6 signaling, and TGF-β, suggesting the existence of an alternative mechanism of NKT-mediated immunoregulation or different subsets of immunoregulatory NKT cells in different tumors (116). The regulatory contribution of type II NKT cells compared to classical Tregs was explored in a murine model of colorectal and renal cancers (117). This work indicated that type II NKT cells and classical Treg cells were equally essential in suppressing antitumor responses (117).

The different cytokine profiles between type I and type II iNKT cells were better characterized in a murine model of B-cell lymphoma, where type I iNKT secreted primarily IFN-γ, and type II iNKT cells secreted TGF-β and IL-13 (78). Furthermore, a balance between the two subsets allowed for adequate tumor immunosurveillance, as demonstrated by the enhanced mortality of tumor-bearing mice that are deficient in type I NKT cells, but retain type II NKT cells (78).

In exploring αGC-induced anergy of iNKT cells, a distinct subset of regulatory iNKT cells was identified, now termed NKT10 cells (118). As the name suggests, this population of NKT cells secretes IL-10 upon antigenic stimulation and is able to increase the tumor burden in mice challenged with B16 melanoma cells (118). In line with these results, a recent report noted that the absence of iNKT cells correlated with reduced number of intestinal polyps in a murine model of colorectal cancer (119). It was shown that IL-10 producing iNKT cells—reminiscent of the NKT10 cell subset—were enriched within polyps (119). Furthermore, these cells lack the NKT cell transcription factor PLZF, in keeping with recent findings that the PLZF is absent in regulatory iNKT cells in adipose tissue, where they secrete IL-2 and IL-10, control the number of classical Tregs, and promote M2 polarization of adipose-resident macrophages (120).

MANIPULATING iNKT CELLS IN CANCER IMMUNOTHERAPY

Given their essential role in antitumor responses, iNKT cells are suitable targets for cancer immunotherapy research. Most studies have mainly focused on the adjuvant behavior of iNKT cells, in particular efficient methods of αGC delivery, often in combination with tumor antigens, to trigger an all-encompassing immune response against the tumor. However, more recent studies have focused on harnessing iNKT cells in new, promising cancer immunotherapies.

Although αGC is a naturally occurring iNKT cell agonist, which enhances the adjuvant effect of iNKT cells in cancer, there is a focus on identifying stronger iNKT cell agonists, either by modifying αGC or identifying novel molecules based on medicinal chemistry programs. Such efforts are supported by the structural knowledge of CD1d bound to αGC and of the iNKT-TCR either in isolation or during cognate interaction with CD1d–lipid complexes (29, 121–123). In screening a panel of αGC analogs for enhanced iNKT cell activation, several iNKT cell agonists were characterized that produce a strong Th1 response from iNKT cells. One of such compounds features an aromatic ring (or, more specifically, a phenyl ring) within the acyl tail (124–127) and is currently entering clinical trials as a vaccine adjuvant (128).

Since the polar head group of CD1d-bound lipids is key for recognition by the iNKT-TCR, cellular enzymes that might either catabolize some iNKT-cell agonists or redirect them away from lipid–antigen presentation pathways might in part drive suboptimal iNKT cell responses. This observation has fueled work that led to the identification of a novel class of iNKT cell agonists that possess non-carbohydrate structures coupled to the ceramide moiety (129). One of these compounds, threitol-ceramide (ThrCer), which was shown to be very efficient in augmenting antigen-specific T cell responses and minimizing iNKT cell overstimulation and iNKT cell-dependent DC lysis, is capable of rectifying the deficiencies of αGC (130). Recent results have shown that incorporating the head group of ThrCer into a conformationally more restricted six-membered ring results in significantly more potent non-glycosidic analogs. In particular, Thr-6-Cer (IMM60) was found to promote strong antitumor responses and to induce a more prolonged stimulation of iNKT cells than does the canonical αGC, achieving an enhanced T-cell response at lower concentrations compared with αGC both *in vitro*, using human iNKT cell lines, and *in vivo*, using C57BL/6 mice (35). The synthetic non-glycolipid IMM60 is currently entering clinical trials in melanoma and non-small-cell lung cancer patients in combination with anti-PD1 blocking antibodies. In addition, given that the coupling of iNKT cell agonists with PLGA nanoparticles enhances their immune adjuvant potential by orders of magnitude (131), a phase I clinical trial in ovarian cancer and prostate cancer patients will be carried out with IMM60 conjugated to PLGA nanoparticles with full length NY-ESO-1 protein.

Efficient delivery of potent lipid iNKT cell agonists is essential in manipulating the adjuvant effects of iNKT cells. Optimizing delivery methods and combining the stimulatory lipid with tumor-specific antigens are critical to ensure that the adjuvanted immune response is targeted predominantly toward the tumor. In that vein, the use of exosomes as a means of codelivering αGC and ovalbumin has proved highly successful in reducing the tumor burden and increasing survival in mice inoculated with OVA-expressing melanoma compared with injection of soluble αGC and OVA together (132). Since exosomes naturally bear surface markers to direct them to a particular destination—and for this reason are utilized by breast cancer cells themselves to create a "metastatic niche" at a location of future metastasis (133)—they make excellent conduits for delivery of this potential "cancer

vaccine" directly to the tumor site, while perhaps protecting the contents from degradation during delivery. Synthetic nanoparticle delivery systems also hold great promise. In a construct similar to exosomes, delivery of αGC or TLR 3 and 7/8 agonists polyI:C and R848, and OVA in biodegradable poly(lactic-co-glycolic acid) nanoparticles proved efficient in stimulating $CD8^+$ antigen-specific T cell responses against OVA-B16 independent of $CD4^+$ T cell help (131). Encapsulation of the contents was essential, as injection of a mixture of αGC, TLR ligands, and OVA did not induce a comparable antitumor T cell response (131).

As previously mentioned, a major pitfall in using glycolipid antigens, specifically αGC, as adjuvants in cancer immunotherapy is the induction of iNKT cell anergy, as defined by reduced IFN-γ secretion upon secondary exposure. The delivery of αGC, typically as an injection of free lipid particles that might be taken up and presented by a variety of antigen-presenting cells, might contribute to this issue. An alternate delivery method would involve the intravenous injection of autologous DCs preloaded with αGC. In a clinical study involving five late-stage cancer patients, injection of αGC-loaded DCs led to the robust proliferation of iNKT cells, sustained IFN-γ secretion, and enhanced antigen-specific $CD8^+$ T-cell expansion *ex vivo*, as compared to injection of unpulsed DCs (134, 135). It is suggested that the extent of anergy induction is dependent on the type of antigen-presenting cells that present αGC, with B cells reportedly inducing a higher degree of anergy than DCs (135, 136). In addition to the augmented Th1 response upon injection of αGC-loaded DCs (137), perhaps upon secondary stimulation with αGC-pulsed DCs, anergic responses would be reduced. Alternatively, targeting iNKT cell agonists to DCs through nanoparticle formulations has been shown to overcome iNKT cell anergy (138).

The recent emergence of monoclonal antibody therapies to checkpoint regulators has revolutionized the field of cancer immunotherapy, particularly antibodies targeting the PD1–PD1L axis and CTLA4. While these therapies are studied predominantly in the context of $CD8^+$ cytotoxic T cells, iNKT cells are not exempt from their influence. Much like conventional T cells, iNKT cells upregulate PD1 on their cell surface upon activation as means of eventually resolving the immune response (139). Blockade of PD1 using anti-PD1 antibodies injected simultaneously with αGC results in iNKT cell activation and prevents iNKT cell anergy, a common occurrence after potent αGC stimulation (139–141). In fact, blockade of PD1 during αGC-mediated iNKT cell activation in a B16 melanoma mouse model leads to a persistent antimetastatic immune response (139).

Another emerging T cell-based cancer immunotherapy centers on the chimeric antigen receptor (CAR) T cell therapy. CAR T cell therapy works on the principle that genetically engineered $CD8^+$ T cells expressing TCRs specific for a tumor antigen fused to their native CD3 domain or modified with the endodomain of a costimulatory molecule can become activated and expand into a population of tumor-specific $CD8^+$ cytotoxic T cells (142). This approach has recently been applied to iNKT cells (143). A CAR specific for GD2 ganglioside, an abundant neuroblastoma antigen, was expressed in primary human iNKT cells (143). CAR.GD2 iNKT cells took on a Th1 profile and localized directly in the tumor site when transplanted in NSG mice (143). CAR.GD2 iNKT cells were highly cytotoxic against neuroblastoma cells, and when fused with CD28 and 41BB endodomains, they increased long-term survival in a murine model of the disease (143). With conventional T cells, a frequent adverse effect of CAR therapy in NSG mice is that adoptive transfer of the engineered T cells can induce graft-versus-host disease (GVHD) (143). However, there is no evidence of GVHD in *in vivo* models utilizing CAR.GD2 iNKT cells (143). For this reason, CAR iNKT cells might become an alternative to conventional T cells as vectors for CAR therapy. So far, however, CAR iNKT cell therapy has not been translated into clinical trials due to a poor understanding of the mechanisms underlying their *in vivo* proliferation and persistence (144). There have been no clear markers to differentiate effector and memory iNKT cells (144). Recently, a subset of iNKT cells that express the adhesion marker CD62L (also found in naïve and central memory T cells) has been identified (144). As expected, this population rapidly expands and can persist upon stimulation (144). In iNKT cells transduced to express a CD19.CAR, it was the $CD62L^+$ population that achieved persistent activation and proliferation *in vivo* and was responsible for lymphoma and neuroblastoma regression (144).

CONCLUSION

Recent results have indicated that therapies harnessing iNKT cells seem generally well tolerated by mice and humans. There are still many unanswered questions in the field of iNKT cell therapies that demand full investigation, such as the optimal route of administration, formulation of dosing intervals, etc. Although preclinical studies in animal models may help answer these questions, ultimately, appropriately designed clinical trials in humans will guide protocol optimization. Our ability to manipulate these cells in antitumor therapeutics is critically dependent on our understanding of iNKT cell biology, including the factors that activate and regulate these cells during sterile and non-sterile conditions; the strong immunomodulatory ability of iNKT cells begs the question as to whether their activation in cancer patients, in combination with immune check point inhibitors, can enhance the frequency and quality of neo-antigen tumor-specific $CD8^+$ and $CD4^+$ T cell responses. The identification, optimization, formulation, and clinical use of iNKT cell agonists that promote Th1 immune responses should be a high priority in future clinical trials.

AUTHOR CONTRIBUTIONS

MB wrote the manuscript, while MS and VC contributed to the writing and editing of the text.

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REFERENCES

1. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases – elimination, equilibrium and escape. *Curr Opin Immunol* (2014) 27:16–25. doi:10.1016/j.coi.2014.01.004
2. Tian Y, Sette A, Weiskopf D. Cytotoxic CD4 T cells: differentiation, function, and application to dengue virus infection. *Front Immunol* (2016) 7:531. doi:10.3389/fimmu.2016.00531
3. Das S, Chowdhury R, Ghosh S, Das S. A recombinant protein of *Salmonella typhi* induces humoral and cell-mediated immune responses including memory responses. *Vaccine* (2017) 35(35 Pt B):4523–31. doi:10.1016/j.vaccine.2017.07.035
4. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol* (2015) 16(11):1114–23. doi:10.1038/ni.3298
5. Salio M, Cerundolo V. Regulation of lipid specific and vitamin specific non-MHC restricted T cells by antigen presenting cells and their therapeutic potentials. *Front Immunol* (2015) 6:388. doi:10.3389/fimmu.2015.00388
6. Griewank K, Borowski C, Rietdijk S, Wang N, Julien A, Wei DG, et al. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* (2007) 27(5):751–62. doi:10.1016/j.immuni.2007.08.020
7. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13(2):101–17. doi:10.1038/nri3369
8. Gapin L. The making of NKT cells. *Nat Immunol* (2008) 9(9):1009–11. doi:10.1038/ni0908-1009
9. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: natural killer T cell defects and human disease. *Nat Rev Immunol* (2011) 11(2):131–42. doi:10.1038/nri2904
10. Jones EY, Salio M, Cerundolo V. T cell receptors get back to basics. *Nat Immunol* (2007) 8(10):1033–5. doi:10.1038/ni1007-1033
11. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci U S A* (2008) 105(32):11287–92. doi:10.1073/pnas.0801631105
12. Wesley JD, Tessmer MS, Chaukos D, Brossay L. NK cell-like behavior of Valpha14i NK T cells during MCMV infection. *PLoS Pathog* (2008) 4(7):e1000106. doi:10.1371/journal.ppat.1000106
13. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* (2003) 198(7):1069–76. doi:10.1084/jem.20030630
14. Beyaz S, Kim JH, Pinello L, Xifaras ME, Hu Y, Huang J, et al. The histone demethylase UTX regulates the lineage-specific epigenetic program of invariant natural killer T cells. *Nat Immunol* (2017) 18(2):184–95. doi:10.1038/ni.3644
15. Kawakami K, Yamamoto N, Kinjo Y, Miyagi K, Nakasone C, Uezu K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. *Eur J Immunol* (2003) 33(12):3322–30. doi:10.1002/eji.200324254
16. Nieuwenhuis EE, Matsumoto T, Exley M, Schleipman RA, Glickman J, Bailey DT, et al. CD1d-dependent macrophage-mediated clearance of *Pseudomonas aeruginosa* from lung. *Nat Med* (2002) 8(6):588–93. doi:10.1038/nm0602-588
17. Park SH, Kyin T, Bendelac A, Carnaud C. The contribution of NKT cells, NK cells, and other gamma-chain-dependent non-T non-B cells to IL-12-mediated rejection of tumors. *J Immunol* (2003) 170(3):1197–201. doi:10.4049/jimmunol.170.3.1197
18. Schmieg J, Yang G, Franck RW, Van Rooijen N, Tsuji M. Glycolipid presentation to natural killer T cells differs in an organ-dependent fashion. *Proc Natl Acad Sci U S A* (2005) 102(4):1127–32. doi:10.1073/pnas.0408288102
19. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* (2003) 171(10):5140–7. doi:10.4049/jimmunol.171.10.5140
20. Barral P, Polzella P, Bruckbauer A, van Rooijen N, Besra GS, Cerundolo V, et al. CD169(+) macrophages present lipid antigens to mediate early activation of iNKT cells in lymph nodes. *Nat Immunol* (2010) 11(4):303–12. doi:10.1038/ni.1853
21. Barral P, Eckl-Dorna J, Harwood NE, De Santo C, Salio M, Illarionov P, et al. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. *Proc Natl Acad Sci U S A* (2008) 105(24):8345–50. doi:10.1073/pnas.0802968105
22. De Santo C, Salio M, Masri SH, Lee LY, Dong T, Speak AO, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest* (2008) 118(12):4036–48. doi:10.1172/JCI36264
23. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* (2003) 198(2):267–79. doi:10.1084/jem.20030324
24. Galli G, Nuti S, Tavarini S, Galli-Stampino L, De Lalla C, Casorati G, et al. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *J Exp Med* (2003) 197(8):1051–7. doi:10.1084/jem.20021616
25. Galli G, Pittoni P, Tonti E, Malzone C, Uematsu Y, Tortoli M, et al. Invariant NKT cells sustain specific B cell responses and memory. *Proc Natl Acad Sci U S A* (2007) 104(10):3984–9. doi:10.1073/pnas.0700191104
26. Arora P, Baena A, Yu KO, Saini NK, Kharkwal SS, Goldberg MF, et al. A single subset of dendritic cells controls the cytokine bias of natural killer T cell responses to diverse glycolipid antigens. *Immunity* (2014) 40(1):105–16. doi:10.1016/j.immuni.2013.12.004
27. Jenkins MK, Moon JJ. The role of naive T cell precursor frequency and recruitment in dictating immune response magnitude. *J Immunol* (2012) 188(9):4135–40. doi:10.4049/jimmunol.1102661
28. Zajonc DM, Cantu C III, Mattner J, Zhou D, Savage PB, Bendelac A, et al. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat Immunol* (2005) 6(8):810–8. doi:10.1038/ni1224
29. Koch M, Stronge VS, Shepherd D, Gadola SD, Mathew B, Ritter G, et al. The crystal structure of human CD1d with and without alpha-galactosylceramide. *Nat Immunol* (2005) 6(8):819–26. doi:10.1038/ni1225
30. Brennan PJ, Tatituri RV, Brigl M, Kim EY, Tuli A, Sanderson JP, et al. Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals. *Nat Immunol* (2011) 12(12):1202–11. doi:10.1038/ni.2143
31. Cox D, Fox L, Tian R, Bardet W, Skaley M, Mojsilovic D, et al. Determination of cellular lipids bound to human CD1d molecules. *PLoS One* (2009) 4(5):e5325. doi:10.1371/journal.pone.0005325
32. Fox LM, Cox DG, Lockridge JL, Wang X, Chen X, Scharf L, et al. Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS Biol* (2009) 7(10):e1000228. doi:10.1371/journal.pbio.1000228
33. Muindi K, Cernadas M, Watts GF, Royle L, Neville DC, Dwek RA, et al. Activation state and intracellular trafficking contribute to the repertoire of endogenous glycosphingolipids presented by CD1d [corrected]. *Proc Natl Acad Sci U S A* (2010) 107(7):3052–7. doi:10.1073/pnas.0915056107
34. Yuan W, Kang SJ, Evans JE, Cresswell P. Natural lipid ligands associated with human CD1d targeted to different subcellular compartments. *J Immunol* (2009) 182(8):4784–91. doi:10.4049/jimmunol.0803981
35. Jukes JP, Gileadi U, Ghadbane H, Yu TF, Shepherd D, Cox LR, et al. Non-glycosidic compounds can stimulate both human and mouse iNKT cells. *Eur J Immunol* (2016) 46(5):1224–34. doi:10.1002/eji.201546114
36. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, et al. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* (1999) 189(7):1121–8. doi:10.1084/jem.189.7.1121
37. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* (1997) 278(5343):1623–6. doi:10.1126/science.278.5343.1623
38. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, et al. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem* (1995) 38(12):2176–87. doi:10.1021/jm00012a018
39. McCarthy C, Shepherd D, Fleire S, Stronge VS, Koch M, Illarionov PA, et al. The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. *J Exp Med* (2007) 204(5):1131–44. doi:10.1084/jem.20062342

40. Van Rhijn I, Godfrey DI, Rossjohn J, Moody DB. Lipid and small-molecule display by CD1 and MR1. *Nat Rev Immunol* (2015) 15(10):643–54. doi:10.1038/nri3889
41. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C III, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* (2005) 434(7032):525–9. doi:10.1038/nature03408
42. Zhou D, Mattner J, Cantu C III, Schrantz N, Yin N, Gao Y, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science* (2004) 306(5702): 1786–9. doi:10.1126/science.1103440
43. Porubsky S, Speak AO, Salio M, Jennemann R, Bonrouhi M, Zafarulla R, et al. Globosides but not isoglobosides can impact the development of invariant NKT cells and their interaction with dendritic cells. *J Immunol* (2012) 189(6):3007–17. doi:10.4049/jimmunol.1201483
44. Porubsky S, Speak AO, Luckow B, Cerundolo V, Platt FM, Grone HJ. Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. *Proc Natl Acad Sci U S A* (2007) 104(14):5977–82. doi:10.1073/pnas.0611139104
45. Speak AO, Salio M, Neville DC, Fontaine J, Priestman DA, Platt N, et al. Implications for invariant natural killer T cell ligands due to the restricted presence of isoglobotrihexosylceramide in mammals. *Proc Natl Acad Sci U S A* (2007) 104(14):5971–6. doi:10.1073/pnas.0607285104
46. Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, Sheehan KC, et al. Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity* (2007) 27(4):597–609. doi:10.1016/j.immuni.2007.08.017
47. Zeissig S, Murata K, Sweet L, Publicover J, Hu Z, Kaser A, et al. Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat Med* (2012) 18(7):1060–8. doi:10.1038/nm.2811
48. Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, et al. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* (2000) 12(2):211–21. doi:10.1016/S1074-7613(00)80174-0
49. Leite-De-Moraes MC, Hameg A, Arnould A, Machavoine F, Koezuka Y, Schneider E, et al. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J Immunol* (1999) 163(11):5871–6.
50. Tyznik AJ, Tupin E, Nagarajan NA, Her MJ, Benedict CA, Kronenberg M. Cutting edge: the mechanism of invariant NKT cell responses to viral danger signals. *J Immunol* (2008) 181(7):4452–6. doi:10.4049/jimmunol.181.7.4452
51. Torreno-Pina JA, Manzo C, Salio M, Aichinger MC, Oddone A, Lakadamyali M, et al. The actin cytoskeleton modulates the activation of iNKT cells by segregating CD1d nanoclusters on antigen-presenting cells. *Proc Natl Acad Sci U S A* (2016) 113(6):E772–81. doi:10.1073/pnas.1514530113
52. Crowe NY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med* (2002) 196(1):119–27. doi:10.1084/jem.20020092
53. Dashtsoodol N, Shigeura T, Ozawa R, Harada M, Kojo S, Watanabe T, et al. Generation of novel Traj18-deficient mice lacking Valpha14 natural killer T cells with an undisturbed T cell receptor alpha-chain repertoire. *PLoS One* (2016) 11(4):e0153347. doi:10.1371/journal.pone.0153347
54. Kammertoens T, Qin Z, Briesemeister D, Bendelac A, Blankenstein T. B-cells and IL-4 promote methylcholanthrene-induced carcinogenesis but there is no evidence for a role of T/NKT-cells and their effector molecules (Fas-ligand, TNF-alpha, perforin). *Int J Cancer* (2012) 131(7):1499–508. doi:10.1002/ijc.27411
55. Moreno M, Molling JW, von Mensdorff-Pouilly S, Verheijen RH, Hooijberg E, Kramer D, et al. IFN-gamma-producing human invariant NKT cells promote tumor-associated antigen-specific cytotoxic T cell responses. *J Immunol* (2008) 181(4):2446–54. doi:10.4049/jimmunol.181.4.2446
56. Silk JD, Hermans IF, Gileadi U, Chong TW, Shepherd D, Salio M, et al. Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. *J Clin Invest* (2004) 114(12):1800–11. doi:10.1172/JCI22046
57. Fujii S, Shimizu K, Hemmi H, Steinman RM. Innate Valpha14(+) natural killer T cells mature dendritic cells, leading to strong adaptive immunity. *Immunol Rev* (2007) 220:183–98. doi:10.1111/j.1600-065X.2007.00561.x
58. De Santo C, Arscott R, Booth S, Karydis I, Jones M, Asher R, et al. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat Immunol* (2010) 11(11):1039–46. doi:10.1038/ni.1942
59. Song L, Asgharzadeh S, Salo J, Engell K, Wu HW, Sposto R, et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. *J Clin Invest* (2009) 119(6):1524–36. doi:10.1172/JCI37869
60. Liu D, Song L, Wei J, Courtney AN, Gao X, Marinova E, et al. IL-15 protects NKT cells from inhibition by tumor-associated macrophages and enhances antimetastatic activity. *J Clin Invest* (2012) 122(6):2221–33. doi:10.1172/JCI59535
61. Gorini F, Azzimonti L, Delfanti G, Scarfo L, Scielzo C, Bertilaccio MT, et al. Invariant NKT cells contribute to chronic lymphocytic leukemia surveillance and prognosis. *Blood* (2017) 129(26):3440–51. doi:10.1182/blood-2016-11-751065
62. Bassiri H, Das R, Guan P, Barrett DM, Brennan PJ, Banerjee PP, et al. iNKT cell cytotoxic responses control T-lymphoma growth in vitro and in vivo. *Cancer Immunol Res* (2014) 2(1):59–69. doi:10.1158/2326-6066.CIR-13-0104
63. Bellone M, Ceccon M, Grioni M, Jachetti E, Calcinotto A, Napolitano A, et al. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. *PLoS One* (2010) 5(1):e8646. doi:10.1371/journal.pone.0008646
64. Fallarini S, Paoletti T, Orsi Battaglini N, Lombardi G. Invariant NKT cells increase drug-induced osteosarcoma cell death. *Br J Pharmacol* (2012) 167(7):1533–49. doi:10.1111/j.1476-5381.2012.02108.x
65. Dhodapkar KM, Cirignano B, Chamian F, Zagzag D, Miller DC, Finlay JL, et al. Invariant natural killer T cells are preserved in patients with glioma and exhibit antitumor lytic activity following dendritic cell-mediated expansion. *Int J Cancer* (2004) 109(6):893–9. doi:10.1002/ijc.20050
66. Hix LM, Shi YH, Brutkiewicz RR, Stein PL, Wang CR, Zhang M. CD1d-expressing breast cancer cells modulate NKT cell-mediated antitumor immunity in a murine model of breast cancer metastasis. *PLoS One* (2011) 6(6):e20702. doi:10.1371/journal.pone.0020702
67. Exley MA, Hou R, Shaulov A, Tonti E, Dellabona P, Casorati G, et al. Selective activation, expansion, and monitoring of human iNKT cells with a monoclonal antibody specific for the TCR alpha-chain CDR3 loop. *Eur J Immunol* (2008) 38(6):1756–66. doi:10.1002/eji.200737389
68. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* (2007) 121(1):1–14. doi:10.1111/j.1365-2567.2007.02587.x
69. Miura S, Kawana K, Schust DJ, Fujii T, Yokoyama T, Iwasawa Y, et al. CD1d, a sentinel molecule bridging innate and adaptive immunity, is downregulated by the human papillomavirus (HPV) E5 protein: a possible mechanism for immune evasion by HPV. *J Virol* (2010) 84(22):11614–23. doi:10.1128/JVI.01053-10
70. Nowak M, Arredouani MS, Tun-Kyi A, Schmidt-Wolf I, Sanda MG, Balk SP, et al. Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with alpha-galactosylceramide. *PLoS One* (2010) 5(6):e11311. doi:10.1371/journal.pone.0011311
71. Tiper IV, Webb TJ. Histone deacetylase inhibitors enhance CD1d-dependent NKT cell responses to lymphoma. *Cancer Immunol Immunother* (2016) 65(11):1411–21. doi:10.1007/s00262-016-1900-z
72. Yang PM, Lin PJ, Chen CC. CD1d induction in solid tumor cells by histone deacetylase inhibitors through inhibition of HDAC1/2 and activation of Sp1. *Epigenetics* (2012) 7(4):390–9. doi:10.4161/epi.19373
73. Spanoudakis E, Hu M, Naresh K, Terpos E, Melo V, Reid A, et al. Regulation of multiple myeloma survival and progression by CD1d. *Blood* (2009) 113(11):2498–507. doi:10.1182/blood-2008-06-161281
74. Zaborsky N, Gassner FJ, Asslaber D, Reinthaler P, Denk U, Flenady S, et al. CD1d expression on chronic lymphocytic leukemia B cells affects disease progression and induces T cell skewing in CD8 positive and CD4CD8 double negative T cells. *Oncotarget* (2016) 7(31):49459–69. doi:10.18632/oncotarget.10372
75. Tiper IV, Temkin SM, Spiegel S, Goldblum SE, Giuntoli RL II, Oelke M, et al. VEGF potentiates GD3-mediated immunosuppression by human ovarian cancer cells. *Clin Cancer Res* (2016) 22(16):4249–58. doi:10.1158/1078-0432.CCR-15-2518
76. Chong TW, Goh FY, Sim MY, Huang HH, Thike AA, Lim WK, et al. CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. *J Clin Pathol* (2015) 68(3):200–5. doi:10.1136/jclinpath-2014-202735
77. Vyth-Dreese FA, Sein J, van de Kasteele W, Dellemijn TA, van den Bogaard C, Nooijen WJ, et al. Lack of anti-tumour reactivity despite

enhanced numbers of circulating natural killer T cells in two patients with metastatic renal cell carcinoma. *Clin Exp Immunol* (2010) 162(3):447–59. doi:10.1111/j.1365-2249.2010.04274.x
78. Renukaradhya GJ, Khan MA, Vieira M, Du W, Gervay-Hague J, Brutkiewicz RR. Type I NKT cells protect (and type II NKT cells suppress) the host's innate antitumor immune response to a B-cell lymphoma. *Blood* (2008) 111(12):5637–45. doi:10.1182/blood-2007-05-092866
79. Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, et al. A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med* (2005) 202(12):1627–33. doi:10.1084/jem.20051381
80. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer* (2007) 7(12):961–7. doi:10.1038/nrc2254
81. Keller CW, Loi M, Ewert S, Quast I, Theiler R, Gannage M, et al. The autophagy machinery restrains iNKT cell activation through CD1D1 internalization. *Autophagy* (2017) 13(6):1025–36. doi:10.1080/15548627.2017.1297907
82. Salio M, Puleston DJ, Mathan TS, Shepherd D, Stranks AJ, Adamopoulou E, et al. Essential role for autophagy during invariant NKT cell development. *Proc Natl Acad Sci U S A* (2014) 111(52):E5678–87. doi:10.1073/pnas.1413935112
83. Pei B, Zhao M, Miller BC, Vela JL, Bruinsma MW, Virgin HW, et al. Invariant NKT cells require autophagy to coordinate proliferation and survival signals during differentiation. *J Immunol* (2015) 194(12):5872–84. doi:10.4049/jimmunol.1402154
84. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* (1994) 12:991–1045. doi:10.1146/annurev.iy.12.040194.005015
85. Muller S, Ronfani L, Bianchi ME. Regulated expression and subcellular localization of HMGB1, a chromatin protein with a cytokine function. *J Intern Med* (2004) 255(3):332–43. doi:10.1111/j.1365-2796.2003.01296.x
86. Yamazaki T, Hannani D, Poirier-Colame V, Ladoire S, Locher C, Sistigu A, et al. Defective immunogenic cell death of HMGB1-deficient tumors: compensatory therapy with TLR4 agonists. *Cell Death Differ* (2014) 21(1):69–78. doi:10.1038/cdd.2013.72
87. Dumitriu IE, Bianchi ME, Bacci M, Manfredi AA, Rovere-Querini P. The secretion of HMGB1 is required for the migration of maturing dendritic cells. *J Leukoc Biol* (2007) 81(1):84–91. doi:10.1189/jlb.0306171
88. Sharma AK, LaPar DJ, Stone ML, Zhao Y, Kron IL, Laubach VE. Receptor for advanced glycation end products (RAGE) on iNKT cells mediates lung ischemia-reperfusion injury. *Am J Transplant* (2013) 13(9):2255–67. doi:10.1111/ajt.12368
89. Askenase PW, Itakura A, Leite-de-Moraes MC, Lisbonne M, Roongapinun S, Goldstein DR, et al. TLR-dependent IL-4 production by invariant Valpha14+Jalpha18+ NKT cells to initiate contact sensitivity in vivo. *J Immunol* (2005) 175(10):6390–401. doi:10.4049/jimmunol.175.10.6390
90. Salio M, Speak AO, Shepherd D, Polzella P, Illarionov PA, Veerapen N, et al. Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. *Proc Natl Acad Sci U S A* (2007) 104(51):20490–5. doi:10.1073/pnas.0710145104
91. Raftery MJ, Winau F, Giese T, Kaufmann SH, Schaible UE, Schonrich G. Viral danger signals control CD1d de novo synthesis and NKT cell activation. *Eur J Immunol* (2008) 38(3):668–79. doi:10.1002/eji.200737233
92. Riteau N, Gasse P, Fauconnier L, Gombault A, Couegnat M, Fick L, et al. Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. *Am J Respir Crit Care Med* (2010) 182(6):774–83. doi:10.1164/rccm.201003-0359OC
93. Rodrigues RJ, Tome AR, Cunha RA. ATP as a multi-target danger signal in the brain. *Front Neurosci* (2015) 9:148. doi:10.3389/fnins.2015.00148
94. Idzko M, Ferrari D, Eltzschig HK. Nucleotide signalling during inflammation. *Nature* (2014) 509(7500):310–7. doi:10.1038/nature13085
95. Cekic C, Linden J. Purinergic regulation of the immune system. *Nat Rev Immunol* (2016) 16(3):177–92. doi:10.1038/nri.2016.4
96. Bastid J, Regairaz A, Bonnefoy N, Dejou C, Giustiniani J, Laheurte C, et al. Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity. *Cancer Immunol Res* (2015) 3(3):254–65. doi:10.1158/2326-6066.CIR-14-0018
97. Subramanian M, Kini R, Madasu M, Ohta A, Nowak M, Exley M, et al. Extracellular adenosine controls NKT-cell-dependent hepatitis induction. *Eur J Immunol* (2014) 44(4):1119–29. doi:10.1002/eji.201343866
98. Nowak M, Lynch L, Yue S, Ohta A, Sitkovsky M, Balk SP, et al. The A2aR adenosine receptor controls cytokine production in iNKT cells. *Eur J Immunol* (2010) 40(3):682–7. doi:10.1002/eji.200939897
99. Xu X, Pocock GM, Sharma A, Peery SL, Fites JS, Felley L, et al. Human iNKT cells promote protective inflammation by inducing oscillating purinergic signaling in monocyte-derived DCs. *Cell Rep* (2016) 16(12):3273–85. doi:10.1016/j.celrep.2016.08.061
100. Felley LE, Sharma A, Theisen E, Romero-Masters JC, Sauer JD, Gumperz JE. Human invariant NKT cells induce IL-1beta secretion by peripheral blood monocytes via a P2X7-independent pathway. *J Immunol* (2016) 197(6):2455–64. doi:10.4049/jimmunol.1600790
101. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* (2010) 10(12):826–37. doi:10.1038/nri2873
102. Hromadnikova I, Li S, Kotlabova K, Dickinson AM. Influence of in vitro IL-2 or IL-15 alone or in combination with Hsp 70 derived 14-mer peptide (TKD) on the expression of NK cell activatory and inhibitory receptors on peripheral blood T cells, B cells and NKT cells. *PLoS One* (2016) 11(3):e0151535. doi:10.1371/journal.pone.0151535
103. Nieda M, Nicol A, Koezuka Y, Kikuchi A, Lapteva N, Tanaka Y, et al. TRAIL expression by activated human CD4(+)V alpha 24NKT cells induces in vitro and in vivo apoptosis of human acute myeloid leukemia cells. *Blood* (2001) 97(7):2067–74. doi:10.1182/blood.V97.7.2067
104. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer* (2008) 8(10):782–98. doi:10.1038/nrc2465
105. Fisher-Owens SA, Gansky SA, Platt LJ, Weintraub JA, Soobader MJ, Bramlett MD, et al. Influences on children's oral health: a conceptual model. *Pediatrics* (2007) 120(3):e510–20. doi:10.1542/peds.2006-3084
106. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *J Exp Med* (2001) 193(6):661–70. doi:10.1084/jem.193.6.661
107. de Bruyn M, Wiersma VR, Helfrich W, Eggleton P, Bremer E. The ever-expanding immunomodulatory role of calreticulin in cancer immunity. *Front Oncol* (2015) 5:35. doi:10.3389/fonc.2015.00035
108. Fucikova J, Truxova I, Hensler M, Becht E, Kasikova L, Moserova I, et al. Calreticulin exposure by malignant blasts correlates with robust anticancer immunity and improved clinical outcome in AML patients. *Blood* (2016) 128(26):3113–24. doi:10.1182/blood-2016-08-731737
109. Jouan-Lanhouet S, Arshad MI, Piquet-Pellorce C, Martin-Chouly C, Le Moigne-Muller G, Van Herreweghe F, et al. TRAIL induces necroptosis involving RIPK1/RIPK3-dependent PARP-1 activation. *Cell Death Differ* (2012) 19(12):2003–14. doi:10.1038/cdd.2012.90
110. Kang YJ, Bang BR, Han KH, Hong L, Shim EJ, Ma J, et al. Regulation of NKT cell-mediated immune responses to tumours and liver inflammation by mitochondrial PGAM5-Drp1 signalling. *Nat Commun* (2015) 6:8371. doi:10.1038/ncomms9371
111. Bettigole SE, Glimcher LH. Endoplasmic reticulum stress in immunity. *Annu Rev Immunol* (2015) 33:107–38. doi:10.1146/annurev-immunol-032414-112116
112. Dougan SK, Salas A, Rava P, Agyemang A, Kaser A, Morrison J, et al. Microsomal triglyceride transfer protein lipidation and control of CD1d on antigen-presenting cells. *J Exp Med* (2005) 202(4):529–39. doi:10.1084/jem.20050183
113. Wang S, Chen Z, Lam V, Han J, Hassler J, Finck BN, et al. IRE1alpha-XBP1s induces PDI expression to increase MTP activity for hepatic VLDL assembly and lipid homeostasis. *Cell Metab* (2012) 16(4):473–86. doi:10.1016/j.cmet.2012.09.003
114. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* (2000) 1(6):515–20. doi:10.1038/82771
115. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* (2003) 198(11):1741–52. doi:10.1084/jem.20022227

116. Terabe M, Khanna C, Bose S, Melchionda F, Mendoza A, Mackall CL, et al. CD1d-restricted natural killer T cells can down-regulate tumor immunosurveillance independent of interleukin-4 receptor-signal transducer and activator of transcription 6 or transforming growth factor-beta. *Cancer Res* (2006) 66(7):3869–75. doi:10.1158/0008-5472.CAN-05-3421
117. Izhak L, Ambrosino E, Kato S, Parish ST, O'Konek JJ, Weber H, et al. Delicate balance among three types of T cells in concurrent regulation of tumor immunity. *Cancer Res* (2013) 73(5):1514–23. doi:10.1158/0008-5472.CAN-12-2567
118. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124(9):3725–40. doi:10.1172/JCI72308
119. Wang Y, Sedimbi S, Lofbom L, Singh AK, Porcelli SA, Cardell SL. Unique invariant natural killer T cells promote intestinal polyps by suppressing TH1 immunity and promoting regulatory T cells. *Mucosal Immunol* (2017). doi:10.1038/mi.2017.34
120. Lynch L, Michelet X, Zhang S, Brennan PJ, Moseman A, Lester C, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue. *Nat Immunol* (2015) 16(1):85–95. doi:10.1038/ni.3047
121. Gadola SD, Koch M, Marles-Wright J, Lissin NM, Shepherd D, Matulis G, et al. Structure and binding kinetics of three different human CD1d-alpha-galactosylceramide-specific T cell receptors. *J Exp Med* (2006) 203(3):699–710. doi:10.1084/jem.20052369
122. Borg NA, Wun KS, Kjer-Nielsen L, Wilce MC, Pellicci DG, Koh R, et al. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature* (2007) 448(7149):44–9. doi:10.1038/nature05907
123. Wun KS, Cameron G, Patel O, Pang SS, Pellicci DG, Sullivan LC, et al. A molecular basis for the exquisite CD1d-restricted antigen specificity and functional responses of natural killer T cells. *Immunity* (2011) 34(3):327–39. doi:10.1016/j.immuni.2011.02.001
124. Hung JT, Huang JR, Yu AL. Tailored design of NKT-stimulatory glycolipids for polarization of immune responses. *J Biomed Sci* (2017) 24(1):22. doi:10.1186/s12929-017-0325-0
125. Chang YJ, Huang JR, Tsai YC, Hung JT, Wu D, Fujio M, et al. Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids. *Proc Natl Acad Sci U S A* (2007) 104(25):10299–304. doi:10.1073/pnas.0703824104
126. Aspeslagh S, Li Y, Yu ED, Pauwels N, Trappeniers M, Girardi E, et al. Galactose-modified iNKT cell agonists stabilized by an induced fit of CD1d prevent tumour metastasis. *EMBO J* (2011) 30(11):2294–305. doi:10.1038/emboj.2011.145
127. Huang JR, Tsai YC, Chang YJ, Wu JC, Hung JT, Lin KH, et al. alpha-Galactosylceramide but not phenyl-glycolipids induced NKT cell anergy and IL-33-mediated myeloid-derived suppressor cell accumulation via upregulation of egr2/3. *J Immunol* (2014) 192(4):1972–81. doi:10.4049/jimmunol.1302623
128. Tefit JN, Crabe S, Orlandini B, Nell H, Bendelac A, Deng S, et al. Efficacy of ABX196, a new NKT agonist, in prophylactic human vaccination. *Vaccine* (2014) 32(46):6138–45. doi:10.1016/j.vaccine.2014.08.070
129. Tashiro T, Sekine-Kondo E, Shigeura T, Nakagawa R, Inoue S, Omori-Miyake M, et al. Induction of Th1-biased cytokine production by alpha-carba-GalCer, a neoglycolipid ligand for NKT cells. *Int Immunol* (2010) 22(4):319–28. doi:10.1093/intimm/dxq012
130. Silk JD, Salio M, Reddy BG, Shepherd D, Gileadi U, Brown J, et al. Cutting edge: nonglycosidic CD1d lipid ligands activate human and murine invariant NKT cells. *J Immunol* (2008) 180(10):6452–6. doi:10.4049/jimmunol.180.10.6452
131. Dolen Y, Kreutz M, Gileadi U, Tel J, Vasaturo A, van Dinther EA, et al. Co-delivery of PLGA encapsulated invariant NKT cell agonist with antigenic protein induce strong T cell-mediated antitumor immune responses. *Oncoimmunology* (2016) 5(1):e1068493. doi:10.1080/2162402X.2015.1068493
132. Gehrmann U, Hiltbrunner S, Georgoudaki AM, Karlsson MC, Naslund TI, Gabrielsson S. Synergistic induction of adaptive antitumor immunity by codelivery of antigen with alpha-galactosylceramide on exosomes. *Cancer Res* (2013) 73(13):3865–76. doi:10.1158/0008-5472.CAN-12-3918
133. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. *Nature* (2015) 527(7578):329–35. doi:10.1038/nature15756
134. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* (2005) 201(9):1503–17. doi:10.1084/jem.20042592
135. Carreno LJ, Saavedra-Avila NA, Porcelli SA. Synthetic glycolipid activators of natural killer T cells as immunotherapeutic agents. *Clin Transl Immunology* (2016) 5(4):e69. doi:10.1038/cti.2016.14
136. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* (2005) 115(9):2572–83. doi:10.1172/JCI24762
137. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* (2002) 3(9):867–74. doi:10.1038/ni827
138. Thapa P, Zhang G, Xia C, Gelbard A, Overwijk WW, Liu C, et al. Nanoparticle formulated alpha-galactosylceramide activates NKT cells without inducing anergy. *Vaccine* (2009) 27(25–26):3484–8. doi:10.1016/j.vaccine.2009.01.047
139. Durgan K, Ali M, Warner P, Latchman YE. Targeting NKT cells and PD-L1 pathway results in augmented anti-tumor responses in a melanoma model. *Cancer Immunol Immunother* (2011) 60(4):547–58. doi:10.1007/s00262-010-0963-5
140. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* (2009) 182(5):2816–26. doi:10.4049/jimmunol.0803648
141. Chang WS, Kim JY, Kim YJ, Kim YS, Lee JM, Azuma M, et al. Cutting edge: programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. *J Immunol* (2008) 181(10):6707–10. doi:10.4049/jimmunol.181.10.6707
142. Fesnak AD, June CH, Levine BL. Engineered T cells: the promise and challenges of cancer immunotherapy. *Nat Rev Cancer* (2016) 16(9):566–81. doi:10.1038/nrc.2016.97
143. Heczey A, Liu D, Tian G, Courtney AN, Wei J, Marinova E, et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* (2014) 124(18):2824–33. doi:10.1182/blood-2013-11-541235
144. Tian G, Courtney AN, Jena B, Heczey A, Liu D, Marinova E, et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. *J Clin Invest* (2016) 126(6):2341–55. doi:10.1172/JCI83476

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REVIEW
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Natural Killer T Cells: An Ecological Evolutionary Developmental Biology Perspective

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Type I natural killer T (NKT) cells are innate-like T lymphocytes that recognize glycolipid antigens presented by the MHC class I-like protein CD1d. Agonistic activation of NKT cells leads to rapid pro-inflammatory and immune modulatory cytokine and chemokine responses. This property of NKT cells, in conjunction with their interactions with antigen-presenting cells, controls downstream innate and adaptive immune responses against cancers and infectious diseases, as well as in several inflammatory disorders. NKT cell properties are acquired during development in the thymus and by interactions with the host microbial consortium in the gut, the nature of which can be influenced by NKT cells. This latter property, together with the role of the host microbiota in cancer therapy, necessitates a new perspective. Hence, this review provides an initial approach to understanding NKT cells from an ecological evolutionary developmental biology (eco-evo-devo) perspective.

Keywords: NKT cells, cancer immunotherapy, microbiota, infectious diseases, evolution

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INTRODUCTION TO TYPE I NKT CELLS

The evolutionary appearance of the vertebrate immune system equipped complex organisms with the ability to resist invasion by pathogenic microbes and to sense and respond to a loss of tissue integrity due to infection, aberrant cell growth, or mechanical injury. As organisms became increasingly more complex and lived beyond their fecund years, a finer ability to discriminate self from non-self was required (1, 2). Thus, the maintenance of homeostasis in such organisms requires the concerted action of multiple cell types that stand poised to respond to a hostile world filled with a seemingly endless array of infectious agents, toxic chemicals, and biologics. The first responders in this elaborate defensive network have historically been classified as members of the more archaic, multi-modular innate immune system. Should the innate defenses prove insufficient, the evolutionarily younger, adaptive immune system—consisting of B and T lymphocytes—is recruited to restore the homeostatic state. The quick-acting cells of the innate immune system senses an altered homeostatic state with pattern recognition receptors to detect conserved molecular structures shared by many pathogens alike (3, 4). By contrast, the slow-responding, adaptive immune system senses alterations in homeostasis by using diverse, clonally distributed B cell receptors (BCR and their secreted counterparts, antibodies), and T cell receptors (TCRs), respectively.

Bridging the gap between innate and adaptive immune responses are the innate-like B and T lymphocytes. These are a group of cells that express a relatively restricted repertoire of receptors generated through somatic recombination, yet unlike conventional T and B cells, exhibit innate-like recognition principles and functional responses (5). Innate-like lymphocytes include both T cells (γδ T cells, natural killer T cells, mucosal-associated invariant T lymphocytes, and CD8αα-expressing intestinal intraepithelial lymphocytes) and B cells (B-1 B cells and marginal zone B cells). The evolutionary appearance of this group of immune cells, including natural killer T cells (NKT cells) endowed upon vertebrates the capacity to initiate and amplify both the innate and adaptive immune responses. By virtue of their immunoregualtory functions, innate-like lymphocytes can fine-tune the nature and magnitude of these immune responses (6). Although each immune module plays a specific role, it is the controlled integration of multiple modules that results in an effective inflammatory response that is essential in maintaining a stable *milieu intérieur* (7).

NKT cells—originally defined as cells that co-express key natural killer (NK) cell surface markers and a conserved αβ TCR repertoire—are thymus-derived, innate-like T lymphocytes. The functions of NKT cells are controlled by self and non-self-lipid agonists presented by CD1d molecules (8). The majority of NKT cells (type I, invariant NKT) express an invariant TCR α-chain (Vα14Jα18 in mice; Vα24Jα18 in humans). The invariant α-chain pairs predominantly with Vβ8.2, Vβ7, or Vβ2 in mouse NKT cells, or Vβ11 almost exclusively in human NKT cells. A small NKT cell population—referred to as type II NKT cells—expresses a more diverse TCR repertoire and recognizes a distinct group of lipid antigens; these, however, are the focus of other reviews (9–14). The recognition of lipid agonists rapidly activates NKT cells, which respond just as quickly by secreting a variety of cytokines and chemokines, and upregulate costimulatory molecules. By acting promptly, NKT cells alert and regulate the effector functions of myeloid and lymphoid cells. In so doing, NKT cells play a critical role in controlling microbial and tumor immunity as well as autoimmune and inflammatory diseases (6, 15–17).

MULTIPLE MECHANISMS ACTIVATE NKT CELL

The functions of NKT cells are controlled by CD1d molecules. CD1d molecules bind to and present a variety of lipid ligands to reactive T cells (18). Numerous *in vitro* and *in vivo* studies using the synthetic lipid α-galactosylceramide (αGalCer, KRN7000) and its analogs (**Table 1** and references therein) has led to our current understanding of NKT cell biology. αGalCer is a natural product isolated from the marine sponge, *A. mauritianus*. The gut bacterium, *Bacteroides fragilis*, and the fungus, *Aspergillus fumigatus*, also biosynthesise αGalCers and/or related compounds (**Table 1** and references therein). Hence, αGalCer and related compounds may be more prevalent in nature than previously thought and the NKT cell biology so gleaned may be highly relevant.

αGalCer is a potent NKT cell agonist, which when presented by CD1d molecules directly activates NKT cells in a TCR-dependent manner without need for additional signals. This activation mechanism is considered TCR-dominated mode of NKT cell activation (**Figure 1**).

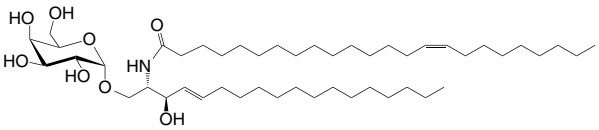
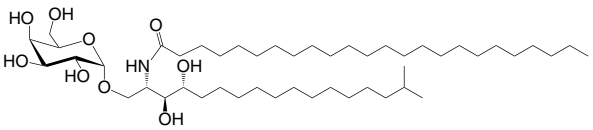
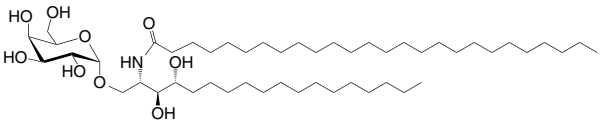
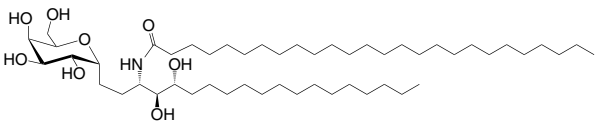
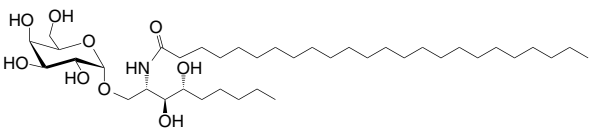
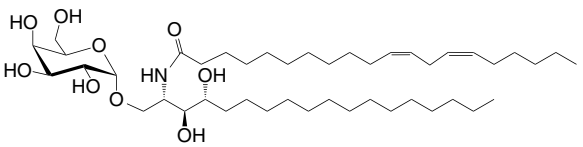
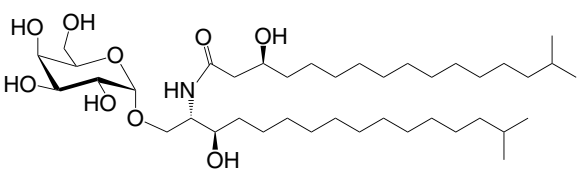
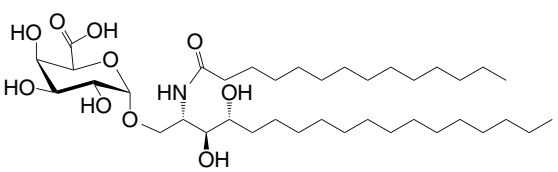
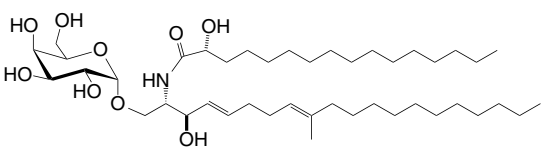
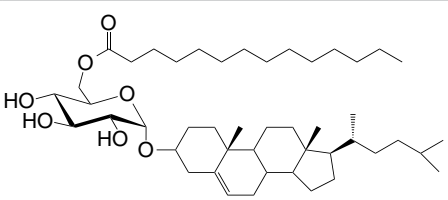
Sphingomonas spp. biosynthesises an αGalCer-related compound, α-galacturonosylceramide (αGalACer). Other weak NKT cell agonists include microbial glycosphingolipid [GSL; e.g., αGalCer-related asparamide B (*A fumigatus*)], diacylglycerolipids [e.g., α-galactosyl- (*Borrelia burgdorferi*—the agent of Lyme disease) and α-glucosyl-diacylglycerol (*Streptococcus pneumoniae*)] and cholesteryl-α-glycoside [e.g., cholesteryl-6-O-acyl α-glucoside (*Helicobacter pylori*)] (**Table 1** and references therein). Being a weak agonist, NKT cell activation by these microbial glycolipids requires a second activation signal from inflammatory cytokines. Such inflammatory cytokines result from dendritic cells (DCs) that are activated through their pattern recognition receptors (45–47). This activation mechanism is considered TCR- and cytokine-mediated mode of NKT cell activation (**Figure 1**)—a feature that is important for NKT cell activation by weak microbial and self-lipid agonists.

NKT cells react to CD1d molecules presenting self-lipids on host APCs in the presence of a second signal (6, 48). The inability to activate NKT cell hybridomas by using artificial APCs lacking βGlcCer synthase (49) and impaired NKT cell development in mice lacking βGlcCer synthase in their thymocytes (50), suggested that a cellular, βGlcCer-derived GSL is an endogenous mouse NKT cell agonist (49, 50). Several microbes—bacteria (e.g., *Staphylococcus aureus*, *Salmonella typhimurium, Listeria monocytogenes*, etc.), fungi (e.g., *A. fumigatus*) and viruses—activate NKT cells but do not biosynthesise NKT cell agonists. Such microbes induce the biosynthesis and/or presentation of self-lipids, which are thought to be mammalian αGalCer and perhaps iGb3 (19, 28, 35). As self-lipids are weak NKT cell agonists, NKT cell activation is bolstered by IL-12 secreted by DCs activated through dectin-1 DCs (31, 47) or toll-like receptor (TLR)-4 (45, 46). This activation mechanism is a variation on the TCR- and cytokine-mediated mode of NKT cell activation and a feature that is important for NKT cell activation by microbes that do not themselves biosynthesise an NKT cell agonist.

Type I interferon (IFN)—produced by DCs activated by the TLR9 ligand CpG—can serve as a second signal for NKT cell activation in conjunction with the presentation of sialylated cellular glycolipids by CD1d molecules (51). This finding is significant because almost all viral infections induce type I IFN response. Even though viruses do not biosynthesise NKT cell agonists, or any lipid for that matter, viral infections also activate NKT cells (52–62). Perchance, in such a circumstance, NKT cell activation occurs *via* the recognition of a self-lipid(s) presented by CD1d in the presence of inflammatory signals relayed by type I IFNs.

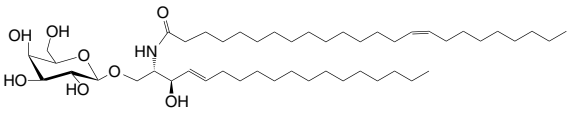
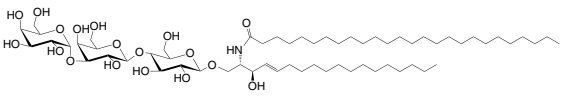
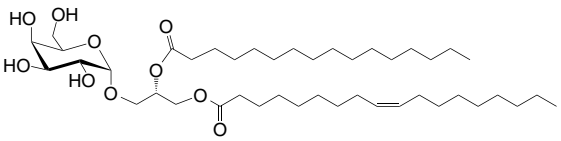
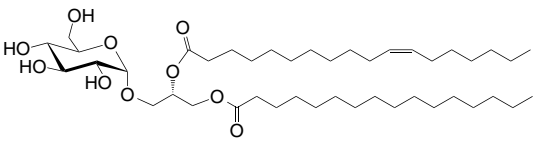
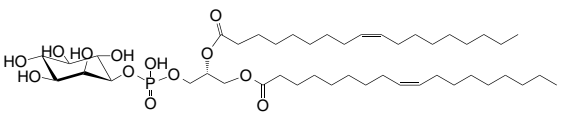
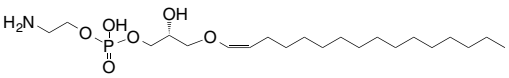
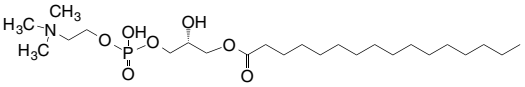
NKT cells are activated by the combined actions of IL-12 and IL-18. Under such conditions, NKT cell activation does not require the recognition of a CD1d-restricted agonist (63–65). This latter mechanism is considered cytokine-driven NKT cell activation (**Figure 1**). This mechanism is important for immunity to cytomegalovirus (65). Summarily, these multiple

TABLE 1 | Synthetic, microbial, and self NKT cell agonists—structures and properties.

Lipid (class)	Chain Length[a]	Structure	Agonist[b]	Reference
αGalCer (GSL)	C18; C24:1		IFN-γ, IL-4 self	(19)
Agel 9b (GSL)	C17 (C_{16}-Me); phyto C24		Anti-tumor; *Agelas mauritianus*	(20, 21)
KRN7000 αGalCer (GSL)	C18-phyto; C26		Very strong; robust IFN-γ IL-4 and other cytokines; synthetic analog of Agel 9b	(22)
αCGal-Cer (GSL)	C18-phyto; C26		Weak (mo)-to-none (hu); IFN-γ; synthetic	(23)
OCH (GSL)	C9-phyto; C24		Weak (mo)-to-none (hu); IL-4 (low-to-no IFN-γ); synthetic	(24)
C20-diene (GSL)	C18-phyto; C20:2		Strong; IL-4 (low-to-no IFN-γ); synthetic	(25)
αGalCer (GSL)	C17-C_3OH; C17		Stimulatory and inhibitory *Bacteroides fragilis*	(26, 27)
αGalU Cer (GSL)	C18-phyto; C14		Weak; *Sphingomonas* spp.	(28–30)
Asp B (GSL)	C20:2-C_9 Me; C16-C_2 OH		Weak; *Aspergillus fumigatus*	(31)
αGlc-6-acyl-Chol	C14		Strong; binds a small NKT cell subset (mo); *Helicobacter pylori*	(32)

(*Continued*)

TABLE 1 | Continued

Lipid (class)	Chain Length[a]	Structure	Agonist[b]	Reference
βGalCer (GSL)	C18; C24:1		Weak; self	(33, 34)
iGb3 (GSL)	C18; C24		Weak (mo)-to-none (hu); self	(35)
αGal-DAG (GGL)	*sn1*-C18:1; *sn2*-C16		Weak (mo)-to-none (hu); *Borrelia burgdorferi*	(36)
αGlc-DAG (GGL)	*sn1*-C18:1; *sn2*-C16		Weak; *Streptococcus pneumoniae*	(37)
PtdIno (GPL)	*sn1*-C18:1; *sn2*-C18:1		Week (mo)-to-no (hu); self	(38, 39)
Plasma-logen (GPL)	*sn1*-C16 vinyl-ether; *sn2*-lyso		Positive selection (mo); self	(40)
Lyso-PtdCho (GPL)	*sn1*-C16; *sn2*-lyso		Weak (hu)-to-none (mo); GM-CSF (no IL-4, IFN-γ); self	(41)

This table is adapted from Ref. (8, 42).

Agel, agelasphin; Asp B, asparamide B; Chol, cholesterol; DAG, diacylglycerol; GalCer, galactosylceramide; GalUCer, galacturonosylceramide; GlcCer, glucosylceramide; PtdCho, phosphatidylcholine; PtdIno, phosphatidylinositol; sn, stereo nomenclature for glycerolipids; GGL, glycoglycerolipid; GPL, glycerophospholipid; GSL, glycosphingolipid; mo, mouse; hu, human.

[a]*Sphingosine/phytosphingosine chain length indicated first and N-acyl chain length second.*

[b]*Agonist strength based on Ref. (43).*

modes of activation suggest that NKT cells have evolved many different mechanisms to sense an altered homeostatic state caused by microbial infections. How activated NKT cells steer downstream innate and adaptive immune responses is described below.

TRANSACTIVATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES BY ACTIVATED NKT CELLS

NKT cells form immune synapses upon recognition of lipid agonists presented by CD1d molecules displayed on APCs or planar membranes. The kinetics NKTCR/ligand interactions determine the functional outcome (66). Positive cooperative engagement of CD1d-lipid agonistic complexes by the NKTCR allows NKT cells to recognize subtle changes in cellular lipid content and to actuate a response (67). Upon activation, NKT cells rapidly polarize IFN-γ and lytic granules to the immune synapse to transmit an effector response (66, 68, 69). The synaptic transmission of effector molecules controls downstream innate and adaptive immune responses as described below.

Akin to the cells of the innate immune system (e.g., neutrophils, Mϕ, DCs, and NK cells), NKT cells respond within the first several hours of agonist recognition and secrete copious amounts of effector cytokines and chemokines (**Figure 2**). The nature of the activating NKT cell agonist controls the nature of the cytokine response (see **Table 1**). For example, the synthetic agonist αGalCer, within 30–90 min, elicits a wide variety of cytokines (**Figure 2**). Nonetheless, αGalCer variants containing different lipid chain length or unsaturation typically induce an IL-4 cytokine response (24, 25). By contrast, other αGalCer variants that have an altered glycosidic linkage, a chemically modified acyl-chain, or a modified sphingoid base, potently induce an IFN-γ response (**Table 1** and references therein). Thus, it is possible to steer desirable immune responses against cancers by harnessing

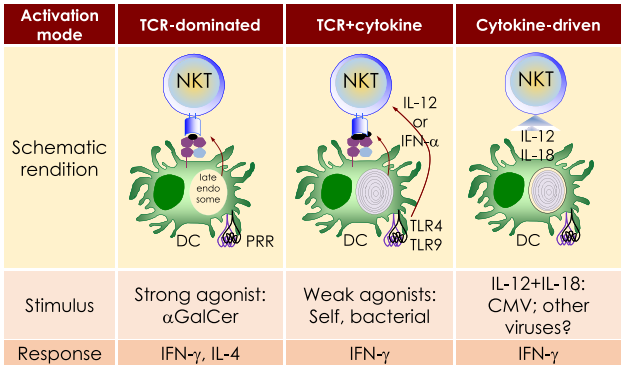


FIGURE 1 | Three distinct strategies activate mouse NKT cells. Potent NKT cell agonists—such as αGalCer—directly activate NKT cells without the need for a second signal, in a T cell receptor (TCR)-signaling dominated fashion (left panel). Alternatively, microbes containing toll-like receptor (TLR) ligands such as LPS activate NKT cells by inducing IL-12 production by DCs, which amplifies weak responses elicited upon the recognition of CD1d bound with self-glycolipids by the NKTCR. Several endogenous lipid agonists have been identified and characterized (see **Table 1**). Some microbes such as *Sphingomonas capsulata*, which are α-Proteobacteria, synthesize α-anomeric glycolipids for their cell walls. These glycolipids, when presented by CD1d, weakly activate NKT cells directly. In the presence of a second signal—generally a pro-inflammatory cytokine such as IL-12—such weak agonists strongly activate NKT cells (middle panel). Intriguingly, NKT cells can be activated solely by cytokines—mainly IL-12— in a TCR-independent manner (right panel). This diagram rendering the different strategies to NKT cells is an adaptation of past reviews (8, 44) and is based on works cited in the text.

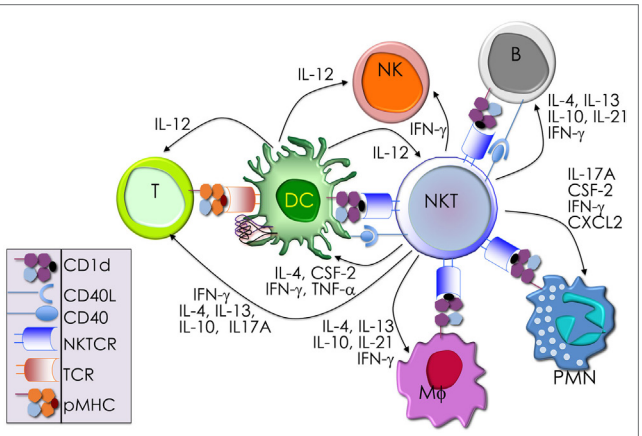


FIGURE 2 | The immunological effector functions of mouse NKT cells. The interactions between the invariant natural killer T (NKT) cell receptor and its cognate antigen, as well as interactions between costimulatory molecules CD28 and CD40 and their cognate ligands CD80/86 (B7.1/7.2) and CD40L, respectively, activate NKT cells. Activated NKT cells participate in crosstalk with members of the innate and the adaptive immune systems by deploying cytokine and chemokine messengers. Upon activation *in vivo*, NKT cells rapidly secrete a variety of cytokines and chemokines, which influence the polarization of $CD4^+$ T cells toward T helper (Th)1 or Th2 cells as well as the differentiation of precursor $CD8^+$ T cells to effector lymphocytes, and B cells to antibody-secreting plasma cells. Some of these mediators facilitate the recruitment, activation and differentiation of macrophages and dendritic cells (DCs), which results in the production of interleukin (IL)-12 and possibly other factors. Interleukin (IL)-12, in turn, stimulates NK cells to secrete interferon (IFN)-γ. Thus, activated NKT cells have the potential to enhance as well as temper the immune response. This schematic rendition of NKT cell effector functions is an adaptation of past reviews (6, 8, 44, 70) and is based on works cited in the text.

lipid agonists that induce therapeutic cytokine responses. This feature of αGalCer variants is further accentuated by the ability of activated NKT cell responses to transactivate cells of the innate and adaptive immune systems as narrated briefly below (see **Figure 2**).

Dendritic cells, especially $CD8\alpha^+$ DCs, which are a major producer of IL-12 (71), play a critical role in glycolipid agonist presentation and NKT cell activation (72–78). Activated NKT cells reciprocate by activating the interacting DCs. DCs so activated rapidly mature. Hence, they upregulate costimulatory molecules CD40, CD80, and CD86; several molecules critical for protein antigen capture and peptide presentation, such as DEC205 and MHC class II molecules (79); and induce the production of IFN-γ, tumor necrosis factor (TNF)-α, and IL-12 (80–83). IFN-γ produced by activated NKT cells coupled with CD154 (CD40 ligand on NKT cells) and CD40 (on DCs) mediate the NKT-DC crosstalk (81, 84). This crosstalk steers multiple downstream immune responses: (1) the number and phenotype of DCs after tumor induction (85). (2) IL-12 and IL-18 resulting from NKT-DC crosstalk transactivates NK cells to produce IFN-γ (82). (3) NKT-DC crosstalk can result in IL-4, IL-6, IL-13, and IL-21, which together can enhance B cells responses to protein antigens by B cells (86–93). (4) NKT-DC cross talk licenses DCs for antigen cross-presentation to $CD8^+$ T cells (94–96), and the activation and differentiation of CD4 and CD8 T cells (79, 95–97). Through these bidirectional interactions, NKT cells and DCs cooperate to amplify and direct both innate and adaptive immune responses. Hence, NKT cells are an attractive target for cancer immunotherapies (98–102).

IMPLICATIONS FOR CANCER IMMUNOTHERAPY

NKT cells have long represented an attractive target for tumor immunotherapy (103, 104). Numerous studies in both humans and mice have demonstrated their ability to directly target CD1d-expressing tumor cells (105–108), recruit and activate anti-tumor effector cells of the innate and adaptive immune systems (100, 109–114), and control the activity of immunosuppressive cells in the tumor microenvironment. After *in vivo* administration of αGalCer, NKT-DC cross-talk-mediated NK cell activation results in IFN-γ response (82) and, potentially, the anti-tumor effect of αGalCer (85, 115).

The potent anti-metastatic activity of αGalCer in mice (20, 116), which is NKT cell mediated (22), prompted investigations in the role of NKT cells in natural immunity against tumors. Such investigations include chemically induced tumors, transplanted tumors, and tumors arising in genetically engineered animals (115). The outcomes of these studies have been promising because NKT cells exhibit natural immunity against different cancer models. Independent studies have sometimes

reported conflicting results as to the importance of NKT cells in the anti-tumor response, particularly with carcinomas induced by the topical carcinogen methylcholanthrene (117, 118). Such conflicting results were likely due to unknown environmental and/or genetic factors present in the mice used as controls in similar experiments by different groups (117). Studies in mice revealed that αGalCer variants that induce type I inflammatory response (see **Table 1**) were protective against tumor metastases. The mechanistic basis of this anti-metastatic effect remains elusive. Nonetheless, the ability of NKT cells activated by αGalCer variants to steer desirable downstream effector functions, such as NK cells, cytotoxic T cells, Th1 and Th17 cells, γδ T cells, IFN-γ, and direct lysis of myeloid lineage cells may underlie the outcome (100, 115). The anti-tumor activities of NKT cell agonists have already been exploited in a variety of clinical trials. The outcomes of these trials have also been promising (103, 104, 119–121).

Genomic Control of NKT Cell Development

NKT cells development and maturation occurs in the thymus (122, 123). Thus, genetically altered mice in which thymocytes do not develop beyond the double-negative (DN)2/DN3 stage also fail to develop NK1.1$^+$ T cells (124). [Note: historically, prior to the development of CD1d-lipid tetramers (125, 126), NKT cells were identified by co-expression of the NK1.1 marker and a TCR. Hence, in pre-tetramer literature, they were referred to as NK1.1$^+$ T cells (127).] Thymic NK1.1$^-$ NKT cells were later recognized as a CD1d tetramer$^+$ NK1.1$^-$ subset that precedes NK1.1$^+$ NKT cells in development (128, 129). Current literature refers to the IFN-γ-producing, mature, stage 3 (st3) NKT cells as NK1.1$^+$ NKT cells (**Figure 3**). Furthermore, NKT cells do not develop in mice harboring mutations in genes (e.g., *Myb*, that encodes the transcription factor c-Myb, *Rorc*, which encodes RORγt, and *Tcf12* that codes for HEB) that impair survival of immature double-positive (DP) thymocytes—cells that co-express both CD4 and CD8 co-receptors— (130–133). Moreover, *Vα14* and *Jα18* rearrangement occurs at a late DP stage (130, 132). Consistent with this finding, NKT cells develop in NKT cell-deficient *Jα18*-deficient (Ja18$^{-/-}$) mice that receive highly purified tetramer-negative, DP-high thymocytes (134). These findings together support the notion that commitment to the NKT cell lineage occurs at the DP stage much alike conventional T cells (135). That notwithstanding, compelling new data indicate that *Vα14* and *Jα18* rearrangement can occur within CD4- and CD8-negative (DN) thymocytes. Additional data indicate that a fraction (~15%) of NKT cells that differentiate into NKT1 cells emerge from DN thymocytes (136). Hence, an alternative precursor can give rise to functional NKT cells.

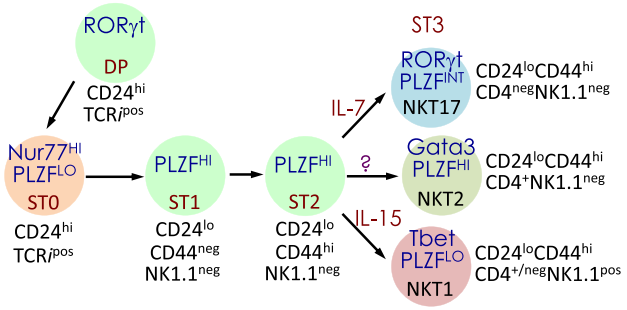


FIGURE 3 | Schematic rendition of mouse NKT cell developmental stages: precursor ST0, immature ST1 and ST2 and mature ST3 and NKT1, 2, and 17 are functional subsets. Early developmental steps are common to both NKT cell and conventional T cell lineages as commitment to the NKT lineage occurs at the CD4 and CD8 double-positive (DP) stage. NKT cell ontogeny begins with rearrangement of the *Vα14* to *Jα18* T cell receptor (TCR) α-chain gene segments and after its interaction with the positively selecting CD1d-self-lipid complex. Stage-specific NKT cell markers—e.g., CD24, CD44, and NK1.1—and subset-specific differentiation signals and transcription factors are indicated. Interleukin (IL)-7 and IL-15 are cytokines that mediate intercellular communication. NKTCR signaling turns-on the master transcription factor promyelocytic leukemia zinc finger (PLZF), which controls multiple molecular events that distinguish NKT cells from all of the other thymus-derived T lymphocytes. Additional molecular cues include Fyn and Lck, which are Src (cellular protein homologous to the *Rous sarcoma virus* oncogene) kinases (protein phosphorylation enzymes) essential for transmitting NKTCR signals from the plasma membrane to inside of the cell. Fyn also transmits signals relayed from SLAM (signaling lymphocyte activation molecule) through the adapter protein SAP (SLAM-associated protein). Protein kinase C (PKC)-θ processes NKTCR signaling and activates the transcription factor nuclear factor-κB (NF-κB). Other transcription factors, such as Egr-2, Ets-1, GATA3, Id2, Id3, MEF, Nur77, RORγt, and T-bet, some of which are also essential for functional differentiation of NKT cell subsets (refer to **Figures 4** and **6**) and act at distinct stages of NKT cell development. This diagrammatic rendition of NKT cell development is an adaptation of a past review (8) and is based on works cited in the text.

Positive selection of NK1.1$^+$ T cells depends on DP thymocytes (122). Developing NKT cell-DP thymocyte interactions involve both self-lipid-bound CD1d/NKTCR (22, 116, 137–139) and signaling lymphocytic activation molecule (SLAM)–SLAM interactions (140–142). These interactions are critical to NKT cell maturation, which involves protein kinase Cθ-NF-κB (143) and NFAT-Egr2 (144–146) activation downstream of the NKTCR, and SLAM-associated protein-Fyn activation downstream of SLAM (140, 141, 147, 148). Signals so transmitted from the cell surface are relayed through multiple signaling nodes in the cytoplasm and integrated in the nucleus into a unique transcriptional program (**Figure 3**). A key nuclear event involves the activation of the zinc finger BTB domain-containing-16 (*Zbtb16*) gene that codes for promyelocytic leukemia zinc finger (PLZF). The PLZF-mediated genomic control distinguishes the unique NKT cell functions from those of the other T lymphocytes (149, 150). NK1.1$^-$ NKT cells undergo several rounds of cell division, retaining some in the thymus with the remaining emigrating and populating the peripheral lymphatic organs. Thence, NK1.1$^-$ NKT cells mature to become NK1.1$^+$ NKT cells, both in the thymus and the periphery (**Figure 3**). A key feature of this maturation process is the acquisition of cytokine secretion function in a less well-understood mechanism (148) and the differentiation into three functional subsets: NKT1, NKT2, and NKT17 (discussed below). These NKT cell subsets marked by the same subset-specific transcription factors and cell surface markers expressed by the corresponding T helper cell subsets (151–156).

Gene regulatory networks (GRNs) are composed of *trans*-regulatory factors—generally made up of transcription factors and regulatory RNA such as microRNAs and long non-coding RNA—and *cis*-regulatory regions generally found upstream of

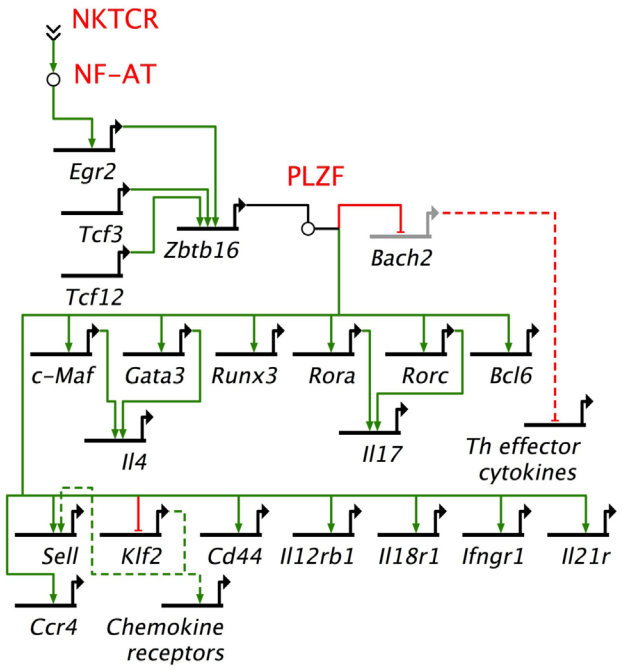


FIGURE 4 | Promyelocytic leukemia zinc finger (PLZF)-driven gene regulatory network directs innate effector function in mouse NKT cells. NKTCR stimulation by self-glycolipid antigen activates downstream nuclear factor of activated T cells (NF-AT), which activates *Egr2* gene. Egr2 is essential for *Zbtb16* gene expression downstream of NKTCR stimulation. PLZF encoded by *Zbtb16* activates T helper (Th) lineage-specific transcription factors, except *Tbx21*, which codes for the Th1 master regulator Tbet. Promyelocytic leukemia zinc finger (PLZF) also binds to multiple *cis*-regulatory elements to repress *Bach2*, which is a repressor of Th cytokine genes. In addition, PLZF binds to *cis*-regulatory elements of a variety of cytokine and chemokine receptor genes. *NF-AT, Tcf3, Tcf12, Egr2, Zbtb16, Bach2, c-Maf, Gata 3, Runx3, Rora, Rorc, Bcl6,* and *Klf2* encode transcription factors. Other genes under PLZF control encode effector proteins, mostly cytokines (e.g., *Il4*), cytokine receptor (*Il12Rb1, Ifngr1, Il21r*), chemockine (*Ccr4*) or cell adhesins [*Cd44*, *Sell* (L-selectin)]. *Bach2* represses the induction of T helper (Th) effector cytokine genes. Thick black lines, *cis*-regulatory elements of genes; solid green lines, enhancer; solid red lines, repressor; dashed green lines, indirect evidence for enhancement; dashed red lines, indirect evidence for repression. Based on Ref. (145, 149, 150, 158).

genes whereupon transcription factors bind to control lineage-specific gene expression. GRNs unveil the origins and evolution of cell lineages (157). Many transcription factors have been studied in relation to NKT cell development and function. Among these, PLZF works as a master transcription factor controlling the development of innate-like functions within NKT cells (**Figure 4**) (149, 150, 158). Mice harboring a loss-of-function PLZF mutation or lacking PLZF demonstrated poor NKT cell development, and those NKT cells that developed were NK1.1$^-$ and homed to lymph nodes but not to tissues such as thymus and liver where they are found abundantly in wild type (wt) mice (149, 150). Additional studies indicated that PLZF binds to *cis* elements of effector cytokine and homing receptor genes to direct their expression within NKT cells (**Figure 4**) (158). Furthermore, forced expression of a *Zbtb16* transgene in all T cells during thymic development resulted in the acquisition of an innate-like phenotype and function in conventional T cells (158). These findings heralded PLZF as a lineage-specific master regulator of transcription (149, 150, 158), and has led to the unveiling of a GRN that controls effector differentiation in developing NKT cells (**Figure 4**).

The induction of *Zbtb16* is controlled in part by acetylated Egr2 (159), which is induced downstream of NKTCR signaling (144). A recent study demonstrated that the gene encoding the histone acetylase GCN (general control non-derepressible) 5 acetylates a critical lysine residue in Egr2. DP thymocyte-specific depletion of GCN5 blocked the progression of NKT cell development from stage 0 to stage 1 in a cell intrinsic manner. This stage 0 to stage 1 developmental block was due to transcriptional downregulation of the lineage driving gene *Zbtb16* and other genes such as *Runx1, Tbx21,* and *Il2rb* that are essential for proper NKT cell development (159). GCN5 itself is an acetylated protein. Whether its function during NKT cell development depends on acetylation is currently unknown. In some models, the function of GCN5 depends on its deactylation (160). Should GCN5 function in NKT cells depend on deacetylation, whether and which sirtuins [silent mating type information regulation 2 homologs 1–7 (160)] play this role in NKT cells remains to be established.

Even though the mouse invariant Vα14i TCR α-chain has the potential to pair with virtually all available TCR β-chains, the peripheral NKT cell repertoire consists of Vα14i paired with a restricted set of β-chains, viz., Vβ8, Vβ7, and Vβ2 (161). There are two views to the events that sculpt this semi-invariant NKTCR repertoire: the predominant view is that such a semi-invariant NKTCR repertoire is built exclusively by positive selection (162). The competing hypothesis—that both positive and negative selections sculpt the semi-invariant NKTCR repertoire—is supported by indirect evidence (163–166).

Two lines of evidence support the notion that positive selection sculpts the NKT cell repertoire. CD1d molecules have a recycling motif in their cytoplasmic tail, which is essential for the endo/lysosomal exchange of CD1d-bound lipids and their subsequent presentation to NKT cells. Transgenic mice expressing a mutant CD1d molecule that has lost the ability to recycle do not develop NKT cells, suggesting that positive selection requires a recycling CD1d molecule (167). Another line of support comes from the study of CD1d-null mice, which contain a small number of CD1d-tetramer$^+$ thymocytes. These pre-selection thymocytes also express only the Vβ8, Vβ7, and Vβ2 β-chains expressed by the post-selection NKT cells. Such pre-selection thymocytes expand the same NKTCR repertoire when stimulated with a putative self-glycolipid called isogloboside-3 *in vitro* (35, 161). These lines of evidence support positive selection as the sole model for sculpting the NKT cell repertoire.

Deletion of the gene coding for NKAP (NF-κB activating protein) in DP thymocytes specifically blocks the development of NKT cells but not conventional T cells (168). NKAP colludes with HDAC3 (histone deacetylase 3) to function as a transcriptional repressor (169). Accordingly, deletion of the *Hdac3* gene in DP thymocytes completely blocks NKT cell development, while conventional T cell development proceeds normally (168). Hence, the repression of target genes at the DP thymocytes stage by the combined action of NKAP and HDAC3 is essential for positive selection of the NKT cell lineage.

Mouse subsets	NKT1	NKT2	NKT17	NKT10
Subset-specific transcription factor				
Surface markers	CD4$^{POS/NEG}$, IL-12R NK1.1 CXCR3, CXCR6	CD4, CD27, IL-25R NKI.1^{NEG}, IL-17RB CCR4, CXCR6	CD127^{HI}, IL-23R, NKI.1^{NEG} , CCR4, CCR6, CXCR6,	CD4, CD49a NKI.1^{NEG}, SLAMF6, PD1
Effectors	IFN-γ TNF-α, (IL-4) granzyme, perforin	IL-4, IL-13 IL-6, (Csf-2)	IL-17A IL-21, IL-22	IL-10 IL-2
Tissue location	Liver Spleen	Lungs Intestine	Lungs, skin Lymph nodes	Adipose tissue Spleen
Immunologic function(s)	Anti-tumor immunity	IL-25 induced AHR	Virus/ozone-induced AHR; tissue repair; limit systemic pathogen spread	Immune tolerance; resolve inflammation

FIGURE 5 | At least four mouse NKT cell subsets divide-up the labor. The four NKT cell subsets reflect T helper (Th)1, Th2, Th17, and Treg subsets. The transcription factors and the prototypic effector molecules as well as the locations and functions, which define the four NKT cell subsets, are represented. The text contains detailed description of each subset. This diagrammatic rendition of NKT cell subsets is an adaptation of a past review (8) and is based on works cited in the text.

Three lines of evidence support a potential role for negative selection in pruning self-reactive NKT cells for sculpting a functional repertoire: first, all available TCR β-chains can pair with the Vα14i TCR α-chain and react with CD1d tetramer, yet only Vβ8, Vβ7, and Vβ2 β-chains are expressed by the post-selection NKT cells (161). This finding can be explained only by negative selection of the majority of the β-chains and not by the failure to survive owing to the inability to interact with CD1d or to failed positive selection (38, 161). Second, transgenic over expression of either mouse or human CD1d in DP thymocytes and thymic myeloid cells results in fewer NKT cells and, those that remain, display altered Vβ usage (163, 170). Furthermore, only wt 16.5-day post-coitus mouse fetal thymic organ cultures (FTOCs), but not FTOCs from CD1d-overexpressing transgenic animals, fostered NKT cell development (163). Finally, exogenous addition of αGalCer, to wt mouse FTOCs resulted in NKT cell depletion (163, 164). Likewise, *in vivo* αGalCer injections into neonatal mice also resulted in the intra-thymic depletion of NKT cells (164). Together, these findings provide compelling evidence, albeit indirect, supporting a role for negative selection in sculpting a functional NKT cell repertoire.

Agonistic ligand(s)—those that positively select in the thymus being similar or identical to ligands that activate in the periphery (19, 27, 171)—selects NKT cells, which strikingly contrast antagonist ligand-mediated selection of conventional T cells. Further, SLAM–SLAM interactions, which activate PKC-θ *via* the SAP-FynT signaling module, mediate persistent interactions between developing NKT cells and the selecting DP cells (140, 141, 147, 172–175). NF-κB provides a survival signal to escape death that could result from these high affinity interactions (166, 176–182). Current evidence suggests that signals relayed through the TCR–PKCθ–CARMA1 axis are integrated by NF-κB to prevent death of developing NKT cells (143, 166, 183). But the signals relayed by the TCR-PKCθ-CARMA1 axis only partially accounts for such death signals. Consistent with this conclusion is the finding that TNF-α ligation of TNF receptor superfamily member 1a (TNFR1) relays caspase 8 and caspase 9 activation signals to mediate NKT cell death. This death signal is also obviated by NF-κB activation (183). Additional signals also mediate NKT cell survival during development (181, 184–192). Hence, escaping cell death from multiple signals may be a key feature of thymic NKT cell development. Whether this cell death is the basis for negative selection of NKT cells currently remains unknown.

NKT cells must tightly regulate NF-κB activation as mice that lack RelA or cannot activate NF-κB poorly develop NKT cells (143, 176, 177). On the other hand, mice that express overactive NF-κB or lack the negative regulator of NF-κB signaling CYLD, develop NKT cells but fail to mature and populate the lymphoid organs and peripheral tissues (181). Hence, NF-κB may function as a rheostat to set the threshold for peripheral NKT cell activation. Such a threshold may be critical as their selection and function are controlled by agonistic ligand(s) so as to prevent autoreactivity. How NF-κB functions as a rheostat in developing NKT cells needs elucidation.

NKT Cell Subsets, Frequency Variation, and Microbial Influences on Function: An Ecological Perspective

Recent findings on NKT cell developmental properties may be best understood from an ecological perspective. These properties include, (a) functional NKT cell subsets and the division of

labor; (b) NKT cell frequency variation; (c) tissue environment-dependent NKT cell subset frequency variation; and (d) gut microbiota-dependent peripheral NKT cell maturation and reciprocal NKT cell control over gut microbiota.

FUNCTIONAL NKT CELL SUBSETS AND THE DIVISION OF LABOUR

NKT cell activation results in rapid secretion of pro-inflammatory and regulatory cytokines and chemokines. This property in conjunction with the capacity to transactivate a variety of innate and adaptive immune cells—see subsection on Transactivation—allows NKT cells to steer downstream immune responses. NKT cells are heterogeneous, consisting of at least four distinct subsets—NKT1, NKT2, NKT10, and NKT17. In addition, at least one induced subset, NKTfh, is also recognized. As with conventional $CD4^+$ T cell subsets, NKT cell subsets are characterized by prototypic cytokine responses and subset-specific transcription factors (**Figure 5**). Each subset is represented at different proportions in various mouse strains (151–155).

MOUSE NKT1 CELLS are marked by either the expression of CD4 or the absence of CD4/CD8 co-receptors. NKT1 cell activation results in a Th1-like cytokine response. The majority of mouse splenic and hepatic NKT cells are NKT1 subset, especially in the C57Bl/6 strain. NKT1 cell differentiation depends on T-bet (*Tbx21*) and IL-15 but less on GATA3 (151, 152, 187, 189–191). Unlike HDAC3 depletion in DP thymocytes, NKT cell lineage-specific deletion of *Hdac3* (derived with the use if *Zbtb-Cre*) results in selective impairment in NKT1 cell development. The selective absence of HDAC3 in NKT cells resulted from reduced autophagy (193–195)—a cytoplasm recycling process essential to T and NKT cell development —and decreased GLUT1, CD71, and CD98 nutrient receptor expression (196). Moreover, the anti-tumor effect of αGalCer (109) is potentially mediated by IFNγ- and TNFα-producing NKT1 cells.

MOUSE NKT2 CELLS express the CD4 co-receptor. NKT2 cell actiation results in a Th2-like cytokine and chemokine response. This subset is enriched in mouse lungs and the intestine (152). IL-13 and IL-4 as well as CCL17, CCL22, CCL10/CCL6, and eosinophil chemotactic factor-L secreted by activated NKT2 cells may mediate airway hyperresponsiveness (151, 197–200). This Th2-type response recruits Mϕs, eosinophils, neutrophils, and lymphocytes into the lungs to incite tissue damage (197). Coincidently, in BALB/c mouse that is sensitive to airway hyper-responsiveness, NKT2 cells predominate (152).

NKT cells constitutively express *Il4* and *Ifng* transcripts. This constitutively expressed cytokine genes may explain the rapid NKT cell response to agonistic stimulation *in vivo* (201). Epigenetic changes in the two cytokine genes control their transcription. For example, the conserved non-coding sequence (CNS) 2 located downstream of the mouse *Il4* locus is constitutively active in NKT cells, which thereby constitutively transcribe the *Il4* gene. CNS 2 activity depends on NOTCH and Rbp-j (recombination signal binding protein for immunoglobulin kappa J region)—a transcriptional regulator of NOTCH signaling. Hence, DP thymocyte-specific deletion of *Rbp-j* abolished CNS 2 activity and the ability to transcribe *Il4* (202).

A similar epigenetic control of the human *Ifng* locus using CNS-30 and CNS +18–20 transcribes the *Ifng* locus in NKT cells (203, 204). Consistent with this finding, NKT cells showed acetylated histone 4 marks upstream and downstream of the *Ifng* coding region only when activated by weak (self agonists) or strong signals (phorbolmyristate acetate + ionomycin) but not in resting NKT cells. Furthermore, NKT cells rested after stimulation returned the *Ifng* locus to an unmarked state (205). H4 acetylation occurs at CNS +18–20, a site essential for human *Ifng* transcription in NKT cells and conserved within the mouse *Ifng* locus (203, 205). These findings notwithstanding, it is unclear whether human NKT cells constitutively transcribe the *Ifng* locus and how mouse NKT cells constitutively transcribe its *Ifng* locus.

MOUSE NKT17 CELLS do not express CD4 or CD8 co-receptors. They are enriched in the lungs, skin, and peripheral lymph nodes, and are poorly represented in the spleen and liver (206–208). These cells require IL-7, not IL-15, for survival (151, 209). The development of NKT17 cells also requires mTORC2 signaling and the transcription factors Runx1 and NKAP (168, 210–213). Thus, NKT cell-specific *Runx1* deletion results in decreased IL-7Rα, BATF, and c-Maf expression against the backdrop of increased Lef and Bcl11b expression (211). On the other hand, how NKAP controls NKT17 cell development is not understood, but appears not to require mTOR, IL-7, and TGF-β signaling (210).

Akin to Th17 cells, NKT17 cells constitutively express RORγt (206), rapidly produce IL-17A in response to certain bacterial infections, and induce airway neutrophilia when challenged with synthetic glycolipid or LPS (37, 206, 214). NKT17 cells may contribute to ozone-induced airway hypersensitivity (215), the development of experimental autoimmune encephalomyelitis (214), and the pathogenesis of acute hepatitis in mice (216).

MOUSE NKT10 CELLS, the PLZF-independent subset (154), are found in low frequency in unchallenged mice and in human peripheral blood mononuclear cells (PBMCs). Upon re-activation, NKT10 cells that previously responded to αGalCer *in vivo*, secrete IL-10 (155). IL-10 produced by activated NKT10 is thought to maintain immune-privilege sites. This NKT cell subset may also control Treg cell functions in adipose tissues (154).

Mouse NKT cells can provide cognate (lipid antigens) or non-cognate (protein antigens) help to B cells and regulate antibody responses (89, 90, 92, 217, 218). Upon immunization with αGalCer a subset of NKT cells acquire a phenotype similar to T follicular helper T cells (TFH) referred to as NKT follicular helper (NKTFH) cells (218–220). NKTFH are characterized by the expression of CXCR5, ICOS, PD1, Bcl6, and BTLA. Their development is dependent on same factors that drive TFH development (219). NKTFH cells induce rapid production of germinal centers through IL-21 production that yields detectable levels of antigen-specific IgG (91, 219, 220). Nonetheless, NKTFH cell-induced antibody responses are short-lived and inferior to TFH cell-induced responses (91, 219, 220). NKTFH cells may play a role in antibody responses against human pathogens such as *Borrelia hermsii*, *Streptococcus pneumoniae*, and *Plasmodium falciparum* (91, 219, 220). NKTFH and TFH cells can act synergistically to

induce robust antigen-specific antibody responses underscoring the use of αGalCer as a vaccine adjuvant (218).

Human NKT cell responses are as diverse as those of mouse (221), yet NKT cell subsets have not been formalized in humans. Functional dichotomy has been reported in human $CD4^+$ and DN NKT cell subsets: activated human $CD4^+$ NKT cells secrete IL-4. A pathological role has been attributed to human $CD4^+$ NKT cells, which accumulate in the lungs of chronic asthmatic patients and produce IL-4 and IL-13 (222). Hence, human $CD4^+$ NKT cell resembles the mouse NKT2 cell subset. On the other hand, the activated DN NKT cells secrete IFN-γ and TNF-α. Furthermore, both CD4 and DN human NKT cell subsets upregulate perforin in the presence of inflammatory signals. The DN NKT cells also upregulate NKG2D expression, which together with perforin may mediate cytotoxicity against infected cells and cancer cells (223, 224). These functions of human NKT cells resemble those of mouse NKT1 cells. Activated human NKT cells can also secrete IL-17 (221), suggesting the presence of an NKT17-like subset.

In summary, mouse NKT cells divide labor into four subsets. Global and single cell transcriptome analyzes demonstrated that the thymic NKT1, NKT2, and NKT17 cells were distinct subsets (156, 225). Even though not formalized, human NKT cells also have the potential to mirror mouse NKT cell subsets, but this requires further investigation. That the tissue environment plays a role in the differentiation of NKT cell subsets is supported by the finding that NKT17 differentiation required mammalian target of rapamycin complex-2 (213) or is suppressed by Tet enzymes that modify 5-methylcytosine in DNA by controlling the expression of Tbet and ThPOK transcription factors (226). Another study using somatic cell nuclear transfer to generate mice with monoclonal NKT cell populations demonstrated that tissue homing pattern, and, to a lesser extent, TCR avidity governed NKT cell subset differentiation (208). That NKT1, NKT2, and NKT17 cells differentiated within peripheral tissues of each of the three monoclonal mouse lines, derived from somatic cell nuclear transfer, suggests that the subsets are perhaps NKT cell "reaktionsnorm [German for reaction norm or norm of reaction; Woltereck 1909 cited in Ref. (227)]" induced by the tissue-specific environment, potentially by local cytokine/chemokine milieu in conjunction with the host microbiota.

NKT CELL FREQUENCY VARIATION

An intriguing property of NKT cells is their frequency variation observed in lymphoid tissues of different inbred strains of similar age: low in 129 and NOD, intermediate in C57Bl/6, and high in BALB/c, CBA, and DBA/2 mice (152, 153, 228–230). Likewise, NKT cells show striking frequency variation that can range from as little as 0.001% to 5–10% within human PBMCs (221, 231, 232).

Mice show inter-strain variation in thymic NKT cell subset numbers (152). C57Bl/6 mice have high proportion of NKT1 cells and low frequency of NKT2 cells, whereas BALB/c have high frequency of NKT2 and NKT17 suggesting an inverse correlation between frequency of NKT1 cells versus NKT2 cells and mouse strains. Curiously, mouse strains that have a high frequency of NKT2 cells (BALB/c, CBA, and DBA/2) showed high numbers of eomesodermin-expressing memory-like $CD8^+$ thymocytes (152) which was attributed to the steady-state production of IL-4 by the expanded NKT2 population in these mice. In an effort to understand whether genetic polymorphisms between mouse strains controlled NKT cell frequency, recombinant inbred and co-isogenic strains begotten from NOD (low NKT cell frequency) X C57Bl/10 (intermediate NKT cell frequency) crosses were analyzed. The outcomes of several such studies indicated that NKT cell frequency segregated with the genetic background of the mouse (153, 229, 230). Whereas this outcome suggests that NKT cell frequency is under genetic control, whether this control is direct or indirect remains to be ascertained.

DEVELOPMENTAL SYMBIOSIS: GUT MICROBIOTA-DEPENDENT PERIPHERAL NKT CELL FREQUENCY AND NKT CELL CONTROL OVER GUT MICROBIOTA

NKT cells surveil barrier mucosae such as that of the small and large intestine (233, 234). The number, phenotype, and functional maturation of NKT cells in the gut epithelium and lamina propria are controlled by neonatal colonization of the gut by bacterial symbionts. Thus, germ-free (GF) mice have high numbers of NKT cells in the gut epithelium and lamina propria that are immature and, hence, hypo-responsive to αGalCer (233). Curiously, reconstitution of young, but not adult mouse gut by bacteria that biosynthesise αGalCer or related compounds reverses the hypo-responsiveness of NKT cells found in GF intestinal mucosae (234). Similarly, GF mice also harbor high hepatic and pulmonary, but not thymic and splenic NKT cell frequencies (234). Additional evidence implicates the CXCR6 ligand CXCL16, whose expression is under the control of gut microbiota, in regulating gut NKT cell frequency and maturation (234, 235). Furthermore, αGalCer compounds (see **Table 1**) synthesized by the bacterial symbiont *Bacteriodes fragilis*, exert either an inhibitory effect preventing proliferation, or are stimulatory on developing NKT cells (26, 27). As the gut microbiota varies between individuals of different genetic, ethnic, and geographic backgrounds (236), the above findings in mice suggest the intriguing possibility that the human symbionts may impart an epistatic control over human NKT cell frequency and maturation as well. Because the frequency and functional status are environmentally controlled even though the genotype of the differentiating NKT cells remains the same, NKT cell frequency and proper maturation are potentially polyphenic (227, 237) properties.

Early-life microbial ecology has implications for health. Thus, GF mice are prone to severe airway hypersensitivity and dextran sodium sulfate-induced colitis (233–235). The latter phenotype is obviated by the interaction of NKT cells with *B. fragilis*-derived glycosphingolipid(s) during early life (26). Not surprisingly, NKT cells can, in turn, control gut microbial ecology and gut physiology (238). Whether similar reciprocal interactions between NKT cells and the gut microbiota occur in humans currently remains unknown.

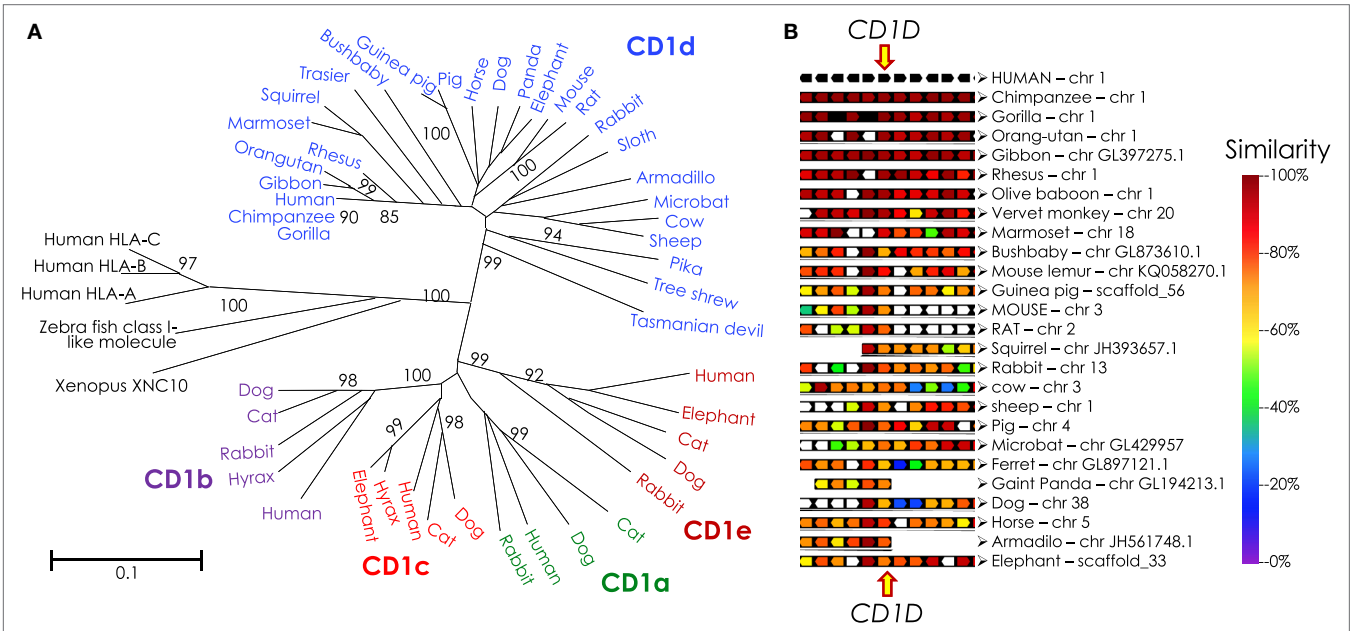


FIGURE 6 | Phylogenetic tree of CD1 and syntenic relationship of eutherian *Cd1d*. **(A)** Protein sequences of α1-α2 domains of mammalian and reptilian CD1, human and zebra fish MHC class I and *Xenopus* XNC10 were retrieved from NCBI using PSI-BLAST. Sequences with *E*-value of ≤0.05 were considered mostly similar to the human CD1D query sequence; significant bootstrap values are indicated for critical nodes. Sequences were aligned using ClustalW. Phylogenetic analysis (pairwise deletion, bootstrap: n = 1,000) was performed to construct the optimal tree with the sum of branch length = 9.42589507. Neighbor-Joining method (245) was used to infer evolutionary history. An optimal tree with the sum of branch length = 9.42589507 is shown. p-distance method was used to compute evolutionary distances (246, 247). The tree is drawn to scale, with branch length units the same as those of the evolutionary distances used to infer the phylogenetic tree. A total of 51 sequences and 210 positions were included in the final dataset. Evolutionary analyses were performed using MEGA6 (248). Mammalian species names according to Ref. (249), and the Order/Family to which they belong according to Ref. (250, 251) as well as homology to human CD1D are as follows: northern brown bandicoot (*Isoodon macrourus*), Peramelemorphia/Peramelidae, 50%; Tasmanian devil (*Sarcophilus harrisii*), Marsupialia/Dasyuridae, 42%; Hoffmann's two-toed sloth (*Choloepus hoffmanni*), Pilosa (Xenarthra)/Megalonychidae, missing various parts of the protein; nine-banded armadillo (*Dasypus novemcinctus*), Cingulata (Xenarthra)/Dasypodidae, 62%; tailless tenrec (*Echinops telfairi*), Afrosoricida (Insectivora)/Tenrecidae, missing N- and C-terminal ends of the protein; western European hedgehog (*Erinaceus europaeus*), Erinaceomorpha (Insectivora)/Erinaceidae; Eurasian shrew (*Sorex araneus*), Soricomorpha (Insectivora)/Soricidae, has only 181 aa, missing N- and C-terminal ends of the protein; northern tree Shrew (*Tupaia belangeri*), Scandentia/Tupiidae, 68%, missing leader sequence; large flying fox (*Pteropus vampyrus*): Chiroptera/Petropodidae; little brown bat (*Myotis lucifugus*), Chiroptera/Vespertilionidae 62%; dog (*Canis lupus familiaris*), Carnivora/Canidae, 57%, missing initiator methionine; giant panda (*Ailuropoda melanoleuca*), Carnivora/Ursidae, 60%, missing initiator methionine; ferret (*Mustela putorius furo*), Carnivora/Mustelidae, 60%, missing leader sequence; cat (*Felis catus*), Carnivora/Filidae; bottlenose dolphin (*Tursiops truncatus*), Cetacea/Delphinidae; African bush elephant (*Loxodonta africana*) Probosidea/Eliphantidae 65%; horse (*Equus caballus*), Perissodactyla/Equidae, 73%; rock hyrax (*Procavia capensis*), Hyracoidea/Procavidae, 64%; wild boar (*Sus scrofa*), Artiodactyla/Suidae, 65%; alpaca—Andean paca (*Vicugna pacos*), Artiodactyla/Camelidae; cow (*Bos taurus*), Artiodactyla/Bovidae, 65%, valine substitution of initiator methionine; squirrel (*Ictidomys tridecemlineatus*), Rodentia/Sciuridae, 64%, missing last 12 aa including the recycling motif; Ord's kangaroo rat (*Dipodomys ordii*): Rodentia/Heteromyidae; brown rat (*Rattus norvegicus*), Rodentia/Muridae; 64%; mouse (*Mus musculus domesticus*), Rodentia/Muridae, 61%; guinea pig (*Cavia porcellus*), Rodentia/Cavidae, 65%; American pika (*Ochotona princeps*), Logomorpha/Ochotonidae, 64%, missing last 17 aa including the recycling motif; European rabbit (*Oryctolagus cuniculus*), Logomorpha/Leporidae, 67%; gray mouse lemur (*Microcebus murinus*), Primates/Cheirogaleidae, 78%; small-eared galago—a bushbaby (*Otolemur garnettii*), Primates/Galagidae (Loridae), 71%, missing last 31 aa including the recycling motif; Philippine tarsier (*Tarsius syrichta*), Primates/Tarsiidae, 71%; white-tufted-ear marmoset (*Callithrix jacchus*), Primates/Callitrichidae, 83%; rhesus monkey/macaque (*Macaca mulatta*), Primates/Cercopithecidae, 88%; northern white-cheeked gibbon (*Nomascus leucogenys*), Primates/Hylobatidae, 95%; western gorilla (*Gorilla gorilla gorilla*), Primates/Hominidae, 99%; Sumatran orang utan (*Pongo abelii*), Primates/Hominidae, 96%; chimpanzee (*Pan troglodytes*), Primates/Hominidae, 98%; human (*Homo sapiens*), Primates/Hominidae. **(B)** Syntenic map was drawn using Genomicus v89.01 Phyloview (252). Human CD1D gene was used as reference gene and species, respectively, Eutheria as the root species. Upstream of CD1D gene are KIRREL, CD5L, FCRL1, FCRL2, and FCRL3 genes, and downstream from CD1D are CD1A, CD1C, CD1B, CD1E, and ORIOT2 genes in the syntenic map.

Microbial ecology has emerged as an important deterministic factor in the outcome of chemotherapy, radiation therapy, and immunotherapy against cancers (239). NKT cells have been targeted in the clinic for immunotherapy (see Implications for Cancer Immunotherapy), but how each of these therapies impact NKT cells is not known. It is noteworthy that a fraction of NKT cells are radiation resistant (130). This feature can be exploited for NKT cell-targeted immunotherapy against lymphomas and leukemias. Clinical trials have shown that the outcome of NKT cell-targeted immunotherapy varied between recipients (103, 104). Hence, what roles the gut microbiota played in the outcome is worthy of investigation. So also, considering that NKT cells can impact microbial ecology (238), what roles NKT cells play in tumorigenesis and metastasis are also worthy of investigation. Insights into how the microbial community assembles and forms the host–symbiont ecosystem

will facilitate an essential understanding of the molecular underpinnings that govern reciprocal interactions between the host and its internal ecosystem. These new insights can, in turn, impact the way by which new cancer therapies are designed, developed, and refined.

Evolution of Type I NKT Cells

> … the struggle against diseases, and especially infectious diseases, has been a very important evolutionary agent and that some of its results have been unlike those of the struggle for life … [(240) within a collection of papers in genetics by Haldane (241)].

Comparative vertebrate genomics, enabled by recent advances in whole-genome sequencing, have revealed molecular signatures of selection upon genes that control many biologic functions, including immune responses. Hence, pathobionts can apply immense selection pressure and play significant roles in the evolution of immune response genes and cells. As early-life symbionts can impact health, microbial ecology may also play roles in the evolution of the immune response genes and cells.

The NKTCR engages its ligand, CD1d-lipid co-complex, with conserved germline-encoded residues in four-to-five of the six complementarity-determining regions of the combined TCR α- and β-chains (242). Hence, phylogenetic studies of genes that encode CD1 molecules and the invariant NKTCR α-chain can reveal the origin and evolution of NKT cells. A recent phylogenomic analysis revealed that the *Cd1* gene is an amniote innovation that evolved in the Mesozoic reptiles and was retained in the extant anapsid (green anole lizard *Anolis carolinensis*) and synapsid (Siamese crocodile *Crocodylus siamensis* and Chinese alligator *Alligator sinensis*) reptilians (243). *Cd1* genes diversified in mammals, wherein evolved the *Cd1d* gene that encodes the lipid agonist presenting molecule that controls the functions of NKT cells in eutherians (of placental mammals; **Figure 6**) (244). Curiously however, the reptilian *Cd1* gene has no orthology with avian or mammalian *Cd1* genes (243), suggesting that *Cd1* genes may have emerged multiple times during amniote evolution. Or alternatively, *Cd1* genes may have evolved rapidly and diverged substantially from the reptilian form within extinct synapsid and mammal-like reptiles prior to stabilization within eutherian species. The latter view is supported by the finding that egg-laying monotremes such as platypuses and echidnas do not have *Cd1* genes while a *CD1d*-like gene exists in a few metatherian (of marsupial mammals) species such as the opossum.

A phylogenetic analysis of TRAV10 (encoding the human Vα24 gene segment) or TRAV11 (encoding the mouse Vα14 gene segment) and TRAJ18 (encoding the Jα18 gene segment) revealed that gene elements related to TRAV10/11 and TRAJ18 were found only in placental mammals (244). This finding suggests that NKT cells are a eutherian innovation. As the host–gut microbiota controls NKT cell terminal functional differentiation and NKT cells impact gut microbial ecology, it is postulated that placental development, sudden perinatal exposure to maternal and environmental microbiota, and lactation may have contributed to the evolution of CD1d-restricted type I NKT cells.

A Final Analysis: Under the Spell of PLZF and Host Microbial Ecology, a Curious Case for a "Limbic Immune System!"

The foregoing discusses recent advances in developmental biology of NKT cells and the environmental context in which it develops, matures and differentiates. A final section discusses their evolutionary path and how developmental biology and ecology may have contributed to this unique developmental plan. In addition, how the eco-evo-devo perspective on NKT cells may contribute to cancer immunotherapy is touched upon. Finally, areas that will benefit from further investigation are also pin pointed in their respective sections. Summarily, such areas include, (a) what early events specify NKT cell lineage commitment and turn on the unique lineage-specific GRN?; (b) what signals do symbionts relay to developing NKT cells to specify physiologic functions?; (c) in turn, what signals do NKT cells relay to the microbial community in the gut, and potentially to the microbionts in skin and lungs, to ensure physiologic community assembly, structure, and organization in early, young, and adult life?; (d) what tissue environmental signals underlie NKT cell subset differentiation?; (e) can radiation resistance of NKT cells be used in cancer immunotherapy?; and (f) what NKT cell intrinsic and environmental signals have retained NKT cells in certain mammalian species but not in others?

As a final note to the devo-eco-evo synthesis, we observed that the unique behavior of a group of innate-like T lymphocytes and innate lymphoid cells (ILCs) are under the control of PLZF (253–255). These include γδ T cells, NKT cells, MAIT cells, and certain ILCs. In addition, the development (MAIT cells, and potentially γδ T cells) and functional differentiation (NKT cells, MAIT cells, and ILCs) of these cells are determined by gut and potentially other barrier (skin and lungs) symbionts. As these immune cells, all of lymphoid origin, function at the edge (limbus in Latin) of the innate and adaptive immune systems, a proposal to group them into the "limbic immune system" is made here. Curiously, γδ T, NK, and NKT cells localize to the inter-follicular region of the lymph nodes, straddling the cells of the innate and adaptive immune systems (256). By virtue of their physiologic functions, other tissue-restricted innate-like lymphocytes, such as CD8αα innate-type lymphocytes (257) as well as B1 cells and NK cells (258), can be included in the "limbic immune system" even though their development and function may not be controlled by PLZF or the microbiota. In other words, the "limbic immune system" is anglicized Latin for the "inbetweeners" (259) and, hence, synonymous with it.

AUTHOR CONTRIBUTIONS

All authors wrote and edited the manuscript.

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REFERENCES

1. Ottaviani E, Valensin S, Franceschi C. The neuro-immunological interface in an evolutionary perspective: the dynamic relationship between effector and recognition systems. *Front Biosci* (1998) 3:d431–5. doi:10.2741/A289
2. Boehm T. Evolution of vertebrate immunity. *Curr Biol* (2012) 22(17):R722–32. doi:10.1016/j.cub.2012.07.003
3. Brubaker SW, Bonham KS, Zanoni I, Kagan JC. Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol* (2015) 33:257–90. doi:10.1146/annurev-immunol-032414-112240
4. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* (1997) 91(3):295–8. doi:10.1016/S0092-8674(00)80412-2
5. Lanier LL. Shades of grey – the blurring view of innate and adaptive immunity. *Nat Rev Immunol* (2013) 13(2):73–4. doi:10.1038/nri3389
6. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13(2):101–17. doi:10.1038/nri3369
7. Kotas ME, Medzhitov R. Homeostasis, inflammation, and disease susceptibility. *Cell* (2015) 160(5):816–27. doi:10.1016/j.cell.2015.02.010
8. Hill TM, Bezbradica JS, Van Kaer L, Joyce S. CD1d-restricted natural killer T cells. In: *eLS*. Chichester: John Wiley & Sons, Ltd (2016). doi:10.1002/9780470015902.a0020180.pub2
9. Marrero I, Ware R, Kumar V. Type II NKT cells in inflammation, autoimmunity, microbial immunity, and cancer. *Front Immunol* (2015) 6:316. doi:10.3389/fimmu.2015.00316
10. Rhost S, Sedimbi S, Kadri N, Cardell SL. Immunomodulatory type II natural killer T lymphocytes in health and disease. *Scand J Immunol* (2012) 76(3):246–55. doi:10.1111/j.1365-3083.2012.02750.x
11. Terabe M, Berzofsky JA. The immunoregulatory role of type I and type II NKT cells in cancer and other diseases. *Cancer Immunol Immunother* (2014) 63(3):199–213. doi:10.1007/s00262-013-1509-4
12. Macho-Fernandez E, Brigl M. The extended family of CD1d-restricted NKT cells: sifting through a mixed Bag of TCRs, antigens, and functions. *Front Immunol* (2015) 6:362. doi:10.3389/fimmu.2015.00362
13. Dasgupta S, Kumar V. Type II NKT cells: a distinct CD1d-restricted immune regulatory NKT cell subset. *Immunogenetics* (2016) 68(8):665–76. doi:10.1007/s00251-016-0930-1
14. Dhodapkar MV, Kumar V. Type II NKT cells and their emerging role in health and disease. *J Immunol* (2017) 198(3):1015–21. doi:10.4049/jimmunol.1601399
15. Cohen NR, Garg S, Brenner MB. Antigen presentation by CD1 lipids, T cells, and NKT cells in microbial immunity. *Adv Immunol* (2009) 102:1–94. doi:10.1016/S0065-2776(09)01201-2
16. Kohlgruber AC, Donado CA, LaMarche NM, Brenner MB, Brennan PJ. Activation strategies for invariant natural killer T cells. *Immunogenetics* (2016) 68(8):649–63. doi:10.1007/s00251-016-0944-8
17. Kim EY, Lynch L, Brennan PJ, Cohen NR, Brenner MB. The transcriptional programs of iNKT cells. *Semin Immunol* (2015) 27(1):26–32. doi:10.1016/j.smim.2015.02.005
18. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol* (2004) 22:817–90. doi:10.1146/annurev.immunol.22.012703.104608
19. Kain L, Webb B, Anderson BL, Deng S, Holt M, Costanzo A, et al. The identification of the endogenous ligands of natural killer T cells reveals the presence of mammalian alpha-linked glycosylceramides. *Immunity* (2014) 41(4):543–54. doi:10.1016/j.immuni.2014.08.017
20. Natori T, Koezuka Y, Higa T. Agelasphins, novel a-galactosylceramides from the marine sponge *Agelas mauritianus*. *Tetrahedron Lett* (1993) 34:5591–2. doi:10.1016/S0040-4039(00)73889-5
21. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, et al. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem* (1995) 38(12):2176–87. doi:10.1021/jm00012a018
22. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* (1997) 278(5343):1626–9. doi:10.1126/science.278.5343.1626
23. Schmieg J, Yang G, Franck RW, Tsuji M. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-galactosylceramide. *J Exp Med* (2003) 198(11):1631–41. doi:10.1084/jem.20031192
24. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* (2001) 413(6855):531–4. doi:10.1038/35097097
25. Yu KO, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, et al. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc Natl Acad Sci U S A* (2005) 102(9):3383–8. doi:10.1073/pnas.0407488102
26. An D, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell* (2014) 156(1–2):123–33. doi:10.1016/j.cell.2013.11.042
27. Penaranda C, Kashyap PC, Williams BB, Clardy J, Kronenberg M, et al. Production of α-galactosylceramide by a prominent member of the human gut microbiota. *PLoS Biol* (2013) 11(7):e1001610. doi:10.1371/journal.pbio.1001610
28. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C III, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* (2005) 434(7032):525–9. doi:10.1038/nature03408
29. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* (2005) 434(7032):520–5. doi:10.1038/nature03407
30. Sriram V, Du W, Gervay-Hague J, Brutkiewicz RR. Cell wall glycosphingolipids of *Sphingomonas paucimobilis* are CD1d-specific ligands for NKT cells. *Eur J Immunol* (2005) 35(6):1692–701. doi:10.1002/eji.200526157
31. Albacker LA, Chaudhary V, Chang YJ, Kim HY, Chuang YT, Pichavant M, et al. Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity. *Nat Med* (2013) 19(10):1297–304. doi:10.1038/nm.3321
32. Chang YJ, Kim HY, Albacker LA, Lee HH, Baumgarth N, Akira S, et al. Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity. *J Clin Invest* (2011) 121(1):57–69. doi:10.1172/JCI44845
33. Ortaldo JR, Young HA, Winkler-Pickett RT, Bere EW Jr, Murphy WJ, Wiltrout RH. Dissociation of NKT stimulation, cytokine induction, and NK activation in vivo by the use of distinct TCR-binding ceramides. *J Immunol* (2004) 172(2):943–53. doi:10.4049/jimmunol.172.2.943
34. Parekh VV, Singh AK, Wilson MT, Olivares-Villagómez D, Bezbradica JS, Inazawa H, et al. Quantitative and qualitative differences in the in vivo response of NKT cells to distinct alpha- and beta-anomeric glycolipids. *J Immunol* (2004) 173(6):3693–706. doi:10.4049/jimmunol.173.6.3693
35. Zhou D, Mattner J, Cantu C III, Schrantz N, Yin N, Gao Y, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science* (2004) 306(5702):1786–9. doi:10.1126/science.1103440
36. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* (2006) 7(9):978–86. doi:10.1038/ni1380
37. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol* (2011) 12(10):966–74. doi:10.1038/ni.2096
38. Mallevaey T, Clarke AJ, Scott-Browne JP, Young MH, Roisman LC, Pellicci DG, et al. A molecular basis for NKT cell recognition of CD1d-self-antigen. *Immunity* (2011) 34(3):315–26. doi:10.1016/j.immuni.2011.01.013
39. Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, et al. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* (2000) 12(2):211–21. doi:10.1016/S1074-7613(00)80174-0
40. Facciotti F, Ramanjaneyulu GS, Lepore M, Sansano S, Cavallari M, Kistowska M, et al. Peroxisome-derived lipids are self antigens that stimulate invariant natural killer T cells in the thymus. *Nat Immunol* (2012) 13(5):474–80. doi:10.1038/ni.2245
41. Fox LM, Cox DG, Lockridge JL, Wang X, Chen X, Scharf L, et al. Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS Biol* (2009) 7(10):e1000228. doi:10.1371/journal.pbio.1000228
42. Kumar A, Bezbradica JS, Stanic AK, Joyce S. Characterization and functional analysis of mouse semi-invariant natural T cells. *Curr Protoc Immunol* (2017) 117:1–14. doi:10.1002/cpim.22
43. Joyce S, Girardi E, Zajonc DM. NKT cell ligand recognition logic: molecular basis for a synaptic duet and transmission of inflammatory effectors. *J Immunol* (2011) 187(3):1081–9. doi:10.4049/jimmunol.1001910

44. Florence WC, Bhat RK, Joyce S. CD1d-restricted glycolipid antigens: presentation principles, recognition logic and functional consequences. *Expert Rev Mol Med* (2008) 10:e20. doi:10.1017/S1462399408000732
45. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* (2003) 4(12):1230–7. doi:10.1038/ni1002
46. Brigl M, Tatituri RV, Watts GF, Bhowruth V, Leadbetter EA, Barton N, et al. Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. *J Exp Med* (2011) 208(6):1163–77. doi:10.1084/jem.20102555
47. Cohen NR, Tatituri RV, Rivera A, Watts GF, Kim EY, Chiba A, et al. Innate recognition of cell wall beta-glucans drives invariant natural killer T cell responses against fungi. *Cell Host Microbe* (2011) 10(5):437–50. doi:10.1016/j.chom.2011.09.011
48. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi:10.1146/annurev.immunol.25.022106.141711
49. Stanic AK, De Silva AD, Park JJ, Sriram V, Ichikawa S, Hirabyashi Y, et al. Defective presentation of the CD1d1-restricted natural Va14Ja18 NKT lymphocyte antigen caused by β-D-glucosylceramide synthase deficiency. *Proc Natl Acad Sci U S A* (2003) 100(4):1849–54. doi:10.1073/pnas.0430327100
50. Popovic ZV, Rabionet M, Jennemann R, Krunic D, Sandhoff R, Gröne HJ, et al. Glucosylceramide synthase is involved in development of invariant natural killer T cells. *Front Immunol* (2017) 8:848. doi:10.3389/fimmu.2017.00848
51. Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, Sheehan KC, et al. Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity* (2007) 27(4):597–609. doi:10.1016/j.immuni.2007.08.017
52. Grubor-Bauk B, Simmons A, Mayrhofer G, Speck PG. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. *J Immunol* (2003) 170(3):1430–4. doi:10.4049/jimmunol.170.3.1430
53. Cornish AL, Keating R, Kyparissoudis K, Smyth MJ, Carbone FR, Godfrey DI. NKT cells are not critical for HSV-1 disease resolution. *Immunol Cell Biol* (2006) 84(1):13–9. doi:10.1111/j.1440-1711.2005.01396.x
54. Ashkar AA, Rosenthal KL. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J Virol* (2003) 77(18):10168–71. doi:10.1128/JVI.77.18.10168-10171.2003
55. Kim EY, Battaile JT, Patel AC, You Y, Agapov E, Grayson MH, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat Med* (2008) 14(6):633–40. doi:10.1038/nm1770
56. Johnson TR, Hong S, Van Kaer L, Koezuka Y, Graham BS. NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. *J Virol* (2002) 76(9):4294–303. doi:10.1128/JVI.76.9.4294-4303.2002
57. Paget C, Ivanov S, Fontaine J, Renneson J, Blanc F, Pichavant M, et al. Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection: potential role in protection against lung epithelial damages. *J Biol Chem* (2012) 287(12):8816–29. doi:10.1074/jbc.M111.304758
58. Paget C, Ivanov S, Fontaine J, Blanc F, Pichavant M, Renneson J, et al. Potential role of invariant NKT cells in the control of pulmonary inflammation and CD8+ T cell response during acute influenza A virus H3N2 pneumonia. *J Immunol* (2011) 186(10):5590–602. doi:10.4049/jimmunol.1002348
59. De Santo C, Salio M, Masri SH, Lee LY, Dong T, Speak AO, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest* (2008) 118(12):4036–48. doi:10.1172/JCI36264
60. Ho LP, Denney L, Luhn K, Teoh D, Clelland C, McMichael AJ. Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection. *Eur J Immunol* (2008) 38(7):1913–22. doi:10.1002/eji.200738017
61. Kok WL, Denney L, Benam K, Cole S, Clelland C, McMichael AJ, et al. Pivotal advance: invariant NKT cells reduce accumulation of inflammatory monocytes in the lungs and decrease immune-pathology during severe influenza A virus infection. *J Leukoc Biol* (2012) 91(3):357–68. doi:10.1189/jlb.0411184
62. Zeissig S, Murata K, Sweet L, Publicover J, Hu Z, Kaser A, et al. Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat Med* (2012) 18(7):1060–8. doi:10.1038/nm.2811
63. Nagarajan NA, Kronenberg M. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. *J Immunol* (2007) 178(5):2706–13. doi:10.4049/jimmunol.178.5.2706
64. Tyznik AJ, Tupin E, Nagarajan NA, Her MJ, Benedict CA, Kronenberg M. The mechanism of invariant NKT cell responses to viral danger signals. *J Immunol* (2008) 181(7):4452–6. doi:10.4049/jimmunol.181.7.4452
65. Wesley JD, Tessmer MS, Chaukos D, Brossay L. NK cell-like behavior of Valpha14i NK T cells during MCMV infection. *PLoS Pathog* (2008) 4(7):e1000106. doi:10.1371/journal.ppat.1000106
66. McCarthy C, Shepherd D, Fleire S, Stronge VS, Koch M, Illarionov PA, et al. The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. *J Exp Med* (2007) 204(5):1131–44. doi:10.1084/jem.20062342
67. Stanic AK, Shashidharamurthy R, Bezbradica JS, Matsuki N, Yoshimura Y, Miyake S, et al. Another view of T cell antigen recognition: cooperative engagement of glycolipid antigens by Va14Ja18 natural T(iNKT) cell receptor [corrected]. *J Immunol* (2003) 171(9):4539–51. doi:10.4049/jimmunol.171.9.4539
68. Bezbradica JS, Gordy LE, Stanic AK, Dragovic S, Hill T, Hawiger J, et al. Granulocyte-macrophage colony-stimulating factor regulates effector differentiation of invariant natural killer T cells during thymic ontogeny. *Immunity* (2006) 25(3):487–97. doi:10.1016/j.immuni.2006.06.017
69. Das R, Bassiri H, Guan P, Wiener S, Banerjee PP, Zhong MC, et al. The adaptor molecule SAP plays essential roles during invariant NKT cell cytotoxicity and lytic synapse formation. *Blood* (2013) 121(17):3386–95. doi:10.1182/blood-2012-11-468868
70. Mashayekhi M, Sandau MM, Dunay IR, Frickel EM, Khan A, Goldszmid RS, et al. α-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat Rev Immunol* (2005) 5(1):31–42. doi:10.1038/nri1531
71. Mashayekhi M, et al. CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by *Toxoplasma gondii* tachyzoites. *Immunity* (2011) 35(2):249–59. doi:10.1016/j.immuni.2011.08.008
72. Bezbradica JS, Stanic AK, Matsuki N, Bour-Jordan H, Bluestone JA, Thomas JW, et al. Distinct roles of dendritic cells and B cells in Va14Ja18 natural T cell activation in vivo. *J Immunol* (2005) 174(8):4696–705. doi:10.4049/jimmunol.174.8.4696
73. Bai L, Constantinides MG, Thomas SY, Reboulet R, Meng F, Koentgen F, et al. Distinct APCs explain the cytokine bias of alpha-galactosylceramide variants in vivo. *J Immunol* (2012) 188(7):3053–61. doi:10.4049/jimmunol.1102414
74. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* (2002) 3(9):867–74. doi:10.1038/ni827
75. Arora P, Baena A, Yu KO, Saini NK, Kharkwal SS, Goldberg MF, et al. A single subset of dendritic cells controls the cytokine bias of natural killer T cell responses to diverse glycolipid antigens. *Immunity* (2014) 40(1):105–16. doi:10.1016/j.immuni.2013.12.004
76. Bialecki E, Macho Fernandez E, Ivanov S, Paget C, Fontaine J, Rodriguez F, et al. Spleen-resident CD4+ and CD4- CD8alpha- dendritic cell subsets differ in their ability to prime invariant natural killer T lymphocytes. *PLoS One* (2011) 6(10):e26919. doi:10.1371/journal.pone.0026919
77. Macho-Fernandez E, Cruz LJ, Ghinnagow R, Fontaine J, Bialecki E, Frisch B, et al. Targeted delivery of alpha-galactosylceramide to CD8alpha+ dendritic cells optimizes type I NKT cell-based antitumor responses. *J Immunol* (2014) 193(2):961–9. doi:10.4049/jimmunol.1303029
78. Zajonc DM, Cantu C III, Mattner J, Zhou D, Savage PB, Bendelac A, et al. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat Immunol* (2005) 6(8):810–8. doi:10.1038/ni1224
79. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* (2003) 198(2):267–79. doi:10.1084/jem.20030324
80. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* (2004) 199(12):1607–18. doi:10.1084/jem.20040317
81. Vincent MS, Leslie DS, Gumperz JE, Xiong X, Grant EP, Brenner MB. CD1-dependent dendritic cell instruction. *Nat Immunol* (2002) 3(12):1163–8. doi:10.1038/ni851

82. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, et al. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* (1999) 163(9):4647–50.
83. Singh N, Hong S, Scherer DC, Serizawa I, Burdin N, Kronenberg M, et al. Cutting edge: activation of NK T cells by CD1d and α-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J Immunol* (1999) 163(5):2373–7.
84. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, et al. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* (1999) 189(7):1121–8. doi:10.1084/jem.189.7.1121
85. Shimizu K, Asakura M, Shinga J, Sato Y, Kitahara S, Hoshino K, et al. Invariant NKT cells induce plasmacytoid dendritic cell (DC) cross-talk with conventional DCs for efficient memory CD8+ T cell induction. *J Immunol* (2013) 190(11):5609–19. doi:10.4049/jimmunol.1300033
86. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci U S A* (2008) 105(32):11287–92. doi:10.1073/pnas.0801631105
87. Chaudhry MS, Karadimitris A. Role and regulation of CD1d in normal and pathological B cells. *J Immunol* (2014) 193(10):4761–8. doi:10.4049/jimmunol.1401805
88. Lang GA, Exley MA, Lang ML. The CD1d-binding glycolipid alpha-galactosylceramide enhances humoral immunity to T-dependent and T-independent antigen in a CD1d-dependent manner. *Immunology* (2006) 119(1):116–25. doi:10.1111/j.1365-2567.2006.02413.x
89. Tonti E, Galli G, Malzone C, Abrignani S, Casorati G, Dellabona P. NKT-cell help to B lymphocytes can occur independently of cognate interaction. *Blood* (2009) 113(2):370–6. doi:10.1182/blood-2008-06-166249
90. Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, et al. NK T cells provide lipid antigen-specific cognate help for B cells. *Proc Natl Acad Sci U S A* (2008) 105(24):8339–44. doi:10.1073/pnas.0801375105
91. King IL, Fortier A, Tighe M, Dibble J, Watts GF, Veerapen N, et al. Invariant natural killer T cells direct B cell responses to cognate lipid antigen in an IL-21-dependent manner. *Nat Immunol* (2012) 13(1):44–50. doi:10.1038/ni.2172
92. Barral P, Eckl-Dorna J, Harwood NE, De Santo C, Salio M, Illarionov P, et al. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. *Proc Natl Acad Sci U S A* (2008) 105(24):8345–50. doi:10.1073/pnas.0802968105
93. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol* (2012) 13(1):35–43. doi:10.1038/ni.2166
94. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, et al. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. *Nat Immunol* (2010) 11(4):313–20. doi:10.1038/ni.1848
95. Gilchuk P, Hill TM, Guy C, McMaster SR, Boyd KL, Rabacal WA, et al. A distinct lung-interstitium-resident memory CD8(+) T cell subset confers enhanced protection to lower respiratory tract infection. *Cell Rep* (2016) 16(7):1800–9. doi:10.1016/j.celrep.2016.07.037
96. Gilchuk P, Spencer CT, Conant SB, Hill T, Gray JJ, Niu X, et al. Discovering naturally processed antigenic determinants that confer protective T cell immunity. *J Clin Invest* (2013) 123(5):1976–87. doi:10.1172/JCI67388
97. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, et al. NKT cells enhance $CD4^+$ and $CD8^+$ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* (2003) 171(10):5140–7. doi:10.4049/jimmunol.171.10.5140
98. Yu KO, Porcelli SA. The diverse functions of CD1d-restricted NKT cells and their potential for immunotherapy. *Immunol Lett* (2005) 100(1):42–55. doi:10.1016/j.imlet.2005.06.010
99. Wu L, Gabriel CL, Parekh VV, Van Kaer L. Invariant natural killer T cells: innate-like T cells with potent immunomodulatory activities. *Tissue Antigens* (2009) 73(6):535–45. doi:10.1111/j.1399-0039.2009.01256.x
100. Van Kaer L, Parekh VV, Wu L. Invariant NK T cells: potential for immunotherapeutic targeting with glycolipid antigens. *Immunotherapy* (2011) 3(1):59–75. doi:10.2217/imt.10.85
101. Singh AK, Gaur P, Das SN. Natural killer T cell anergy, co-stimulatory molecules and immunotherapeutic interventions. *Hum Immunol* (2014) 75(3):250–60. doi:10.1016/j.humimm.2013.12.004
102. Carreno LJ, Kharkwal SS, Porcelli SA. Optimizing NKT cell ligands as vaccine adjuvants. *Immunotherapy* (2014) 6(3):309–20. doi:10.2217/imt.13.175
103. Nair S, Dhodapkar MV. Natural killer T cells in cancer immunotherapy. *Front Immunol* (2017) 8:1178. doi:10.3389/fimmu.2017.01178
104. Waldowska M, Bojarska-Junak A, Roliński J. A brief review of clinical trials involving manipulation of invariant NKT cells as a promising approach in future cancer therapies. *Cent Eur J Immunol* (2017) 42(2):181–95. doi:10.5114/ceji.2017.69361
105. Bassiri H, Das R, Guan P, Barrett DM, Brennan PJ, Banerjee PP, et al. iNKT cell cytotoxic responses control T-lymphoma growth in vitro and in vivo. *Cancer Immunol Res* (2014) 2(1):59–69. doi:10.1158/2326-6066.CIR-13-0104
106. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178 dependent and is correlated with antigenic potency. *J Immunol* (2010) 185(5):2721–9. doi:10.4049/jimmunol.1001018
107. Hagihara M, Gansuvd B, Ueda Y, Tsuchiya T, Masui A, Tazume K, et al. Killing activity of human umbilical cord blood-derived TCRValpha24+ NKT cells against normal and malignant hematological cells in vitro: a comparative study with NK cells or OKT3 activated T lymphocytes or with adult peripheral blood NKT cells. *Cancer Immunol Immunother* (2002) 51(1):1–8. doi:10.1007/s00262-001-0246-2
108. Haraguchi K, Takahashi T, Nakahara F, Matsumoto A, Kurokawa M, Ogawa S, et al. CD1d expression level in tumor cells is an important determinant for anti-tumor immunity by natural killer T cells. *Leuk Lymphoma* (2006) 47(10):2218–23. doi:10.1080/10428190600682688
109. Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J Exp Med* (2005) 202(9):1279–88. doi:10.1084/jem.20050953
110. Keller CW, Freigang S, Lünemann JD. Reciprocal crosstalk between dendritic cells and natural killer T cells: mechanisms and therapeutic potential. *Front Immunol* (2017) 8:570. doi:10.3389/fimmu.2017.00570
111. Gottschalk C, Mettke E, Kurts C. The role of invariant natural killer T cells in dendritic cell licensing, cross-priming, and memory cd8(+) t cell generation. *Front Immunol* (2015) 6:379. doi:10.3389/fimmu.2015.00379
112. Chung Y, Kim BS, Kim YJ, Ko HJ, Ko SY, Kim DH, et al. CD1d-restricted T cells license B cells to generate long-lasting cytotoxic antitumor immunity in vivo. *Cancer Res* (2006) 66(13):6843–50. doi:10.1158/0008-5472.CAN-06-0889
113. Kim Y-J, Ko HJ, Kim YS, Kim DH, Kang S, Kim JM, et al. α-Galactosylceramide-loaded, antigen-expressing B cells prime a wide spectrum of antitumor immunity. *Int J Cancer* (2008) 122(12):2774–83. doi:10.1002/ijc.23444
114. Iyoda T, Yamasaki S, Hidaka M, Kawano F, Abe Y, Suzuki K, et al. Amelioration of NK cell function driven by Vα24+ invariant NKT cell activation in multiple myeloma. *Clin Immunol* (2017). doi:10.1016/j.clim.2017.10.007
115. Fujii S, Shimizu K, Okamoto Y, Kunii N, Nakayama T, Motohashi S, et al. NKT cells as an ideal anti-tumor immunotherapeutic. *Front Immunol* (2013) 4:409. doi:10.3389/fimmu.2013.00409
116. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* (1997) 278(5343):1623–6. doi:10.1126/science.278.5343.1623
117. Kammertoens T, Qin Z, Briesemeister D, Bendelac A, Blankenstein T. B-cells and IL-4 promote methylcholanthrene-induced carcinogenesis but there is no evidence for a role of T/NKT-cells and their effector molecules (Fas-ligand, TNF-alpha, perforin). *Int J Cancer* (2012) 131(7):1499–508. doi:10.1002/ijc.27411
118. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* (2000) 191(4):661–8. doi:10.1084/jem.191.4.661
119. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* (2009) 182(4):2492–501. doi:10.4049/jimmunol.0800126
120. Kunii N, Horiguchi S, Motohashi S, Yamamoto H, Ueno N, Yamamoto S, et al. Combination therapy of in vitro-expanded natural killer T cells and

alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* (2009) 100(6):1092–8. doi:10.1111/j.1349-7006.2009.01135.x
121. Lam PY, Nissen MD, Mattarollo SR. Invariant natural killer T cells in immune regulation of blood cancers: harnessing their potential in immunotherapies. *Front Immunol* (2017) 8:1355. doi:10.3389/fimmu.2017.01355
122. Bendelac A. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J Exp Med* (1995) 182(6):2091–6. doi:10.1084/jem.182.6.2091
123. Coles MC, Raulet DH. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J Immunol* (2000) 164(5):2412–8. doi:10.4049/jimmunol.164.5.2412
124. Boesteanu A, Silva AD, Nakajima H, Leonard WJ, Peschon JJ, Joyce S. Distinct roles for signals relayed through the common cytokine receptor gamma chain and interleukin 7 receptor alpha chain in natural T cell development. *J Exp Med* (1997) 186(2):331–6. doi:10.1084/jem.186.2.331
125. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J Exp Med* (2000) 191(11):1895–903. doi:10.1084/jem.191.11.1895
126. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* (2000) 192(5):741–54. doi:10.1084/jem.192.5.741
127. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol* (2004) 4(3):231–7. doi:10.1038/nri1309
128. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NK T cell lineage. *Science* (2002) 296(5567):553–5. doi:10.1126/science.1069017
129. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *J Exp Med* (2002) 195(7):835–44. doi:10.1084/jem.20011544
130. Bezbradica JS, Hill T, Stanic AK, Van Kaer L, Joyce S. Commitment toward the natural T (iNKT) cell lineage occurs at the CD4+8+ stage of thymic ontogeny. *Proc Natl Acad Sci U S A* (2005) 102(14):5114–9. doi:10.1073/pnas.0408449102
131. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An essential role for the transcription factor HEB in thymocyte survival, Tcra rearrangement and the development of natural killer T cells. *Nat Immunol* (2010) 11(3):240–9. doi:10.1038/ni.1845
132. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, et al. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* (2005) 22(6):705–16. doi:10.1016/j.immuni.2005.03.011
133. Hu T, Simmons A, Yuan J, Bender TP, Alberola-Ila J. The transcription factor c-Myb primes CD4+CD8+ immature thymocytes for selection into the iNKT lineage. *Nat Immunol* (2010) 11(5):435–41. doi:10.1038/ni.1865
134. Gapin L, Matsuda JL, Surh CD, Kronenberg M. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat Immunol* (2001) 2(10):971–8. doi:10.1038/ni710
135. Godfrey DI, Berzins SP. Control points in NKT-cell development. *Nat Rev Immunol* (2007) 7(7):505–18. doi:10.1038/nri2116
136. Dashtsoodol N, Shigeura T, Aihara M, Ozawa R, Kojo S, Harada M, et al. Alternative pathway for the development of Valpha14(+) NKT cells directly from CD4(-)CD8(-) thymocytes that bypasses the CD4(+)CD8(+) stage. *Nat Immunol* (2017) 18(3):274–82. doi:10.1038/ni.3668
137. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. *Immunity* (1997) 6(4):459–67. doi:10.1016/S1074-7613(00)80289-7
138. Mendiratta SK, Martin WD, Hong S, Boesteanu A, Joyce S, Van Kaer L. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* (1997) 6(4):469–77. doi:10.1016/S1074-7613(00)80290-3
139. Smiley ST, Kaplan MH, Grusby MJ. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science* (1997) 275(5302):977–9. doi:10.1126/science.275.5302.977
140. Griewank K, Borowski C, Rietdijk S, Wang N, Julien A, Wei DG, et al. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* (2007) 27(5):751–62. doi:10.1016/j.immuni.2007.08.020
141. Wang N, Satoskar A, Faubion W, Howie D, Okamoto S, Feske S, et al. The cell surface receptor SLAM controls T cell and macrophage functions. *J Exp Med* (2004) 199(9):1255–64. doi:10.1084/jem.20031835
142. Jordan MA, Fletcher JM, Jose R, Chowdhury S, Gerlach N, Allison J, et al. Role of SLAM in NKT cell development revealed by transgenic complementation in NOD mice. *J Immunol* (2011) 186(7):3953–65. doi:10.4049/jimmunol.1003305
143. Stanic AK, Bezbradica JS, Park JJ, Van Kaer L, Boothby MR, Joyce S. Cutting edge: the ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase C theta and NF-kappa B. *J Immunol* (2004) 172(8):4667–71. doi:10.4049/jimmunol.172.8.4667
144. Lazarevic V, Zullo AJ, Schweitzer MN, Staton TL, Gallo EM, Crabtree GR, et al. The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nat Immunol* (2009) 10(3):306–13. doi:10.1038/ni.1696
145. Seiler MP, Mathew R, Liszewski MK, Spooner CJ, Barr K, Meng F, et al. Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. *Nat Immunol* (2012) 13(3):264–71. doi:10.1038/ni.2230
146. Qi Q, Kannan AK, August A. Tec family kinases: Itk signaling and the development of NKT alphabeta and gammadelta T cells. *FEBS J* (2011) 278(12):1970–9. doi:10.1111/j.1742-4658.2011.08074.x
147. Nichols KE, Hom J, Gong SY, Ganguly A, Ma CS, Cannons JL, et al. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med* (2005) 11(3):340–5. doi:10.1038/nm1189
148. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nat Immunol* (2010) 11(3):197–206. doi:10.1038/ni.1841
149. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* (2008) 29(3):391–403. doi:10.1016/j.immuni.2008.07.011
150. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, et al. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol* (2008) 9(9):1055–64. doi:10.1038/ni.1641
151. Watarai H, Sekine-Kondo E, Shigeura T, Motomura Y, Yasuda T, Satoh R, et al. Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines. *PLoS Biol* (2012) 10(2):e1001255. doi:10.1371/journal.pbio.1001255
152. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol* (2013) 14(11):1146–54. doi:10.1038/ni.2731
153. Hammond KJ, Pellicci DG, Poulton LD, Naidenko OV, Scalzo AA, Baxter AG, et al. CD1d-restricted NKT cells: an interstrain comparison. *J Immunol* (2001) 167(3):1164–73. doi:10.4049/jimmunol.167.3.1164
154. Lynch L, Michelet X, Zhang S, Brennan PJ, Moseman A, Lester C, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of Treg cells and macrophages in adipose tissue. *Nat Immunol* (2015) 16(1):85–95. doi:10.1038/ni.3047
155. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124(9):3725–40. doi:10.1172/JCI72308
156. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol* (2016) 17(6):728–39. doi:10.1038/ni.3437
157. Peter I, Davidson E. *Genomic Control Process: Development and Evolution*. London: Academic Press (2015).
158. Mao AP, Constantinides MG, Mathew R, Zuo Z, Chen X, Weirauch MT, et al. Multiple layers of transcriptional regulation by PLZF in NKT-cell development. *Proc Natl Acad Sci U S A* (2016) 113(27):7602–7. doi:10.1073/pnas.1601504113
159. Wang Y, Yun C, Gao B, Xu Y, Zhang Y, Wang Y, et al. The lysine acetyltransferase GCN5 is required for iNKT cell development through EGR2 acetylation. *Cell Rep* (2017) 20(3):600–12. doi:10.1016/j.celrep.2017.06.065
160. Dominy JE Jr, Lee Y, Jedrychowski MP, Chim H, Jurczak MJ, Camporez JP, et al. The deacetylase Sirt6 activates the acetyltransferase GCN5

and suppresses hepatic gluconeogenesis. *Mol Cell* (2012) 48(6):900–13. doi:10.1016/j.molcel.2012.09.030
161. Mallevaey T, Scott-Browne JP, Matsuda JL, Young MH, Pellicci DG, Patel O, et al. T cell receptor CDR2 beta and CDR3 beta loops collaborate functionally to shape the iNKT cell repertoire. *Immunity* (2009) 31(1):60–71. doi:10.1016/j.immuni.2009.05.010
162. Wei DG, Curran SA, Savage PB, Teyton L, Bendelac A. Mechanisms imposing the Vbeta bias of Valpha14 natural killer T cells and consequences for microbial glycolipid recognition. *J Exp Med* (2006) 203(5):1197–207. doi:10.1084/jem.20060418
163. Chun T, et al. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J Exp Med* (2003) 197(7):907–18. doi:10.1084/jem.20021366
164. Pellicci DG, Uldrich AP, Kyparissoudis K, Crowe NY, Brooks AG, Hammond KJ, et al. Intrathymic NKT cell development is blocked by the presence of alpha-galactosylceramide. *Eur J Immunol* (2003) 33(7):1816–23. doi:10.1002/eji.200323894
165. Schümann J, Pittoni P, Tonti E, Macdonald HR, Dellabona P, Casorati G. Targeted expression of human CD1d in transgenic mice reveals independent roles for thymocytes and thymic APCs in positive and negative selection of Valpha14i NKT cells. *J Immunol* (2005) 175(11):7303–10. doi:10.4049/jimmunol.175.11.7303
166. Stanic AK, Bezbradica JS, Park JJ, Matsuki N, Mora AL, Van Kaer L, et al. NF-kappa B controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. *J Immunol* (2004) 172(4):2265–73. doi:10.4049/jimmunol.172.4.2265
167. Chiu YH, Park SH, Benlagha K, Forestier C, Jayawardena-Wolf J, Savage PB, et al. Multiple defects in antigen presentation and T cell development by mice expressing cytoplasmic tail-truncated CD1d. *Nat Immunol* (2002) 3(1):55–60. doi:10.1038/ni740
168. Thapa P, Das J, McWilliams D, Shapiro M, Sundsbak R, Nelson-Holte M, et al. The transcriptional repressor NKAP is required for the development of iNKT cells. *Nat Commun* (2013) 4:1582. doi:10.1038/ncomms2580
169. Pajerowski AG, Nguyen C, Aghajanian H, Shapiro MJ, Shapiro VS. NKAP is a transcriptional repressor of notch signaling and is required for T cell development. *Immunity* (2009) 30(5):696–707. doi:10.1016/j.immuni.2009.02.011
170. Napolitano A, Pittoni P, Beaudoin L, Lehuen A, Voehringer D, MacDonald HR, et al. Functional education of invariant NKT cells by dendritic cell tuning of SHP-1. *J Immunol* (2013) 190(7):3299–308. doi:10.4049/jimmunol.1203466
171. Wei DG, Lee H, Park SH, Beaudoin L, Teyton L, Lehuen A, et al. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *J Exp Med* (2005) 202(2):239–48. doi:10.1084/jem.20050413
172. Cannons JL, Yu LJ, Hill B, Mijares LA, Dombroski D, Nichols KE, et al. SAP regulates TH2 differentiation and PKC-θ-mediated activation of NF-κB1. *Immunity* (2004) 21(5):693–706. doi:10.1016/j.immuni.2004.09.012
173. Huang B, Gomez-Rodriguez J, Preite S, Garrett LJ, Harper UL, Schwartzberg PL. CRISPR-mediated triple knockout of SLAMF1, SLAMF5 and SLAMF6 supports positive signaling roles in NKT cell development. *PLoS One* (2016) 11(6):e0156072. doi:10.1371/journal.pone.0156072
174. Chen S, Cai C, Li Z, Liu G, Wang Y, Blonska M, et al. Dissection of SAP-dependent and SAP-independent SLAM family signaling in NKT cell development and humoral immunity. *J Exp Med* (2017) 214(2):475–89. doi:10.1084/jem.20161312
175. Michel ML, Lenoir C, Massot B, Diem S, Pasquier B, Sawa S, et al. SLAM-associated protein favors the development of iNKT2 over iNKT17 cells. *Eur J Immunol* (2016) 46(9):2162–74. doi:10.1002/eji.201646313
176. Vallabhapurapu S, Hammond KJL, Howells N, Pfeffer K, Weih F. Differential requirement for Rel/nuclear factor κB family members in natural killer T cell development. *J Exp Med* (2003) 197(12):1613–21. doi:10.1084/jem.20022234
177. Vallabhapurapu S, Powolny-Budnicka I, Riemann M, Schmid RM, Paxian S, Pfeffer K, et al. Rel/NF-kappaB family member RelA regulates NK1.1- to NK1.1+ transition as well as IL-15-induced expansion of NKT cells. *Eur J Immunol* (2008) 38(12):3508–19. doi:10.1002/eji.200737830
178. Elewaut D, Shaikh RB, Hammond KJ, De Winter H, Leishman AJ, Sidobre S, et al. NIK-dependent RelB activation defines a unique signaling pathway for the development of V alpha 14i NKT cells. *J Exp Med* (2003) 197(12):1623–33. doi:10.1084/jem.20030141
179. Schmidt-Supprian M, Tian J, Grant EP, Pasparakis M, Maehr R, Ovaa H, et al. Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. *Proc Natl Acad Sci U S A* (2004) 101(13):4566–71. doi:10.1073/pnas.0400885101
180. Stankovic S, Gugasyan R, Kyparissoudis K, Grumont R, Banerjee A, Tsichlis P, et al. Distinct roles in NKT cell maturation and function for the different transcription factors in the classical NF-kappaB pathway. *Immunol Cell Biol* (2011) 89(2):294–303. doi:10.1038/icb.2010.93
181. Lee AJ, Zhou X, Chang M, Hunzeker J, Bonneau RH, Zhou D, et al. Regulation of natural killer T-cell development by deubiquitinase CYLD. *EMBO J* (2010) 29(9):1600–12. doi:10.1038/emboj.2010.31
182. Qi Q, Huang W, Bai Y, Balmus G, Weiss RS, August A. A unique role for ITK in survival of invariant NKT cells associated with the p53-dependent pathway in mice. *J Immunol* (2012) 188(8):3611–9. doi:10.4049/jimmunol.1102475
183. Kumar A, Gordy LE, Bezbradica JS, Stanic AK, Hill TM, Boothby MR, et al. NF-kappaB protects NKT cells from tumor necrosis factor receptor 1-induced death. *Sci Rep* (2017) 7(1):15594. doi:10.1038/s41598-017-15461-y
184. Crawford G, Enders A, Gileadi U, Stankovic S, Zhang Q, Lambe T, et al. DOCK8 is critical for the survival and function of NKT cells. *Blood* (2013) 122(12):2052–61. doi:10.1182/blood-2013-02-482331
185. Okamura K, Kitamura A, Sasaki Y, Chung DH, Kagami S, Iwai K, et al. Survival of mature T cells depends on signaling through HOIP. *Sci Rep* (2016) 6:36135. doi:10.1038/srep36135
186. Lan P, Fan Y, Zhao Y, Lou X, Monsour HP, Zhang X, et al. TNF superfamily receptor OX40 triggers invariant NKT cell pyroptosis and liver injury. *J Clin Invest* (2017) 127(6):2222–34. doi:10.1172/JCI91075
187. Gordy LE, Bezbradica JS, Flyak AI, Spencer CT, Dunkle A, Sun J, et al. IL-15 regulates homeostasis and terminal maturation of NKT cells. *J Immunol* (2011) 187(12):6335–45. doi:10.4049/jimmunol.1003965
188. Matsuda JL, Gapin L, Sidobre S, Kieper WC, Tan JT, Ceredig R, et al. Homeostasis of V[alpha]14i NKT cells. *Nat Immunol* (2002) 3(10):966–74. doi:10.1038/ni837
189. Castillo EF, Acero LF, Stonier SW, Zhou D, Schluns KS. Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation. *Blood* (2010) 116(14):2494–503. doi:10.1182/blood-2010-03-277103
190. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible defects in natural killer and memory Cd8 T cell lineages in interleukin 15–deficient mice. *J Exp Med* (2000) 191(5):771–80. doi:10.1084/jem.191.5.771
191. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* (1998) 9(5):669–76. doi:10.1016/S1074-7613(00)80664-0
192. Anderson CK, Salter AI, Toussaint LE, Reilly EC, Fugère C, Srivastava N, et al. Role of SHIP1 in invariant NKT cell development and functions. *J Immunol* (2015) 195(5):2149–56. doi:10.4049/jimmunol.1500567
193. Parekh VV, Wu L, Boyd KL, Williams JA, Gaddy JA, Olivares-Villagómez D, et al. Impaired autophagy, defective T cell homeostasis, and a wasting syndrome in mice with a T cell-specific deletion of Vps34. *J Immunol* (2013) 190(10):5086–101. doi:10.4049/jimmunol.1202071
194. Pei B, Zhao M, Miller BC, Véla JL, Bruinsma MW, Virgin HW, et al. Invariant NKT cells require autophagy to coordinate proliferation and survival signals during differentiation. *J Immunol* (2015) 194(12):5872–84. doi:10.4049/jimmunol.1402154
195. Salio M, Puleston DJ, Mathan TS, Shepherd D, Stranks AJ, Adamopoulou E, et al. Essential role for autophagy during invariant NKT cell development. *Proc Natl Acad Sci U S A* (2014) 111(52):E5678–87. doi:10.1073/pnas.1413935112
196. Thapa P, Romero Arocha S, Chung JY, Sant'Angelo DB, Shapiro VS. Histone deacetylase 3 is required for iNKT cell development. *Sci Rep* (2017) 7(1):5784. doi:10.1038/s41598-017-06102-5
197. Terashima A, Watarai H, Inoue S, Sekine E, Nakagawa R, Hase K, et al. A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J Exp Med* (2008) 205(12):2727–33. doi:10.1084/jem.20080698
198. Matangkasombut P, Marigowda G, Ervine A, Idris L, Pichavant M, Kim HY, et al. Natural killer T cells in the lungs of patients with asthma. *J Allergy Clin Immunol* (2009) 123(5):1181–5. doi:10.1016/j.jaci.2009.02.013

199. Kim HY, Pichavant M, Matangkasombut P, Koh YI, Savage PB, DeKruyff RH, et al. The development of airway hyperreactivity in T-bet-deficient mice requires CD1d-restricted NKT cells. *J Immunol* (2009) 182(5):3252–61. doi:10.4049/jimmunol.0803339
200. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* (2003) 9:582. doi:10.1038/nm851
201. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* (2003) 198(7):1069–76. doi:10.1084/jem.20030630
202. Tanaka S, Tsukada J, Suzuki W, Hayashi K, Tanigaki K, Tsuji M, et al. The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. *Immunity* (2006) 24(6):689–701. doi:10.1016/j.immuni.2006.04.009
203. Collins PL, Chang S, Henderson M, Soutto M, Davis GM, McLoed AG, et al. Distal regions of the human IFNG locus direct cell type-specific expression. *J Immunol* (2010) 185(3):1492–501. doi:10.4049/jimmunol.1000124
204. Collins PL, Henderson MA, Aune TM. Lineage-specific adjacent IFNG and IL26 genes share a common distal enhancer element. *Genes Immun* (2012) 13(6):481–8. doi:10.1038/gene.2012.22
205. Wang X, Bishop KA, Hegde S, Rodenkirch LA, Pike JW, Gumperz JE. Human invariant natural killer T cells acquire transient innate responsiveness via histone H4 acetylation induced by weak TCR stimulation. *J Exp Med* (2012) 209(5):987–1000. doi:10.1084/jem.20111024
206. Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, et al. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med* (2007) 204(5):995–1001. doi:10.1084/jem.20061551
207. Doisne JM, Becourt C, Amniai L, Duarte N, Le Luduec JB, Eberl G, et al. Skin and peripheral lymph node invariant NKT cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions. *J Immunol* (2009) 183(3):2142–9. doi:10.4049/jimmunol.0901059
208. Clancy-Thompson E, Chen GZ, Tyler PM, Servos MM, Barisa M, Brennan PJ, et al. Monoclonal invariant NKT (iNKT) cell mice reveal a role for both tissue of origin and the TCR in development of iNKT functional subsets. *J Immunol* (2017) 199(1):159–71. doi:10.4049/jimmunol.1700214
209. Webster KE, Kim HO, Kyparissoudis K, Corpuz TM, Pinget GV, Uldrich AP, et al. IL-17-producing NKT cells depend exclusively on IL-7 for homeostasis and survival. *Mucosal Immunol* (2014) 7(5):1058–67. doi:10.1038/mi.2013.122
210. Thapa P, Chen MW, McWilliams DC, Belmonte P, Constans M, Sant'Angelo DB, et al. NKAP regulates invariant NKT cell proliferation and differentiation into ROR-gammat-expressing NKT17 cells. *J Immunol* (2016) 196(12):4987–98. doi:10.4049/jimmunol.1501653
211. Thapa P, Manso B, Chung JY, Romera Arocha S, Xue HH, Angelo DBS, et al. The differentiation of ROR-gammat expressing iNKT17 cells is orchestrated by Runx1. *Sci Rep* (2017) 7(1):7018. doi:10.1038/s41598-017-07365-8
212. Sklarz T, Guan P, Gohil M, Cotton RM, Ge MQ, Haczku A, et al. mTORC2 regulates multiple aspects of NKT-cell development and function. *Eur J Immunol* (2017) 47(3):516–26. doi:10.1002/eji.201646343
213. Wei J, Yang K, Chi H. Cutting edge: discrete functions of mTOR signaling in invariant NKT cell development and NKT17 fate decision. *J Immunol* (2014) 193(9):4297–301. doi:10.4049/jimmunol.1402042
214. Price AE, Reinhardt RL, Liang HE, Locksley RM. Marking and quantifying IL-17A-producing cells in vivo. *PLoS One* (2012) 7(6):e39750. doi:10.1371/journal.pone.0039750
215. Pichavant M, Goya S, Meyer EH, Johnston RA, Kim HY, Matangkasombut P, et al. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med* (2008) 205(2):385–93. doi:10.1084/jem.20071507
216. Milosavljevic N, Gazdic M, Simovic Markovic B, Arsenijevic A, Nurkovic J, Dolicanin Z, et al. Mesenchymal stem cells attenuate acute liver injury by altering ratio between interleukin 17 producing and regulatory natural killer T cells. *Liver Transpl* (2017) 23(8):1040–50. doi:10.1002/lt.24784
217. Galli G, Pittoni P, Tonti E, Malzone C, Uematsu Y, Tortoli M, et al. Invariant NKT cells sustain specific B cell responses and memory. *Proc Natl Acad Sci U S A* (2007) 104(10):3984–9. doi:10.1073/pnas.0700191104
218. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly – TFH cells in human health and disease. *Nat Rev Immunol* (2013) 13(6):412–26. doi:10.1038/nri3447
219. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol* (2011) 13(1):35–43. doi:10.1038/ni.2166
220. Tonti E, Fedeli M, Napolitano A, Iannacone M, von Andrian UH, Guidotti LG, et al. Follicular helper NKT cells induce limited B cell responses and germinal center formation in the absence of CD4(+) T cell help. *J Immunol* (2012) 188(7):3217–22. doi:10.4049/jimmunol.1103501
221. Chan AC, Leeansyah E, Cochrane A, d'Udekem d'Acoz Y, Mittag D, Harrison LC, et al. Ex-vivo analysis of human natural killer T cells demonstrates heterogeneity between tissues and within established CD4(+) and CD4(-) subsets. *Clin Exp Immunol* (2013) 172(1):129–37. doi:10.1111/cei.12045
222. Akbari O, Faul JL, Hoyte EG, Berry GJ, Wahlström J, Kronenberg M, et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N Engl J Med* (2006) 354(11):1117–29. doi:10.1056/NEJMoa053614
223. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* (2002) 195(5):625–36. doi:10.1084/jem.20011786
224. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med* (2002) 195(5):637–41. doi:10.1084/jem.20011908
225. Cohen NR, Brennan PJ, Shay T, Watts GF, Brigl M, Kang J, et al. Shared and distinct transcriptional programs underlie the hybrid nature of iNKT cells. *Nat Immunol* (2013) 14(1):90–9. doi:10.1038/ni.2490
226. Tsagaratou A, González-Avalos E, Rautio S, Scott-Browne JP, Togher S, Pastor WA, et al. TET proteins regulate the lineage specification and TCR-mediated expansion of iNKT cells. *Nat Immunol* (2017) 18(1):45–53. doi:10.1038/ni.3630
227. Gilbert SF, Epel D. *Ecological Development al Biology*. Sunderland, MA: Sinaur (2015). 576 p.
228. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity* (2015) 43(3):566–78. doi:10.1016/j.immuni.2015.06.025
229. Matsuki N, Stanic AK, Embers ME, Van Kaer L, Morel L, Joyce S. Genetic dissection of V alpha 14J alpha 18 natural T cell number and function in autoimmune-prone mice. *J Immunol* (2003) 170(11):5429–37. doi:10.4049/jimmunol.170.11.5429
230. Poulton LD, Smyth MJ, Hawke CG, Silveira P, Shepherd D, Naidenko OV, et al. Cytometric and functional analyses of NK and NKT cell deficiencies in NOD mice. *Int Immunol* (2001) 13(7):887–96. doi:10.1093/intimm/13.7.887
231. Lee PT, Putnam A, Benlagha K, Teyton L, Gottlieb PA, Bendelac A. Testing the NKT cell hypothesis of human IDDM pathogenesis. *J Clin Invest* (2002) 110(6):793–800. doi:10.1172/JCI0215832
232. Motsinger A, Haas DW, Stanic AK, Van Kaer L, Joyce S, Unutmaz D. CD1d-restricted human natural killer T cells are highly susceptible to human immunodeficiency virus 1 infection. *J Exp Med* (2002) 195(7):869–79. doi:10.1084/jem.20011712
233. Wingender G, Stepniak D, Krebs P, Lin L, McBride S, Wei B, et al. Intestinal microbes affect phenotypes and functions of invariant natural killer T cells in mice. *Gastroenterology* (2012) 143(2):418–28. doi:10.1053/j.gastro.2012.04.017
234. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* (2012) 336(6080):489–93. doi:10.1126/science.1219328
235. Zeissig S, Blumberg RS. Commensal microbiota and NKT cells in the control of inflammatory diseases at mucosal surfaces. *Curr Opin Immunol* (2013) 25(6):690–6. doi:10.1016/j.coi.2013.09.012
236. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* (2012) 486(7402):207–14. doi:10.1038/nature11234
237. Sultan SE. *Organism & Environment: Ecological Development, Niche Construction, and Adaptation*. Oxford, UK: Oxford University Press (2015).
238. Selvanantham T, Lin Q, Guo CX, Surendra A, Fieve S, Escalante NK, et al. NKT cell-deficient mice harbor an altered microbiota that fuels

intestinal inflammation during chemically induced colitis. *J Immunol* (2016) 197(11):4464–72. doi:10.4049/jimmunol.1601410
239. Roy S, Trinchieri G. Microbiota: a key orchestrator of cancer therapy. *Nat Rev Cancer* (2017) 17(5):271–85. doi:10.1038/nrc.2017.13
240. Haldane JBS. Disease and evolution. *La ricerca scientifico, supplemento* (1949) 19:68–75.
241. Dronamraju KR, editor. *Selected Genetic Papers of J.B.S. Haldane*. New York/London: Garland Publishing (1990). 542 p.
242. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T cell antigen receptor recognition of antigen-presenting molecules. *Annu Rev Immunol* (2015) 33:169–200. doi:10.1146/annurev-immunol-032414-112334
243. Yang Z, Wang C, Wang T, Bai J, Zhao Y, Liu X, et al. Analysis of the reptile CD1 genes: evolutionary implications. *Immunogenetics* (2015) 67(5–6):337–46. doi:10.1007/s00251-015-0837-2
244. Boudinot P, Mondot S, Jouneau L, Teyton L, Lefranc MP, Lantz O. Restricting nonclassical MHC genes coevolve with TRAV genes used by innate-like T cells in mammals. *Proc Natl Acad Sci U S A* (2016) 113(21):E2983–92. doi:10.1073/pnas.1600674113
245. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* (1987) 4(4):406–25.
246. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* (1985) 39(4):783–91. doi:10.1111/j.1558-5646.1985.tb00420.x
247. Nei M, Kumar S. *Molecular Evolution and Phylogenetics*. New York: Oxford University Press (2000).
248. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* (2013) 30(12):2725–9. doi:10.1093/molbev/mst197
249. Wund M, Myers P. *The Animal Diversity Web*. (2017). Available from: http://animaldiversity.org/accounts/Mammalia/
250. Corbert G, Hill J. *A World List of Mammalian Species*. New York/London: Facts on File Publications/British Museum of Natural History (1991).
251. Palmer D. *Evolution: The Story of Life*. Berkley, Los Angeles: University of California (2009).
252. Louis A, Nguyen NT, Muffato M, Roest Crollius H. Genomicus update 2015: KaryoView and MatrixView provide a genome-wide perspective to multispecies comparative genomics. *Nucleic Acids Res* (2015) 43(Database issue):D682–9. doi:10.1093/nar/gku1112
253. Alonzo ES, Sant'Angelo DB. Development of PLZF-expressing innate T cells. *Curr Opin Immunol* (2011) 23(2):220–7. doi:10.1016/j.coi.2010.12.016
254. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol* (2013) 25(2):161–7. doi:10.1016/j.coi.2013.01.003
255. Ishizuka IE, Constantinides MG, Gudjonson H, Bendelac A. The innate lymphoid cell precursor. *Annu Rev Immunol* (2016) 34:299–316. doi:10.1146/annurev-immunol-041015-055549
256. Kastenmuller W, Torabi-Parizi P, Subramanian N, Lammermann T, Germain RN. A spatially-organized multicellular innate immune response in lymph nodes limits systemic pathogen spread. *Cell* (2012) 150(6):1235–48. doi:10.1016/j.cell.2012.07.021
257. Van Kaer L, Algood HMS, Singh K, Parekh VV, Greer MJ, Piazuelo MB, et al. CD8alphaalpha(+) innate-type lymphocytes in the intestinal epithelium mediate mucosal immunity. *Immunity* (2014) 41(3):451–64. doi:10.1016/j.immuni.2014.08.010
258. Bendelac A, Bonneville M, Kearney JF. Autoreactivity by design: innate B and T lymphocytes. *Nat Rev Immunol* (2001) 1(3):177–86. doi:10.1038/35105052
259. Multiple. Focus on: the inbetweeners: innate-like lymphocytes. *Nat Rev Immunol* (2013) 13(February):73–149.

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Neurofibromin 1 Impairs Natural Killer T-Cell-Dependent Antitumor Immunity against a T-Cell Lymphoma

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Neurofibromin 1 (NF1) is a tumor suppressor gene encoding a Ras GTPase that negatively regulates Ras signaling pathways. Mutations in NF1 are linked to neurofibromatosis type 1, juvenile myelomonocytic leukemia and Watson syndrome. In terms of antitumor immunity, CD1d-dependent natural killer T (NKT) cells play an important role in the innate antitumor immune response. Generally, Type-I NKT cells protect (and Type-II NKT cells impair) host antitumor immunity. We have previously shown that CD1d-mediated antigen presentation to NKT cells is regulated by cell signaling pathways. To study whether a haploinsufficiency in NF1 would affect CD1d-dependent activation of NKT cells, we analyzed the NKT-cell population as well as the functional expression of CD1d in *Nf1*$^{+/-}$ mice. *Nf1*$^{+/-}$ mice were found to have similar levels of NKT cells as wild-type (WT) littermates. Interestingly, however, reduced CD1d expression was observed in *Nf1*$^{+/-}$ mice compared with their WT littermates. When inoculated with a T-cell lymphoma *in vivo*, *Nf1*$^{+/-}$ mice survived longer than their WT littermates. Furthermore, blocking CD1d *in vivo* significantly enhanced antitumor activity in WT, but not in *Nf1*$^{+/-}$ mice. In contrast, a deficiency in Type-I NKT cells increased antitumor activity in *Nf1*$^{+/-}$ mice, but not in WT littermates. Therefore, these data suggest that normal NF1 expression impairs CD1d-mediated NKT-cell activation and antitumor activity against a T-cell lymphoma.

Keywords: neurofibromin 1, CD1d, natural killer T cells, T-cell lymphoma, antitumor immunity

INTRODUCTION

Neurofibromatosis type 1 is an autosomal-dominant disorder caused by a mutation in a tumor suppressor gene encoding the protein neurofibromin 1 (NF1) (1), affecting 1 in 3,500 individuals worldwide. NF1 is a p21^{ras} (Ras) guanosine triphosphatase (GTP)-activating protein (GAP). It catalyzes the hydrolysis of Ras-GTP, thus negatively regulating multiple Ras-dependent cellular signaling pathways (1). Mutations in *NF1* are associated with many diseases, including hematopoietic cancers such as myeloid leukemia and diffuse plexiform neurofibromas (2). Extensive studies from human tissue analyses and mouse models have discovered that loss of heterogyzosity (LOH) of *NF1* in Schwann cells and a heterozygous *NF1* microenvironment are both important for the formation of neurofibromas (3, 4). LOH may also explain the localized formation of tumors in patients with neurofibromatosis type 1 (1).

Ras-dependent signaling pathways have been shown to be important for αβ T-cell positive selection (5). Because NF1 is a negative regulatory GAP and highly expressed in leukocytes (6), the absence of NF1 may affect T-cell development. An *Nf1*$^{-/-}$ mutation is embryonic lethal (1). Therefore, the method of *Nf1*$^{-/-}$ fetal liver reconstitution to immune-deficient mice, such as Rag1 KO mice, has been used to study T-cell development in the absence of NF1 (7). Although an *nf1* deficiency in mice increases T-cell numbers in both thymus and spleen, it also causes impaired proliferation of T cells in response to *in vitro* stimulation (7). Moreover, antigen receptor-induced proliferation is also defective in NF1-deficient peripheral B cells (8), implicating a positive (but unknown) role for NF1 in regulating B and T-cell receptor (TCR)-induced proliferation. An earlier study indicated that NF1 promotes thymocyte positive selection, but has no effect on negative selection (9). Increasing evidence also suggests that NF1 may function in other cellular processes besides negatively regulating Ras function (10). For example, the Sec14-homology domain of NF1 is involved in forming a bipartite lipid-binding module, and possibly binds to cellular glycerophospholipid ligands (11). The loss of NF1 in *Drosophila* causes a reduction in body size, which is rescued by increasing cAMP protein kinase (PKA) signaling; this suggests that NF1 may also regulate the cAMP signaling pathway in a GAP-independent manner (12).

Natural killer T (NKT) cells express both natural killer (NK) and T-cell markers. Unlike conventional T cells which recognize peptide antigens presented by MHC class I and II molecules, NKT cells are activated by lipid antigens presented by the MHC class I-like molecule, CD1d. CD1d-deficient mice lack NKT cells and NKT-cell development requires positive selection in the thymus, similar to conventional T-cell development (13). Ras/mitogen-activated protein kinase (MAPK) signaling pathways, which are important for αβ T-cell positive selection (5), have also been shown to be critical for NKT-cell development (14). Furthermore, previous work from our laboratory has demonstrated that stimulation of MAPK pathways affects CD1d-mediated antigen presentation (15, 16). We have found that activation of the p38 pathway inhibits, whereas activation of ERK pathway increases, CD1d-mediated antigen presentation to NKT cells, likely through regulating the trafficking of CD1d molecules in antigen-presenting cells (15). In line with this, we reported that anthrax toxin inhibits CD1d-mediated antigen presentation by targeting the ERK pathway (16).

Based on TCR usage, NKT cells can be divided into two groups: Type-I (invariant) and Type-II (other CD1d-restricted) NKT cells. Type-I NKT (also called *i*NKT) cells express an invariant TCR α-chain rearrangement (Vα14Jα18 in mice and Vα24Jα18 in humans) that is associated with β-chains of limited diversity (Vβ8.2, Vβ7, and Vβ2 in mice; Vβ11 in humans). The glycolipid α-galactosylceramide (α-GalCer or PBS57), originally derived from a marine sponge, has been shown to be a specific activator of *i*NKT cells in a CD1d-dependent manner (17, 18). Type-II NKT cells are less well-defined, due to a paucity of ligands identified that are recognized by these NKT cells (19, 20). However, by studying CD1d-deficient (lacking both Type-I and Type-II NKT cells) and Jα18-deficient mice (lacking only Type-I NKT cells), it is believed that Type-II NKT cells are similar to T regulatory cells (Tregs) and are mostly immunosuppressive (21). In line with this idea, Type-II NKT cells have been shown to impair tumor immunosurveillance in a CD1d-dependent manner (22).

In the current study, we asked whether NF1, a negative regulator of Ras/MAPK pathways, impacts CD1d-dependent antitumor activity by NKT cells. Because an *Nf1*$^{-/-}$ mutation is embryonic lethal, a haploinsufficient (*Nf1*$^{+/-}$) mouse model is commonly used for the study of NF1 function *in vivo*. We analyzed NKT-cell activity as well as the functional expression of CD1d in *Nf1*$^{+/-}$ mice, in order to determine whether a haploinsufficiency in NF1 would affect the CD1d/NKT-cell axis in the context of NKT-cell-mediated antitumor activity.

MATERIALS AND METHODS

Animals

Female C57BL/6 wildtype (WT) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Male *Nf1*$^{+/-}$ mice were kindly provided by Dr. Wade Clapp (Indiana University, Indianapolis, IN, USA). *CD1d1* KO (*CD1d1*$^{-/-}$) mice on the C57BL/6 background (23) were a kind gift from Dr. Luc Van Kaer (Vanderbilt University, Nashville, TN, USA). Jα18-deficient C57BL/6 mice were also obtained from Dr. Van Kaer, with permission from Professor M. Taniguchi (Chiba University, Chiba, Japan). All mice were bred in specific pathogen-free facilities at the Indiana University School of Medicine. *Nf1*$^{+/-}$ mice were backcrossed to *CD1d1*$^{-/-}$ mice or *Jα18*$^{-/-}$ to obtain *Nf1*$^{+/-}$/*CD1d1*$^{-/-}$ and *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ mice, respectively. All mice were age- and sex-matched littermates, both males and females were utilized, and used in all experiments between 8 and 16 weeks of age. All animal procedures were approved by the Indiana University School of Medicine's Institutional Animal Care and Use Committee.

Cell Lines

The Tap 2-deficient RMA/S T-cell lymphoma cell line was kindly provided by Drs. J. Yewdell and J. Bennink (National Institutes of Health, Bethesda, MD, USA). These cells were transfected with the pcDNA3.1-neo vector alone (RMA/S-V) or the vector with a mouse *cd1d1* cDNA insert (RMA/S-CD1d) as previously described (23). MC57G–CD1d cells were generated by transfecting the methylcholanthrene-induced fibrosarcoma cell line MC57G with a pSRα vector encoding mouse *cd1d1* cDNA (a kind gift from Dr. S. Balk, Harvard University, Cambridge, MA, USA).

Antibodies and Reagents

Allophycocyanin (APC)-conjugated, PBS57-loaded, and unloaded CD1d tetramers were provided by the NIH Tetramer Core Facility (Atlanta, GA, USA). APC-, Phycoerythrin (PE)-, and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAb) against murine NK cell-, B-cell- or T-cell-specific markers, including NK1.1, MHC class II, CD11c, B220, CD1d (1B1), CD4, CD8, and TCRβ, were purchased from BD Biosciences (San Diego, CA, USA). PE/Cy7-conjugated

anti-CD21 and PerCP/Cy5.5-conjugated anti-CD23 were from Biolegend (San Diego, CA, USA). The mouse CD1d-specific mAb 1H6 generated by our laboratory has been previously described (24). The isotype control mAb TW2.3 was kindly provided by Drs. J. Yewdell and J. Bennink (NIH, Bethesda, MD, USA). 1H6 and TW2.3 hybridoma supernatants were purified by immobilized protein A agarose beads for *in vivo* use.

Flow Cytometry

Thymocytes and splenocytes were harvested using standard procedures. Liver mononuclear cells (LMNCs) were harvested as described previously (25). To obtain bone marrow-derived dendritic cells (BMDCs), bone marrow cells obtained from mouse femurs and tibias were cultured in the presence of IL-4 (10 ng/mL) and GM-CSF (10 ng/mL) for 7 days. For flow cytometry analyses, single-cell suspensions of all indicated cell types were prepared, and 1×10^6 cells were incubated at 4°C for 30 min with various mAb as indicated. The cells were washed three times with HBSS containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA). All cells were fixed with 1% paraformaldehyde in PBS and analyzed on a FACSCalibur or LSRII (Becton Dickinson, San Jose, CA, USA).

T-Cell Stimulation Assays

Bone marrow-derived dendritic cells from *Nf1*$^{+/-}$ mice and their littermates were incubated with the mouse Type-I NKT hybridoma N38-2C12 (26) or Type-II NKT hybridoma N37-1A12 (27) [both hybridomas kindly provided by Dr. K Hayakawa (Fox Chase Cancer Center, Philadelphia, PA, USA)]. 5×10^4 hybridoma cells and 5×10^5 BMDCs were added to triplicate wells in 96-well microtiter plates for 24 h. Secreted IL-2 levels in the supernatants were measured by ELISA.

Western Blot Analysis

Thymocytes and splenocytes were lysed, separated on a 10% SDS-PAGE gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). The blot was then probed with phospho-JNK1/2 or ERK1/2-specific antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA), and developed using chemiluminescence prior to exposure on film. The same membrane was then stripped and reprobed with total JNK1/2- or ERK1/2-specific antibodies (Cell Signaling Technology Inc.). Images were quantified using ImageJ (1.37v; National Institutes of Health, Bethesda, MD, USA).

In Vitro Stimulation of NKT Cells

Liver mononuclear cells (2.5×10^5 cells/well) from *Nf1*$^{+/-}$ mice or WT littermates were cocultured with α-GalCer-pulsed MC57G–CD1d cells (5×10^5 cells/well) in triplicate wells of a 96-well microtiter plate. After culture at 37°C for 48 h, the supernatants were collected for the analysis of NKT-cell production of IFN-γ, IL-4, and IL-13 by ELISA.

Tumor Inoculation

Nf1$^{+/-}$, *CD1d1*$^{-/-}$, *Nf1*$^{+/-}$/*CD1d1*$^{-/-}$, *Jα18*$^{-/-}$, *Nf1*$^{+/-}$/*Jα18*$^{-/-}$, and their WT littermates were inoculated intraperitoneally (i.p.) with 5×10^5 RMA/S-V or RMA/S-CD1d cells in 500-μL IMDM media supplemented with 5% FBS. The mice were monitored for up to 60 days posttumor inoculation, as previously described (23). To block CD1d *in vivo*, the mice were injected i.p. with 50 μg/mouse of purified mouse CD1d-specific antibody (1H6) or isotype control mAb in PBS on days 1, 5, 10, and 20 posttumor inoculation.

Statistics

Graphs were generated and statistics calculated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The mean of triplicates of a representative assay is shown with error bars representing the SEM, using Student's *t*-test analyses. For the statistical analysis of survival rate, the log-rank test was performed. A p-value < 0.05 was considered significant.

RESULTS

Increased JNK and ERK Activation in *Nf1*$^{+/-}$ Mice

Previous reports have shown increased Ras-GTP levels in unstimulated thymocytes from *Nf1*$^{+/-}$ mice when compared with WT mice (7). In our study, we also observed elevated ERK phosphorylation in splenocytes and thymocytes when they were stimulated with Phorbol 12-myristate 13-acetate (**Figure 1A**; Figures S7A,B in Supplementary Material), suggesting elevated activation of the Ras/ERK pathway in *Nf1*$^{+/-}$ mice. Compared with their WT littermates, *Nf1*$^{+/-}$ mice were also found to have elevated JNK activation in the spleen and thymus (**Figure 1B**; Figures S7C,D in Supplementary Material). We did not observe any hyperactivation of p38 in the thymus or spleen from *Nf1*$^{+/-}$ mice (data not shown). These data indicate that elevated Ras-GTP activity causes hyperactivation of the ERK and JNK pathways in *Nf1*$^{+/-}$ mice.

Comparable *i*NKT-Cell Population in WT and *Nf1*$^{+/-}$ Mice

Previous studies have suggested that an NF1 deficiency increases the number of immature and mature conventional T cells *in vivo*, but reduces cell proliferation in response to TCR and IL-2 stimulation *in vitro* (7). NF1 promotes thymocyte positive selection, a process that is also required for NKT-cell development (9, 28). To determine if an NF1 deficiency affects *i*NKT-cell development, we compared the *i*NKT-cell populations in thymus, spleen, and liver from *Nf1*$^{+/-}$ mice to those from WT littermates. We found there were comparable levels of *i*NKT cells in WT and *Nf1*$^{+/-}$ mice (**Figure 2**), suggesting that a haploinsufficiency in NF1 has a minimal effect on NKT-cell development and their numbers in the periphery.

Lower CD1d Expression on BMDCs from *Nf1*$^{+/-}$ Mice

Although a haploinsufficiency in NF1 did not seem to affect *i*NKT-cell development, we found that BMDCs from *Nf1*$^{+/-}$ mice expressed lower levels of CD1d (but similar amounts of

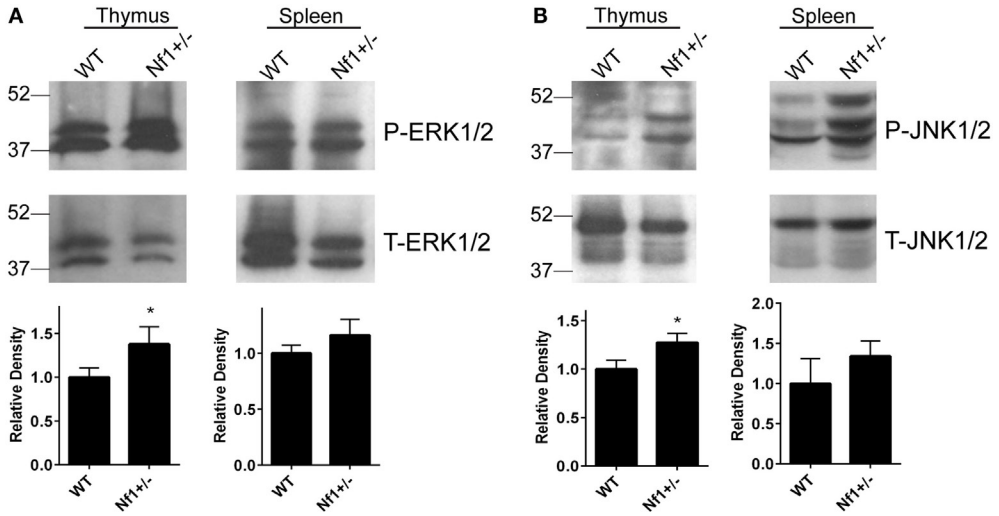


FIGURE 1 | Increased activation of ERK and JNK in the spleen and thymus of *Nf1*$^{+/-}$ mice. Splenocytes and thymocytes were treated with Phorbol 12-myristate 13-acetate (100 ng/mL) for 30 min. The cells were then lysed and resolved on a 10% SDS-PAGE gel for the detection of phosphorylated and total ERK1/2 **(A)** and JNK1/2 **(B)** expression by Western blot analysis. The relative levels of phosphorylated ERK1/2 and JNK1/2 compared with the total respective proteins were quantified by densitometry. Combined results from multiple experiments are shown in the bar graphs. The data are plotted as the mean ± SD. *$p < 0.05$.

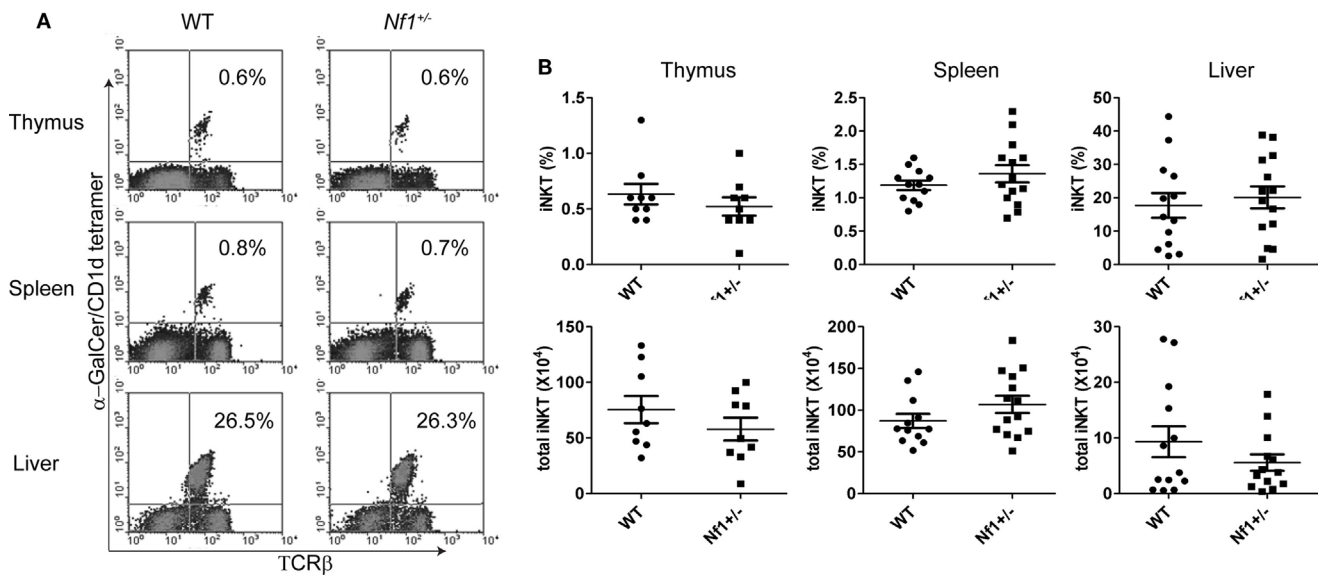


FIGURE 2 | Comparable numbers of *i*NKT cells in wildtype (WT) and *Nf1*$^{+/-}$ mice. **(A)** Thymocytes, splenocytes, and liver mononuclear cells from *Nf1*$^{+/-}$ mice and WT littermates were stained with α-GalCer-loaded CD1d tetramers and a TCR-β-specific antibody for the identification of *i*NKT cells, identified in the upper right quadrant. **(B)** Percentages (upper) and total numbers (lower) of *i*NKT cells are summarized for the thymus, spleen and liver. Pooled data from three independent experiments are shown. Each dot represents an individual mouse. The data are plotted as mean ± SEM.

MHC class I and II) on the cell surface compared with WT BMDCs (**Figure 3A**; Figure S1 in Supplementary Material). Furthermore, similar to the reduced CD1d expression observed in *Nf1*$^{+/-}$ BMDCs, there was also a significant decrease in the splenic B220^{+}CD21^{hi}CD23^{int}CD1d^{hi} population in *Nf1*$^{+/-}$ mice (**Figures 3B,C**). These cells express a high level of CD21 and low to intermediate levels of CD23, suggesting they are marginal zone B (MZB) cells. We also analyzed DCs (MHC II^{+} CD11c^{+}) and macrophages (MHC II^{+} F4/80^{+}) for CD1d expression, but there were no differences between WT and *Nf1*$^{+/-}$ mice (data not shown). Although BMDCs from *Nf1*$^{+/-}$ mice expressed less CD1d on their surface, they were similar to WT BMDCs in their ability to activate both Type-I and Type-II NKT-cell hybridomas (**Figure 3D**). Interestingly, thymocytes from both WT and *Nf1*$^{+/-}$ mice express similar levels of CD1d and have a comparable ability in stimulating NKT cells (Figure S8 in

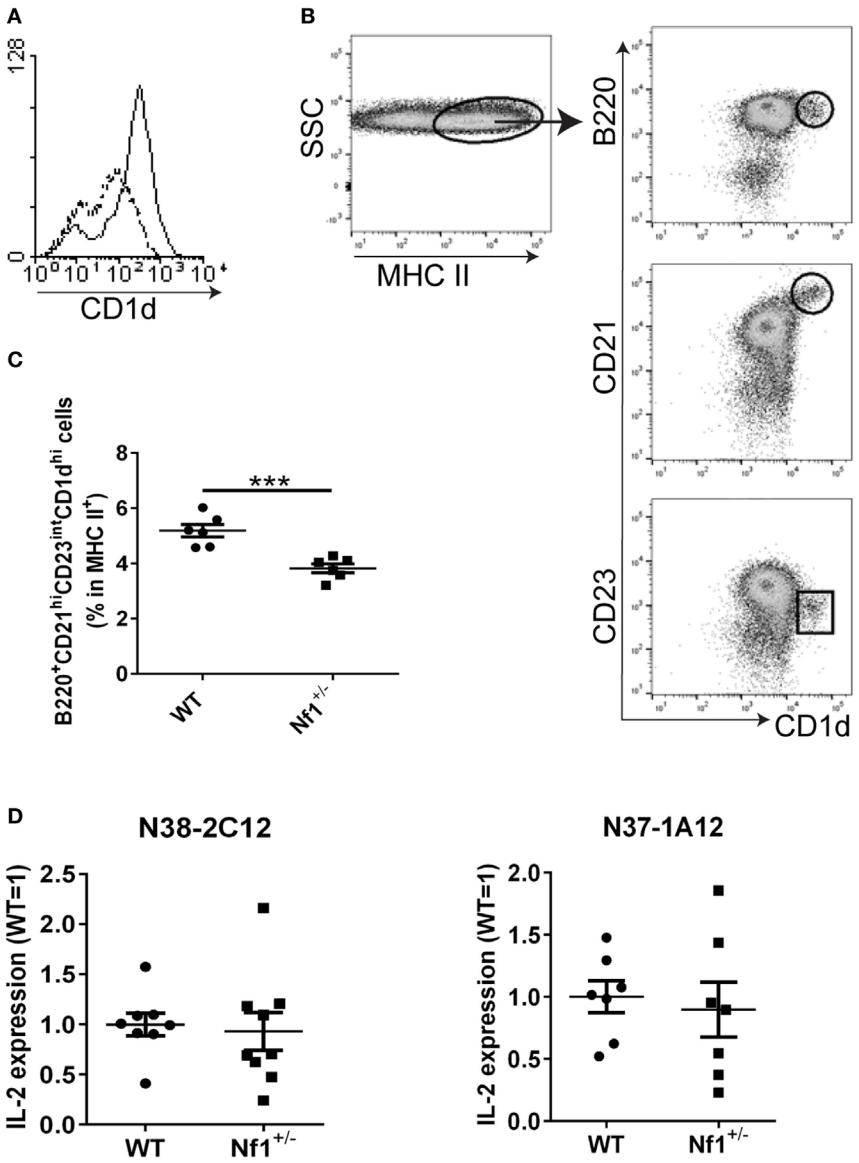


FIGURE 3 | Lower CD1d expression on cells from *Nf1*$^{+/-}$ mice. **(A)** Bone marrow-derived dendritic cells (BMDCs) from *Nf1*$^{+/-}$ and wildtype (WT) mice were fixed and stained with the anti-CD1d mAb, 1B1. CD1d-specific staining from a representative *Nf1*$^{+/-}$ mouse (dotted line) was overlaid with that of a WT littermate (solid line). **(B)** Splenocytes from *Nf1*$^{+/-}$ mice or WT littermates were stained with MHC II-, B220-, CD21-, CD23-, and CD1d-specific antibodies. MHC II^{+} cells were gated and further analyzed for B220, CD21, CD23, and CD1d expression by flow cytometry. The circled population corresponds to B220^{+}CD21^{hi}CD23^{int}CD1d^{hi} splenocytes. Combined results from multiple experiments are shown in **(C)**. Each dot represents an individual mouse. ***$p < 0.001$. **(D)** BMDCs from *Nf1*$^{+/-}$ mice or WT littermates were cocultured with the NKT-cell hybridomas, N38-2C12 and N37-1A12. The activation of NKT cells by BMDCs was determined by ELISA, measuring IL-2 secretion in the supernatants. The relative levels of IL-2 production in *Nf1*$^{+/-}$ BMDCs compared with WT (WT = 1) are indicated. Combined results from multiple experiments are shown. The data are plotted as the mean ± SEM. Each dot represents an individual mouse.

Supplementary Material); this suggests that a haploinsufficiency in NF1 does not alter the positive selection of NKT cells in the thymus. Overall, we conclude that a haploinsufficiency in NF1 reduces CD1d surface expression, but the decrease in CD1d expression in *Nf1*$^{+/-}$ cells is likely still above the normal threshold level necessary to activate NKT cells. This may help explain why *Nf1*$^{+/-}$ mice have a similar level of *i*NKT cells *in vivo* as their WT littermates.

Increased Activation of *i*NKT Cells from *Nf1*$^{+/-}$ LMNCs

Because there was decreased CD1d expression on APCs from *Nf1*$^{+/-}$ mice, we next wanted to find out whether NKT cells from *Nf1*$^{+/-}$ mice were functionally normal *in vitro* and *in vivo*. To test *i*NKT-cell function *in vitro*, LMNCs were cocultured with CD1d-expressing MC57G cells (derived from histocompatible H-2^{b} mice) in the presence of the *i*NKT-cell ligand, α-GalCer.

LMNCs from *Nf1*$^{+/-}$ mice were more responsive to CD1d-mediated antigen presentation than those from their WT littermates (**Figures 4A,B**). The addition of an anti-CD1d antibody blocked the activation of *i*NKT cells (**Figure 4C**), demonstrating that the NKT-cell activation was CD1d-specific. In contrast to antigen-specific activation, when LMNCs from *Nf1*$^{+/-}$ mice were stimulated with anti-CD3 and anti-CD28 antibodies, they secreted a similar level of cytokines as their WT littermates (Figure S2 in Supplementary Material). Thus, these data demonstrate that *i*NKT cells (but not conventional T cells) from *Nf1*$^{+/-}$ mice are more activated than those from WT littermates upon exogenous lipid Ag stimulation *in vitro*.

To determine whether the NF1 haploinsufficiency would affect NKT-cell function *in vivo*, we injected the *i*NKT-cell ligand α-GalCer to *Nf1*$^{+/-}$ mice and their WT littermates. At different time points, sera were harvested and circulating IL-4 and IFN-γ were measured. *Nf1*$^{+/-}$ mice produced similar levels of these cytokines as their WT littermates (Figure S3 in Supplementary Material). Therefore, these results suggest that *i*NKT cells are functionally normal in *Nf1*$^{+/-}$ mice.

Nf1$^{+/-}$ Mice Bearing RMA/S Tumors Surviving Longer Than WT Mice

Because we observed decreased CD1d expression but increased *i*NKT-cell activity *in vitro* in *Nf1*$^{+/-}$ mice, it was important to determine what the impact of a haploinsufficiency of NF1 would be on CD1d-dependent antitumor activity. Previous reports have suggested that *Nf1*$^{+/-}$ mice are predisposed to developing multiple cancers after 1 year of age, and thus have a shorter life span compared with WT mice (1). To address this question, we inoculated *Nf1*$^{+/-}$ mice and their WT littermates with the RMA/S T cell lymphoma transfected with an empty vector or the murine *cd1d1* cDNA (23). They were then observed for tumor incidence and survival rate. Surprisingly, *Nf1*$^{+/-}$ mice had a better survival rate and longer median survival time (MST) than their WT littermates when they were challenged with either CD1d-positive or CD1d-negative RMA/S tumor cells although, in this experiment, the difference between *Nf1*$^{+/-}$ and WT mice was not statistically significant (Figure S4 in Supplementary Material). Thus, in terms of survival, the antitumor activity in *Nf1*$^{+/-}$ mice exceeds that of their WT littermates.

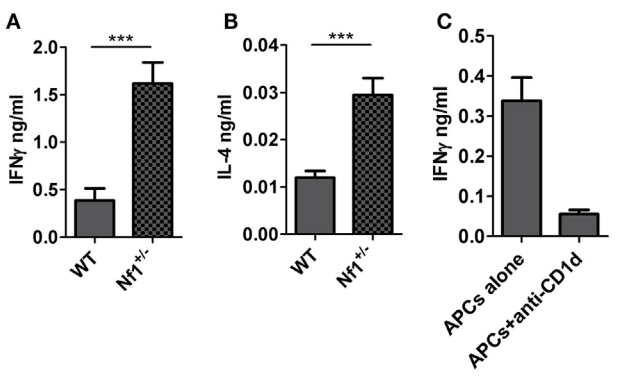


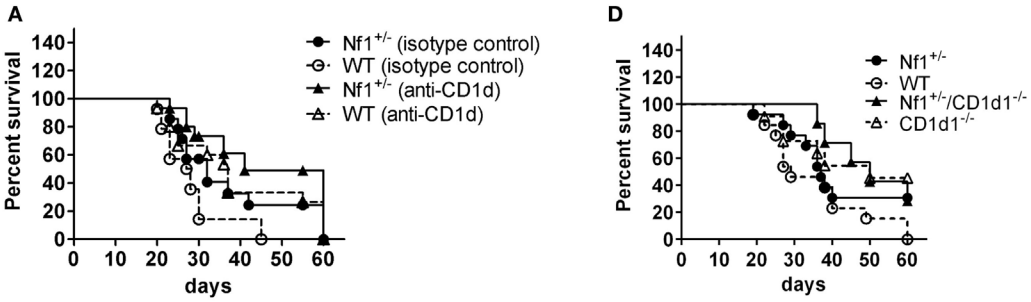
FIGURE 4 | Increased activation of liver *i*NKT cells from *Nf1*$^{+/-}$ mice. Liver mononuclear cells (LMNCs) from individual *Nf1*$^{+/-}$ mice or wildtype (WT) littermates were cocultured with MC57G–mCD1d cells in the presence of α-GalCer for 48 h. Activation of *i*NKT cells was measured by IFN-γ **(A)** and IL-4 **(B)** production into the supernatants. ***$p < 0.001$. **(C)** MC57G–mCD1d cells were cocultured with LMNCs from WT mice in the presence or absence of the murine CD1d-specific antibody, 1H6, for 48 h. Production of IFN-γ into the supernatants was measured by ELISA. The data are shown as the mean ± SEM. The results are representative of three independent experiments.

Blocking CD1d *In Vivo* Enhancing Antitumor Activity in WT But Not *Nf1*$^{+/-}$ Mice

Our previous studies have shown enhanced survival in CD1d-deficient mice when they were inoculated with RMA/S T-cell lymphoma cells (23). It was possible that the reduced CD1d expression in *Nf1*$^{+/-}$ mice altered host antitumor activity. To test this hypothesis, *Nf1*$^{+/-}$ and WT mice were treated with an anti-CD1d antibody or isotype control at various times before and after they were inoculated with RMA/S-CD1d cells. Blocking CD1d expression by a CD1d-specific antibody significantly enhanced antitumor activity in WT mice. The CD1d-specific antibody treatment in *Nf1*$^{+/-}$ mice also increased the survival rate of tumor-bearing mice. Although reproducible, the difference was not statistically significant in this experiment (**Figures 5A–C**). In a parallel experiment, CD1d was also genetically deleted from *Nf1*$^{+/-}$ mice by back-crossing *Nf1*$^{+/-}$ mice to *CD1d1*$^{-/-}$ mice. Thus, *Nf1*$^{+/-}$, *CD1d1*$^{-/-}$, *Nf1*$^{+/-}$/*CD1d1*$^{-/-}$, and WT mice were inoculated with RMA/S-CD1d cells, WT mice had the lowest survival rate among these four different strains of mice (**Figures 5D,E**). As was observed when CD1d was blocked by antibody *in vivo*, deleting CD1d genetically from WT (but not *Nf1*$^{+/-}$) mice significantly enhanced their survival rate (**Figure 5F**). Therefore, reduced CD1d expression in *Nf1*$^{+/-}$ mice very likely contributes to host antitumor activity in this model system.

NF1-Haploinsufficient Type-I (But Not Type II) NKT Cells Suppressing Antitumor Immunity *In Vivo*

CD1d-deficient mice lack both Type-I and Type-II NKT cells, whereas *Jα18*$^{-/-}$ mice only have Type-II NKT cells (29). To determine the impact of NF1 on the antitumor activity of Type-I and Type-II NKT cells, *Nf1*$^{+/-}$ mice were crossed with *Jα18*$^{-/-}$ mice to generate *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ mice. These mice, together with their WT, *Nf1*$^{+/-}$ and *Jα18*$^{-/-}$ littermates were inoculated with RMA/S-CD1d cells. While WT and *Jα18*$^{-/-}$ mice died at a similar rate, as we observed in multiple experiments, *Nf1*$^{+/-}$ mice survived significantly longer than their WT littermates (**Figures 6A,C,D**). Interestingly, *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ mice had the highest survival rate among the four experimental groups (**Figures 6A,C**). Thus, the deletion of Type-I NKT cells in *Nf1*$^{+/-}$ mice significantly enhanced survival (**Figure 6D**), which suggests NF1-haploinsufficent Type-I NKT cells may actually impair antitumor activity. However, as *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ mice survived much longer than their *Jα18*$^{-/-}$ littermates, this would indicate



B

	Isotype control (n=14/group)		Anti-CD1d (n=15/group)	
	MST	% Survival	MST	% Survival
WT	27.5	7.1%	37	13.3%
Nf1$^{+/-}$	32	21.4%	41	46.7%

C

	p Value	Significance
WT treated with isotype control vs. anti-CD1d	0.013	S
Nf1$^{+/-}$ treated with isotype control vs. anti-CD1d	0.177	NS

E

	MST	% Survival
WT (n=13)	29	0%
Nf1$^{+/-}$ (n=13)	37	30.8%
CD1d1$^{-/-}$ (n=11)	50	45.5%
Nf1$^{+/-}$/*CD1d1*$^{-/-}$ (n=7)	50	28.6%

F

	p Value	Significance
WT vs. *CD1d1*$^{-/-}$	0.026	S
Nf1$^{+/-}$ vs. *Nf1*$^{+/-}$/*CD1d1*$^{-/-}$	0.480	NS

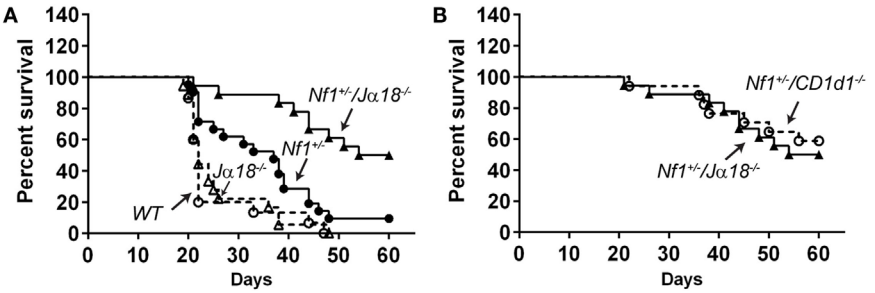
FIGURE 5 | Blocking CD1d *in vivo* enhancing antitumor activity in wildtype (WT) (but not *Nf1*$^{+/-}$) mice. **(A)** *Nf1*$^{+/-}$ mice (black symbols) and their WT littermates (white symbols) were treated i.p. with 50 μg of anti-CD1d antibody 1H6 (triangles) or isotype control (circles) on day 1, and days 5, 10, and 20 posttumor inoculation. The mice were inoculated i.p. with 5 × 10^5 RMA/S-CD1d cells on day 0 and survival was monitored for up to 60 days posttumor inoculation. Pooled data from three independent experiments are shown. *N* = 14–15 per group. The median survival time (MST) and percent survival on the final day were determined and summarized in **(B)**. Statistical analyses of the survival curves between the different groups are shown in **(C)**. The *p*-values were based on a log-rank test comparing the survival curves of the indicated two groups of mice. S, significant, $p < 0.05$; NS, not significant, $p > 0.05$. **(D)** *Nf1*$^{+/-}$ (black circles), *Nf1*$^{+/-}$/*CD1d1*$^{-/-}$ (black triangles) *CD1d1*$^{-/-}$ (white triangles), and their WT littermates (white circles) were inoculated with 5 × 10^5 RMA/S-CD1d cells on day 0 and their survival was monitored for up to 60 days posttumor inoculation. Pooled data from three independent experiments are shown. *N* = 7–13 per group. The MST and percent survival on the final day were determined and summarized in **(E)**. Statistical analyses of the survival curves between the different groups are shown in **(F)**. The *p*-values were based on a Log-rank test comparing the survival curve of the indicated two groups of mice. S, significant, $p < 0.05$; NS, not significant, $p > 0.05$.

that a haploinsufficiency of NF1 also results in a reduction of the immunosuppressive activity of Type-II NKT cells. In fact, *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ and *Nf1*$^{+/-}$/*CD1d1*$^{-/-}$ mice had similar survival rates posttumor inoculation (**Figures 6B–D**); this suggests that NF1-haploinsufficent Type-II NKT cells in *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ mice are less able to suppress antitumor immunity as compared with WT.

DISCUSSION

Neurofibromatosis type 1 is a disease caused by mutations in the *NF1* gene, a negative regulator of the Ras signaling pathway. Elevated Ras/ERK activation has been reported in *Nf1*$^{+/-}$ mice and reconstituted *NF1*$^{-/-}$ mice, as well as cells from NF1 patients (7, 30). Hyperactivation of the Ras/ERK pathway was also confirmed in the current study by the detection of increased phospho-ERK in the thymus and spleen of *Nf1*$^{+/-}$ mice. Although hyperactivation of the Ras/ERK pathway has been reported to be associated with a defect in NKT-cell development (31), we did not observe any defect in NKT-cell development in *Nf1*$^{+/-}$ mice. Instead, our work has demonstrated that NKT cells from *Nf1*$^{+/-}$ mice actually have enhanced CD1d-dependent activation, compared with those from their WT littermates. We observed similar levels of circulating cytokines in *Nf1*$^{+/-}$ mice after *in vivo* treatment with α-GalCer, even though APCs from *Nf1*$^{+/-}$ mice expressed lower levels of CD1d compared with their WT littermates. This may be explained by the increased responsiveness of NKT cells *in vitro*.

On the other hand, the reduced CD1d expression found in BMDCs from *Nf1*$^{+/-}$ mice suggests that NF1 positively regulates CD1d expression. It is worthwhile to point out that this effect might be due to *in vitro* cultures, as we did not observe a difference in CD1d expression in splenic DCs. It is well-known that NF1 is a negative regulator of the Ras/MAPK pathway (1). We not only observed hyperactivation of the Ras/ERK pathway but also detected elevated Ras/JNK pathway activation in the thymus and spleen of *Nf1*$^{+/-}$ mice. Daginakatte et al. (32) also reported increased JNK activation in *Nf1*$^{+/-}$ microglia cells, but not *Nf1*$^{-/-}$ astrocytes, which likely contributed to the increased proliferation of *Nf1*$^{+/-}$ microglia cells in that study. Consistent with our findings, they also did not observe increased p38 activation in these tissues (32). Our results are particularly interesting because we found that blocking the JNK pathway increases (and activation of JNK decreases) CD1d-mediated antigen presentation (Liu et al., manuscript in preparation). In contrast, we previously reported that elevated ERK activation enhances CD1d-mediated antigen presentation during a viral infection by



C

	MST	% Survival
WT (n=15)	22	0%
Jα18$^{-/-}$ (n=18)	22	0%
Nf1$^{+/-}$ (n=21)	37	9.52%
Nf1$^{+/-}$/*Jα18*$^{-/-}$ (n=18)	57	50%
Nf1$^{+/-}$/*CD1d1*$^{-/-}$ (n=18)	58	58%

D

	p Value	Significance
WT vs. *Nf1*$^{+/-}$	0.016	S
Jα18$^{-/-}$ vs. *Nf1*$^{+/-}$/*Jα18*$^{-/-}$	<0.001	S
WT vs. *Nf1*$^{+/-}$	0.66	NS
Nf1$^{+/-}$ vs. *Nf1*$^{+/-}$/*Jα18*$^{-/-}$	0.006	S
Nf1$^{+/-}$/*CD1d1*$^{-/-}$ vs. *Nf1*$^{+/-}$/*Jα18*$^{-/-}$	0.09	NS

FIGURE 6 | NF1-haploinsufficient Type-I (but not Type-II) NKT cells suppressing antitumor immunity *in vivo*. **(A)** *Nf1*$^{+/-}$ (black circles), *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ (black triangles), *Jα18*$^{-/-}$ (white triangles), and WT littermates (white circles) were inoculated with 5 × 10^5 RMA/S-CD1d cells on day 0 and survival was monitored for up to 60 days posttumor inoculation. Pooled data from two independent experiments are shown. N = 15–21 per group. **(B)** *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ and *Nf1*$^{+/-}$/*CD1d1*$^{-/-}$ mice were inoculated with RMA/S-CD1d cells and their survival was monitored as shown in **(A)**. Pooled data from two independent experiments are shown. N =18 per group. The MST and percent survival on the final day were determined and summarized in **(C)**. Statistical analyses of the survival curves between the different groups are shown in **(D)**. The p-values were based on a Log-rank test comparing the survival curve of the indicated two groups of mice. S, significant, $p < 0.05$; NS, not significant, $p > 0.05$.

regulating intracellular CD1d trafficking (15). Thus, it is very likely that the reduction of CD1d expression in *Nf1*$^{+/-}$ APCs is an outcome of the combined regulation of multiple signaling pathways as a consequence of the NF1 haploinsufficiency. It is worthwhile to mention that we did not observe any difference in CD1d recycling (Figure S5 in Supplementary Material) or CD1d distribution by confocal microscopy between BMDCs from *Nf1*$^{+/-}$ or WT littermates (data not shown). This suggests that the reduced CD1d expression on the cell surface of *Nf1*$^{+/-}$ APCs is not due to modified CD1d intracellular trafficking caused by an NF1 haploinsufficiency.

Marginal zone B cells, which express a high level of CD1d, are reduced in *Nf1*$^{+/-}$ mice. Moreover, it is known that Notch2 is indispensable to MZB development (33). A recent report suggests that Notch is the effector of NF1 in neurological tissue (34). Thus, a haploinsufficiency of NF1 may affect Notch2 and thereby alter MZB development. However, NF1 may have other unknown functions, not just as a Ras-GAP. A 2,839-amino-acid protein, NF1 contains two major functional domains: a Ras-GAP-related domain (Ras-GRD) and a Sec14-interactive domain. The Sec14-interactive domain is involved in forming a bipartite lipid-binding module and possibly binds to a cellular glycerophospholipid ligand (11). Further investigations are needed to determine how NF1 regulates CD1d expression.

We observed increased antitumor activity in *Nf1*$^{+/-}$ mice compared with their WT littermates. Although the differences were not always statistically significant, *Nf1*$^{+/-}$ mice consistently survived longer than their WT littermates. Treatment with a CD1d-specific mAb has shown to protect mice from tumor metastasis by several groups (22, 35, 36). It has been suggested that the CD1d mAb may block the activation of "immunosuppressive" Type-II NKT cells (22). Crosslinking CD1d by a specific mAb can also activate antigen-presenting cells, such as DCs, to produce the proinflammatory cytokines IL-12 and IFN-γ (35, 36). Reduced CD1d expression in *Nf1*$^{+/-}$ mice may be somewhat like CD1d-deficient mice (or CD1d mAb-treated mice), in that there is a dysfunction in (or reduced activity of) "immunosuppressive" Type-I NKT cells; this could explain the increased antitumor activity observed in *Nf1*$^{+/-}$ mice. It is also consistent with the findings that blocking CD1d *in vivo* enhanced antitumor activity in WT but *Nf1*$^{+/-}$ mice. In the current study, *WT* and *Jα18*$^{-/-}$ mice died at similar rates and *CD1d1*$^{-/-}$ mice survived longer than WT mice. The results suggest that Type-I NKT cells have little impact on antitumor activity whereas Type-I NKT cells are *immunosuppressive* in this model system (**Figure 7**). *Nf1*$^{+/-}$/*Jα18*$^{-/-}$ mice survived much longer than *Nf1*$^{+/-}$ littermates, suggesting that NF1-haploinsufficient Type-I NKT cells, although demonstrating increased activity *in vitro*, suppressed antitumor activity *in vivo*.

Type-I NKT cells can directly destroy tumor cells, especially those expressing CD1d on their surface, by performing cytolysis *via* perforin, granzyme B, Fas ligand (FasL), and TRAIL (37). Type-I NKT cells can also suppress the function of myeloid-derived suppressor cells (MDSC) and suppressive IL-10-producing neutrophils, to enhance antitumor immunity (20). Type-I NKT cells are capable of producing both Th1 and Th2 cytokines (38). The avidity and stability of antigen/TCR complex determines the type of cytokine production. Strong antigen/TCR interaction causes

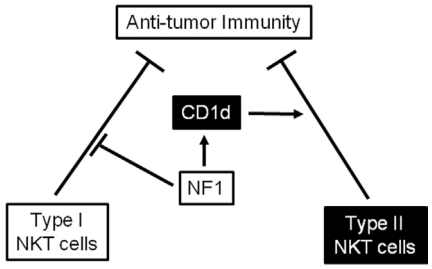


FIGURE 7 | A working model illustrating that neurofibromin 1 (NF1) plays distinct roles in regulating the antitumor activity of Type-I and Type-II NKT cells *in vivo*. NF1 reduces the immunosuppressive activity of Type-I NKT cells, as NF1-haploinsufficient Type-I NKT cells suppress antitumor immunity. In contrast, NF1 expression upregulates CD1d levels and enhances the immunosuppressive activity of Type-II NKT cells. In line with this, a haploinsufficiency in NF1 causes a reduction in CD1d expression and decreases the immunosuppressive activity of Type-II NKT cells, augmenting antitumor immunity.

NKT cells to produce Th1 cytokines, whereas weak antigen/TCR interaction results in Th2 cytokines from NKT cells (39). Th1-biased and IFNγ-producing Type-I NKT cells greatly boost antitumor immunity (20). On the other hand, Type-I NKT cells have been reported to be immunosuppressive by supporting Tregs and/or suppressing tumor-specific $CD8^+$ T cells (40, 41). In the current study, the reduced CD1d expression observed in $Nf1^{+/-}$ mice may further cause Type-I NKT cells to become Th2-biased and thereby suppress the antitumor activity of CTL and NK cells. In conclusion, NF1-haploinsufficient Type-I NKT cells are more immunosuppressive compared with WT Type-I NKT cells, through a currently unknown mechanism.

In contrast, $Nf1^{+/-}/J\alpha18^{-/-}$ mice survived much longer than their $J\alpha18^{-/-}$ littermates, indicating that Type-II NKT cells in $Nf1^{+/-}$ mice are not as immunosuppressive as WT Type-II NKT cells. $Nf1^{+/-}/J\alpha18^{-/-}$ mice survived at a similar rate as $Nf1^{+/-}/CD1^{-/-}$ mice, further confirming that Type-II NKT cells in $Nf1^{+/-}$ mice are not immunosuppressive. Our work suggests that NF1 is required for the immunosuppressive activity of Type-II NKT cells. The reduced CD1d expression in $Nf1^{+/-}$ mice may functionally alter Type-II NKT cells, moving from suppressing to enhancing antitumor activity.

A recent publication has suggested that Tregs are also important in the balance of antitumor activity involving Type-I/Type-II NKT cells (29). We did not observe any changes in Tregs in our studies (data not shown). Further studies are needed to investigate tumor immunosurveillance by Type-I/Type-II NKT cells as well as Tregs in $Nf1^{+/-}$ mice.

One question raised from our tumor challenge study is the identity of the effector cells that are responsible for removing the tumor cells *in vivo*. Because the RMA/S cell line is Tap-2 deficient, they express a very low level of MHC I molecules on their surface (42). Thus, the effector cells for eliminating RMA/S cells are unlikely to be $CD8^+$ cytotoxic T cells. NKT cells have also been shown to exhibit cytotoxicity activity against $CD1d^+$ cells (23). However, because $Nf1^{+/-}$ mice are more resistant to both $CD1d^+$ and $CD1d^-$ RMA/S cells, it is unlikely that the sole effector cells are NKT cells. Another population of cytotoxic cells that could play a role here are NK cells. RMA/S cells are highly susceptible to NK cell-mediated lysis (43). We speculate that Type-I and Type-II NKT cells may impact the function of NK cells in the RMA/S tumor model (**Figure 7**). On the other hand, the cytolytic activity of NK cells is also regulated by many signaling pathways (44). It has been reported that NK cells from vav-1 (a GEF)-deficient mice have reduced cytotoxicity (45), suggesting that NK cell activity may be impacted by changes in Ras/MAPK pathways. However, in the current study, we did not observe increased cytotoxicity by NK cells in $Nf1^{+/-}$ mice (Figure S6 in Supplementary Material). Of further interest, a recent report suggests that the inoculation of mice with RMA/S cells causes NK cell anergy and escape from antitumor immunity (46). Importantly, NK cell anergy only occurs in the tumor proximal environment and is likely due to impaired ERK activation downstream of activating receptors on NK cells (46). It is possible that NK cells in $Nf1^{+/-}$ mice may be compensated for by reduced ERK phosphorylation and rescue of MAPK/ERK signaling in the tumor microenvironment; thus, $Nf1^{+/-}$ mice would exhibit an increase in antitumor immunity and have enhanced survival. Further studies will be focused on how NF1 regulates the antitumor activity of NKT and NK cells in $Nf1^{+/-}$ mice.

Neurofibromas are derived from a broad range of cells, including hyperproliferative Schwann cells, fibroblasts, mast cells and perineural cells (47). Loss of heterogyzosity of NF1 in Schwann cells and a heterozygous NF1 microenvironment are both important for the formation of neurofibromas (3). Schwann cells have been shown to express CD1d and can activate NKT cells to secrete anti-inflammatory cytokines (48). We speculate that the absence of NF1 in Schwann cells from NF1 patients may cause a deficiency in CD1d expression. It would be interesting to study the tumor immunosurveillance activity of Type-I and Type-II NKT cells within the neurofibroma microenvironment, where Schwann cells are NF1-deficient. Further studies are necessary to understand the role of the CD1d/NKT-cell axis in NF1-dependent disease progression.

In summary, we have found reduced CD1d expression but increased antitumor activity in a haploinsufficiency model of NF1. This is likely due to reduced immunosuppressive activity by Type-II NKT cells, rather than by an increase in antitumor activity by Type-I NKT cells. The results support the hypothesis that NF1 regulates CD1d-mediated NKT-cell activation and consequent antitumor activity (**Figure 7**). Future work will focus on investigating how NF1 may regulate the antitumor activity of NKT cells. Our study may therefore provide mechanistic support to target NF1 to improve CD1d/NKT-cell-based immunetherapy.

ETHICS STATEMENT

All animal procedures were approved by the Indiana University School of Medicine's Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

RB and JL contributed to the concept and design of the paper. JL, RG, MK, and GR contributed to the acquisition of data. JL, RG,

and MK contributed to the analysis and interpretation of data. JL and RB contributed to the writing, review, and/or revision of the manuscript. JL, RG, MK, and GR contributed to the administrative, technical, or material support of the study. RB contributed to the supervision of the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01901/full#supplementary-material.

REFERENCES

1. Staser K, Yang FC, Clapp DW. Mast cells and the neurofibroma microenvironment. *Blood* (2010) 116(2):157–64. doi:10.1182/blood-2009-09-242875
2. Brems H, Beert E, de Ravel T, Legius E. Mechanisms in the pathogenesis of malignant tumours in neurofibromatosis type 1. *Lancet Oncol* (2009) 10(5):508–15. doi:10.1016/S1470-2045(09)70033-6
3. Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. *Science* (2002) 296(5569):920–2. doi:10.1126/science.1068452
4. Yang FC, Ingram DA, Chen S, Zhu Y, Yuan J, Li X, et al. Nf1-dependent tumors require a microenvironment containing Nf1±- and c-kit-dependent bone marrow. *Cell* (2008) 135(3):437–48. doi:10.1016/j.cell.2008.08.041
5. Alberola-Ila J, Hogquist KA, Swan KA, Bevan MJ, Perlmutter RM. Positive and negative selection invoke distinct signaling pathways. *J Exp Med* (1996) 184(1):9–18. doi:10.1084/jem.184.1.9
6. Gutmann DH, Wood DL, Collins FS. Identification of the neurofibromatosis type 1 gene product. *Proc Natl Acad Sci U S A* (1991) 88(21):9658–62. doi:10.1073/pnas.88.21.9658
7. Ingram DA, Zhang L, McCarthy J, Wenning MJ, Fisher L, Yang FC, et al. Lymphoproliferative defects in mice lacking the expression of neurofibromin: functional and biochemical consequences of Nf1 deficiency in T-cell development and function. *Blood* (2002) 100(10):3656–62. doi:10.1182/blood-2002-03-0734
8. Kim TJ, Cariappa A, Iacomini J, Tang M, Shih S, Bernards A, et al. Defective proliferative responses in B lymphocytes and thymocytes that lack neurofibromin. *Mol Immunol* (2002) 38(9):701–8. doi:10.1016/S0161-5890(01)00101-8
9. Oliver JA, Lapinski PE, Lubeck BA, Turner JS, Parada LF, Zhu Y, et al. The Ras GTPase-activating protein neurofibromin 1 promotes the positive selection of thymocytes. *Mol Immunol* (2013) 55(3–4):292–302. doi:10.1016/j.molimm.2013.03.005
10. Le LQ, Parada LF. Tumor microenvironment and neurofibromatosis type I: connecting the GAPs. *Oncogene* (2007) 26(32):4609–16. doi:10.1038/sj.onc.1210261
11. Welti S, Fraterman S, D'Angelo I, Wilm M, Scheffzek K. The sec14 homology module of neurofibromin binds cellular glycerophospholipids: mass spectrometry and structure of a lipid complex. *J Mol Biol* (2007) 366(2):551–62. doi:10.1016/j.jmb.2006.11.055
12. The I, Hannigan GE, Cowley GS, Reginald S, Zhong Y, Gusella JF, et al. Rescue of a *Drosophila* NF1 mutant phenotype by protein kinase A. *Science* (1997) 276(5313):791–4. doi:10.1126/science.276.5313.791
13. Gapin L, Godfrey DI, Rossjohn J. Natural killer T cell obsession with self-antigens. *Curr Opin Immunol* (2013) 25(2):168–73. doi:10.1016/j.coi.2013.01.002
14. Krishna S, Zhong XP. Regulation of lipid signaling by diacylglycerol kinases during T cell development and function. *Front Immunol* (2013) 4:178. doi:10.3389/fimmu.2013.00178
15. Renukaradhya GJ, Webb TJ, Khan MA, Lin YL, Du W, Gervay-Hague J, et al. Virus-induced inhibition of CD1d1-mediated antigen presentation: reciprocal regulation by p38 and ERK. *J Immunol* (2005) 175(7):4301–8. doi:10.4049/jimmunol.175.7.4301
16. Khan MA, Gallo RM, Brutkiewicz RR. Anthrax lethal toxin impairs CD1d-mediated antigen presentation by targeting the ERK1/2 MAPK pathway. *Infect Immun* (2010) 78(5):1859–63. doi:10.1128/IAI.01307-09
17. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of $V_{\alpha}14$ NKT cells by glycosylceramides. *Science* (1997) 278(5343):1626–9. doi:10.1126/science.278.5343.1626
18. Brutkiewicz RR. CD1d ligands: the good, the bad, and the ugly. *J Immunol* (2006) 177(2):769–75. doi:10.4049/jimmunol.177.2.769
19. Marrero I, Ware R, Kumar V. Type II NKT cells in inflammation, autoimmunity, microbial immunity, and cancer. *Front Immunol* (2015) 6:316. doi:10.3389/fimmu.2015.00316
20. Robertson FC, Berzofsky JA, Terabe M. NKT cell networks in the regulation of tumor immunity. *Front Immunol* (2014) 5:543. doi:10.3389/fimmu.2014.00543
21. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4-STAT6 pathway. *Nat Immunol* (2000) 1(6):515–20. doi:10.1038/82771
22. Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, et al. A nonclassical non-Vα14Jα18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med* (2005) 202(12):1627–33. doi:10.1084/jem.20051381
23. Renukaradhya GJ, Sriram V, Du W, Gervay-Hague J, Van Kaer L, Brutkiewicz RR. Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* (2006) 118(12):3045–53. doi:10.1002/ijc.21764
24. Roberts TJ, Sriram V, Spence PM, Gui M, Hayakawa K, Bacik I, et al. Recycling CD1d1 molecules present endogenous antigens processed in an endocytic compartment to NKT cells. *J Immunol* (2002) 168(11):5409–14. doi:10.4049/jimmunol.168.11.5409
25. Tupin E, Kronenberg M. Activation of natural killer T cells by glycolipids. *Methods Enzymol* (2006) 417:185–201. doi:10.1016/S0076-6879(06)17014-7
26. Gui M, Li J, Wen LJ, Hardy RR, Hayakawa K. TCR β chain influences but does not solely control autoreactivity of Vα14Jα281 T cells. *J Immunol* (2001) 167(11):6239–46. doi:10.4049/jimmunol.167.11.6239
27. Burdin N, Brossay L, Koezuka Y, Smiley ST, Grusby MJ, Gui M, et al. Selective ability of mouse CD1 to present glycolipids: α-galactosylceramide specifically stimulates $V\alpha14^{+}$ NK T lymphocytes. *J Immunol* (1998) 161:3271–81.
28. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol* (2004) 4(3):231–7. doi:10.1038/nri1309
29. Izhak L, Ambrosino E, Kato S, Parish ST, O'Konek JJ, Weber H, et al. Delicate balance among three types of T cells in concurrent regulation of tumor immunity. *Cancer Res* (2013) 73(5):1514–23. doi:10.1158/0008-5472.CAN-12-2567
30. Lee PR, Cohen JE, Fields RD. Immune system evasion by peripheral nerve sheath tumor. *Neurosci Lett* (2006) 397(1–2):126–9. doi:10.1016/j.neulet.2005.12.027

31. Shen S, Wu J, Srivatsan S, Gorentla BK, Shin J, Xu L, et al. Tight regulation of diacylglycerol-mediated signaling is critical for proper invariant NKT cell development. *J Immunol* (2011) 187(5):2122–9. doi:10.4049/jimmunol.1100495
32. Daginakatte GC, Gianino SM, Zhao NW, Parsadanian AS, Gutmann DH. Increased c-Jun-NH2-kinase signaling in neurofibromatosis-1 heterozygous microglia drives microglia activation and promotes optic glioma proliferation. *Cancer Res* (2008) 68(24):10358–66. doi:10.1158/0008-5472.CAN-08-2506
33. Saito T, Chiba S, Ichikawa M, Kunisato A, Asai T, Shimizu K, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* (2003) 18(5):675–85. doi:10.1016/S1074-7613(03)00111-0
34. Lopez-Juarez A, Titus HE, Silbak SH, Pressler JW, Rizvi TA, Bogard M, et al. Oligodendrocyte Nf1 controls aberrant notch activation and regulates myelin structure and behavior. *Cell Rep* (2017) 19(3):545–57. doi:10.1016/j.celrep.2017.03.073
35. Teng MW, Yue S, Sharkey J, Exley MA, Smyth MJ. CD1d activation and blockade: a new antitumor strategy. *J Immunol* (2009) 182(6):3366–71. doi:10.4049/jimmunol.0802964
36. Yue SC, Shaulov A, Wang R, Balk SP, Exley MA. CD1d ligation on human monocytes directly signals rapid NF-κB activation and production of bioactive IL-12. *Proc Natl Acad Sci U S A* (2005) 102(33):11811–6. doi:10.1073/pnas.0503366102
37. Nair S, Dhodapkar MV. Natural killer T cells in cancer immunotherapy. *Front Immunol* (2017) 8:1178. doi:10.3389/fimmu.2017.01178
38. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol* (2004) 22:817–90. doi:10.1146/annurev.immunol.22.012703.104608
39. Rossjohn J, Pellicci DG, Patel O, Gapin L, Godfrey DI. Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* (2012) 12(12):845–57. doi:10.1038/nri3328
40. Jiang S, Game DS, Davies D, Lombardi G, Lechler RI. Activated CD1d-restricted natural killer T cells secrete IL-2: innate help for CD4+CD25+ regulatory T cells? *Eur J Immunol* (2005) 35(4):1193–200. doi:10.1002/eji.200425899
41. Bjordahl RL, Gapin L, Marrack P, Refaeli Y. iNKT cells suppress the CD8+ T cell response to a murine Burkitt's-like B cell lymphoma. *PLoS One* (2012) 7(8):e42635. doi:10.1371/journal.pone.0042635
42. Aldrich CJ, Waltrip R, Hermel E, Attaya M, Lindahl KF, Monaco JJ, et al. T cell recognition of QA-1b antigens on cells lacking a functional Tap-2 transporter. *J Immunol* (1992) 149(12):3773–7.
43. Chang CS, Brossay L, Kronenberg M, Kane KP. The murine nonclassical class I major histocompatibility complex-like CD1.1 molecule protects target cells from lymphokine-activated killer cell cytolysis. *J Exp Med* (1999) 189(3):483–91. doi:10.1084/jem.189.3.483
44. Lanier LL. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol* (2008) 8(4):259–68. doi:10.1038/nri2276
45. Chan G, Hanke T, Fischer KD. Vav-1 regulates NK T cell development and NK cell cytotoxicity. *Eur J Immunol* (2001) 31(8):2403–10. doi:10.1002/1521-4141(200108)31:8<2403::AID-IMMU2403>3.0.CO;2-O
46. Ardolino M, Azimi CS, Iannello A, Trevino TN, Horan L, Zhang L, et al. Cytokine therapy reverses NK cell anergy in MHC-deficient tumors. *J Clin Invest* (2014) 124(11):4781–94. doi:10.1172/JCI74337
47. Serra E, Rosenbaum T, Winner U, Aledo R, Ars E, Estivill X, et al. Schwann cells harbor the somatic NF1 mutation in neurofibromas: evidence of two different Schwann cell subpopulations. *Hum Mol Genet* (2000) 9(20):3055–64. doi:10.1093/hmg/9.20.3055
48. Im JS, Tapinos N, Chae GT, Illarionov PA, Besra GS, DeVries GH, et al. Expression of CD1d molecules by human Schwann cells and potential interactions with immunoregulatory invariant NK T cells. *J Immunol* (2006) 177(8):5226–35. doi:10.4049/jimmunol.177.8.5226

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REVIEW
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The Yin and Yang of Invariant Natural Killer T Cells in Tumor Immunity—Suppression of Tumor Immunity in the Intestine

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CD1d-restricted invariant natural killer T (iNKT) cells are known as early responding, potent regulatory cells of immune responses. Besides their established role in the regulation of inflammation and autoimmune disease, numerous studies have shown that iNKT cells have important functions in tumor immunosurveillance and control of tumor metastasis. Tumor-infiltrating T helper 1 (TH1)/cytotoxic T lymphocytes have been associated with a positive prognosis. However, inflammation has a dual role in cancer and chronic inflammation is believed to be a driving force in many cancers as exemplified in patients with inflammatory bowel disease that have an increased risk of colorectal cancer. Indeed, NKT cells promote intestinal inflammation in human ulcerative colitis, and the associated animal model, indicating that NKT cells may favor tumor development in intestinal tissue. In contrast to other cancers, recent data from animal models suggest that iNKT cells promote tumor formation in the intestine by supporting an immunoregulatory tumor microenvironment and suppressing TH1 antitumor immunity. Here, we review the role of iNKT cells in suppression of tumor immunity in light of iNKT-cell regulation of intestinal inflammation. We also discuss suppression of immunity in other situations as well as factors that may influence whether iNKT cells have a protective or an immunosuppressive and tumor-promoting role in tumor immunity.

Keywords: natural killer T cells, CD1d, tumor immunity, immunosuppression, intestinal inflammation, intestinal polyposis

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INTRODUCTION

CD1d-restricted natural killer T (NKT) lymphocytes belong to a diverse group of non-conventional T cells that recognize non-peptide antigens. These T cells have some general features that distinguish them from conventional T cells that are activated by foreign peptides presented on MHC class-I and -II molecules (1, 2). Non-conventional T cells often have a memory/pre-activated phenotype and respond more rapidly to stimulation and can be activated in the absence of T-cell receptor (TCR) signals. They are generally not recirculating but preferentially localize to particular tissues and have a reduced TCR diversity compared with conventional T cells. These characteristics make

Abbreviations: AOM, azoxymethane; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; NKT, natural killer T; dNKT, NKT cell with diverse TCR; iNKT, NKT cell with invariant TCR; TCR, T-cell receptor; TH, T helper; TLR, toll-like receptor; Treg, regulatory T.

non-conventional T cells rapid responders to infection, inflammatory signals, and tissue damage and enable them to regulate the quality and quantity of immune responses. Recent progress in our understanding of the activation and function of these cells has led to an increased appreciation of their role in health and disease. The evolutionary conservation of CD1d and NKT cells between mouse and humans, and the essentially non-polymorphic nature of CD1d, has made the CD1d-NKT-cell system attractive to explore for the development of targeted immunomodulation.

Natural killer T cells recognize lipid and glycolipid antigens of self or microbial origin presented on the MHC class-I-like CD1d molecule. The term "NKT cells" is now generally used synonymously with CD1d-restricted T cells that carry TCRαβ (3). This definition will be used here. NKT cells can be divided into two subsets based on the TCR expressed. The first is type I or invariant NKT (iNKT) cells. These display an invariant TCR α-chain (Vα14-Jα18 in the mouse, Vα24-Jα18 in humans) paired with TCR β-chains using a limited set of Vβ segments but diverse CDR3 (4–6). In contrast, type II or diverse NKT (dNKT) cells have diverse TCR (7–9). The relative roles of these NKT-cell subsets have been explored in mice lacking all NKT cells (*CD1d*$^{-/-}$ mice) and mice lacking only iNKT cells (*Jα18*$^{-/-}$ mice). Studies of iNKT cells have also been greatly facilitated by the use of CD1d multimers loaded with the iNKT-cell ligand α-galactosylceramide (αGalCer) (10) which detects essentially all iNKT cells with high specificity. In contrast, while the role of dNKT cells can be studied in TCR transgenic mice (11) and deduced from comparisons of *CD1d*$^{-/-}$ and *Jα18*$^{-/-}$ mice, there are no specific reagents that detect all dNKT cells. Thus, studies of dNKT cells are more challenging. From studies of animal models and humans, both subsets of NKT cells have been suggested to play a role in diverse immune settings including autoimmunity, immunity to infections, and tumor immunity. Sometimes the two subsets have a similar function, while in other immune responses they counteract each other (12, 13). Besides the division of NKT cells based on their TCR, different functional programs have been identified in iNKT cells (14). For example, the iNKT1, iNKT2, and iNKT17 subsets express distinct sets of transcription factors. This makes them poised for the production of certain cytokines analogous to the T helper (TH) 1, TH2, and TH17 conventional T-cell subsets and corresponding subsets of innate lymphoid cells. More recently, iNKT cells with immunosuppressive function have been described that do not fit into this classification, as discussed below (15–17).

The majority or data from animal models and inferred from studies of human cancers show that iNKT cells enhance TH1 tumor immunity and combat tumors. However, in some situations, iNKT cells have surprisingly have demonstrated the opposite effect and promoted tumor development (**Figure 1**) (17–19). A recent example of iNKT-cell promotion of tumors is from a spontaneous mouse model for human colon cancer (17). We recently demonstrated that deletion of iNKT cells in this model reduces spontaneous intestinal polyp formation by 75%. Here, we will discuss our findings in light of the role of iNKT cells in intestinal immunity and compare tumor models that demonstrate tumor suppressive and tumor-promoting effects of iNKT cells.

NKT CELLS ENHANCING TUMOR IMMUNITY IN MANY MODELS

The potent role of iNKT cells in tumor immunosurveillance and suppression of tumor metastasis was demonstrated many years ago. The enhancement of tumor immunity by iNKT cells has been

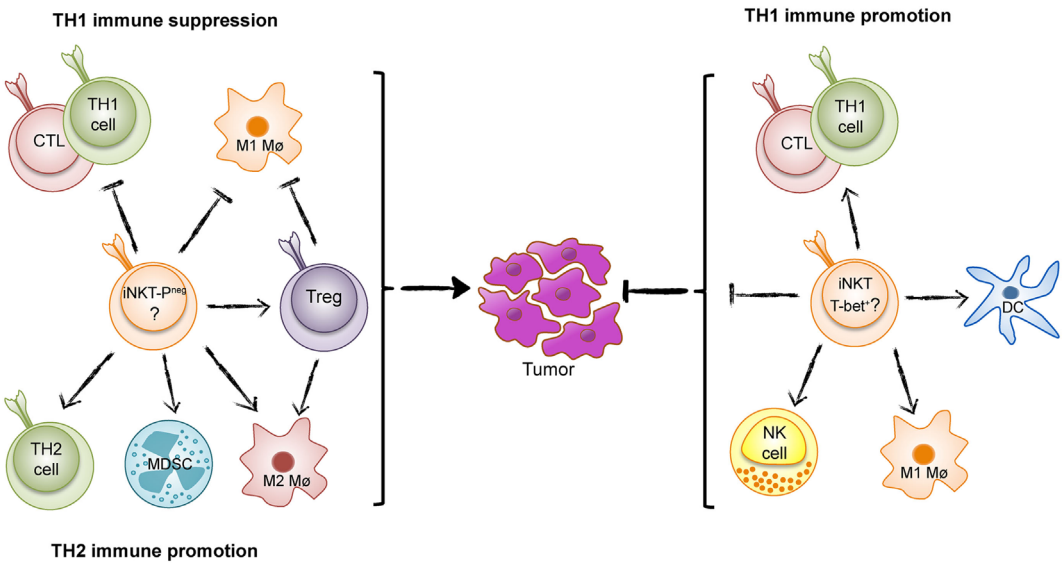

FIGURE 1 | The Yin and Yang of invariant natural killer T (iNKT) cells in tumor immunity. iNKT cells can either suppress T helper 1 (TH1) tumor immunity and promote an immunosurveillance environment as found in intestinal polyposis (Yin, left) (PLZF-neg; PLZF-negative), or enhance TH1 immunity to tumors as found in many tumor models and inferred from studies of human cancer (Yang, right).

described in several publications and discussed in many reviews [see for example Ref. (20)], and has led to intense studies aimed at targeting iNKT cells to combat cancer. The strong agonist iNKT-cell ligand αGalCer very efficiently activates iNKT cells leading to reduction or eradication of tumors in several animal models (13). Subsequent studies have addressed iNKT-cell function in tumor immunity after treatment with αGalCer and structural variants (21). iNKT cells can directly kill cancer cells, but the most important role of iNKT cells may be to activate other immune cells involved in tumor immunity. This includes activation of NK cells, maturation, and activation of dendritic cells, promotion of cytotoxic T cells and TH1 cells, and generally relies on the production of IFN-γ and TH1-related cytokines (**Figure 1**). iNKT cells can also counteract immunosuppressive cells. In patients, there is a reduced number of circulating iNKT cells in some cancers, and this reduction has been associated with poor survival in head and neck cancer and acute myeloid leukemia. These findings have led to clinical trials with iNKT-cell-directed immunotherapy in human cancer (22). In addition, iNKT cells perform natural tumor immunosurveillance in some animal models, suppressing the appearance of tumors and leukemia even in the absence of treatments that activate iNKT cells (23–26). In contrast to the usual protective function of iNKT cells, a natural immunosuppressive effect has been described for dNKT cells that promote tumor growth (27, 28).

iNKT CELLS SUPPRESSING TUMOR IMMUNITY IN MURINE INTESTINAL POLYPOSIS

The influence of NKT cells on tumor immunity or tumor growth and metastasis has frequently been investigated in tumor cell transplantation models such as the B16 melanoma model. These models have some advantages, including a coordinated initiation of growth, the availability of well-characterized tumor cell lines for transplantation as well as the possibility to genetically alter the tumor cells and design specific tools to study the tumor immune response. On the other hand, induction of tumors by injection of cell lines has the disadvantages of lacking the early steps of natural cancer initiation and the natural heterogeneity of tumors. Tumor growth is also usually at a site different from the origin of the tumor. These parameters may influence the immune response to the tumor, and the regulation of tumor immunity.

It is well known that cancer is promoted by inflammation. For example, the chronic inflammation in patients with inflammatory bowel disease (IBD) leads to increased risk for colorectal cancer. It has been described that iNKT cells promote inflammation in a mouse model for IBD (29), suggesting that iNKT cells might promote tumor formation in the intestine. To investigate this, we have studied iNKT-cell-mediated regulation of tumor immunity in the orthotopic spontaneous model for early stages of intestinal cancer, the *Apc*$^{min/+}$ mouse (30). *Apc*$^{min/+}$ mice are heterozygous for a truncating mutation in the adenomatous polyposis coli (*Apc*) tumor suppressor gene, and spontaneously develop polyps in the small and large intestines (31). The APC protein is part of an inhibitory complex associated with the canonical Wnt signaling pathway that ubiquitously controls cell differentiation and proliferation. Mutation of the *APC* gene is an early event in 80% of sporadic colorectal cancers in humans and is the mutated gene inherited in familial adenomatous polyposis. In the *Apc*$^{min/+}$ mouse, polyps develop following additional genetic events such as loss of heterozygosity of the *Apc* gene (31).

Using mice deficient in either all NKT cells or specifically lacking iNKT cells, we found a dramatic reduction of intestinal polyps, demonstrating that iNKT cells favor polyp growth in this tissue (17). Detailed investigation of immune parameters revealed that iNKT cells suppressed the expression of genes associated with TH1 immunity, including IFN-γ, inducible nitric oxide synthase (iNOS), IL-12p40, T-bet, and granzyme B. A TH1-type immune response has been shown to prevent tumors in the *Apc*$^{min/+}$ mouse model as well as in human colorectal cancer (32–34). In contrast, the presence of iNKT cells increased the proportion and activation of FoxP3-expressing regulatory T (Treg) cells specifically in polyps (17). The infiltration of high proportions of Treg cells in tumors is generally associated with suppressed tumor immunity and poor prognosis in cancer patients (35). Although Treg cells have a complex role in colon carcinogenesis, likely reflecting different functions of Treg cells at different stages of disease (36), deletion of Treg cells in the *Apc*$^{min/+}$ mouse resulted in enhanced accumulation of conventional CXCR3^{+} T lymphocytes in polyps (37). Moreover, iNKT cells augmented the proportion of M2-like macrophages, a cell type that is associated with tumor promotion. They also elevated the numbers of myeloid-derived suppressor cells, especially those with a polymorphonuclear phenotype (17). Taken together, we concluded that iNKT cells support an immunosuppressive microenvironment, most pronounced in the polyps, and directly and/or through the promotion of Treg cells, inhibited a TH1 antitumor immune response (**Figure 1**).

It should be noted that the immune microenvironment changes in tumors of colon cancer as the disease progresses. The role of Treg cells in human colon cancer has been controversial, and different studies have associated high densities of infiltrating Treg cells with either a better or worse outcome. A possible explanation for this was recently provided, as it was demonstrated that at later stages of colon cancer, Treg cells become positive for the transcription factor RORγt and acquire the capacity to produce the tumor-promoting cytokine IL-17 (36). RORγt-expressing Treg cells retained the capacity to suppress T lymphocytes, but were no longer anti-inflammatory. Similar Treg cells were found in the *Apc*$^{min/+}$ mouse, and ablation of RORγt in Treg cells in this model resulted in improved suppression of inflammation and attenuated polyposis. A study of human colorectal cancer demonstrated an increased infiltration of cells positive for Vα24, the TCR α-segment used by human iNKT cells, in carcinomas compared with unaffected tissue (38). A higher density of Vα24^{+} cells correlated with increased patient survival, suggesting that iNKT cells may be protective. This is in contrast to the findings in *Apc*$^{min/+}$ mice where iNKT cells promote polyps. Further studies, using specific reagents such as αGalCer-loaded CD1d multimers, are required to investigate whether iNKT cells, analogous to Treg cells, perform different function at different stages of intestinal cancer.

Quite surprisingly, the characteristics of iNKT cells in polyps of *Apc*$^{min/+}$ mice differed significantly from iNKT cells in lymphoid organs (17). Most importantly, they were negative for PLZF, which has been considered a master transcription factor for NKT cells (39). Polyp iNKT cells also had lower expression of CD4, NK1.1, and CD44, and were enriched for IL-10- and IL-17-producing cells. They also had a lower production of pro-inflammatory cytokines compared with iNKT cells in other organs. These distinct characteristics of intestinal polyp iNKT cells likely underpin their unusual function to suppress tumor immunity.

THE ROLE OF iNKT CELLS IN THE GUT DURING INTESTINAL INFLAMMATION AND INFLAMMATION-DRIVEN INTESTINAL CANCER

In the steady state, there does not seem to be a role for iNKT cells to maintain immune homeostasis in the intestine, as to our knowledge, there are no reports of evident inflammation or alteration in the inflammatory microenvironment in the gut in normally housed and unchallenged *Jα18*- or *CD1d*-deficient mice (also, our unpublished data). In contrast, iNKT cells have been shown to be involved in the regulation of inflammation in murine models of induced IBD and induced inflammation-associated colon cancer.

For example, the murine oxazalone-induced colitis model shares immunological features with human ulcerative colitis. It was demonstrated that iNKT cells had an important role in driving inflammation in this model (29) and IL-13 produced by iNKT cells was essential for disease to develop. A similar function was proposed for CD1d-restricted dNKT cells in patients with ulcerative colitis (40). To establish a model for inflammation-induced colon cancer, a chronic form of oxazalone-induced colitis was combined with the genotoxic agent azoxymethane (AOM) resulting in colon tumors. Tumor development was associated with the appearance of F4/80^{+}CD11b^{high}Gr1^{low} M2 macrophages that produced the tumor-promoting factors IL-6 and EGF, in a CD1d- and IL-13-dependent manner (41). Interestingly, while oxazalone + AOM treatment resulted in robust colitis in mice lacking the major signaling pathway for toll-like receptors (TLR) (MyD88$^{-/-}$ mice), these mice did not develop colon tumors. A dependence on MyD88 for polyp formation has also been demonstrated in *Apc*$^{min/+}$ mice (42), indicating that signaling from the microbial flora plays a major role in tumor development. The immunoregulatory functions of iNKT cells in the oxazalone colitis and the oxazalone + AOM models appear similar to what has been described for iNKT cells in the murine intestinal polyposis model (*Apc*$^{min/+}$ mice) (17). The similarity in regulatory function performed by iNKT cells in these models indicates that tissue location may influence local iNKT-cell behavior.

Both murine and human intestinal epithelial cells express CD1d (43, 44), which upon cross-linking by iNKT TCR mediates retrograde signaling in epithelial cells inducing their IL-10 production (45). Epithelial cell-specific deletion of IL-10 or CD1d leads to more severe oxazalone colitis, illustrating protective anti-inflammatory cross talk between intestinal epithelium and iNKT cells (46). In contrast, IL-13-mediated inflammation in this model was dependent on CD1d expression on bone-marrow-derived cells. Thus, in the oxazalone-induced IBD model, iNKT cells are activated to produce pro-inflammatory IL-13 by CD1d expressed by bone-marrow-derived cells, while interaction with CD1d on epithelial cells results in epithelial cell production of protective IL-10.

A somewhat different picture is found in the dextran sodium sulfate (DSS) colitis model of IBD, which results from disruption of the colonic epithelial barrier. Inflammation in this model differs from that of the oxazalone model in that it is mediated by innate cells and is not dependent on adaptive immunity (47). If present, however, T cells can promote the inflammatory response as exemplified by a TH1 or mixed TH1/2 response has been found in an acute or chronic DSS colitis, respectively. In the acute DSS model, activation of iNKT cells ameliorated disease after administration of αGalCer or OCH, a structural variant of αGalCer that skews the cytokine response toward TH2 (48, 49). It was therefore speculated that the protection was due to anti-inflammatory TH2-type cytokines induced by the activation of iNKT cells. The natural effect of iNKT cells on DSS colitis (without experimental activation of iNKT cells) was not directly addressed in these studies. In another model, repeated administration of DSS has been applied together with AOM to induce inflammation-driven colon cancer development. In these studies, an increased number of colon tumors and more severe inflammation were found in mice lacking iNKT cells compared with control mice (50). Administration of αGalCer, but not the TH2-skewing iNKT-cell ligand OCH, led to reduced inflammation, reduced colon tumor formation and decreased IL-13. This suggests that iNKT cells protected the mice from colitis, and consequently from the downstream development of colon tumors in the AOM-DSS model, through a mechanism that includes suppression of IL-13 production. It is possible that the disruption of the epithelial barrier and leakage of bacteria/bacterial fragments into the intestinal tissue in this model results in an inflammatory microenvironment that is different from that of the oxazalone colitis model. This may influence the function of iNKT cells, as NKT cells can be activated by inflammatory cytokines produced by TLR-stimulated dendritic cells, and IL-12 strongly skews NKT cells toward the production of TH1 cytokines (51).

INTERPLAY BETWEEN iNKT CELLS AND THE INTESTINAL MICROBIOTA

The commensal intestinal microbiota is essential for the development and function of the host immune system (52). The interplay between the intestinal microbiota and iNKT cells in health and disease was recently discussed in a comprehensive review (53). iNKT cells are established in the intestinal mucosa in an IL-12- and MyD88-independent manner. However, microbial colonization affects the phenotype and function of systemic iNKT cells and increases their immune responsiveness. This occurs independently of MyD88 and is thought instead to depend on agonistic iNKT cell ligands produced by bacteria

such as *Sphingomonas* (54). In another study, neonatal microbial colonization limited iNKT-cell numbers in the adult mouse colon, which reduced sensitivity to oxazalone-induced intestinal inflammation (55). This was shown to depend on an abundant inhibitory glycosphingolipid from *Bacteroides fragilis* that bound CD1d but failed to activate iNKT cells (56). At the same time, NKT cells influence the growth of the commensal microflora (57). Mice lacking NKT cells demonstrate an accelerated microbial colonization and an altered composition of the intestinal microbiota. NKT cells also provide protection to bacterial infections, as recently reviewed (51, 58). Thus, iNKT cells are strongly affected by different species of bacteria that colonize the intestine. It is unclear, however, to what extent the intestinal microbial flora can skew the functional program in local iNKT cells, as has been described for conventional T cells (52).

SUPPRESSION OF TUMOR IMMUNITY BY INVARIANT AND dNKT CELLS IN OTHER TUMOR MODELS

Similar to their immunosuppression in intestinal polyposis, iNKT cells have been shown to suppress immunity in some other tumor models. However, the mechanisms underlying NKT-cell suppression of tumor immunity has been most exhaustively studied for dNKT cells. A series of elegant publications by Terabe and Berzofsky and coworkers detail how dNKT cells suppressed CD8 T-cell tumor immunity to different transplanted tumors (27, 59, 60). In these models, it was shown that dNKT cells produced IL-13 that activated $CD11b^+Gr\text{-}1^+$ myeloid cells to produce TGF-β. This suppressed cytotoxic T-cell activity, resulting in tumor recurrence. Tumor recurrence was prevented in mice deficient of all NKT cells (but not in mice lacking iNKT cells only), or by blocking TGF-β or depleting $Gr\text{-}1^+$ cells. A similar mechanism may underlie the dNKT-cell suppression of immunity to a B lymphoma where increased levels of IL-13, TGF-β, and myeloid-derived suppressor cells correlated with enhanced tumor growth (28). In contrast, lack of dNKT cells and reduced tumor growth was associated with increased IFN-γ and IL-12. In these models, iNKT cells had a protective effect, suggesting that dNKT cells and iNKT cells counteracted each other in the regulation of immunity to this tumor. In myeloma patients, it has been proposed that also human dNKT cells can have suppressive role in tumor immunity (61).

Interestingly, as suggested from two lymphoma models, sometimes iNKT cells seem to be able to support suppression of tumor immunity by mechanisms similar to those described above for dNKT cells. In a transplantable B-cell lymphoma model it was found that iNKT cells suppressed antitumor $CD8^+$ T cells required for lymphoma eradication (19). While the majority of WT mice succumbed to the lymphoma, mice lacking iNKT cells cleared the tumor cells. In another study, the survival of WT mice inoculated with CD1d-transfected T lymphoma RMA-S cells was significantly lower than inoculated $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice (18). Improved survival in iNKT-cell-deficient mice was associated with increased production of IFN-γ, while tumor growth in WT mice correlated with higher IL-13 production. Lymphoma growth in WT mice was not observed after inoculation of untransfected RMA-S cells, suggesting that lymphoma overexpression of CD1d-induced immunosuppressive activities of iNKT cells. Interestingly, studies of patients with B-cell-derived chronic lymphatic leukemia indicate a similar scenario. Here, CD1d expression on tumor cells was higher in patients with unfavorable prognosis, and the level of CD1d on leukemia cells was inversely correlated with iNKT-cell frequencies (26, 62). Higher levels of CD1d on lymphoma cells were also correlated with reduced IFN-γ production by remaining iNKT cells. However, it was not reported in these studies whether these iNKT cells instead produce other cytokines such as IL-10 or IL-13 (26). Thus, in some tumor models, iNKT cells express immunosuppressive effector functions similar to those described for dNKT cells. These studies indicate that suppressive iNKT cells may be more likely to be induced in a situation where the tumor cells express high levels of CD1d.

IMMUNOSUPPRESSION BY ADIPOSE TISSUE iNKT CELLS

The mechanisms that underpin the iNKT-cell-mediated regulation of tumor immunity in the $Apc^{min/+}$ model also bear striking resemblance to what has been described in adipose tissue homeostasis (16). iNKT cells are highly enriched in this tissue in both mice and humans, and are suggested to protect against obesity-induced inflammation. It was suggested that in the lean state, production of IL-2 by iNKT cells promoted Treg cells in adipose tissue. Furthermore, mice deficient in iNKT cells had a reduced frequency of Treg cells that proliferated less at this site. It is notable that in the prevention of autoreactivity, iNKT cells have also been shown to support Treg cells in several different settings of autoimmunity and tolerance induction, through a variety of mechanisms (13). Moreover, adipose tissue iNKT cells had high expression of E4BP4, a transcription factor that determines production of IL-10 in T cells (16). iNKT cells were localized juxtaposed to macrophages in adipose tissue, and IL-10 produced by activated iNKT cells enhanced M2 and reduced M1 phenotype in adipose tissue macrophages. This strongly suggests that comparable subsets of regulatory iNKT cells regulate immunity by very similar mechanisms in intestinal polyps of $Apc^{min/+}$ mice and in adipose tissue of lean WT mice.

THE PLZF-NEGATIVE/ADIPOSE/iNKT10 SUBSET OF iNKT CELLS WITH IMMUNOSUPPRESSIVE CAPACITIES

A unique feature in adipose tissue iNKT cells (16) that is shared with intestinal polyp iNKT cells (17) is a lack of the NKT-cell transcription factor PLZF. In fact, adipose iNKT cells demonstrated some similarities to the reduced population of iNKT cells that remain in PLZF-deficient mice (16). Moreover, polyp and adipose iNKT cells share the production of IL-10. IL-10-producing iNKT cells, then coined iNKT10 cells, were also increased in αGalCer-injected mice, and were enriched in adipose tissue after injection of αGalCer (15). This latter

study showed that a single treatment of mice with αGalCer led to expansion of IL-10-producing iNKT cells concomitant with reduced production of pro-inflammatory cytokines by iNKT cells. Strikingly, the αGalCer-induced iNKT10 cells could suppress tumor immunity and ameliorate experimental autoimmune encephalomyelitis (15). These iNKT10 cells thus appear similar to adipose iNKT cells and intestinal polyp iNKT cells. Taken together, these cell types seem to constitute a unique regulatory subset(s) of iNKT cells that is not yet well defined, and that has not been taken into account in previous studies.

DIFFERENTIATION OF SUPPRESSIVE iNKT CELLS—THYMICALLY DETERMINED OR AN EFFECT OF THE LOCAL MICROENVIRONMENT?

It is yet to be revealed what determines the development of immunosuppressive PLZF-negative iNKT cells, and how these cells localize to the tissue where they suppress immunity. Do these cells develop during thymic maturation followed by selective homing to specific tissues? Or is their function influenced during peripheral activation by tissue-derived signals such as those from local CD1d-expressing cells and other cues in the tissue microenvironment? A recent study found that mutating a hydrophobic patch in the iNKT-cell TCR resulted in thymic selection of functionally altered iNKT cells that accumulated in adipose tissue (63). In the thymus, these iNKT cells already expressed some characteristics of adipose iNKT cells. This suggests that iNKT cells with the immunosuppressive PLZF-negative phenotype may be committed during thymic development, as has been proposed for other functional subsets of iNKT cells (14). If this is the case, there may be a selective expression of homing receptors on this subset of iNKT cells that guide their localization to adipose tissue and intestinal polyps or other tumor or inflammatory sites where they may suppress immunity. Adipose tissue-specific recruitment is unlikely to occur constitutively at a high rate, however, as adipose iNKT cells are mostly resident and non-recirculating (16). On the other hand, it is also likely that the tissue microenvironment will alter the function of immune cells that enter the tissue (64). Further transcriptomic and epigenetic analysis of iNKT-cell subsets will provide important information in this respect (65). The finding that the immune response to syngeneic colon cancer cells is significantly different when the cells grow in the intestine compared with subcutaneous growth demonstrates that the immune microenvironment varies between tissues (66). In the $Apc^{min/+}$ model, we showed that transfer of hepatic iNKT cells predominantly of the iNKT1 type to iNKT-cell-deficient $Ja18^{-/-}Apc^{min/+}$ mice reconstituted immunosuppressive effects of iNKT cells in intestinal polyps (17). This indicates that the polyp microenvironment might alter the function of iNKT cells. Highly relevant in this context is the recent interest in immunometabolism and investigations of how the metabolism of immune cells is modulated in fat tissue and tumors, resulting in alterations of immune cell function (67, 68). It is important to investigate whether metabolic states of iNKT cells correlate with their programs and their immunosuppressive activities in different situations.

ACTIVATION OF IMMUNOSUPPRESSIVE iNKT CELLS—A ROLE FOR THE CD1d-EXPRESSING CELL AND CD1d-PRESENTED LIGANDS?

It seems feasible that a major importance for determining iNKT-cell function will be attributed to the CD1d-expressing cell type that activates iNKT cells in the periphery. Induced effector functions are likely different if CD1d is presented to iNKT cells on a professional antigen-presenting cell such as dendritic cells, which may produce pro-inflammatory cytokines like IL-12 and provide other accessory signals, or if iNKT cells are activated by CD1 expressed by non-professional antigen-presenting cells such as adipocytes, epithelium, or tumor cells of different origin. Activation by non-professional antigen-presenting cells may be more likely to induce an anti-inflammatory function in iNKT cells. In the oxazalone-induced colitis model, iNKT cells were induced to produce IL-13 by bone-marrow-derived cells. In contrast, IL-13 production was reduced by iNKT-cell interaction with CD1d-expressing intestinal epithelial cells through induction of IL-10 secretion by the epithelial cells (46). The adipose tissue iNKT cells expressed high levels of Nur77 (16), a nuclear receptor that is upregulated upon TCR ligation. This suggests that adipose iNKT cells may be under constant TCR stimulation. Adipocytes express high levels of CD1d that is required for iNKT-cell regulation of immune homeostasis in adipose tissue (64), indicating that adipocytes act as non-professional antigen-presenting cells for iNKT cells. Whether adipose tissue-specific signals or CD1d-presented lipids determine the functional phenotype of adipose iNKT cells remains to be determined. Interestingly, a role for CD1d on tumor cells was also suggested (18, 26, 62). CD1d expression on tumor cells was associated with the induction of immunosuppressive functions in iNKT cells or the induction of "non-functional" iNKT cells.

The activating ligand presented on CD1d may also play a role in selective induction of iNKT subsets and functions. Considering the dynamic nature of glycolipid metabolism and modulations of these processes in activated or stressed cells, it seems feasible that cancer cells contain an altered set of lipids that are potential iNKT ligands. Such ligands could be presented on CD1d, either on the cancer cells themselves, if CD1d-positive, or on antigen-presenting cells and result in different outcomes. An intriguing example is the disialoganglioside GD3. This is expressed only in a few normal tissues at low levels but accumulates in human melanoma, and in ascites fluid in ovarian cancer patients (69, 70). Using a mouse model, one study found that GD3 was an activating ligand for a small subset of iNKT cells that was only detectable in immunized mice (69). Interestingly, these iNKT cells produced IL-4, but not IFN-γ, in response to immunization with GD3-pulsed dendritic cells. Another study found that GD3 bound both human and mouse CD1d with high affinity (70). However, in contrast to the previous study, GD3 was not found to stimulate iNKT cells. It rather inhibited αGC stimulation of iNKT cells

in vitro and *in vivo*, and GD3-loaded CD1d multimers did not bind iNKT cells. The latter study may have missed the small GD3-reactive iNKT-cell subset, as these cells were not detectable in non-immunized mice. Thus, GD3 enriched in some cancers seems to prevent induction of TH1 tumor immunity by iNKT cells in two ways: it inhibits the majority iNKT cells from activation with agonist ligands by binding to CD1d with high affinity, while at the same time stimulating a small subset of GD3-specific iNKT cells to secrete IL-4. Another glycolipid that inhibited iNKT-cell activation, gangliotriaosylceramide, was found to be shed from a T-cell lymphoma line (71). Besides these examples, there is little information available about the contribution of CD1d-presented lipids that are cancer specific or upregulated in cancer to activation of NKT cells in tumor immunity.

Research ongoing in many laboratories explore and refine iNKT-cell-directed immunotherapy strategies using αGalCer and structurally related ligands that have an improved induction of tumor immunity, often by the enhancement of TH1 cytokines [for recent reviews, see Ref. (72, 73)]. However, many factors influence the outcome of iNKT-cell activation in tumor immunity. For example, Wingender et al. demonstrated that αGalCer as well as the TH1-biasing structural variant of αGalCer, C-glycoside, could induce the expansion of iNKT10 cells, while a TH2-biasing variant, OCH, did not induce their expansion (74). Another study identified β-mannosylceramide as a ligand for mouse and human iNKT cells (75). Administration of β-mannosylceramide protected mice from tumors in a manner that was dependent on TNF-α and nitric oxide synthase and only partially dependent on IFN-γ. In contrast, protection afforded by αGalCer was completely dependent on IFN-γ. To investigate whether iNKT-cell-targeted immunotherapy with agonist ligands could counteract the immunosuppressive effects of iNKT cells in the *Apc*$^{min/+}$ intestinal polyposis model, we treated mice repeatedly with αGalCer or the TH2-biasing structural variant of αGalCer, C20:2 (17). Despite the natural tumor-promoting effect of iNKT cells in untreated mice, treatment with αGalCer resulted in decreased polyp numbers. This suggests that αGalCer may either modulate the function of polyp iNKT cells, or overcome their immunosuppressive effect by activating iNKT cells systemically. As expected, mice treated with C20:2 had increased numbers of polyps. This is consistent with a TH2 cytokine profile induced by this ligand, resulting in suppression of a TH1 antitumor immune response. Quite surprisingly, treatment with the TH1-biasing ligand C-glycoside had no effect on polyp counts (76). As C-glycoside has been shown to induce TH1 cytokines, this treatment would be expected to reduce polyp counts, and be even more effective than αGalCer. It is possible that the lack of effect on polyps after repeated treatment of *Apc*$^{min/+}$ with C-glycoside is a consequence of expansion of iNKT10 cells by this ligand, as demonstrated by Wingender and coworkers (74). Our own data from the *Apc*$^{min/+}$ model also show that different time periods of treatment with iNKT ligands can result in opposite effects (76). Taken together, these data show that modulation of tumor immunity by treatment with iNKT-cell ligands is not always predictable. They also warrant some caution and motivate further studies of the effects of iNKT-cell-directed therapy on iNKT-cell function and downstream regulation of tumor immunity.

CONCLUDING REMARKS AND OUTSTANDING QUESTIONS

Natural killer T cells are important players in the regulation of tumor immunity. iNKT cells are an expanded subset of NKT cells with potent and rapid effector response that show great promise as targets for tumor immunotherapy. However, the effect of iNKT cells, activated naturally or by artificial ligands, on tumor immunity is influenced by many factors. This makes the outcome of iNKT-cell-targeted therapy difficult to predict. The recent identification of a distinct subset of iNKT cells with immunosuppressive properties, shown to suppress tumor immunity and promote tumor growth, calls for more detailed investigation of iNKT cells in different cancer settings. There are a number of outstanding questions that will be important to resolve to clarify the differentiation, activation, and function of immunosuppressive iNKT cells. Is the lack of PLZF a common feature of immunosuppressive iNKT cells in different tumor settings? Is this also the case for regulatory iNKT cells in inflammation and autoimmune diseases? Is the PLZF-negative functional phenotype determined during thymic development, or is imposed by activation and/or the tissue microenvironment under specific conditions? What is the role of the tumor microenvironment, local cytokines, and other signals in modulating the iNKT-cell phenotype, and in the attraction of functionally different iNKT-cell subsets? What is the role of the CD1d-expressing antigen-presenting cell for induced immunosuppressive iNKT-cell functions? What is the effect of iNKT-targeting immunotherapy on tumor-associated iNKT cells? What is the influence of the intestinal microbiota and inflammation on local iNKT cells? Undoubtedly, our knowledge regarding these issues will be significantly expanded in the coming years, and will hopefully contribute to improved strategies for iNKT-cell-directed tumor immunotherapy.

AUTHOR CONTRIBUTIONS

Both authors contributed substantially to the work and approved it for publication.

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REFERENCES

1. Fan X, Rudensky AY. Hallmarks of tissue-resident lymphocytes. *Cell* (2016) 164:1198–211. doi:10.1016/j.cell.2016.02.048
2. Mori L, Lepore M, De Libero G. The immunology of CD1- and MR1-restricted T cells. *Annu Rev Immunol* (2016) 34:479–510. doi:10.1146/annurev-immunol-032414-112008
3. Godfrey DI, Macdonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol* (2004) 4:231–7. doi:10.1038/nri1309
4. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med* (1993) 178:1–16. doi:10.1084/jem.178.1.1
5. Dellabona P, Padovan E, Casorati G, Brockhaus M, Lanzavecchia A. An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med* (1994) 180:1171. doi:10.1084/jem.180.3.1171
6. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science* (1995) 268:863–5. doi:10.1126/science.7538697
7. Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med* (1995) 182:993–1004. doi:10.1084/jem.182.4.993
8. Park S-H, Roark JH, Bendelac A. Tissue-specific recognition of mouse CD1 molecules. *J Immunol* (1998) 160:3128–34.
9. Behar SM, Podrebarac T, Roy C, Wang CR, Brenner MB. Diverse TCRs recognize murine CD1. *J Immunol* (1999) 162:161–7.
10. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of Va14 NKT cells by glycosylceramides. *Science* (1997) 278:1626–9. doi:10.1126/science.278.5343.1626
11. Skold M, Faizunnessa NN, Wang CR, Cardell S. CD1d-specific NK1.1+ T cells with a transgenic variant TCR. *J Immunol* (2000) 165:168–74. doi:10.4049/jimmunol.165.1.168
12. Rhost S, Sedimbi S, Kadri N, Cardell SL. Immunomodulatory type II natural killer T lymphocytes in health and disease. *Scand J Immunol* (2012) 76:246–55. doi:10.1111/j.1365-3083.2012.02750.x
13. Terabe M, Berzofsky JA. The immunoregulatory role of type I and type II NKT cells in cancer and other diseases. *Cancer Immunol Immunother* (2014) 63:199–213. doi:10.1007/s00262-013-1509-4
14. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol* (2013) 25:161–7. doi:10.1016/j.coi.2013.01.003
15. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124:3725–40. doi:10.1172/JCI72308
16. Lynch L, Michelet X, Zhang S, Brennan PJ, Moseman A, Lester C, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of Treg cells and macrophages in adipose tissue. *Nat Immunol* (2015) 16:85–95. doi:10.1038/ni.3047
17. Wang Y, Sedimbi S, Lofbom L, Singh AK, Porcelli SA, Cardell SL. Unique invariant natural killer T cells promote intestinal polyps by suppressing TH1 immunity and promoting regulatory T cells. *Mucosal Immunol* (2018) 11(1):131–43. doi:10.1038/mi.2017.34
18. Renukaradhya GJ, Sriram V, Du W, Gervay-Hague J, Van Kaer L, Brutkiewicz RR. Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* (2006) 118:3045–53. doi:10.1002/ijc.21764
19. Bjordahl RL, Gapin L, Marrack P, Refaeli Y. iNKT cells suppress the CD8+ T cell response to a murine Burkitt's-like B cell lymphoma. *PLoS One* (2012) 7:e42635. doi:10.1371/journal.pone.0042635
20. Robertson FC, Berzofsky JA, Terabe M. NKT cell networks in the regulation of tumor immunity. *Front Immunol* (2014) 5:543. doi:10.3389/fimmu.2014.00543
21. Carreno LJ, Saavedra-Avila NA, Porcelli SA. Synthetic glycolipid activators of natural killer T cells as immunotherapeutic agents. *Clin Transl Immunology* (2016) 5:e69. doi:10.1038/cti.2016.14
22. McEwen-Smith RM, Salio M, Cerundolo V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res* (2015) 3:425–35. doi:10.1158/2326-6066.CIR-15-0062
23. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* (2000) 191:661–8. doi:10.1084/jem.191.4.661
24. Swann JB, Uldrich AP, Van Dommelen S, Sharkey J, Murray WK, Godfrey DI, et al. Type I natural killer T cells suppress tumors caused by p53 loss in mice. *Blood* (2009) 113:6382–5. doi:10.1182/blood-2009-01-198564
25. Bellone M, Ceccon M, Grioni M, Jachetti E, Calcinotto A, Napolitano A, et al. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. *PLoS One* (2010) 5:e8646. doi:10.1371/journal.pone.0008646
26. Gorini F, Azzimonti L, Delfanti G, Scarfo L, Scielzo C, Bertilaccio MT, et al. Invariant NKT cells contribute to chronic lymphocytic leukemia surveillance and prognosis. *Blood* (2017) 129:3440–51. doi:10.1182/blood-2016-11-751065
27. Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, et al. A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med* (2005) 202:1627–33. doi:10.1084/jem.20051381
28. Renukaradhya GJ, Khan MA, Vieira M, Du W, Gervay-Hague J, Brutkiewicz RR. Type I NKT cells protect (and type II NKT cells suppress) the host's innate antitumor immune response to a B-cell lymphoma. *Blood* (2008) 111:5637–45. doi:10.1182/blood-2007-05-092866
29. Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity* (2002) 17:629–38. doi:10.1016/S1074-7613(02)00453-3
30. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* (1990) 247:322–4. doi:10.1126/science.2296722
31. Yamada Y, Mori H. Multistep carcinogenesis of the colon in Apc(Min/+) mouse. *Cancer Sci* (2007) 98:6–10. doi:10.1111/j.1349-7006.2006.00348.x
32. Scott DJ, Hull MA, Cartwright EJ, Lam WK, Tisbury A, Poulsom R, et al. Lack of inducible nitric oxide synthase promotes intestinal tumorigenesis in the ApcMin/+ mouse. *Gastroenterology* (2001) 121:889–99. doi:10.1053/gast.2001.27994
33. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* (2011) 71:1263–71. doi:10.1158/0008-5472.CAN-10-2907
34. Wang L, Wang Y, Song Z, Chu J, Qu X. Deficiency of interferon-gamma or its receptor promotes colorectal cancer development. *J Interferon Cytokine Res* (2015) 35:273–80. doi:10.1089/jir.2014.0132
35. Oleinika K, Nibbs RJ, Graham GJ, Fraser AR. Suppression, subversion and escape: the role of regulatory T cells in cancer progression. *Clin Exp Immunol* (2013) 171:36–45. doi:10.1111/j.1365-2249.2012.04657.x
36. Blatner NR, Mulcahy MF, Dennis KL, Scholtens D, Bentrem DJ, Phillips JD, et al. Expression of RORγt marks a pathogenic regulatory T cell subset in human colon cancer. *Sci Transl Med* (2012) 4:164ra159. doi:10.1126/scitranslmed.3004566
37. Akeus P, Langenes V, Kristensen J, Von Mentzer A, Sparwasser T, Raghavan S, et al. Treg-cell depletion promotes chemokine production and accumulation of CXCR3(+) conventional T cells in intestinal tumors. *Eur J Immunol* (2015) 45:1654–66. doi:10.1002/eji.201445058
38. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, et al. Increased intratumor V 24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res* (2005) 11:7322–7. doi:10.1158/1078-0432.CCR-05-0877
39. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* (2008) 29:391–403. doi:10.1016/j.immuni.2008.07.011
40. Fuss IJ, Heller F, Boirivant M, Leon F, Yoshida M, Fichtner-Feigl S, et al. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* (2004) 113:1490–7. doi:10.1172/JCI19836
41. Schiechl G, Bauer B, Fuss IJ, Lang SA, Moser C, Ruemmele P, et al. Tumor development in murine ulcerative colitis depends on MyD88 signaling of colonic F4/80+CD11bhighGr1low macrophages. *J Clin Invest* (2011) 121:1692–708. doi:10.1172/JCI42540

42. Lee SH, Hu LL, Gonzalez-Navajas J, Seo GS, Shen C, Brick J, et al. ERK activation drives intestinal tumorigenesis in Apc(min/+) mice. *Nat Med* (2010) 16:665–70. doi:10.1038/nm.2143
43. Bleicher PA, Balk SP, Hagen SJ, Blumberg RS, Flotte TJ, Terhorst C. Expression of murine CD1 on gastrointestinal epithelium. *Science* (1990) 250:679–82. doi:10.1126/science.1700477
44. Blumberg RS, Terhorst C, Bleicher PA, Mcdermott FV, Allan CH, Landau SB, et al. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. *J Immunol* (1991) 147:2518–24.
45. Colgan SP, Hershberg RM, Furuta GT, Blumberg RS. Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. *Proc Natl Acad Sci U S A* (1999) 96:13938–43. doi:10.1073/pnas.96.24.13938
46. Olszak T, Neves JF, Dowds CM, Baker K, Glickman J, Davidson NO, et al. Protective mucosal immunity mediated by epithelial CD1d and IL-10. *Nature* (2014) 509:497–502. doi:10.1038/nature13150
47. Kiesler P, Fuss IJ, Strober W. Experimental models of inflammatory bowel diseases. *Cell Mol Gastroenterol Hepatol* (2015) 1:154–70. doi:10.1016/j.jcmgh.2015.01.006
48. Saubermann LJ, Beck P, De Jong YP, Pitman RS, Ryan MS, Kim HS, et al. Activation of natural killer T cells by α-galactosylceramide in the presence of CD1d provides protection against colitis in mice. *Gastroenterology* (2000) 119:119–28. doi:10.1053/gast.2000.9114
49. Ueno Y, Tanaka S, Sumii M, Miyake S, Tazuma S, Taniguchi M, et al. Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of Vα14 natural killer T cells in mice. *Inflamm Bowel Dis* (2005) 11:35–41. doi:10.1097/00054725-200501000-00005
50. Yoshioka K, Ueno Y, Tanaka S, Nagai K, Onitake T, Hanaoka R, et al. Role of natural killer T cells in the mouse colitis-associated colon cancer model. *Scand J Immunol* (2012) 75:16–26. doi:10.1111/j.1365-3083.2011.02607.x
51. Kohlgruber AC, Donado CA, Lamarche NM, Brenner MB, Brennan PJ. Activation strategies for invariant natural killer T cells. *Immunogenetics* (2016) 68:649–63. doi:10.1007/s00251-016-0944-8
52. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* (2012) 336:1268–73. doi:10.1126/science.1223490
53. Dowds CM, Blumberg RS, Zeissig S. Control of intestinal homeostasis through crosstalk between natural killer T cells and the intestinal microbiota. *Clin Immunol* (2015) 159:128–33. doi:10.1016/j.clim.2015.05.008
54. Wingender G, Stepniak D, Krebs P, Lin L, Mcbride S, Wei B, et al. Intestinal microbes affect phenotypes and functions of invariant natural killer T cells in mice. *Gastroenterology* (2012) 143:418–28. doi:10.1053/j.gastro.2012.04.017
55. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell funciton. *Science* (2012) 336:489–93. doi:10.1126/science.1219328
56. An D, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell* (2014) 156:123–33. doi:10.1016/j.cell.2013.11.042
57. Nieuwenhuis EE, Matsumoto T, Lindenbergh D, Willemsen R, Kaser A, Simons-Oosterhuis Y, et al. Cd1d-dependent regulation of bacterial colonization in the intestine of mice. *J Clin Invest* (2009) 119:1241–50. doi:10.1172/JCI36509
58. Crosby CM, Kronenberg M. Invariant natural killer T cells: front line fighters in the war against pathogenic microbes. *Immunogenetics* (2016) 68:639–48. doi:10.1007/s00251-016-0933-y
59. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* (2000) 1:515–20. doi:10.1038/82771
60. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* (2003) 198:1741–52. doi:10.1084/jem.20022227
61. Dhodapkar MV, Kumar V. Type II NKT cells and their emerging role in health and disease. *J Immunol* (2017) 198:1015–21. doi:10.4049/jimmunol.1601399
62. Bojarska-Junak A, Hus I, Chocholska S, Tomczak W, Wos J, Czubak P, et al. CD1d expression is higher in chronic lymphocytic leukemia patients with unfavorable prognosis. *Leuk Res* (2014) 38:435–42. doi:10.1016/j.leukres.2013.12.015
63. Vieth JA, Das J, Ranaivoson FM, Comoletti D, Denzin LK, Sant'angelo DB. TCRalpha-TCRbeta pairing controls recognition of CD1d and directs the development of adipose NKT cells. *Nat Immunol* (2017) 18:36–44. doi:10.1038/ni.3622
64. van Eijkeren RJ, Krabbe O, Boes M, Schipper HS, Kalkhoven E. Endogenous lipid antigens for invariant natural killer T cells hold the reins in adipose tissue homeostasis. *Immunology* (2017). doi:10.1111/imm.12839
65. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol* (2016) 17:728–39. doi:10.1038/ni.3437
66. Zhao X, Li L, Starr TK, Subramanian S. Tumor location impacts immune response in mouse models of colon cancer. *Oncotarget* (2017) 8:54775–87. doi:10.18632/oncotarget.18423
67. Kohlgruber AC, Lamarche NM, Lynch L. Adipose tissue at the nexus of systemic and cellular immunometabolism. *Semin Immunol* (2016) 28:431–40. doi:10.1016/j.smim.2016.09.005
68. Kouidhi S, Elgaaied AB, Chouaib S. Impact of metabolism on T-cell differentiation and function and cross talk with tumor microenvironment. *Front Immunol* (2017) 8:270. doi:10.3389/fimmu.2017.00270
69. Wu DY, Segal NH, Sidobre S, Kronenberg M, Chapman PB. Cross-presentation of disialoganglioside GD3 to natural killer T cells. *J Exp Med* (2003) 198:173–81. doi:10.1084/jem.20030446
70. Webb TJ, Li X, Giuntoli RL, Lopez PHH, Heuser C, Schnaar RL, et al. Molecular identification of GD3 as a suppressor of the innate immune response in ovarian cancer. *Cancer Res* (2012) 72:3744–52. doi:10.1158/0008-5472.CAN-11-2695
71. Sriram V, Cho S, Li P, O'donnell PW, Dunn C, Hayakawa K, et al. Inhibition of glycolipid shedding rescues recognition of a CD1+ T cell lymphoma by natural killer T (NKT) cells. *Proc Natl Acad Sci U S A* (2002) 99:8197–202. doi:10.1073/pnas.122636199
72. Shissler SC, Bollino DR, Tiper IV, Bates JP, Derakhshandeh R, Webb TJ. Immunotherapeutic strategies targeting natural killer T cell responses in cancer. *Immunogenetics* (2016) 68:623–38. doi:10.1007/s00251-016-0928-8
73. Waldowska M, Bojarska-Junak A, Rolinski J. A brief review of clinical trials involving manipulation of invariant NKT cells as a promising approach in future cancer therapies. *Cent Eur J Immunol* (2017) 42:181–95. doi:10.5114/ceji.2017.69361
74. Wingender G, Birkholz AM, Sag D, Farber E, Chitale S, Howell AR, et al. Selective conditions are required for the induction of invariant NKT cell hyporesponsiveness by antigenic stimulation. *J Immunol* (2015) 195:3838–48. doi:10.4049/jimmunol.1500203
75. O'Konek JJ, Illarionov P, Khursigara DS, Ambrosino E, Izhak L, Castillo BF II, et al. Mouse and human iNKT cell agonist beta-mannosylceramide reveals a distinct mechanism of tumor immunity. *J Clin Invest* (2011) 121:683–94. doi:10.1172/JCI42314
76. Wang Y. *Natural Killer T (NKT) Lymphocytes Regulate Intestinal Tumor Immunity [Ph.D. Thesis]*. Gothenburg, Sweden: University of Gothenburg (2017). Available from: http://hdl.handle.net/2077/52418

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REVIEW
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Possible Therapeutic Application of Targeting Type II Natural Killer T Cell-Mediated Suppression of Tumor Immunity

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Natural killer T (NKT) cells are a unique T cell subset that exhibits characteristics from both the innate immune cells and T cells. There are at least two subsets of NKT cells, type I and type II. These two subsets of NKT cells have opposite functions in antitumor immunity. Type I NKT cells usually enhance and type II NKT cells suppress antitumor immunity. In addition, these two subsets of NKT cells cross-regulate each other. In this review, we mainly focus on immunosuppressive NKT cells, type II NKT cells. After summarizing their definition, experimental tools to study them, and subsets of them, we will discuss possible therapeutic applications of type II NKT cell pathway targeted therapies.

Keywords: natural killer T cell, type II natural killer T cell, tumor immunology, immune regulation, immunosuppression, immunotherapy, lipid antigens, transforming growth factor beta

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INTRODUCTION

One of the successful recent approaches to cancer immunotherapy is to overcome immunosuppressive signaling pathways, such as the CTLA-4 or PD-1 pathways. These pathways are endogenous regulatory systems to suppress excessive immunity. Since tumor antigens (Ags) are autologous, antitumor immunity is a kind of autoimmune reaction and is inhibited by those immunosuppressive mechanisms. Thus, to induce antitumor immunity of sufficient magnitude, it is important to overcome them.

In the tumor microenvironment, multiple immune cells form an interacting network. In addition, considering that the suppression mechanisms differ among the mouse tumor models, the mechanisms may also differ among cancer types in humans. Moreover, it is known that even cells of the same mouse tumor cell line growing in different organs are subject to different dominant immunosuppressive mechanisms (1). These findings indicate that the most appropriate immunotherapy for cancer patients with distant metastasis may be different among the metastatic sites of cancer. Altogether, a detailed understanding of suppression mechanisms is important to establish appropriate strategies to control them.

In this review, we mainly focus on immunosuppressive natural killer T (NKT) cells, type II NKT cells. Type II NKT cells play a suppressive role in many diseases, including autoimmune and inflammatory diseases, as well as the tumor setting. Although multiple experimental tools have been used, no tools can analyze the entire population of type II NKT cells so far. Thus, when we discuss type II NKT cells, it is important to understand the advantages and limitations of each experimental tool, as well as the definition of type II NKT cells used in the study.

DEFINITION OF NKT CELLS, TYPE I NKT CELLS, AND TYPE II NKT CELLS

Natural killer T cells are a unique T cell subset that exhibits characteristics from both innate immune cells and T cells. Similar to innate immune cells, NKT cells react quickly to stimuli and produce a large amount of various kinds of cytokines and chemokines to modulate the immune response (2, 3). Also, like T cells, NKT cells express a T cell receptor (TCR) and respond in an Ag-specific manner. Thus, the nomenclature of NKT cells may be misleading, as they are not related to natural killer (NK) cells. The name originally came from the expression of the NK1.1 marker by many of them (4), but this was an unreliable characteristic and the definition was changed to define any T cell expressing a TCR that recognized a lipid presented by CD1d as an NKT cell (5).

With this feature of lipid–CD1d specificity, NKT cells can be distinguished from conventional T cells that recognize peptide Ags presented by conventional MHC molecules. Therefore, NKT cells expand the repertoire of Ags that the cellular immune system can recognize beyond the proteins detected by conventional T cells. Thus, the current definition of NKT cells is any CD1d-restricted T cell, which can recognize lipid Ag presented by CD1d through its TCR.

Type I NKT cells are defined as CD1d-restricted T cells, which express a TCR α-chain that utilizes Vα14-Jα18 gene segments in mice and Vα24-Jα18 gene segments in humans. This semiinvariant TCR α-chain was initially discovered as a quite unusual TCR α-chain across several hybridoma lines (6), because the TCRα chain expressed by these NKT cells has very few or no nucleotide insertions in the CDR3 region. Thus, type I NKT cells are also referred to as invariant or iNKT cells.

Type II NKT cells are defined as CD1d-restricted T cells that express diverse TCR α-chains other than the semiinvariant one expressed by type I NKT cells. This definition means all CD1d-restricted T cells except for type I NKT cells are type II NKT cells. Thus, type II NKT cells can be a mixture of a variety of different subsets. However, currently, no experimental tools exist to identify or analyze the entire population of type II NKT cells. When we discuss type II NKT cells, it is important to understand the advantage and limitation of each experimental tool, as well as the definition of type II NKT cells in the discussion. The frequency of the entire type II NKT cell population is not known yet. However, sulfatide-reactive type II NKT cells are reported to be approximately 4.5% of the mononuclear cell fraction of cells in the liver (7). Taking into consideration that these type II NKT cells have the same Ag specificity in naive mice, this T cell population is not trivial in size compared to the population of naive conventional T cells specific for a single Ag or epitope. In this review, we first summarize the experimental tools and subsets of type II NKT cells, and next, we will focus on their role in tumor immunity.

EXPERIMENTAL TOOLS FOR TYPE II NKT CELL ANALYSIS

Type II NKT cells were originally discovered from MHC-II-deficient mice. Cardell et al. discovered that MHC-II-deficient mice unexpectedly had a significant population of peripheral $CD4^+$ T cells (8), even though conventional $CD4^+$ T cells are absent. The authors created several $CD4^+$ T cell hybridomas from MHC-II-deficient mice and found that many of them were CD1d restricted. These type II NKT cell hybridomas are useful tools for *in vitro* analysis. For example, many lipid Ags for type II NKT cells have been discovered using NKT cell hybridomas (9–11). Also, analysis using type II NKT hybridomas demonstrated that type II NKT cells did not recognize α-galactosylceramide (α-GalCer), which is a potent stimulator for type I NKT cells (12), suggesting that the lipid Ags recognized by type I NKT cells and type II NKT cells are different.

For *in vivo* analysis, the function of different NKT cell subsets has been assessed by comparison of immune responses of WT mice that have both type I and II NKT cells to those of $J\alpha18^{-/-}$ mice that lack type I NKT cells but retain type II and to those of CD1d $KO^{-/-}$ mice, which lack all NKT cells. Although this model can provide only indirect evidence of type II NKT cell function, currently, this is the only strategy that can analyze the *in vivo* function of the entire type II NKT cell population.

For direct analysis of type II NKT cells, three experimental tools have been reported, 24αβ-TCR transgenic mice, 4get $J\alpha18^{-/-}$ mice, and lipid Ag-loaded CD1d tetramers. Although none of them can identify the entire population of type II NKT cells *in vivo*, these tools can provide direct evidence of *in vivo* function of at least a subset of type II NKT cells. These experimental tools are summarized in **Table 1**.

SULFATIDE-REACTIVE TYPE II NKT CELLS

The first lipid Ag for murine type II NKT cells, sulfatide, was reported in 2004 (7) (**Figure 1**). Sulfatide, 3′-*O*-sulfogalactosylceramide, is an endogenous glycolipid, which is abundant in the myelin in the nervous system, as well as the pancreas, kidney, and liver (13). Notably, the authors created sulfatide-loaded CD1d tetramers and identified sulfatide-reactive type II NKT cells in the liver and spleen. This is the first report that type II NKT cells in *ex vivo* mononuclear cells were visualized. Subsequently, using sulfatide-loaded CD1d tetramers, the TCR repertoire of sulfatide-reactive type II NKT cells in the liver was analyzed (14). As expected, the TCR repertoire of sulfatide-reactive type II NKT was diverse, but most frequently employed alpha gene segments from Vα1 and Vα3 and paired with Vβ8.1/Vβ8.3.

Although sulfatide-loaded CD1d tetramers were reported in 2004, the analysis of sulfatide-reactive type II NKT cells has not been as rapid as that of type I NKT cells. This may be partly due to the fact that sulfatide-loaded CD1d tetramers are not widely available, because making stable sulfatide-loaded CD1d tetramers to stain sulfatide-reactive type II NKT cells is technically difficult. Recently, we have overcome these problems (Kato et al., manuscript in preparation). We found that a significant number of sulfatide-reactive type II NKT cells exist in the lung, which is a major target organ for tumor metastasis. This population can produce IL-13 after activation, consistent with the previous observation in the analysis of their suppressive effect in tumor

TABLE 1 | Experimental tools to analyze type II NKT cells.

Tools	Advantage	Limitation
Type II NKT cell hybridomas	Easy to handle	Limited to *in vitro* experiments and specific clones available, not representative of all populations
Comparison of WT mice, Jα18$^{-/-}$ mice, and CD1d KO$^{-/-}$ mice	This model can provide *in vivo* behavior of the entire type II NKT cell population	This model can provide only indirect evidence of type II NKT cell function
24αβ-TCR transgenic mice	This model enables identification of type II NKT cells *in vivo*	This model can provide behavior of type II NKT cells with one TCR repertoire, not representative of the majority of type II NKT cells
	This model can provide *in vivo* behavior of type II NKT cells	The majority of other T cells are absent in this model
4get Jα18$^{-/-}$ mice	This model enables identification of type II NKT cells *in vivo*	Not all type II NKT cells may be identified in this model, only ones in which the IL-4 gene is activated
		Type I NKT cells are absent in this model
	More conventional T cells are present than in 24αβ-TCR transgenic mice	Once other T cells are activated, type II NKT cells can no longer be distinguished from other T cells as other T cells may express GFP
Lipid antigen-loaded CD1d tetramers	These tools can provide direct identification and evidence of type II NKT cell function	Currently, no reagents can identify all type II NKT cells, just ones with receptors recognizing the lipid–CD1d combinations available
		Some reagents are technically difficult to create

NKT, natural killer T cell; TCR, T cell receptor.

immunity (15). A transcriptome analysis of sulfatide-reactive type II NKT cells indicated that this cell type has a gene expression profile distinct from but similar to that of type I NKT cells, in contrast to Th2, Th0, and innate-like lymphoid cells (ILCs)/NK cells.

24αβ-TCR TRANSGENIC MICE

The 24αβ-TCR was identified as one of the TCRs in the repertoire of murine type II NKT cells from the CD4$^+$ type II NKT cell hybridoma VIII24 that expresses a Vα3.2 and Vβ9 rearrangement (8). For *in vivo* analysis of type II NKT cells, TCR transgenic mice carrying the 24αβ-TCR were developed (16). In 24αβ-TCR transgenic mice, the majority of αβ-T cells express the 24αβ-TCR. They express NK1.1, CD122, intermediate levels of TCR and are CD4/CD8 double negative or CD4$^+$. Upon activation *in vitro*, they secrete large amounts of IL-4 and IFN-γ, as this is characteristic of NKT cells.

The gene expression profiling of 24αβ-TCR cells revealed that the cells expressed genes predominantly associated with Th1 effector functions comparable to type I NKT cells (17). Also, the 24αβ-TCR cell signature genes, such as annexin A2, Ly6C and c-kit, have all been shown to be augmented in CD8αα thymic cells obtained by re-aggregation thymic organ culture (18), which have self-reactive TCRs and phenotypic characteristics of innate immune cells. Taken together, the pattern of genes highly expressed in 24αβ-TCR cells indicates that the cells have characteristics of innate immune cells and Th1 cells.

Although the gene profile suggests that 24αβ-TCR cells have features of cytotoxic T cells (CTLs), their immune-suppressive effect has been reported in multiple mouse disease models, including type I diabetes (19) and autoimmune colitis (20). The functions of 24αβ-TCR cells in tumor settings are unclear.

Although VIII24 was reported not to react to sulfatide, interestingly, we found that lung sulfatide-reactive type II NKT cells were partially positive for Ly6C and c-kit, similar to 24αβ-TCR cells (Kato et al., manuscript in preparation). These findings suggest that these two subsets of type II NKT cells may share some characteristics.

4get Jα18$^{-/-}$ MICE

Other approaches to detect type II NKT cells *in vivo* involve using constitutive expression of cytokine mRNA for their marker. The IL-4 GFP enhanced transcript (4get) mice were used to identify type II NKT cells *in vivo* based on the hypothesis that similar to type I NKT cells, which constitutively express IL-4 mRNA, type II NKT cells must express IL-4 at a steady state (21, 22). TCRβ$^+$GFP$^+$α-GalCer/CD1d tetramer-negative cells were sorted from liver mononuclear cells of 4get Jα18$^{-/-}$ mice. This population produced IFN-γ when co-cultured with CD1d-expressing bone marrow-derived dendritic cells (DCs), suggesting the cells reacted with self-Ags presented by CD1d, and thus they were type II NKT cells. This sorted population reacted with lipid ligands, which had been previously shown to be ligands for type II NKT cells, such as β-glucosylceramide (β-GlcCer) and β-GalCer (10, 23) (**Figure 1**). The sorted β-GlcCer reactive type II NKT cells did not respond to sulfatide and favored TCR gene segments from Vα8 and Vβ8.1/8.2, a combination that is distinct from sulfatide-reactive type II NKT cells (22).

OTHER TYPE II NKT CELLS

Type II NKT cells recognize both glycolipids and phospholipids derived from self as well as microbes. In addition to sulfatide, other self-glycolipids, β-GlcCer, β-GalCer, and lysophosphatidylcholine (LPC) have been reported to be recognized by type II

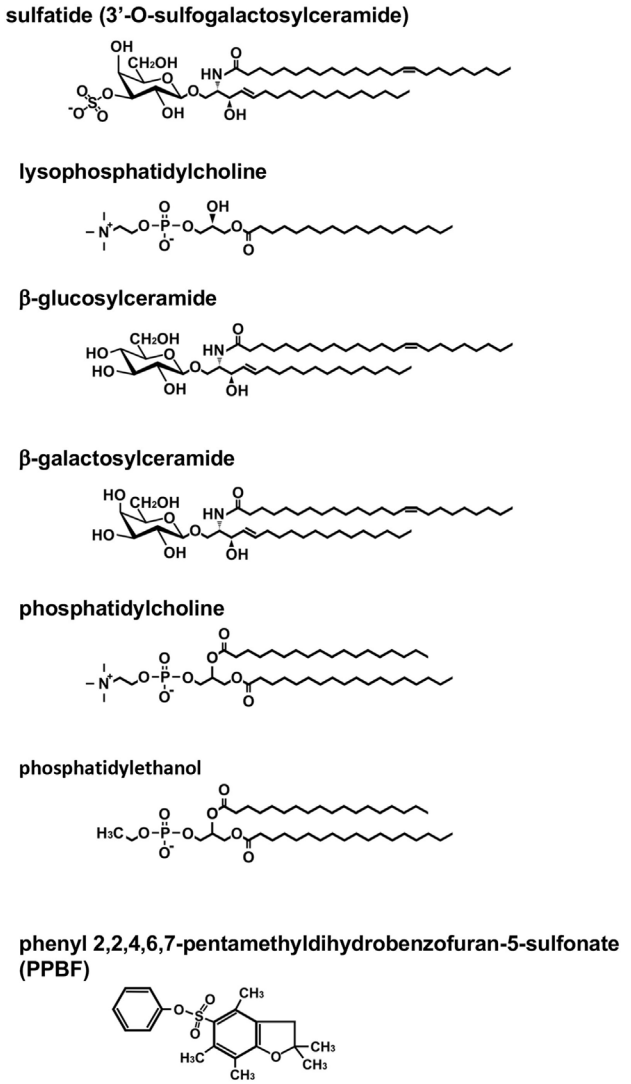

FIGURE 1 | Structure of lipid antigens for type II natural killer T (NKT) cells. Type II NKT cells can recognize a broad range of both endogenous and exogenous lipid antigens. The representative structures for each lipid are shown. Pollen grain phospholipids, such as phosphatidylcholine and phosphatidylethanol, are recognized by human type II NKT cells.

NKT cells (10, 23, 24) (**Figure 1**). The lipid Ags from microbial sources, such as glycolipids from *Mycobacterium tuberculosis* or *Corynebacterium glutamicum* (11) and phosphatidylglycerol from *Listeria monocytogenes* (25), have also been reported to be Ags for type II NKT cells other than those described above. Observations that some Ags recognized by a fraction of type II NKT cells or type II NKT cell hybridomas are not always recognized by other fractions of the cells suggest that the type II NKT cell population contains multiple cell subsets specific for distinct Ags. However, the functional diversity of type II NKT cells recognizing distinct Ags remains to be explored.

TYPE II NKT CELLS IN HUMANS

Human type II NKT cells have been studied as CD1d-reactive T cells expressing diverse TCRs and were found to be more frequent than type I NKT cells in bone marrow, liver, and inflamed intestines of patients with ulcerative colitis (26–28).

Also, some lipid ligands have been reported to be recognized by both murine and human type II NKT cells, such as β-GlcCer 22:0, glucosylsphingosine (29), sulfatide, and lysosulfatide (30, 31). LPC was discovered from the plasma of myeloma patients and has been shown to be recognized by both human and murine type II NKT cells (24, 32–34). Human type II NKT cells are also reported to recognize non-lipid small molecules, such as phenyl pentamethyldihydrobenzofuran (35) (**Figure 1**). Interestingly, although most lipid ligands for type II NKT cells are not recognized by type I NKT cells, LPC is reported to be recognized by a few human type I NKT cell clones. However, it is not recognized by murine type I NKT cells (32, 36–38).

In addition to the use of αβ-TCRs, NKT cells using γδ-TCRs have been described. In humans, γδ-T cells that recognize lipid Ag presented by CD1d were discovered in peripheral blood and nasal mucosa of cypress pollen-sensitive subjects (39–41). These cells recognize phospholipids, such as phosphatidylcholine and phosphatidylethanol, extracted from pollen grains (41) (**Figure 1**). Surprisingly, although sulfatide-reactive type II NKT cells in mice use αβ-TCRs, at least some sulfatide-reactive CD1d-restricted T cells in humans have been shown to express γδ-TCRs (42, 43). In addition, although sulfatide has been considered as a specific Ag for type II NKT cells, a recent report demonstrated that human, but not mouse, type I NKT cells could recognize sulfatide presented by CD1d (44). These findings suggest that sulfatide-reactive type II NKT cells in humans and mice may comprise distinct populations, so we may need to subdivide them for further analysis.

THE MECHANISM OF TYPE II NKT CELL-MEDIATED SUPPRESSION OF TUMOR IMMUNITY

The initial observation that NKT cells can suppress tumor immunity was reported in 2000 (15). In a 15-12RM fibrosarcoma tumor model in which tumors show a growth-regression-recurrence pattern, IL-13 had a key role for downregulation of CTLs, and CD1d$^{-/-}$ mice had decreased IL-13 production and resistance to the recurrence. Subsequently, type II NKT cells were shown to be sufficient for the suppression of tumor immunity in multiple mouse tumor models, in which CD4^{+}CD25^{+} regulatory T cells (Tregs) do not play a critical role in the regulation of immunosurveillance (24, 45).

These represented indirect evidence of suppressive roles of type II NKT cells, which were obtained by comparing WT mice, Jα18$^{-/-}$ mice, and CD1d KO$^{-/-}$ mice. After the discovery of sulfatide as a ligand for type II NKT cells, direct evidence of the suppressive role of type II NKT cells was obtained. The administration of sulfatide to activate sulfatide-reactive type II

NKT cells enhanced tumor growth in a CD1d-dependent manner in a murine colon cancer cell line, CT26, lung metastasis model (46). Although it is not clear whether all type II NKT cells suppress tumor immunity, the studies suggest that type II NKT cells in the absence of type I NKT cells in Ja18$^{-/-}$ mice are sufficient to suppress tumor immunity. However, among subsets of type II NKT cells, we have detailed knowledge only of sulfatide-reactive ones, and even among those, we cannot be sure that all of them are immunosuppressive.

One of the mechanisms of type II NKT cell-mediated suppression is through cross talk with myeloid-derived suppressor cells (MDSCs). In the 15-12RM fibrosarcoma tumor model, although IL-13 was necessary for downregulation of CTL-mediated tumor immunosurveillance, it could not directly downregulate CTL activity as T cells do not have receptors for IL-13. IL-13 induced transforming growth factor beta (TGF-β) production by the CD11b^{+}Gr1^{+} population of myeloid suppressor cells, and blocking TGF-β or depleting Gr1^{+} cells *in vivo* could inhibit the suppression of tumor immunity by type II NKT cells (47) (**Figure 2**). This IL-13 signaling is mediated through an IL-4 receptor α, which forms a heterodimer with IL-13 receptor α1, and the STAT6 pathway. There is another receptor that can bind IL-13, IL-13 receptor α2, whose expression is induced by tumor necrosis factor-α (TNF-α) along with the STAT6 signal from IL-4 or IL-13. Because a TNF-α-neutralizing agent was shown to be able to inhibit the suppression of tumor immunity by type II NKT cells, TNF-α is also involved in this signaling pathway (48). It was shown that induction of TGF-β requires a two-step process in which TNF-α and IL-13/4/STAT6 synergistically upregulated the IL-13Rα2, which then responded to IL-13 to induce TGF-β production through AP-1 signaling. This interaction with MDSCs was also reported in a CD1d-overexpressing B cell lymphoma model (49). Interestingly, reports of similar IL-13-mediated cross talk with MDSCs by group 2 innate lymphoid cells have been recently published (50, 51). Therefore, this IL-13-mediated cross talk with MDSCs is not limited to type II NKT cells, and multiple kinds of immune cells that can produce IL-13 may be involved in this immunosuppressive loop. However, it should be pointed out that CD1$^{-/-}$ mice that lack NKT cells with facilitated tumor immunity retain ILCs, so the effects lost in these mice must be dependent on NKT cells rather than ILCs.

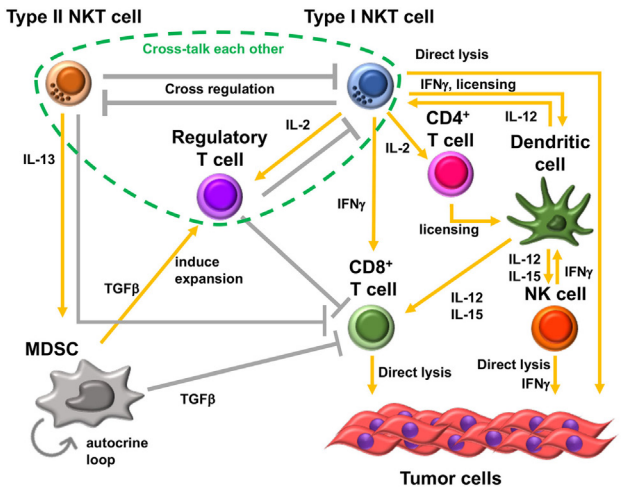

FIGURE 2 | Immunosuppressive cell network in tumor microenvironment. Type II natural killer T (NKT) cells and type I NKT cells cross-regulate each other. Type II NKT cells cross talk with myeloid-derived suppressor cells (MDSCs) through production of IL-13. Transforming growth factor beta (TGF-β) produced by MDSCs suppresses CD8^{+} T cells, induces immunosuppressive regulatory T cells (Tregs), and enhances development of additional tumor-associated MDSCs by an autocrine loop. In addition, type II NKT cells can suppress CD8^{+} T cells by unknown mechanisms other than through cross talk with MDSCs. Type I NKT cells enhance the antitumor function of CD8^{+} T cells and are able to directly lyse tumor cells. Type I NKT cells' interaction with dendritic cells (DCs) through CD1d-TCR and CD40-CD40L induces activation and maturation of DCs. The interaction licenses DCs to be able to prime CD8^{+} T cells and produce IL-12 and IL-15. IL-12 and IL-15 production by DCs stimulates natural killer (NK), type I NKT, and/or CD8^{+} T cells. IL-2 produced by activated type I NKT cells induces the proliferation of memory CD4^{+} T cells, which support the activation of CD8^{+} T cells. However, type I NKT cells also support Tregs through IL-2 production. Treg cells suppress type I NKT cells and CD8^{+} T cells. Three kinds of T cells, type II NKT cells, type I NKT cells, and Tregs, cross talk with each other.

Another mechanism may exist for suppression of tumor immunity by type II NKT cells. In a K7M2 mouse osteosarcoma model, CD1d$^{-/-}$ mice showed higher resistance to growth of osteosarcoma primary tumors than WT mice. The protection was shown to be CD8^{+} T cell dependent, and CD1d$^{-/-}$ mice had significantly higher numbers of tumor-infiltrating lymphocytes. In this model, TGF-β and IL-13 were not the drivers of immunosuppression (52). Thus, alternative pathways exist for immunosuppression mediated by type II NKT cells.

CROSS-REGULATION OF TYPE I NKT CELLS AND TYPE II NKT CELLS

As mentioned above, type I and type II NKT cells generally have opposite function in tumor immunity. In addition, these two subsets of NKT cells cross-regulate each other (**Figure 2**). In the CT26 pulmonary metastasis model, selective stimulation of type II NKT cells by sulfatide enhanced tumor growth. In addition, when both type I and type II NKT cells were activated simultaneously by α-GalCer and sulfatide, respectively, the tumor immunity by activated type I NKT cells was inhibited by concurrent activation of type II NKT cells. This finding suggested that activated type II NKT cells may suppress type I NKT cell-mediated enhancement of tumor immunity (46). Analogously, in *in vitro* analysis, α-GalCer-induced cytokine production by type I NKT cells was inhibited by type II NKT cells stimulated with sulfatide (46, 53). This suppressive effect of type II NKT cells against type I NKT cells was also reported in mouse models of other diseases. For example, in a model of concanavalin A-induced hepatitis, activation of type II NKT cells by sulfatide induced anergy, or hyporesponsiveness, of type I NKT cells (54).

In the CT26 pulmonary metastasis model, Jα18$^{-/-}$ mice showed lower and CD1d$^{-/-}$ mice showed higher resistance to tumor growth than WT mice. Consistent with this finding, Jα18$^{-/-}$ mice showed a weaker specific CTL response than did

WT mice against tumor Ag-pulsed cells, whereas CD1d$^{-/-}$ mice showed a stronger tumor-Ag-specific cytotoxic response than WT mice. These results suggested that type I NKT cells inhibit the function of type II NKT cells in tumor immunity to suppress CTL activity (46). Altogether, therefore, type I and type II NKT cells form a novel immunoregulatory axis of cells with opposing roles that counteract each other, in which the balance affects the tone of local immunity, in that sense (but not in specific cytokines) analogs to the Th1–Th2 axis that so profoundly affected immunology (55). Because NKT cells act early in immune responses, the balance along this axis can have profound effects on subsequent conventional T cell or antibody responses (56).

CROSS TALK AMONG THREE TYPES OF IMMUNOREGULATORY T CELLS

In addition to cross-regulation of two subsets of NKT cells, type II NKT cells may also cross talk with CD4^{+}Foxp3^{+} Tregs. In a CT26 subcutaneous tumor model, this cross-regulation of two subsets of NKT cells helps determine the primary suppressive cell in tumor immunity. Although Tregs are known not to have suppressive effects in the CT26 pulmonary metastasis model, they do have a suppressive effect in the CT26 subcutaneous tumor model. In the CT26 subcutaneous tumor model, Treg blockade was sufficient to protect against tumor outgrowth in WT type and CD1d$^{-/-}$ mice. However, Treg blockade was insufficient for protection in Jα18$^{-/-}$ mice in which type II NKT cells are unopposed (57, 58). It was demonstrated that in WT mice, type II NKT cells are neutralized by type I NKT cells, leaving Tregs as the primary suppressor in this model. In mice lacking type I NKT cells, in which type II are not neutralized by type I NKT cells, type II NKT cells could suppress tumor immunity even when Treg cells are blocked. Thus, type I NKT cells regulate the balance between other regulatory cells, regulating the regulators. This situation may apply in human cancer patients, as it was reported that myeloma patients have deficient type I NKT cell function (59), and other studies of type I NKT cells in cancer patients have reported either decreased numbers or decreased cytokine production (59–64). This altered balance of type I versus II NKT cells in patients with cancer may affect the dominant immune-suppressive cells in the patients.

POTENTIAL ROLE OF TYPE II NKT CELL IN CHRONIC INFLAMMATION-MEDIATED CANCER

Type II NKT cells may play an important role in inflammation-induced cancers. Because type II NKT cells react to endogenous lipids, they may be activated when endogenous lipids are released from autologous organs damaged by inflammation. In addition, unusual lipid accumulation caused by metabolic disorders may induce the activation of type II NKT cells. Gaucher's disease (GD) is an inherited metabolic disorder characterized by lysosomal storage of β-GlcCer (d18:1/C22:0; βGL1-22) and glucosylsphingosine (Lyso-GL1; LGL1) (65). It has been reported that the overall cancer risk is increased in GD patients (66–68). Especially, the association of GD and multiple myeloma is most striking, with the risk estimated at almost 37-fold compared to the general population (68).

Studies in human GD patients revealed that human type II NKT cells react to βGL1-22 and LGL1 that accumulate in these patients. Also, both βGL1-22/LGL1-reactive type II NKT cells express markers of T follicular helper cells (CXCR5^{hi} PD1^{hi} ICOS^{hi} BCL6^{+}IL-21^{+}) and promote plasma cell differentiation in human T-B cocultures (29). In addition, it is reported that the clonal immunoglobulin in patients with GD is reactive against LGL1. Furthermore, administration of anti-LGL1 antibodies ameliorates GD-associated gammopathy in mice, suggesting that long-term immune activation by LGL1 may underlie GD-associated gammopathies (69). These findings suggest that type II NKT cells are activated due to abnormal accumulation of lipid Ags and provide help for B cell activation in patients with GD. This chronic lipid-mediated and type II NKT cell-mediated B cell activation may underlie the increased risk of plasma cell tumors in GD.

Notably, dysregulation of glucosphingolipids has been demonstrated not only in inherited metabolic disorders but also in obesity (70). Recently, obesity is viewed as a chronic low-grade inflammatory disease that is also associated with cancer risk (71). The relationship between NKT cells and obesity is unclear, because three different outcomes for the involvement of NKT cells in the development of obesity are reported. Some groups reported a protective role and demonstrated that type I NKT cells in adipose tissue produce anti-inflammatory cytokines, such as IL-4 and IL-10 (72–74). On the other hand, other groups reported their aggravating role and demonstrated that type I NKT cells produced pro-inflammatory cytokines, such as IFN-γ, in response to lipid excess in the body (75, 76). In addition, another group reported a neutral role and stated NKT cells have no active role for skewing the environment toward either a Th1- or Th2-bias during the development of obesity (77). Regarding the involvement of type II NKT cells, one of the reports demonstrated that type II NKT cells exacerbated diet-induced obesity in the absence of type I NKT cells (78). Thus, type II NKT cells may be activated during obesity-induced chronic inflammation and may have a role for exacerbation of obesity and carcinogenesis associated with obesity.

POTENTIAL ROLE OF TYPE II NKT CELLS IN CANCER IMMUNOEDITING

In the tumor microenvironment, cancer cells affect and modulate antitumor immunity to escape immunosurveillance. Some cancer cells are reported to express CD1d, suggesting that they may affect NKT cell-mediated antitumor immunity. Considering that type I NKT cells have been reported to be able to eliminate CD1d-expressing tumor cells *via* multiple pathways (79–83), it seems that CD1d on the cancer cells is mainly recognized by type I NKT cells, resulting in enhancement of tumor immunity. However, conversely, CD1d expression in human cancer has been reported to be correlated with poor prognosis in human renal cell carcinoma and multiple

human hematopoietic malignancies (84–87). This finding suggests that CD1d on the cancer cells may be recognized by type II NKT cells and that activated type II NKT cells may induce suppression of tumor immunity.

TGF-β BLOCKADE AS A THERAPEUTIC APPLICATION OF BLOCKING TYPE II NKT CELL-MEDIATED IMMUNE SUPPRESSION

One of the candidates for therapeutic targets for cancer immunotherapy is type II NKT cell-MDSC cross talk, which results in TGF-β-mediated CTL downregulation. A role for TGF-β in cancer-mediated immunosuppression was demonstrated in 1990 for the first time (88). Initial studies to inhibit TGF-β signaling by antibodies demonstrated enhanced cancer cell-specific immune responses (89) and reduced tumorigenicity of a human breast cancer cell line in athymic mice (90).

In addition, to enhance the effect of TGF-β blockade on tumor immunity, combination therapies have been studied. In a murine B16 melanoma model, neutralizing antibodies to TGF-β combined with IL-2 therapy could decrease the number of metastases (91). Similar synergistic effect of TGF-β blockade in conjunction with a cancer vaccine has been reported in multiple tumor models with multiple vaccine platforms (92–97). In humans, an antibody that neutralizes all three isoforms of active TGF-β has shown clinical benefit in some patients with metastatic malignant melanoma (98, 99). Of course, TGF-β can be made by many cell types, not just MDSCs. For example, TGF-β can be important in induction of some types of Treg cells and can play a role in their function (100). It can also be made by tumor cells themselves. Thus, TGF-β blockade can promote antitumor immunity through a plethora of complementary mechanisms. Nevertheless, despite their limited numbers, type II NKT cells may play a key role, as a frequency of as much as 4.5% of sulfatide-reactive type II NKT cells in the liver (7) is actually a substantial number when one considers that the steady-state frequency of conventional T cells with a single-Ag specificity is orders of magnitude lower.

In addition to synergy with IL-2 therapy or a cancer vaccine, TGF-β blockade may have multiple benefits for the induction of adequate immune response to tumor cells. Because TGF-β is a pleiotropic cytokine, it has multiple roles in tumor immunity. First, as mentioned above, TGF-β production by MDSCs directly suppresses other immune cells, such as CTLs. Second, TGF-β produced by MDSCs also feeds into an autocrine loop to enhance the development of additional tumor-associated MDSCs (101). Third, MDSCs induce expansion of immunosuppressive, tumor-specific Tregs (102), resulting in stronger suppression of CTLs. Altogether, TGF-β blockade could interrupt these autocrine and paracrine loops driving suppression of tumor immunity (101, 103).

OTHER POSSIBLE THERAPEUTIC APPLICATIONS OF BLOCKING TYPE II NKT CELL-MEDIATED IMMUNE SUPPRESSION

One of the possible therapeutic approaches targeting type II NKT cell-mediated immune suppression is development of an antagonistic Ag for type II NKT cells. The development of antagonistic Ags for type II NKT cells that have higher affinity for CD1d than tumor Ags may enable blocking the signaling between tumor lipid Ags and type II NKT cells. The candidates for the antagonistic Ags are structural analogs of type II NKT cell Ags since it is reported that the affinity between lipid Ag and CD1d differ according to the structure of the fatty acid chain of the glycolipid Ags (104, 105). To this end, it is important to carry out structure–function studies of the tumor-derived and other lipid Ags recognized by type II NKT cells in cancer patients. Such studies are underway in our laboratory.

CONCLUSION

After development of immunotherapy targeting a CTLA-4 or a PD-1 signaling pathway, multiple combination therapies have been studied. More detailed understanding of the roles and cross talk among immune cells in the tumor microenvironment will be necessary for the development of effective combination therapies. In addition to the more widely studied immunoregulatory cells such as Tregs and MDSCs, here we have reviewed abundant evidence that type II NKT cells play a major role in regulating immunity against cancer. Furthermore, the dominant immunosuppressive cells may differ among different types of cancer or sites of metastasis. Thus, the development of diagnostic methods to determine dominant immunosuppressive cells and proper targeting of cells or pathways for individual patients is needed to relieve this suppression and allow the full efficacy of the immune system to be marshaled to treat cancer. This "precision diagnosis of immunosuppressor cells in the tumor microenvironment" would help enhance the efficacy and decrease adverse effects of cancer immunotherapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. O'Konek JO, Ambrosino E, Bloom AC, Pasquet L, Massilamany C, Xia M, et al. Differential regulation of T-cell mediated anti-tumor memory and cross-protection against the same tumor in lungs versus skin. *OncoImmunol* (2018). (in press).
2. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* (2000) 192:741–54. doi:10.1084/jem.192.5.741
3. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest* (2004) 114:1379–88. doi:10.1172/JCI200423594

4. Makino Y, Kanno R, Ito T, Higashino K, Taniguchi M. Predominant expression of invariant V alpha 14+ TCR alpha chain in NK1.1+ T cell populations. *Int Immunol* (1995) 7:1157–61. doi:10.1093/intimm/7.7.1157
5. Godfrey DI, Macdonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol* (2004) 4:231–7. doi:10.1038/nri1309
6. Imai K, Kanno M, Kimoto H, Shigemoto K, Yamamoto S, Taniguchi M. Sequence and expression of transcripts of the T-cell antigen receptor alpha-chain gene in a functional, antigen-specific suppressor-T-cell hybridoma. *Proc Natl Acad Sci U S A* (1986) 83:8708–12. doi:10.1073/pnas.83.22.8708
7. Jahng A, Maricic I, Aguilera C, Cardell S, Halder RC, Kumar V. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* (2004) 199:947–57. doi:10.1084/jem.20031389
8. Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med* (1995) 182:993–1004. doi:10.1084/jem.182.4.993
9. Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, et al. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* (2000) 12:211–21. doi:10.1016/S1074-7613(00)80174-0
10. Rhost S, Lofbom L, Rynmark BM, Pei B, Mansson JE, Teneberg S, et al. Identification of novel glycolipid ligands activating a sulfatide-reactive, CD1d-restricted, type II natural killer T lymphocyte. *Eur J Immunol* (2012) 42:2851–60. doi:10.1002/eji.201142350
11. Tatituri RV, Watts GF, Bhowruth V, Barton N, Rothchild A, Hsu FF, et al. Recognition of microbial and mammalian phospholipid antigens by NKT cells with diverse TCRs. *Proc Natl Acad Sci U S A* (2013) 110:1827–32. doi:10.1073/pnas.1220601110
12. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* (1997) 278:1626–9. doi:10.1126/science.278.5343.1626
13. Takahashi T, Suzuki T. Role of sulfatide in normal and pathological cells and tissues. *J Lipid Res* (2012) 53:1437–50. doi:10.1194/jlr.R026682
14. Arrenberg P, Halder R, Dai Y, Maricic I, Kumar V. Oligoclonality and innate-like features in the TCR repertoire of type II NKT cells reactive to a beta-linked self-glycolipid. *Proc Natl Acad Sci U S A* (2010) 107:10984–9. doi:10.1073/pnas.1000576107
15. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* (2000) 1:515–20. doi:10.1038/82771
16. Skold M, Faizunnessa NN, Wang CR, Cardell S. CD1d-specific NK1.1+ T cells with a transgenic variant TCR. *J Immunol* (2000) 165:168–74. doi:10.4049/jimmunol.165.1.168
17. Rolf J, Berntman E, Stenstrom M, Smith EM, Mansson R, Stenstad H, et al. Molecular profiling reveals distinct functional attributes of CD1d-restricted natural killer (NK) T cell subsets. *Mol Immunol* (2008) 45:2607–20. doi:10.1016/j.molimm.2007.12.022
18. Yamagata T, Mathis D, Benoist C. Self-reactivity in thymic double-positive cells commits cells to a CD8 alpha alpha lineage with characteristics of innate immune cells. *Nat Immunol* (2004) 5:597–605. doi:10.1038/ni1070
19. Kadri N, Korpos E, Gupta S, Briet C, Lofbom L, Yagita H, et al. CD4(+) type II NKT cells mediate ICOS and programmed death-1-dependent regulation of type 1 diabetes. *J Immunol* (2012) 188:3138–49. doi:10.4049/jimmunol.1101390
20. Liao CM, Zimmer MI, Shanmuganad S, Yu HT, Cardell SL, Wang CR. Dysregulation of CD1d-restricted type ii natural killer T cells leads to spontaneous development of colitis in mice. *Gastroenterology* (2012) 142 (326–334):e321–2. doi:10.1053/j.gastro.2011.10.030
21. Zeissig S, Murata K, Sweet L, Publicover J, Hu Z, Kaser A, et al. Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat Med* (2012) 18:1060–8. doi:10.1038/nm.2811
22. Zhao J, Weng X, Bagchi S, Wang CR. Polyclonal type II natural killer T cells require PLZF and SAP for their development and contribute to CpG-mediated antitumor response. *Proc Natl Acad Sci U S A* (2014) 111:2674–9. doi:10.1073/pnas.1323845111
23. Roy KC, Maricic I, Khurana A, Smith TR, Halder RC, Kumar V. Involvement of secretory and endosomal compartments in presentation of an exogenous self-glycolipid to type II NKT cells. *J Immunol* (2008) 180:2942–50. doi:10.4049/jimmunol.180.5.2942
24. Chang DH, Deng H, Matthews P, Krasovsky J, Ragupathi G, Spisek R, et al. Inflammation-associated lysophospholipids as ligands for CD1d-restricted T cells in human cancer. *Blood* (2008) 112:1308–16. doi:10.1182/blood-2008-04-149831
25. Wolf BJ, Tatituri RV, Almeida CF, Le Nours J, Bhowruth V, Johnson D, et al. Identification of a potent microbial lipid antigen for diverse NKT cells. *J Immunol* (2015) 195:2540–51. doi:10.4049/jimmunol.1501019
26. Exley MA, Tahir SM, Cheng O, Shaulov A, Joyce R, Avigan D, et al. A major fraction of human bone marrow lymphocytes are Th2-like CD1d-reactive T cells that can suppress mixed lymphocyte responses. *J Immunol* (2001) 167:5531–4. doi:10.4049/jimmunol.167.10.5531
27. Exley MA, He Q, Cheng O, Wang RJ, Cheney CP, Balk SP, et al. Cutting edge: compartmentalization of Th1-like noninvariant CD1d-reactive T cells in hepatitis C virus-infected liver. *J Immunol* (2002) 168:1519–23. doi:10.4049/jimmunol.168.4.1519
28. Fuss IJ, Heller F, Boirivant M, Leon F, Yoshida M, Fichtner-Feigl S, et al. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* (2004) 113:1490–7. doi:10.1172/JCI19836
29. Nair S, Boddupalli CS, Verma R, Liu J, Yang R, Pastores GM, et al. Type II NKT-TFH cells against Gaucher lipids regulate B-cell immunity and inflammation. *Blood* (2015) 125:1256–71. doi:10.1182/blood-2014-09-600270
30. Yang SH, Lee JP, Jang HR, Cha RH, Han SS, Jeon US, et al. Sulfatide-reactive natural killer T cells abrogate ischemia-reperfusion injury. *J Am Soc Nephrol* (2011) 22:1305–14. doi:10.1681/ASN.2010080815
31. Fuss IJ, Joshi B, Yang Z, Degheidy H, Fichtner-Feigl S, De Souza H, et al. IL-13Ralpha2-bearing, type II NKT cells reactive to sulfatide self-antigen populate the mucosa of ulcerative colitis. *Gut* (2014) 63:1728–36. doi:10.1136/gutjnl-2013-305671
32. Fox LM, Cox DG, Lockridge JL, Wang X, Chen X, Scharf L, et al. Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS Biol* (2009) 7:e1000228. doi:10.1371/journal.pbio.1000228
33. Maricic I, Girardi E, Zajonc DM, Kumar V. Recognition of lysophosphatidylcholine by type II NKT cells and protection from an inflammatory liver disease. *J Immunol* (2014) 193:4580–9. doi:10.4049/jimmunol.1400699
34. Macho-Fernandez E, Brigl M. The extended family of CD1d-restricted NKT cells: sifting through a mixed bag of TCRs, antigens, and functions. *Front Immunol* (2015) 6:362. doi:10.3389/fimmu.2015.00362
35. Van Rhijn I, Young DC, Im JS, Levery SB, Illarionov PA, Besra GS, et al. CD1d-restricted T cell activation by nonlipidic small molecules. *Proc Natl Acad Sci U S A* (2004) 101:13578–83. doi:10.1073/pnas.0402838101
36. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* (2002) 195:625–36. doi:10.1084/jem.20011786
37. Pei B, Speak AO, Shepherd D, Butters T, Cerundolo V, Platt FM, et al. Diverse endogenous antigens for mouse NKT cells: self-antigens that are not glycosphingolipids. *J Immunol* (2011) 186:1348–60. doi:10.4049/jimmunol.1001008
38. Gapin L, Godfrey DI, Rossjohn J. Natural Killer T cell obsession with self-antigens. *Curr Opin Immunol* (2013) 25:168–73. doi:10.1016/j.coi.2013.01.002
39. Agea E, Russano A, Bistoni O, Mannucci R, Nicoletti I, Corazzi L, et al. Human CD1-restricted T cell recognition of lipids from pollens. *J Exp Med* (2005) 202:295–308. doi:10.1084/jem.20050773
40. Russano AM, Agea E, Corazzi L, Postle AD, De Libero G, Porcelli S, et al. Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted gamma delta T cells. *J Allergy Clin Immunol* (2006) 117:1178–84. doi:10.1016/j.jaci.2006.01.001
41. Dieude M, Striegl H, Tyznik AJ, Wang J, Behar SM, Piccirillo CA, et al. Cardiolipin binds to CD1d and stimulates CD1d-restricted gammadelta T cells in the normal murine repertoire. *J Immunol* (2011) 186:4771–81. doi:10.4049/jimmunol.1000921
42. Bai L, Picard D, Anderson B, Chaudhary V, Luoma A, Jabri B, et al. The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vdelta1 TCR. *Eur J Immunol* (2012) 42:2505–10. doi:10.1002/eji.201242531

43. Luoma AM, Castro CD, Mayassi T, Bembinster LA, Bai L, Picard D, et al. Crystal structure of Vdelta1 T cell receptor in complex with CD1d-sulfatide shows MHC-like recognition of a self-lipid by human gammadelta T cells. *Immunity* (2013) 39:1032–42. doi:10.1016/j.immuni.2013.11.001
44. Stax AM, Tuengel J, Girardi E, Kitano N, Allan LL, Liu V, et al. Autoreactivity to sulfatide by human invariant NKT cells. *J Immunol* (2017) 199:97–106. doi:10.4049/jimmunol.1601976
45. Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, et al. A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med* (2005) 202:1627–33. doi:10.1084/jem.20051381
46. Ambrosino E, Terabe M, Halder RC, Peng J, Takaku S, Miyake S, et al. Cross-regulation between type I and type II NKT cells in regulating tumor immunity: a new immunoregulatory axis. *J Immunol* (2007) 179:5126–36. doi:10.4049/jimmunol.179.8.5126
47. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* (2003) 198:1741–52. doi:10.1084/jem.20022227
48. Fichtner-Feigl S, Terabe M, Kitani A, Young CA, Fuss I, Geissler EK, et al. Restoration of tumor immunosurveillance via targeting of interleukin-13 receptor-alpha 2. *Cancer Res* (2008) 68:3467–75. doi:10.1158/0008-5472.CAN-07-5301
49. Renukaradhya GJ, Khan MA, Vieira M, Du W, Gervay-Hague J, Brutkiewicz RR. Type I NKT cells protect (and type II NKT cells suppress) the host's innate antitumor immune response to a B-cell lymphoma. *Blood* (2008) 111:5637–45. doi:10.1182/blood-2007-05-092866
50. Chevalier MF, Trabanelli S, Racle J, Salome B, Cesson V, Gharbi D, et al. ILC2-modulated T cell-to-MDSC balance is associated with bladder cancer recurrence. *J Clin Invest* (2017) 127:2916–29. doi:10.1172/JCI89717
51. Trabanelli S, Chevalier MF, Martinez-Usatorre A, Gomez-Cadena A, Salome B, Lecciso M, et al. Tumour-derived PGD2 and NKp30-B7H6 engagement drives an immunosuppressive ILC2-MDSC axis. *Nat Commun* (2017) 8:593. doi:10.1038/s41467-017-00678-2
52. Terabe M, Khanna C, Bose S, Melchionda F, Mendoza A, Mackall CL, et al. CD1d-restricted natural killer T cells can down-regulate tumor immunosurveillance independent of interleukin-4 receptor-signal transducer and activator of transcription 6 or transforming growth factor-beta. *Cancer Res* (2006) 66:3869–75. doi:10.1158/0008-5472.CAN-05-3421
53. Kanamori M, Tasumi Y, Iyoda T, Ushida M, Inaba K. Sulfatide inhibits alpha-galactosylceramide presentation by dendritic cells. *Int Immunol* (2012) 24:129–36. doi:10.1093/intimm/dxr108
54. Halder RC, Aguilera C, Maricic I, Kumar V. Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. *J Clin Invest* (2007) 117:2302–12. doi:10.1172/JCI31602
55. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* (1989) 7:145–73. doi:10.1146/annurev.iy.07.040189.001045
56. Terabe M, Berzofsky JA. The immunoregulatory role of type I and type II NKT cells in cancer and other diseases. *Cancer Immunol Immunother* (2014) 63:199–213. doi:10.1007/s00262-013-1509-4
57. Izhak L, Ambrosino E, Kato S, Parish ST, O'konek JJ, Weber H, et al. Delicate balance among three types of T cells in concurrent regulation of tumor immunity. *Cancer Res* (2013) 73:1514–23. doi:10.1158/0008-5472.CAN-12-2567
58. Izhak L, Berzofsky JA, Terabe M. Balance is a key for happiness. *Oncoimmunology* (2013) 2:e24211. doi:10.4161/onci.24211
59. Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J Exp Med* (2003) 197:1667–76. doi:10.1084/jem.20021650
60. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, et al. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol* (2001) 167:4046–50. doi:10.4049/jimmunol.167.7.4046
61. Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. *J Immunol* (2002) 168:6494–9. doi:10.4049/jimmunol.168.12.6494
62. Fujii S, Shimizu K, Klimek V, Geller MD, Nimer SD, Dhodapkar MV. Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br J Haematol* (2003) 122:617–22. doi:10.1046/j.1365-2141.2003.04465.x
63. Molling JW, Kolgen W, Van Der Vliet HJ, Boomsma MF, Kruizenga H, Smorenburg CH, et al. Peripheral blood IFN-gamma-secreting Valpha24+ Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *Int J Cancer* (2005) 116:87–93. doi:10.1002/ijc.20998
64. Yoneda K, Morii T, Nieda M, Tsukaguchi N, Amano I, Tanaka H, et al. The peripheral blood Valpha24+ NKT cell numbers decrease in patients with haematopoietic malignancy. *Leuk Res* (2005) 29:147–52. doi:10.1016/j.leukres.2004.06.005
65. Liu J, Halene S, Yang M, Iqbal J, Yang R, Mehal WZ, et al. Gaucher disease gene GBA functions in immune regulation. *Proc Natl Acad Sci U S A* (2012) 109:10018–23. doi:10.1073/pnas.1200941109
66. Rosenbloom BE, Weinreb NJ, Zimran A, Kacena KA, Charrow J, Ward E. Gaucher disease and cancer incidence: a study from the Gaucher registry. *Blood* (2005) 105:4569–72. doi:10.1182/blood-2004-12-4672
67. Taddei TH, Kacena KA, Yang M, Yang R, Malhotra A, Boxer M, et al. The underrecognized progressive nature of N370S Gaucher disease and assessment of cancer risk in 403 patients. *Am J Hematol* (2009) 84:208–14. doi:10.1002/ajh.21362
68. Mistry PK, Taddei T, Vom Dahl S, Rosenbloom BE. Gaucher disease and malignancy: a model for cancer pathogenesis in an inborn error of metabolism. *Crit Rev Oncog* (2013) 18:235–46. doi:10.1615/CritRevOncog.2013006145
69. Nair S, Branagan AR, Liu J, Boddupalli CS, Mistry PK, Dhodapkar MV. Clonal immunoglobulin against lysolipids in the origin of myeloma. *N Engl J Med* (2016) 374:555–61. doi:10.1056/NEJMoa1508808
70. Bikman BT. A role for sphingolipids in the pathophysiology of obesity-induced inflammation. *Cell Mol Life Sci* (2012) 69:2135–46. doi:10.1007/s00018-012-0917-5
71. Divella R, De Luca R, Abbate I, Naglieri E, Daniele A. Obesity and cancer: the role of adipose tissue and adipo-cytokines-induced chronic inflammation. *J Cancer* (2016) 7:2346–59. doi:10.7150/jca.16884
72. Lynch L, Nowak M, Varghese B, Clark J, Hogan AE, Toxavidis V, et al. Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* (2012) 37:574–87. doi:10.1016/j.immuni.2012.06.016
73. Schipper HS, Rakhshandehroo M, Van De Graaf SF, Venken K, Koppen A, Stienstra R, et al. Natural killer T cells in adipose tissue prevent insulin resistance. *J Clin Invest* (2012) 122:3343–54. doi:10.1172/JCI62739
74. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124:3725–40. doi:10.1172/JCI72308
75. Ohmura K, Ishimori N, Ohmura Y, Tokuhara S, Nozawa A, Horii S, et al. Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. *Arterioscler Thromb Vasc Biol* (2010) 30:193–9. doi:10.1161/ATVBAHA.109.198614
76. Wu L, Parekh VV, Gabriel CL, Bracy DP, Marks-Shulman PA, Tamboli RA, et al. Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice. *Proc Natl Acad Sci U S A* (2012) 109:E1143–52. doi:10.1073/pnas.1200498109
77. Mantell BS, Stefanovic-Racic M, Yang X, Dedousis N, Sipula IJ, O'Doherty RM. Mice lacking NKT cells but with a complete complement of CD8+ T-cells are not protected against the metabolic abnormalities of diet-induced obesity. *PLoS One* (2011) 6:e19831. doi:10.1371/journal.pone.0019831
78. Satoh M, Andoh Y, Clingan CS, Ogura H, Fujii S, Eshima K, et al. Type II NKT cells stimulate diet-induced obesity by mediating adipose tissue inflammation, steatohepatitis and insulin resistance. *PLoS One* (2012) 7:e30568. doi:10.1371/journal.pone.0030568
79. Dao T, Mehal WZ, Crispe IN. IL-18 augments perforin-dependent cytotoxicity of liver NK-T cells. *J Immunol* (1998) 161:2217–22.

80. Leite-De-Moraes MC, Hameg A, Arnould A, Machavoine F, Koezuka Y, Schneider E, et al. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J Immunol* (1999) 163:5871–6.
81. Hagihara M, Gansuvd B, Ueda Y, Tsuchiya T, Masui A, Tazume K, et al. Killing activity of human umbilical cord blood-derived TCRValpha24(+) NKT cells against normal and malignant hematological cells in vitro: a comparative study with NK cells or OKT3 activated T lymphocytes or with adult peripheral blood NKT cells. *Cancer Immunol Immunother* (2002) 51:1–8. doi:10.1007/s00262-001-0246-2
82. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol* (2010) 185:2721–9. doi:10.4049/jimmunol.1001018
83. Bassiri H, Das R, Guan P, Barrett DM, Brennan PJ, Banerjee PP, et al. iNKT cell cytotoxic responses control T-lymphoma growth in vitro and in vivo. *Cancer Immunol Res* (2014) 2:59–69. doi:10.1158/2326-6066.CIR-13-0104
84. Fais F, Tenca C, Cimino G, Coletti V, Zanardi S, Bagnara D, et al. CD1d expression on B-precursor acute lymphoblastic leukemia subsets with poor prognosis. *Leukemia* (2005) 19:551–6. doi:10.1038/sj.leu.2403671
85. Anastasiadis A, Kotsianidis I, Papadopoulos V, Spanoudakis E, Margaritis D, Christoforidou A, et al. CD1d expression as a prognostic marker for chronic lymphocytic leukemia. *Leuk Lymphoma* (2014) 55:320–5. doi:10.3109/10428194.2013.803222
86. Bojarska-Junak A, Hus I, Chocholska S, Tomczak W, Wos J, Czubak P, et al. CD1d expression is higher in chronic lymphocytic leukemia patients with unfavorable prognosis. *Leuk Res* (2014) 38:435–42. doi:10.1016/j.leukres.2013.12.015
87. Chong TW, Goh FY, Sim MY, Huang HH, Thike AA, Lim WK, et al. CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. *J Clin Pathol* (2015) 68:200–5. doi:10.1136/jclinpath-2014-202735
88. Torre-Amione G, Beauchamp RD, Koeppen H, Park BH, Schreiber H, Moses HL, et al. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. *Proc Natl Acad Sci U S A* (1990) 87:1486–90. doi:10.1073/pnas.87.4.1486
89. Maeda H, Shiraishi A. TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. *J Immunol* (1996) 156:73–8.
90. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* (1993) 92:2569–76.
91. Wojtowicz-Praga S, Verma UN, Wakefield L, Esteban JM, Hartmann D, Mazumder A. Modulation of B16 melanoma growth and metastasis by anti-transforming growth factor beta antibody and interleukin-2. *J Immunother Emphasis Tumor Immunol* (1996) 19:169–75. doi:10.1097/00002371-199605000-00001
92. Gunn GR, Zubair A, Peters C, Pan ZK, Wu TC, Paterson Y. Two Listeria monocytogenes vaccine vectors that express different molecular forms of human papilloma virus-16 (HPV-16) E7 induce qualitatively different T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16. *J Immunol* (2001) 167:6471–9. doi:10.4049/jimmunol.167.11.6471
93. Kim S, Buchlis G, Fridlender ZG, Sun J, Kapoor V, Cheng G, et al. Systemic blockade of transforming growth factor-beta signaling augments the efficacy of immunogene therapy. *Cancer Res* (2008) 68:10247–56. doi:10.1158/0008-5472.CAN-08-1494
94. Terabe M, Ambrosino E, Takaku S, O'konek JJ, Venzon D, Lonning S, et al. Synergistic enhancement of CD8+ T cell-mediated tumor vaccine efficacy by an anti-transforming growth factor-beta monoclonal antibody. *Clin Cancer Res* (2009) 15:6560–9. doi:10.1158/1078-0432.CCR-09-1066
95. Ueda R, Fujita M, Zhu X, Sasaki K, Kastenhuber ER, Kohanbash G, et al. Systemic inhibition of transforming growth factor-beta in glioma-bearing mice improves the therapeutic efficacy of glioma-associated antigen peptide vaccines. *Clin Cancer Res* (2009) 15:6551–9. doi:10.1158/1078-0432.CCR-09-1067
96. Takaku S, Terabe M, Ambrosino E, Peng J, Lonning S, Mcpherson JM, et al. Blockade of TGF-beta enhances tumor vaccine efficacy mediated by CD8(+) T cells. *Int J Cancer* (2010) 126:1666–74. doi:10.1002/ijc.24961
97. Terabe M, Robertson FC, Clark K, De Ravin E, Bloom A, Venzon DJ, et al. Blockade of only TGF-beta 1 and 2 is sufficient to enhance the efficacy of vaccine and PD-1 checkpoint blockade immunotherapy. *Oncoimmunology* (2017) 6:e1308616. doi:10.1080/2162402X.2017.1308616
98. Morris JC, Tan AR, Olencki TE, Shapiro GI, Dezube BJ, Reiss M, et al. Phase I study of GC1008 (fresolimumab): a human anti-transforming growth factor-beta (TGFbeta) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. *PLoS One* (2014) 9:e90353. doi:10.1371/journal.pone.0090353
99. Lacouture ME, Morris JC, Lawrence DP, Tan AR, Olencki TE, Shapiro GI, et al. Cutaneous keratoacanthomas/squamous cell carcinomas associated with neutralization of transforming growth factor beta by the monoclonal antibody fresolimumab (GC1008). *Cancer Immunol Immunother* (2015) 64(4):437–46. doi:10.1007/s00262-015-1653-0
100. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* (2006) 24:677–88. doi:10.1016/j.immuni.2006.06.002
101. Pang Y, Gara SK, Achyut BR, Li Z, Yan HH, Day CP, et al. TGF-beta signaling in myeloid cells is required for tumor metastasis. *Cancer Discov* (2013) 3:936–51. doi:10.1158/2159-8290.CD-12-0527
102. Serafini P, Mgebroff S, Noonan K, Borrello I. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res* (2008) 68:5439–49. doi:10.1158/0008-5472.CAN-07-6621
103. Moses HL, Yang EY, Pietenpol JA. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* (1990) 63:245–7. doi:10.1016/0092-8674(90)90155-8
104. Zajonc DM, Cantu C III, Mattner J, Zhou D, Savage PB, Bendelac A, et al. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat Immunol* (2005) 6:810–8. doi:10.1038/ni1224
105. Wu D, Zajonc DM, Fujio M, Sullivan BA, Kinjo Y, Kronenberg M, et al. Design of natural killer T cell activators: structure and function of a microbial glycosphingolipid bound to mouse CD1d. *Proc Natl Acad Sci U S A* (2006) 103:3972–7. doi:10.1073/pnas.0600285103

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REVIEW
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Novel Approaches to Exploiting Invariant NKT Cells in Cancer Immunotherapy

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iNKT cells are a subset of innate-like T cells that utilize an invariant TCR alpha chain complexed with a limited repertoire of TCR beta chains to recognize specific lipid antigens presented by CD1d molecules. Because iNKT cells have an invariant TCR, they can be easily identified and targeted in both humans and mice via standard reagents, making this a population of T cells that has been well characterized. iNKT cells are some of the first cells to respond during an infection. By making different types of cytokines in response to different infection stimuli, iNKT cells help determine what kind of immune response then develops. It has been shown that iNKT cells are some of the first cells to respond during infection with a pathogen and the type of cytokines that iNKT cells make help determine the type of immune response that develops in various situations. Indeed, along with immunity to pathogens, pre-clinical mouse studies have clearly demonstrated that iNKT cells play a critical role in tumor immunosurveillance. They can mediate anti-tumor immunity by direct recognition of tumor cells that express CD1d, and/or via targeting CD1d found on cells within the tumor microenvironment. Multiple groups are now working on manipulating iNKT cells for clinical benefit within the context of cancer and have demonstrated that targeting iNKT cells can have a therapeutic benefit in patients. In this review, we briefly introduce iNKT cells, then discuss preclinical data on roles of iNKT cells and clinical trials that have targeted iNKT cells in cancer patients. We finally discuss how future trials could be modified to further increase the efficacy of iNKT cell therapies, in particular CAR-iNKT and rTCR-iNKT cells.

Keywords: NKT cells, CD1d, iNKT cells, cancer immunotherapy, monoclonal antibody

INTRODUCTION

T cells utilize their unique T cell receptor (TCR) αβ or γδ chain pairs to recognize the universe of antigens. Although many TCRs with extensive somatic V-D-J gene rearrangements recognize peptide antigens within the context of MHC molecules, this is not the only type of antigen that can be recognized. T cells can also utilize near-germline V-J TCR rearrangements to recognize vitamin metabolites, small phosphoantigens, and lipid antigens presented within various highly conserved and non-polymorphic MHC-I like molecules (1–3). Collectively, these non-peptide-recognizing T cells are called "innate-like" T cells and make up a significant proportion of the mammalian T cell compartment (1–4). Importantly, new research is suggesting that these innate-like T cells have important roles in regulating immune reactions not only to pathogens but also to tumors, making them potentially exploitable T cell populations for immunotherapy (1–6). One of the best

characterized innate-like T cell subsets that is being leveraged in immuno-oncology are Natural Killer T (NKT) cells, which recognize lipid antigens bound within the antigen presentation molecule CD1d. The best-characterized subset of NKT cells is "invariant" or "iNKT" cells (1–6). Mouse and human iNKT cells are sufficiently conserved that they can respond to each other's CD1d (7).

OVERVIEW OF iNKT CELL BIOLOGY

NKT cells are a heterogenous population of innate-like CD1d-restricted T cells, the best known of which are invariant NKT (iNKT) cells (1–9). iNKT cells utilize a near-germline TCRα rearrangement (Vα24-Jα18 in humans and Vα14-Jα18 in mice) combined with a limited TCRβ repertoire (1–4). iNKT cells were originally named because of expression of NK1.1 (CD161C) in some mouse strains (CD161A in humans) but this does not accurately define iNKT cells (5, 6). Instead, iNKT cells are functionally defined by their ability to respond to the lipid antigen α-galactosylceramide (α-GalCer) when bound within CD1d molecules and/or by utilizing monoclonal antibodies against the human invariant TCRα chain (3–8). By utilizing (imperfect) co-expression of NK and T cell markers, absence in CD1d or Jα18 KO mice, CD1d tetramers loaded with α-GalCer, or other methods, iNKTs were discovered to make up a significant proportion of T cells within the mouse liver (~20–50%) and adipose tissue (~10–25%) and are present in significant numbers (~0.5–2%) within the murine thymus, spleen, blood, and bone marrow (3–6). Within humans, iNKT cells are represented at similar frequencies to mice in adipose tissue, but are much less frequent in the liver and other organs where "non-invariant" or "diverse" NKT cells predominate (6, 8, 9). In human peripheral blood, iNKT cells range between undetectable to over 1% of circulating T cells in rare individuals, with a median percentage of approximately 0.05% (6, 8, 9).

Unlike peptide-MHC restricted T cells, which emerge from the thymus "naïve," iNKT cells leave the thymus fully matured and able to perform their effector functions without priming (3–6). Within the periphery, iNKT cells respond to lipid antigen and/or cytokine (e.g., IL-12/18) exposure by rapid secretion of multiple cytokines (3–6). Depending on how the iNKT cells are activated, this can include both regulatory cytokines (e.g., IL-4, IL-10, by analogy with Th2, Treg, etc., especially from NKT2, NKT10) (3–6) and/or pro-inflammatory cytokines (e.g., IL-2, IL-17, TNFα, and/or IFNγ, particularly NKT1 or NKT17) (3–6). Since iNKT cells respond rapidly and without the need for priming, they are some of the first cells within an immune response to be activated and therefore act as a "bridge" between the innate and adaptive immune systems. Indeed, iNKT cell activation via TCR engagement or IL-12 or both causes iNKT cells to upregulate IL-12 receptor [which is already basally expressed at a higher level than in NK cells (6, 8)] and CD40L, while also inducing maturation and production of IL-12 in dendritic cells (DCs). This IL-12 release then in turn greatly increases IFNγ production by iNKT cells, leading to a positive feedback loop for Th1 immunity (**Figure 1**) (3–6, 8, 9). Additionally, this maturation of DCs leads to trans-activation of NK cells and increased MHC class I and II antigen presentation to T cells as well as direct cognate B cell "help," allowing for both innate and adaptive immune responses to be established (3–6).

Invariant NKT cells can be further subdivided into additional subsets based on anatomical location or by surface activation

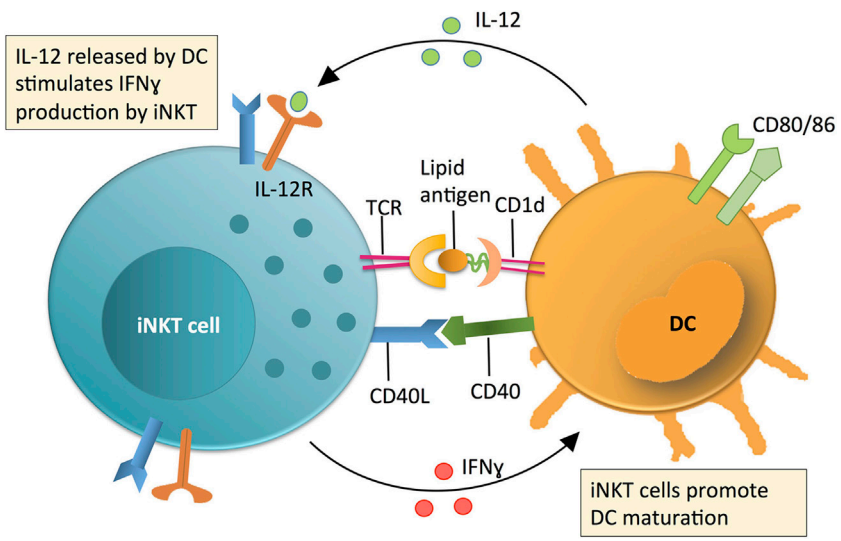

FIGURE 1 | Invariant NKT (iNKT) cell contribution to initiating immune reactions via a positive feedback loop with dendritic cells (DCs). The contribution of iNKT cells to immune surveillance is linked to DC maturation. DCs produce IL-12 and present lipid antigens on CD1d (an MHC class 1 homolog) to stimulate iNKT cell production of IFNγ. The relative contribution of IL-12 or lipid-CD1d to iNKT cell activation is variable and context dependent. Activated iNKT cells produce IFNγ and co-stimulate DCs via CD40L-CD40 interactions to promote DCs to produce IL-12. This IL-12 then further activates iNKT cells in a positive feedback loop. Following activation of iNKT cells, iNKT cell-produced IFNγ and other iNKT-DC interactions (e.g., CD40L-CD40) mature DCs and promote production of IL-12, which further activates iNKT cells in a positive feedback loop.

markers and transcription factors (1–6). The key master transcription factor of at least iNKT cell development and present in most mature iNKT populations is PLZF (4). Unlike most MHC-restricted T cells that are either CD4 + or CD8α + , iNKT cells in mice are either CD4 + or CD4/CD8αβ double negative (DN) (3–6). In humans, a minor population of iNKT cells (typically 1–5%) can instead express CD8αβ (3, 10). Additionally, CD8αα homodimers are expressed by other activated human T cells, although at lower levels on activated CD4 + T cell subsets lacking CD8β (1, 2, 11). In general, CD4 + iNKT cells are able to express more Th2-related cytokines like IL-4, although they can express as much Th1 cytokines at the same time (3–9). Human CD8αβ + and DN iNKT cells are biased toward a Th1-related phenotype, more cytotoxic and preferentially make IFNγ. In both cases, these are plastic definitions and CD4 + iNKT cells can make IFNγ and DN iNKT cells can make IL-4, at least partially depending on the stimuli given (3–6, 8, 9). Mouse iNKT cells are less clearly biased, although liver iNKT cells have greater anti-tumor activity than other organ iNKT cells (12). Indeed, within different organs, different iNKT phenotypes tend to dominate. Relatively Th1-like iNKT cells tend to be enriched within the spleen and liver, while Th2-like iNKT cells are associated with the lungs and intestine (3–6). There are also Th17-like iNKT cells that express cytokines like IL-17 and are enriched within the lungs, intestine, lymph nodes, and skin (13). Finally, a subset has recently been described in adipose tissue. Adipose iNKT tend to make anti-inflammatory cytokines like IL-10 and unlike other mature iNKT cells, lack PLZF (14, 15).

Because iNKT cells can rapidly produce IFNγ, IL-4, or both, they have been found to play a role in various diseases by establishing a Th1- or Th2-based immune response. In bacterial and viral infections, iNKT cells typically help in early control of the pathogen by establishing a productive Th1 response (1–6, 9, 13, 16). In both mouse and human studies, roles for iNKT cells have been described in diseases associated with excessive Th1 responses like type 1 diabetes (9) and chronic obstructive pulmonary disease (17, 18). Roles have also been described for iNKT cells helping to suppress Th1 responses and drive tolerogenic responses to grafts. As an example, following hematopoietic stem cell transfer, the presence of iNKT cells is predictive for survival with a reduction in graft versus host disease (GvHD) in patients and preclinical models (8, 9, 19–21).

iNKT CELLS IN CANCER

Within the context of cancer, the frequency and/or function of iNKT cells (either within the tumor or in circulation) can be selectively and highly correlative with overall survival. In human studies, this has been demonstrated in prostate cancer, medulloblastoma and neuroblastoma, melanoma, colon, lung, breast, and head and neck squamous cell carcinomas (8, 9, 22–37). The largest numbers of patient samples and/or longest follow-ups were analyzed for tumor in neuroblastoma and circulating in head and neck squamous cell carcinomas, respectively (26, 37). Consistent with reduced numbers, proliferative response defects of iNKT cells have been noted in cancer patient iNKT cells (23, 28, 37). Decreased numbers of circulating iNKT cells can be accompanied by decreases in IFNγ production and a concurrent increase in IL-4 production (22, 24, 25, 35). Importantly, all of these defects including the shift toward an iNKT cell Th2 phenotype can be reversed *in vitro*. Activation via 2 strong stimuli, such as α-GalCer and IL-12, increases iNKT cell IFNγ production, promotes tumor rejection, and protects from development of metastasis in multiple mouse models and enhances cancer patient iNKT Th1 responses *in vitro* (8, 22, 24, 25, 35). However, such stimuli do not reverse iNKT defects individually (particularly in advanced disease) (8, 22, 24, 25, 35). Additionally, injection of α-GalCer-pulsed DCs (particularly mature DC) can provide a strong anti-tumor effect (31, 34, 35).

ROLE OF iNKT CELLS IN CANCER: PRE-CLINICAL MOUSE MODELS

While the human data is correlative, the role for iNKT cells in providing tumor surveillance has been well-characterized in mouse models. Examples of iNKT-mediated tumor clearance were demonstrated by the lab of Taniguchi et al. (31, 38) as well as those of Smyth and Godfrey (9, 12). iNKT cells were found to be essential for anti-tumor responses induced by α-GalCer (12, 30, 38). Treatment with carcinogen or transfer of carcinogen-induced tumor cell lines in mice lacking iNKT cells (via TCR Jα18 deletion, Jα18-KO) caused tumors to appear at a much higher frequency than in wild-type (WT) mice (39). Additionally, transfer of iNKT cells into Jα18-KO mice was sufficient to cause protection against tumors to a level like WT mice, unless the iNKT cells came from an IFNγ KO mouse (39). Together, these and other results show that even in the absence of exogenous antigens like α-GalCer, iNKT cells can establish a Th1 response to some tumors and can contribute to tumor clearance (8, 9, 29, 32, 39). Further support for iNKT cell-mediated tumor surveillance was obtained with the spontaneous prostate cancer mouse strain: transgenic adenocarcinoma of the mouse prostate (TRAMP). By back-crossing Jα18-KO to TRAMP mice, Bellone et al. suggested that lack of iNKT cells led to accelerated tumor generation and quicker mortality than was detected in WT TRAMP mice (39), consistent with earlier human *in vitro* data (22). However, more recently, a caveat of studies using the original Jα18 KO mice (38) has come to light, most notably the inability of these mice to express TCR Jα regions past Jα19 (40). This impacts the TCR repertoire of conventional T cells, which could also impact *in vivo* immune responses, so new Jα18 KO mice have been developed that do not share this defect (41, 42).

While some CD1d-expressing tumors can probably cause Th1-biased iNKT cell activation, progressive chronic tumor cell growth can also apparently directly cause Th2-biased iNKT cell activation. By utilizing the same TRAMP prostate cancer model as a source of primary prostate tumors, we demonstrated that CD1d-expressing prostate tumor cells can directly activate iNKT cells, but biased them toward making Th2 cytokines (43). While addition of α-GalCer or IL-12 can usually help bias an iNKT cell toward a Th1 phenotype, neither of these stimuli on their own were enough to reverse the tumor cell driven Th2 bias in iNKT cells. However, pulsing the tumor cells with α-GalCer

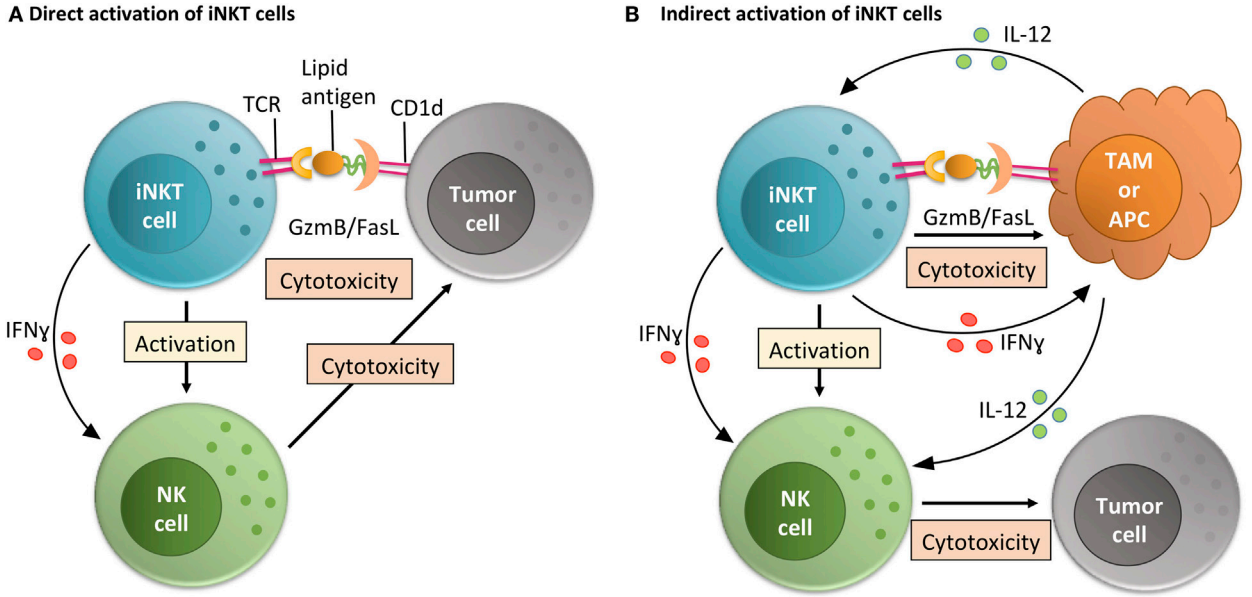

FIGURE 2 | Invariant NKT (iNKT) cell activity within the tumor microenvironment (TME). iNKT cells can function within the TME via direct or indirect interactions with tumor cells. **(A)** In the direct activation pathway, iNKT cells recognize lipid-CD1d complexes on the surface of tumor cells and then directly mediate killing of tumor cells. By making inflammatory cytokines like IFNγ, this also helps TME-resident NK cells perform their anti-tumor cell effector functions (24, 35, 36, 43, 44). **(B)** In the indirect activation pathway, iNKT cells recognize lipid-CD1d complexes on the surface of TME-resident antigen presenting cells (APCs) or tissue-associated macrophages (TAMs) (29). This interaction leads to iNKT cell-mediated killing of immunosuppressive TAMs, leading to a less immunosuppressive environment where tumor-infiltrating NK cells can better perform their functions. Alternately, if the CD1d is on the surface of a TME-resident APC, the iNKT cell can activate that APC and stimulate production of IL-12, helping resident effector cells like NK cells overcome the immunosuppressive state of the tumor (32).

and adding IL-12 at the same time synergized to allow for IFNγ production to occur (43).

In both the models described above and in humans, activation of iNKT cells and tumor rejection can occur in one of two ways (**Figure 2**). The first is that iNKT cells directly recognize and kill CD1d-expressing tumor cells. This can occur in a significant portion of lymphomas, early myeloma, myeloid leukemias, medulloblastoma, and prostate cancers (24, 35, 36, 43, 44). The second is by activation of iNKT cells by other CD1d-expressing cells in the tumor microenvironment (TME) (29, 32). In this indirect system, iNKT cell activation by CD1d-expressing TME cells leads to trans-activation of NK cells and/or killing of immunosuppressive cells like tumor-associated Macrophages (TAMs) (29, 32, 45). When we directly tested the ability of iNKT cells to respond to CD1d-expressing prostate tumor cells from TRAMP mice *in vitro*, we found that they did not elicit a Th1 phenotype that would be indicative of killing (43). However, Bellone et al. found that iNKT cells do help delay tumor growth within intact TRAMP mice over periods of months (39). The differences in these two studies may include that within the intact mouse there is also a role for iNKT cells in killing CD1d-expressing TAMs independent of any direct anti-tumor interactions (29, 32). Therefore, relieving some of the immunosuppression within the primary tumor by killing TAMs may be a key role for iNKT cells *in vivo*. However, in progressive clinical cancer, TAMs can apparently overwhelm iNKT cells (29, 32). Reversing these as well as tumor cell-driven iNKT defects is the goal of the groups working on clinical trials targeting iNKT cells worldwide.

PRECLINICAL AND CLINICAL TRIALS TARGETING iNKT CELLS

Pre-clinical murine models have shown similar defects in iNKT cells as have been seen in humans and demonstrated that iNKT stimulation *in vivo* or adoptive transfer can induce strong antitumor immune responses (38, 43, 46–51). This has been shown to be the case for stimulation of iNKT cells via α-GalCer infusion and when α-GalCer has been loaded on DCs (46–51). Additionally, as iNKT cells play a key role in generating a positive feedback loop for IL-12 production by DCs, low and moderate-dose IL-12 therapy in animal models is also dependent on iNKT cells (46, 50, 51). Either stimulation causes iNKT cells to rapidly produce a strong cytokine response, including large amounts of IFN-γ that stimulates NK cells, B cells, and that also enhances the generation of classical cytotoxic T cell responses (39, 49, 51). Strong antitumor immune responses to α-GalCer and/or IL-12 have been observed in most murine models, including colon carcinoma, lymphomas, sarcoma, melanoma, prostate, and lung carcinoma (39, 41, 46–51). Together, these observations indicate that restoration of iNKT cell function in humans with cancer may stimulate potent antitumor immune responses.

Clinical Trials Targeting iNKT Cells via Stimulation with α-GalCer

The pre-clinical antitumor effects of α-GalCer stimulated a phase 1 clinical study in advanced-stage cancer patients (52).

Administration of α-GalCer was not accompanied by dose limiting toxicity. In this phase 1 study, as in other analyses (9, 28, 29, 37), circulating iNKT cell numbers were found to be decreased in cancer patients (52). The relevance of the decreased size of the iNKT cell pool was demonstrated in the same trial as immunological responses to α-GalCer administration (increases in GM-CSF and TNF-α) were only observed in those patients with higher iNKT cell levels comparable to healthy controls (52).

This initial clinical study and preclinical studies outlined above implied that antitumor effects of α-GalCer in cancer patients would be limited by both qualitative and quantitative defects in iNKT cells, necessitating the evaluation of alternative approaches to exploit this natural antitumor system. In mice, administration of α-GalCer-loaded DC resulted in a more powerful antitumor immune response (48, 49). Several phase 1 clinical studies have used α-GalCer-loaded immature or matured monocyte-derived dendritic cells (Mo-DC) or other monocyte-derived antigen-presenting cell (APC) preparations leading to clinically relevant antitumor responses (53–58).

In the first published autologous Mo-DC transfer clinical study, Nieda et al. investigated the transfer of purified α-GalCer-pulsed immature Mo-DC in a variety of different malignancies (53). They found that adoptive transfer of α-GalCer-pulsed Mo-DC led to minor systemic side effects in 9 of 12 patients such as fever, malaise, lethargy, and headache (53). These side effects were temporary and expected when eliciting an immune response by activating iNKT cells. Several patients experienced temporary exacerbation of tumor symptoms that were interpreted as inflammatory responses to the tumor (e.g., enlargement of tumor deposits or associated lymph nodes, bone pain, and respiratory symptoms in subjects with pulmonary metastases) (53). These exacerbated tumor symptoms had a strong temporal and reproducible relationship in terms of timing and nature with treatment cycles, were transient (generally lasting 1 to 3 days), and were absent outside of the study period. In four of the patients, there were decreases in tumor markers, and in one patient, there was extensive tumor necrosis (53). This study was also important in that it was the first to provide clinical interventional data for the role of iNKT cells as the "bridge" between the innate and adaptive immune systems in humans, as has been seen in multiple human *in vitro* and murine *in vivo* studies (39, 43, 46–51). In this clinical trial, activation of human iNKT cells *in vivo* by adoptive transfer of α-GalCer-loaded Mo-DC reproducibly initiated an activation program wherein iNKT cell activation led to subsequent activation of B cells, T cells, NK cells, and increased serum levels of IL-12 and IFN-γ (53).

Ishikawa et al. investigated the effects of adoptive transfer of autologous cell preparations that were enriched for α-GalCer-pulsed DCs in 11 patients with recurrent lung cancer or advanced non-small cell lung cancer (54). No serious adverse events were reported. Importantly, in several patients, an increase in the circulating number of iNKT cells was also detected. Notably, as reported previously, immunological responses were restricted to patients having "normal" pretreatment iNKT cell numbers. No patients exhibited complete or partial responses in this study, but two patients had stable disease (54).

Chang et al. performed a clinical trial where five cancer patients were treated with α-GalCer-pulsed mature Mo-DC (55). The trial was focused on evaluating the number and phenotype of iNKT cells following stimulation via DC transfer. A more than 100-fold expansion of circulating iNKT cell numbers was observed in all five patients, and this expansion was sustained for up to 6 months post-vaccination (55). Additionally, the data suggested a boost in adaptive T cell immunity, as it was accompanied by an increase in antigen-specific memory CD8 + T cells (55). In this study, no more than grade 1 toxicity was observed, and although one patient developed rheumatoid factor and transient positive anti-nuclear antibody at follow up, no clinical evidence of autoimmunity was observed (55).

In addition to these trials above, several subsequent trials have used APC (i.e., adherent monocytic cells treated with GM-CSF and IL-2) loaded with α-GalCer and have shown increasing effectiveness as dose and targeting have been improved, particularly so far in lung cancer and head and neck cancers (56–58). Specifically in a lung cancer trial, patients who had circulating iNKT able to produce IFNγ had a threefold longer lifespan (57).

Clinical Trials Boosting Endogenous iNKT Cell Numbers via Adoptive Transfer

Another (and complementary) approach to α-GalCer-based treatments involves the adoptive transfer of activated iNKT cells to restore iNKT cell numbers and potentially iNKT cell function in cancer patients. This approach has been tested in preclinical models of melanoma and lung cancer and shown to be more effective compared to the i.v. administration of α-GalCer (50). Trials of iNKT-enriched PBMC have supported direct use of iNKT with evidence for immunological and objective clinical responses (59–62).

The first of these adoptive iNKT cell therapies targeted six patients with non-small cell lung cancer (59). To grow out iNKT cells, bulk PBMCs were stimulated two to three times via addition of α-GalCer to the cultured cells. These iNKT cell-enriched products were then infused back into the patient, and the iNKT cell numbers, persistence, and phenotype were measured. In most patients, there was a transient but not long-term increase in iNKT cell number within the blood, and this coincided with the ability to detect IFNγ production *ex vivo* via α-GalCer stimulation of PBMCs. Only minor adverse effects were seen in this first trial, demonstrating that adoptive cell therapy of iNKT cells is likely to be safe. In this study, no partial or complete responses were seen (59).

The next adoptive iNKT cell-based therapy studies combined autologous iNKT cell-enriched product with *in vivo* boosting. In a Phase I and subsequent Phase II study, the trial group first treated head and neck squamous cell carcinoma (HNSCC) patients with two doses of α-GalCer-loaded DCs followed by an iNKT cell infusion (60). In the Phase I trial, three patients showed partial responses, four had stable disease, and one had progressive disease (60). Of the eight patients, only one had grade 3 adverse events and that patient also had a partial response: a fistula formed within the tumor apparently due to rapid tumor killing (60). In the follow-up Phase II trial for 10 patients with HNSCC, patients

were first given nasal submucosal administration of α-GalCer loaded DCs followed by iNKT cell infusion directly into the tumor-feeding arteries, so that iNKT cells were more likely to end up in the tumor site (61). Adverse events were minimal and limited to grade 2 or below, five patients had a partial response, and five patients had stable disease (61). iNKT cell numbers within the tumor and in the peripheral blood were measured, and while iNKT cell numbers in the blood did increase in 9 of 10 patients post-treatment, this did not correlate with outcome. Instead, a high number of tumor-infiltrating iNKT cells correlated with an objective response of patients (61).

With clinical colleagues at Harvard Cancer Center, we performed a Phase I clinical trial of autologous purified [with the iNKTCR mAb 6B11 (62)] and expanded iNKT cells in nine melanoma cancer patients (62). In our study, iNKT cells were isolated from PBMCs with a protocol based on a monoclonal antibody that specifically recognizes the invariant TCR of iNKT cells and then expanded *in vitro* with plate bound anti-CD3 antibody (62–64). Compared to previous studies using α-GalCer stimulated PBMCs as a source of iNKT cells (59–61), this study transferred in generally higher purity and/or larger numbers of iNKT cells (3 doses at up to 250 million iNKT cells per dose). Since iNKT cells are activated via interaction with CD1d on APC, after the first three patients had no significant toxicities, subsequent patients were pre-treated with GM-CSF to enhance DC functions before iNKT infusion cycles 2 and 3. Like in the other studies, we noted a transient increase in circulating iNKT cell numbers following adoptive cell transfer and increased activation of other T cells and myeloid cells in some patients, and toxicities were minor and readily treatable (Grade 1 & 2 only) (62). In terms of responses at the end of the study, three patients had no evidence of disease or stable disease, three eventually progressed and responded to subsequent treatment, and three died of disease (one removed from study after infusions, two at 2 or more years post-treatment). Overall, our trial confirms that iNKT cell adoptive therapy is safe and well-tolerated, but modified treatment regimens are likely required to demonstrate efficacy. These could include further conditioning with stimulations like α-GalCer (on APC or free) and/or IL-12 *in vivo*.

FUTURE CLINICAL TRIALS: CAR-iNKT AND rTCR-iNKT

Current clinical trials with either α-GalCer-loaded Mo-DCs or adoptive transfer of iNKT cells have produced partial and complete responses, but few if any cures in late stage patients. In contrast, T cells expressing chimeric antigen receptors (CAR-T) targeting surface proteins like CD19 have shown complete response rates of up to 90% in specific diseases such as B-ALL, leading to the first approvals for these treatments (65). Additional T cell therapies are utilizing recombinant TCR (rTCR-T) expressing T cells to be able to target peptides from tumor-associated intracellular proteins within the context of HLA molecules and are reporting similar complete response rates in myeloma (66) and solid malignancies.

One of the major drawbacks of CAR-T/rTCR-T cell therapy is a very high rate of serious adverse effects, including cytokine release syndrome (CRS) and lethal neurotoxicity (65, 67). Other risks include antigen selection issues (e.g., off-tumor on-target) and GvHD caused by TCR mispairing in rTCR-expressing cells and via allogeneic cell therapy (67). Interestingly, while GvHD is a common concern for both CAR-T and rTCR-T, iNKT cells have been shown in pre-clinical models to suppress, not cause GvHD and are associated with reduced GvHD in the clinic (19–21, 68, 69), making for a potentially safer therapeutic approach. In light of both the promises and drawbacks of CAR-T and rTCR-T cells, there is growing interest in utilizing iNKT cells as an ideal platform for CAR or rTCR therapies (CAR-iNKT or CAR-rTCR).

One of the main benefits of considering iNKT cells as an ideal vector for CAR/rTCR therapies is that iNKT cells have an endogenous TCR that is confirmed to have intrinsic anti-tumor capabilities (**Figure 3**). While the "random" endogenous TCRs on a bulk polyclonal T cell preparations are unlikely to contribute to anti-tumor effects, it is likely that iNKT cells could utilize both their endogenous TCR and their CAR/rTCR to target the tumor with two different targeting moieties. As mentioned previously, this could be by direct CD1d targeting either on the surface of the tumor cell or a bystander tumor-promoting myeloid cell, or via removing immunosuppression by killing CD1d + TAMs. Since strong TCR signaling cascades (as evidenced by potent iNKT cell antigens like α-GalCer) help cause Th1-based iNKT cell responses, having a second TCR signaling pathway engaged within the iNKT cell may help ensure that the iNKT cells remain Th1-biased *in vivo*. Another advantage for CAR-iNKT or rTCR-iNKT cell therapies is that iNKT cells naturally migrate into non-lymphoid tissues (70), suggesting that they would be ideal cells to target non-lymphoid tumors. Indeed, PLZF expression seems to drive innate T cells tissue homing in general (4, 71). iNKT cells are known to respond to tissue chemokines CCL2 (72) and CCL20 (45). While suggestive of intrinsic benefits of iNKT cells, these points remain to be formally tested in the context of CARs in the clinic.

The Metelitsa group has pioneered CAR-iNKT cells for tumor therapies. In pre-clinical models, they have tested human iNKT cells purified with the iNKTCR mAb 6B11 for their ability to express GD2 CARs (against neuroblastomas) and CD19 CARs (against B cell lymphomas) (73, 74). Importantly, they demonstrated that iNKT cells could stably express either CAR construct, and that the CAR-iNKT cells kill relevant antigen-expressing tumor cell lines *in vitro*. With GD2 CAR-iNKT, this included killing of both GD2 + CD1d− cells and GD2− CD1d + cells, demonstrating that the endogenous iNKT TCR was still functional within GD2 CAR-iNKT cells (73). Importantly, CAR-iNKT cell homing and killing *in vivo* of either the solid tumor xenograft model GD2-expressing neuroblastoma or liquid xenograft B cell lymphoma was greatly increased over non-transduced iNKT cells, leading to substantially increased survival of CAR-iNKT treated mice (73).

As tumor homing and GvHD are concerns for both CAR-T and rTCR-T, they further measured if CAR-iNKT cells had better trafficking to the tumor and what effect placing the CAR into bulk T cells or iNKT cells had on GvHD. CAR-iNKT cells homed to the tumor at an even higher frequency than CAR-T cells, providing evidence that iNKT cells do indeed have better tumor

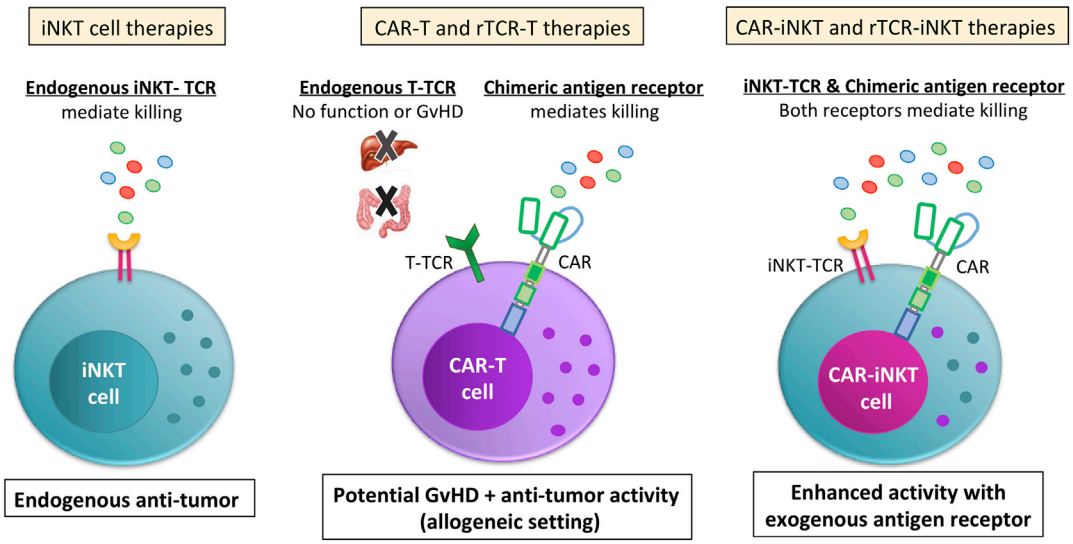

FIGURE 3 | Invariant NKT (iNKT) cells are a viable cell vector for emerging CAR and rTCR therapies. Unlike bulk T cell preparations that are currently used for chimeric antigen receptor (CAR) or recombinant TCR (rTCR) T cell therapies, the endogenous iNKT-TCR has anti-tumor function (left side). In contrast, the bulk T cell endogenous TCRs do not contribute to the anti-tumor function of the cell (middle picture). Instead, the bulk T cell endogenous TCR is a liability, endowing the cells with the potential for graft versus host disease (GvHD) or other off-target effects. While GvHD is a common concern for both CAR-T and rTCR-T allogeneic therapy and at least a possibility due to mispairing for autologous rTCR-iNKT, iNKT cells are shown in pre-clinical models to suppress, not cause GvHD during allogeneic transfer, making for a safer therapeutic approach. Additionally, iNKT cells expressing a CAR or rTCR (right picture) would be endowed with two different anti-tumor receptors. This would allow iNKT cells to target the tumor directly with the CAR or rTCR while its endogenous iNKT-TCR would be able to target the tumor microenvironment and/or the tumor itself, depending on if the tumor expressed CD1d. Either way, CAR-iNKT and rTCR-iNKT therapies would have an endogenous TCR that contributes to tumor clearance instead of be a hindrance.

homing than bulk T cells (73). To model GvHD within the context of a xenogeneic cell transfer, CAR-iNKT or CAR-T cells were transferred into humanized mice and monitored for GvHD. As would be expected when transferring in xenogeneic T cells, CAR-T cells caused severe GvHD in the livers and lungs of the mice. In contrast, CAR-iNKT cells did not cause GvHD of these organs during xenogeneic transfer (73), consistent with the GvHD suppressing activities of iNKT described above. Finally, additional work suggested that in GD2 CARs, expression of both the CD28 and 4-1BB costimulatory domains led to longer CAR-iNKT persistence and increased survival of mice compared to single CD28 or 4-1BB costimulatory domains (73). As well as the Metelitsa group, Karadimitris et al. reported in a review otherwise of potential myeloma treatments that CD19-CAR iNKT had promising preclinical anti-tumor activity in their hands also (75). Both groups are planning clinical trials in the near future.

Many groups are looking at transducing subsets of T cells for CAR/rTCR therapies, and some studies have suggested that CD62L+ (central memory) T cells are superior to other T cell subsets (76). In the Metelitsa group CD19 CAR-iNKT study, Tian et al. separated CD62L+ and CD62L− CAR-iNKT cells and measured their persistence and anti-tumor ability (74). CD62L+ CAR-iNKT cells had superior proliferation, *in vivo* persistence, and antitumor activity as compared to the CD62L− CAR-iNKT cells (74), suggesting that even with iNKT cells, it may be worthwhile to target a defined subset for CAR-iNKT or rTCR-iNKT therapies. Interestingly, the majority of iNKT express CD62L until they are repeatedly stimulated *in vitro* (74).

While these CAR-iNKT preclinical studies are extremely valuable, critical issues should be generalized within future studies. First, multiple *in vivo* comparisons of tumor killing and survival of CAR-iNKT to bulk T cell CAR-T cells should be performed. Second, as *in vivo* administration of α-GalCer is well tolerated in humans, it should be determined if α-GalCer administration could help either expand CAR-iNKT *in vivo* and/or cause an additive/synergistic increase in anti-tumor activity. Planned clinical studies will begin to address these issues in the near future.

Jiang et al. have provided the first evidence of iNKT cells being able to express a second recombinant TCR (77). In this study, a HLA class I-restricted TCR (TCR-Vα9 TCR-Vβ5) against the *Mycobacterium tuberculosis* (Mtb) 38-kDa protein was cloned and expressed in iNKT cells. Using autologous 38-kDa protein pulsed Mo-DCs as APC, they confirmed that only rTCR-expressing iNKT cells recognized and killed these cells. The relative killing efficiency of α-GalCer-pulsed Mo-DCs was similar to the killing of 38-kDa pulsed Mo-DCs, suggesting that the endogenous iNKT TCR was still fully functional (77). However, it was not determined if the recombinant TCR and the endogenous TCR could both signal at the same time to cause additive or synergistic effects. Nor was it determined if expression of the recombinant TCR came at the expense of some endogenous iNKT-TCR, as could happen due to competition for CD3 complexes. Finally, as it has been reported that the anti-Mtb activity of iNKT cells is due to production of GM-CSF and not production of IFNγ or infected cell lysis (78), it is unclear what additional role(s) rTCR-iNKT cells would play during Mtb infection. Future studies are

needed to determine if expressing this rTCR in iNKT cells skews their function *in vivo* during Mtb infection, either by helping trafficking of iNKT cells to the site of infection or otherwise. Clearly, iNKT expressing anti-tumor rTCR could also gain augmented activity, as is currently being addressed by some groups.

CONCLUSION

iNKT cells provide a novel alternative to standard T cells in cancer immunotherapy, as described above. Their tissue (and therefore also tumor) tropism, inherent direct and indirect anti-tumor activities and our ability to manipulate them *in vitro* and *in vivo* (e.g., with α-GalCer, analogs thereof, or the iNKTCR mAb 6B11) combined with reversible defects in cancer patients suggest that they can be exploited to treat a range of solid and hematological malignancies.

An important potential caveat in exploiting iNKT cells has been the observation that repeated stimulation of mouse iNKT cells (though less so with human iNKT) with α-GalCer can lead to an anergic-like state (3–6, 8, 79–81). Interestingly, this state can be reversed by PD-1/PD-L1 blockade (79), commonly now used in the clinic to overcome conventional anti-tumor responses. Furthermore, it may reflect a polarization to IL-10 producing "NKT10" that have been found in mice and man (81). Another promising approach in general, which may also overcome NKT cell anergy/polarization, is differential use of co-stimulation alongside direct invariant TCR stimulation (82).

There may well be more total "non-invariant" diverse CD1d-restricted NKT cells in the body than iNKT and their ability to make Th2 cytokines appears to impair tumor immunity (30), whereas such NKT making IFNγ stratifies with cancer patient survival (83), as does iNKT (24, 25, 54). However, non-invariant CD1d-restricted NKT cell manipulation is much more challenging and their understanding lags far behind iNKT cells. Finally, unlike other innate lymphocytes like NK and γδ T cells, iNKT are also relatively rare, so substantially increasing their numbers should be safe and is both very feasible (as described above) and has more potential to change the milieu (the other populations at ~5% of total lymphocytes probably cannot be increased more than ~10-fold without concomitant loss of conventional T cells). The next few years should provide an opportunity for iNKT cells to "put up or shut up"!

AUTHOR CONTRIBUTIONS

BW, JC, and ME contributed to the writing and production of this manuscript. JC designed the figures with editorial input from BW and ME.

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REFERENCES

1. Kronenberg M. When less is more: T lymphocyte populations with restricted antigen receptor diversity. *J Immunol* (2014) 193(3):975–6. doi:10.4049/jimmunol.1401491
2. Van Rhijn I, Moody DB. Donor unrestricted T cells: a shared human T cell response. *J Immunol* (2015) 195(5):1927–32. doi:10.4049/jimmunol.1500943
3. Salio M, Silk JD, Yvonne Jones E, Cerundolo V. Biology of CD1- and MR1-restricted T cells. *Annu Rev Immunol* (2014) 32:323–66. doi:10.1146/annurev-immunol-032713-120243
4. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol* (2013) 25(2):161–7. doi:10.1016/j.coi.2013.01.003
5. Rossjohn J, Pellicci DG, Patel O, Gapin L, Godfrey DI. Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* (2012) 12(12):845–57. doi:10.1038/nri3328
6. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13(2):101–17. doi:10.1038/nri3369
7. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, et al. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med* (1998) 188:1521–8. doi:10.1084/jem.188.8.1521
8. Exley MA, Lynch L, Varghese B, Nowak M, Alatrakchi N, Balk S. Developing understanding of the roles of CD1d-restricted T cell subsets in cancer: reversing tumor-induced defects. *Clin Immunol* (2011) 140:184–95. doi:10.1016/j.clim.2011.04.017
9. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: natural killer T cell defects and human disease. *Nat Rev Immunol* (2011) 11:131–42. doi:10.1038/nri2904
10. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med* (2002) 195:637–41. doi:10.1084/jem.20011908
11. Park Y, Moon SJ, Lee SW. Lineage re-commitment of CD4CD8αα intraepithelial lymphocytes in the gut. *BMB Rep* (2016) 49(1):11–7. doi:10.5483/BMBRep.2016.49.1.242
12. Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J Exp Med* (2005) 202(9):1279–88. doi:10.1084/jem.20050953
13. Monteiro M, Graca L. iNKT cells: innate lymphocytes with a diverse response. *Crit Rev Immunol* (2014) 34(1):81–90. doi:10.1615/CritRevImmunol.2014010088
14. Lynch L, O'Shea D, Winter DC, Geoghegan J, Doherty DG, O'Farrelly C. Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. *Eur J Immunol* (2009) 39(7):1893–901. doi:10.1002/eji.200939349
15. Exley MA, Hand L, O'Shea D, Lynch L. Interplay between the immune system and adipose tissue in obesity. *J Endocrinol* (2014) 223(2):R41–8. doi:10.1530/JOE-13-0516
16. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* (2003) 4:1230–7. doi:10.1038/ni1002
17. Kim EY, Battaile JT, Patel AC, You Y, Agapov E, Grayson MH, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat Med* (2008) 14(6):633–40. doi:10.1038/nm1770
18. Nembrini C, Marsland BJ, Kopf M. IL-17-producing T cells in lung immunity and inflammation. *J Allergy Clin Immunol* (2009) 123(5):986–94. doi:10.1016/j.jaci.2009.03.033
19. Kohrt HE, Pillai AB, Lowsky R, Strober S. NKT cells, Treg, and their interactions in bone marrow transplantation. *Eur J Immunol* (2010) 40(7):1862–9. doi:10.1002/eji.201040394
20. Karadimitris A, Chaidos A. The role of invariant NKT cells in allogeneic hematopoietic stem cell transplantation. *Crit Rev Immunol* (2012) 32(2):157–71. doi:10.1615/CritRevImmunol.v32.i2.40

21. Guan P, Bassiri H, Patel NP, Nichols KE, Das R. Invariant natural killer T cells in hematopoietic stem cell transplantation: killer choice for natural suppression. *Bone Marrow Transplant* (2016) 51(5):629–37. doi:10.1038/bmt.2015.335
22. Tahir M, Cheng O, Shaulov A, Bubley G, Balk S, Exley M. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol* (2001) 167:4046–50. doi:10.4049/jimmunol.167.7.4046
23. Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. *J Immunol* (2002) 168(12):6494–9. doi:10.4049/jimmunol.168.12.6494
24. Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J Exp Med* (2003) 197:1667–76. doi:10.1084/jem.20021650
25. Fujii S, Shimizu K, Klimek V, Geller MD, Nimer SD, Dhodapkar MV. Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br J Haematol* (2003) 122(4):617–22. doi:10.1046/j.1365-2141.2003.04465.x
26. Metelitsa LS, Wu HW, Wang H, Yang Y, Warsi Z, Asgharzadeh S, et al. Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2. *J Exp Med* (2004) 199(9):1213–21. doi:10.1084/jem.20031462
27. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, et al. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res* (2005) 11(20):7322–7. doi:10.1158/1078-0432.CCR-05-0877
28. Molling JW, Kolgen W, van der Vliet HJ, Boomsma MF, Kruizenga H, Smorenburg CH, et al. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *Int J Cancer* (2005) 116:87–93. doi:10.1002/ijc.20998
29. Song L, Asgharzadeh S, Salo J, Engell K, Wu HW, Sposto R, et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. *J Clin Invest* (2009) 119(6):1524–36. doi:10.1172/JCI37869
30. Berzofsky JA, Terabe M. The contrasting roles of NKT cells in tumor immunity. *Curr Mol Med* (2009) 9(6):667–72. doi:10.2174/156652409788970706
31. Taniguchi M, Tashiro T, Dashtsoodol N, Hongo N, Watarai H. The specialized iNKT cell system recognizes glycolipid antigens and bridges the innate and acquired immune systems with potential applications for cancer therapy. *Int Immunol* (2010) 22(1):1–6. doi:10.1093/intimm/dxp104
32. Metelitsa LS. Anti-tumor potential of type-I NKT cells against CD1d-positive and CD1d-negative tumors in humans. *Clin Immunol* (2011) 140(2):119–29. doi:10.1016/j.clim.2010.10.005
33. Schneiders FL, Scheper RJ, von Blomberg BM, Woltman AM, Janssen HL, van den Eertwegh AJ, et al. Clinical experience with α-galactosylceramide (KRN7000) in patients with advanced cancer and chronic hepatitis B/C infection. *Clin Immunol* (2010) 140:130–41. doi:10.1016/j.clim.2010.11.010
34. Motohashi S, Okamoto Y, Yoshino I, Nakayama T. Anti-tumor immune responses induced by iNKT cell-based immunotherapy for lung cancer and head and neck cancer. *Clin Immunol* (2011) 140:167–76. doi:10.1016/j.clim.2011.01.009
35. Dhodapkar MV, Richter J. Harnessing natural killer T (NKT) cells in human myeloma: progress and challenges. *Clin Immunol* (2011) 140:160–6. doi:10.1016/j.clim.2010.12.010
36. Liu D, Song L, Brawley VS, Robison N, Wei J, Gao X, et al. Medulloblastoma expresses CD1d and can be targeted for immunotherapy with NKT cells. *Clin Immunol* (2013) 149(1):55–64. doi:10.1016/j.clim.2013.06.005
37. Schneiders FL, de Bruin RC, van den Eertwegh AJ, Scheper RJ, Leemans CR, Brakenhoff RH, et al. Circulating invariant natural killer T-cell numbers predict outcome in head and neck squamous cell carcinoma: updated analysis with 10-year follow-up. *J Clin Oncol* (2012) 30(5):567–70. doi:10.1200/JCO.2011.38.8819
38. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* (1997) 278:1623–6. doi:10.1126/science.278.5343.1623
39. Bellone M, Ceccon M, Grioni M, Jachetti E, Calcinotto A, Napolitano A, et al. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. *PLoS One* (2010) 5(1):e8646. doi:10.1371/journal.pone.0008646
40. Bedel R, Matsuda JL, Brigl M, White J, Kappler J, Marrack P, et al. Lower TCR repertoire diversity in Traj18-deficient mice. *Nat Immunol* (2012) 13(8):705–6. doi:10.1038/ni.2347
41. Dashtsoodol N, Shigeura T, Ozawa R, Harada M, Kojo S, Watanabe T, et al. Generation of novel Traj18-deficient mice lacking Vα14 natural killer T cells with an undisturbed T cell receptor α-chain repertoire. *PLoS One* (2016) 11(4):e0153347. doi:10.1371/journal.pone.0153347
42. Zhang J, Bedel R, Krovi SH, Tuttle KD, Zhang B, Gross J, et al. Mutation of the Traj18 gene segment using TALENs to generate natural killer T cell deficient mice. *Sci Rep* (2016) 6:27375. doi:10.1038/srep27375
43. Nowak M, Arredouani MS, Tun-Kyi A, Schmidt-Wolf I, Sanda MG, Balk SP, et al. Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with alpha- galactosylceramide. *PLoS One* (2010) 5(6):e11311. doi:10.1371/journal.pone.0011311
44. Chaudhry MS, Karadimitris A. Role and regulation of CD1d in normal and pathological B cells. *J Immunol* (2014) 193(10):4761–8. doi:10.4049/jimmunol.1401805
45. Liu D, Song L, Wei J, Courtney AN, Gao X, Marinova E, et al. IL-15 protects NKT cells from inhibition by tumor-associated macrophages and enhances antimetastatic activity. *J Clin Invest* (2012) 122(6):2221–33. doi:10.1172/JCI59535
46. Kawano T, Nakayama T, Kamada N, Kaneko Y, Harada M, Ogura N, et al. Antitumor cytotoxicity mediated by ligand-activated human V alpha24 NKT cells. *Cancer Res* (1999) 59:5102–5.
47. Nakagawa R, Motoki K, Ueno H, Iijima R, Nakamura H, Kobayashi E, et al. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an α-galactosylceramide, KRN7000. *Cancer Res* (1998) 58:1202–7.
48. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* (1999) 163:2387–91.
49. Nishimura T, Kitamura H, Iwakabe K, Yahata T, Ohta A, Sato M, et al. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol* (2000) 12:987–94. doi:10.1093/intimm/12.7.987
50. Shin T, Nakayama T, Akutsu Y, Motohashi S, Shibata Y, Harada M, et al. Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Vα14 NKT cells. *Int J Cancer* (2001) 91:523–8. doi:10.1002/1097-0215(20010215)91:4<523::AID-IJC1087>3.0.CO;2-L
51. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, et al. Sequential production of interferon-γ by NK1.1+ T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. *Blood* (2002) 99:1259–66. doi:10.1182/blood.V99.4.1259
52. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* (2002) 8:3702–9.
53. Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, et al. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* (2004) 103:383–9. doi:10.1182/blood-2003-04-1155
54. Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, et al. A phase 1 study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* (2005) 11:1910–7. doi:10.1158/1078-0432.CCR-04-1453
55. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosylceramide loaded mature dendritic cells in cancer patients. *J Exp Med* (2005) 201:1503–17. doi:10.1084/jem.20042592
56. Uchida T, Horiguchi S, Tanaka Y, Yamamoto H, Kunii N, Motohashi S, et al. Phase I study of alpha-galactosylceramide-pulsed antigen presenting cells administration to the nasal submucosa in unresectable or recurrent head and neck cancer. *Cancer Immunol Immunother* (2008) 57(3):337–45. doi:10.1007/s00262-007-0373-5
57. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* (2009) 182(4):2492–501. doi:10.4049/jimmunol.0800126

58. Nagato K, Motohashi S, Ishibashi F, Okita K, Yamasaki K, Moriya Y, et al. Accumulation of activated invariant natural killer T cells in the tumor microenvironment after α-galactosylceramide-pulsed antigen presenting cells. *J Clin Immunol* (2012) 32(5):1071–81. doi:10.1007/s10875-012-9697-9
59. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* (2006) 12:6079–86. doi:10.1158/1078-0432.CCR-06-0114
60. Kunii N, Horiguchi S, Motohashi S, Yamamoto H, Ueno N, Yamamoto S, et al. Combination therapy of in vitro-expanded natural killer T cells and alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* (2009) 100(6):1092–8. doi:10.1111/j.1349-7006.2009.01135.x
61. Yamasaki K, Horiguchi S, Kurosaki M, Kunii N, Nagato K, Hanaoka H, et al. Induction of NKT cell-specific immune responses in cancer tissues after NKT cell-targeted adoptive immunotherapy. *Clin Immunol* (2011) 138(3):255–65. doi:10.1016/j.clim.2010.11.014
62. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, et al. Adoptive transfer of invariant NKT cells as immunotherapy for advanced melanoma: a phase I clinical trial. *Clin Cancer Res* (2017) 23(14):3510–9. doi:10.1158/1078-0432.CCR-16-0600
63. Exley MA, Hou R, Shaulov A, Tonti E, Dellabona P, Casorati G, et al. Selective activation, expansion, and monitoring of human iNKT cells with a mAb specific for TCR alpha-chain CDR3 loop. *Eur J Immunol* (2008) 20:1756–1756. doi:10.1002/eji.200737389
64. Exley MA, Wilson SB, Balk SP. Isolation and functional use of human NKT cells. *Curr Protoc Immunol* (2017) 119:1–14. doi:10.1002/cpim.33
65. Neelapu SS, Tummala S, Kebriaei P, Wierda W, Gutierrez C, Locke FL, et al. Chimeric antigen receptor T-cell therapy – assessment and management of toxicities. *Nat Rev Clin Oncol* (2018) 15(1):47–62. doi:10.1038/nrclinonc.2017.148
66. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med* (2015) 21(8):914–21. doi:10.1038/nm.3910
67. Bedoya F, Frigault MJ, Maus MV. The flipside of the power of engineered T cells: observed and potential toxicities of genetically modified T cells as therapy. *Mol Ther* (2017) 25(2):314–20. doi:10.1016/j.ymthe.2016.11.011
68. Pillai AB, George TI, Dutt S, Teo P, Strober S. Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol* (2007) 178(10):6242–51. doi:10.4049/jimmunol.178.10.6242
69. Dellabona P, Casorati G, de Lalla C, Montagna D, Locatelli F. On the use of donor-derived iNKT cells for adoptive immunotherapy to prevent leukemia recurrence in pediatric recipients of HLA haploidentical HSCT for hematological malignancies. *Clin Immunol* (2011) 140(2):152–9. doi:10.1016/j.clim.2010.11.015
70. Slauenwhite D, Johnston B. Regulation of NKT cell localization in homeostasis and infection. *Front Immunol* (2015) 6:255. doi:10.3389/fimmu.2015.00255
71. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* (2008) 29(3):391–403. doi:10.1016/j.immuni.2008.07.011
72. Song L, Ara T, Wu HW, Woo CW, Reynolds CP, Seeger RC, et al. Oncogene MYCN regulates localization of NKT cells to the site of disease in neuroblastoma. *J Clin Invest* (2007) 117(9):2702–12. doi:10.1172/JCI30751
73. Heczey A, Liu D, Tian G, Courtney AN, Wei J, Marinova E, et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* (2014) 124(18):2824–33. doi:10.1182/blood-2013-11-541235
74. Tian G, Courtney AN, Jena B, Heczey A, Liu D, Marinova E, et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. *J Clin Invest* (2016) 126(6):2341–55. doi:10.1172/JCI83476
75. Rotolo A, Caputo V, Karadimitris A. The prospects and promise of chimeric antigen receptor immunotherapy in multiple myeloma. *Br J Haematol* (2016) 173(3):350–64. doi:10.1111/bjh.13976
76. Sommermeyer D, Hudecek M, Kosasih PL, Gogishvili T, Maloney DG, Turtle CJ, et al. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. *Leukemia* (2016) 30(2):492–500. doi:10.1038/leu.2015.247
77. Jiang ZM, Luo W, Wen Q, Liu SD, Hao PP, Zhou CY, et al. Development of genetically engineered iNKT cells expressing TCRs specific for the *M. tuberculosis* 38-kDa antigen. *J Transl Med* (2015) 13:141. doi:10.1186/s12967-015-0502-4
78. Rothchild AC, Jayaraman P, Nunes-Alves C, Behar SM. iNKT cell production of GM-CSF controls *Mycobacterium tuberculosis*. *PLoS Pathog* (2014) 10(1):e1003805. doi:10.1371/journal.ppat.1003805
79. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* (2009) 182(5):2816–26. doi:10.4049/jimmunol.0803648
80. Kojo S, Elly C, Harada Y, Langdon WY, Kronenberg M, Liu YC. Mechanisms of NKT cell anergy induction involve Cbl-b-promoted monoubiquitination of CARMA1. *Proc Natl Acad Sci U S A* (2009) 106(42):17847–51. doi:10.1073/pnas.0904078106
81. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124(9):3725–40. doi:10.1172/JCI72308
82. van den Heuvel MJ, Garg N, Van Kaer L, Haeryfar SM. NKT cell costimulation: experimental progress and therapeutic promise. *Trends Mol Med* (2011) 17(2):65–77. doi:10.1016/j.molmed.2010.10.007
83. Shaulov A, Yue S, Wang R, Joyce RM, Balk SP, Kim HT, et al. Peripheral blood progenitor cell product contains Th1-biased noninvariant CD1d-reactive natural killer T cells: implications for posttransplant survival. *Exp Hematol* (2008) 36(4):464–72. doi:10.1016/j.exphem.2007.12.010

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α-GalCer and iNKT Cell-Based Cancer Immunotherapy: Realizing the Therapeutic Potentials

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NKT cells are CD1d-restricted innate-like T cells expressing both T cell receptor and NK cell markers. The major group of NKT cells in both human and mice is the invariant NKT (iNKT) cells and the best-known function of iNKT cells is their potent anti-tumor function in mice. Since its discovery 25 years ago, the prototype ligand of iNKT cells, α-galactosylceramide (α-GalCer) has been used in over 30 anti-tumor clinical trials with mostly suboptimal outcomes. To realize its therapeutic potential, numerous preclinical models have been developed to optimize the scheme and strategies for α-GalCer-based cancer immunotherapies. Nevertheless, since there is no standard protocol for α-GalCer delivery, we reviewed the preclinical studies with a focus on B16 melanoma model in the goal of identifying the best treatment schemes for α-GalCer treatment. We then reviewed the current progress in developing more clinically relevant mouse models for these preclinical studies, most notably the generation of new mouse models with a humanized CD1d/iNKT cell system. With ever-emerging novel iNKT cell ligands, invention of novel α-GalCer delivery strategies and significantly improved preclinical models for optimizing these new strategies, one can be hopeful that the full potential of anti-tumor potential for α-GalCer will be realized in the not too distant future.

Keywords: iNKT cell, cancer immonotherapy, preclinical modeling, humanized mice, α-GalCer

Natural Killer T (NKT) cells are CD1d-restricted innate-like T cells expressing both T cell receptor and NK cell markers (1). Invariant NKT (iNKT) cells are the major group of NKT cells in both human and mice. They express the invariant Vα24-Jα18 chains and Vα14-Jα18 TCRα chains in human and mice, respectively (2–4). Since their discovery in the early 1990s, the best-studied function of iNKT cells has been their anti-tumor function. Activated iNKT cells rapidly secrete both Th1 and Th2 cytokines and activate NK and other immune cells to stimulate anti-tumor immune responses (5, 6). The prototypical iNKT cell ligand, α-galactosylceramide (α-GalCer), is a sphingolipid that was first isolated from the marine sponge *Agelas mauritianas* in 1994 by chloroform extraction and HPLC purification techniques (7). Mice injected with free α-GalCer demonstrated potent anti-tumor activity against metastatic B16 melanoma cells (7, 8). Furthermore, α-GalCer demonstrated synergistic anti-tumor effects when co-administered with another chemotherapy agent, adriamycin (8), suggesting α-GalCer has a different target other than the tumor cells themselves. Interestingly, early researchers believed that α-GalCer was a non-specific immunostimulatory agent (8). However, by 1998, studies using knockout mice had concluded that α-GalCer's anti-tumor properties were mediated by CD1d-restricted iNKT cells (9, 10). Several excellent reviews have been published recently on the anti-tumor function of α-GalCer and iNKT cells (5, 6, 11–15). Here we aim to review commonly used preclinical mouse

models for α-GalCer and iNKT cell-based cancer immunotherapy to compare and contrast the different approaches in mobilizing iNKT cells for anti-tumor therapies. We specifically focus on the syngeneic mouse B16 melanoma model, a well-established model for human melanoma (16). For a comprehensive review of preclinical modeling of α-GalCer-based cancer therapy targeting diverse tumor types, readers are referred to a recent review by Nair and Dhodapkar (6).

HOW EFFECTIVE IS α-GALCER IN ANTI-TUMOR IMMUNITY IN EXPERIMENTAL MICE?

Despite extensive literature on the anti-tumor function of α-GalCer and iNKT cells, no standard procedure has been established in delivering the iNKT cell ligands. Procedures vary widely between different research groups. The glycolipid ligands can be administered prior to, simultaneously or after the inoculation of tumor cells at different time points, via intravenous, intraperitoneal or subcutaneous routes, and in free or vehicled forms. Clearly, the scheme, time points, routes and forms for glycolipid deliveries all have a significant impact on the ensuing immune response and therefore the interpretation of the results.

In initial reports on the anti-tumor function of α-GalCer [KRN7000, a close analog of original AGL9b (8)], the glycolipid was injected post B16 cell inoculation. For example, Morita et al. (8) reported that α-GalCer injected 1, 5, and 9 days after subcutaneous inoculation of B16 cells suppressed the tumor volume growth by about 50%. Glycolipid treatment before tumor inoculation represents a prophylactic treatment and may be particularly applicable for future tumor vaccination with specific neoantigens, while the post-tumor inoculation studies are more clinically relevant for anti-cancer therapies.

α-GalCer Treatment Prior to B16 Melanoma Inoculation

While several reports have demonstrated that pre-treatment with α-GalCer can lead to an anti-tumor response in mice, one study found that injecting α-GalCer immediately before tumor inoculation does not show an anti-tumor effect (17) (**Table 1**). However, pre-administration of a single-dose α-GalCer 2 days prior to B16 cell inoculation leads to powerful anti-metastatic effect (20). This has been confirmed by ours and other studies (14, 21–23). It is unclear how long the anti-tumor response can last, but it is unlikely to last too long, for example 30 days, as the NKT cells will become anergic by then (17, 25). More kinetic experiments are warranted to determine the duration of this anti-tumor response before the anergy induction because the information will be important for future repetitive administration of α-GalCer and its analogs in clinics.

α-GalCer Treatment Post B16 Melanoma Inoculation

Several reports showed that one single injection of α-GalCer either simultaneously or shortly after the B16 melanoma inoculation does not inhibit tumor growth (17–19, 24). Similarly, a single α-GalCer treatment 4 days (19), or seven days after B16 cell inoculation (24) had little beneficial effect on suppressing tumor growth or mouse survival. Therefore, most reports investigating anti-B16 function of α-GalCer have utilized multiple dosages of α-GalCer, typically in a three-dose scheme at days 0, 4, and 8 post B16 inoculation (25–28). In one study, repetitive administration of α-GalCer was initiated at different time points post B16 inoculation (29). α-GalCer was administrated every other day until the end of the experiment on day 14. The free α-GalCer glycolipid demonstrated anti-B16 function as late as 3 days after tumor inoculation, but not beyond 5 days after (29). This may be due to immune-suppression by the established B16 tumors as reported (30). On the other hand, DC-vehicled α-GalCer clearly can extend this treatment window to at least seven days after B16 inoculation (29), suggesting that the vehicled α-GalCer is more efficient in boosting immune response and/or overcoming tumor-led immune suppression.

APPROACHES TO IMPROVE THE ANTI-TUMOR EFFICACY OF α-GALCER

Many possible mechanisms have been proposed for the suboptimal efficacies of α-GalCer in anti-tumor clinical trials (5, 6, 11), such as the induction of anergy, the secretion of both Th1 and Th2 cytokines by iNKT cells and immune suppression by the tumors in the microenvironment (30). Many novel α-GalCer analogs have been designed to increase the Th1/Th2 ratio and enhance the anti-tumor immunity (22, 37, 38). While we focus on the anti-tumor function of the prototypic α-GalCer, the chemistry and anti-tumor efficacy and mechanism for these novel α-GalCer analogs have been elegantly reviewed elsewhere (38).

Approaches to Suppress the Induction of iNKT Cell Anergy

Pioneering work from Fujii and Van Kaer groups demonstrated the induction of long-lasting anergy post α-GalCer activation of iNKT cells (17, 25). The anergy induction not only makes further activation of iNKT cells inefficient, anergic iNKT cells can actually exacerbate tumorigenesis upon further stimulation by glycolipids (25).

The arguably best approach by far to overcome iNKT cell anergy is to load the α-GalCer to dendritic cells (17). Although the absolute amounts of Th1/Th2 cytokines secreted post DC-loaded α-GalCer were not as high as that of free α-GalCer and the cytokines were secreted at a delayed kinetics, the DC-vehicled α-GalCer stimulated higher numbers of cytokine-secreting splenocytes. Importantly, DC-loaded α-GalCer does not lead to iNKT cell anergy (17). More importantly, the DC-vehicled α-GalCer showed more potent anti-tumor activity than free α-GalCer in the B16 melanoma model (17). Interestingly, in this study, both the free α-GalCer and DC-loaded α-GalCer were administered simultaneously with the B16 melanoma cells. While co-injected α-GalCer does not induce immediate anti-tumor activity as discussed

TABLE 1 | Preclinical studies of α-GalCer and iNKT cell-mediated anti-tumor therapies.

Treatment agent	Treatment regime	Administration	α-GalCer amount/Cell number per mouse	Cancer type/mouse model	Outcome	References
Injection of free α-GalCer						
α-GalCer	Once, immediately before B16 inoculation	Intravenous	2 μg	B16 melanoma	Very little anti-tumor effect	(17)
α-GalCer	Once, shortly after B16 inoculation	Intravenous	100 ng or 500 ng	B16 melanoma	Very little anti-tumor effect	(18, 19)
α-GalCer	Once, simultaneously with B16 inoculation	Intravenous	2 μg	B16 melanoma	Very little anti-tumor effect	(17)
α-GalCer	Once, 2 days prior to B16 inoculation	Intravenous or intraperitoneal	2 μg or 4 nmol	B16 melanoma	Potent anti-tumor effect	(20–23)
α-GalCer	Once, 7 days post B16 inoculation	Intraperitoneal	2 μg	B16 melanoma	Very little anti-tumor effect	(24)
α-GalCer	Multiple, days 0, 4, 8 post B16 inoculation	Intravenous or intraperitoneal	2 or 5 μg	B16 melanoma	Potent anti-tumor effect	(25–28)
α-GalCer	Multiple, from day 3 post B16 inoculation, every other day	Intravenous	2 μg	B16 melanoma	Effective anti-tumor response	(29)
α-GalCer	Multiple, days 1, 5, 9 post B16 inoculation	Intraperitoneal	2 μg	B16 melanoma	Tumor growth inhibition	(9)
α-GalCer	Once and together with anti-PD-1/PD-L1/L2 antibodies	Intraperitoneal	2 μg	B16 melanoma	Enhanced anti-tumor effect, suppressing iNKT cell anergy	(28)
α-GalCer	Once, 7 days post B16 inoculation	Intraperitoneal	2 μg	B16 melanoma/iNOS-KO	Tumor growth inhibition	(24)
α-GalCer	Multiple, every 4 days post B16 inoculation plus ATRA treatment	Intraperitoneal	2 μg	B16 melanoma	Enhanced anti-tumor effect, reducing $CD11b^{+}$ $Gr\text{-}1^{+}$ cells	(30)
Vehicled α-GalCer						
DC-loaded α-GalCer	Once, simultaneously with B16 inoculation	Intravenous	$6 \times 10e^{5}$	B16 melanoma	Enhanced anti-tumor effect, no induction of iNKT cell anergy	(17)
DC-loaded α-GalCer	Multiple, from day 7 post B16 inoculation, every other day	Intravenous	$3 \times 10e^{6}$	B16 melanoma	Extended therapeutic window with DC-loaded α-GalCer	(29)
DC-loaded α-GalCer	Multiple, days−7, 14, 21 from tumor cell inoculation	Subcutaneous	$6 \times 10e^{5}$	PancO2 pancreatic cancer	Suppressing tumor growth	(31)
DC-loaded α-GalCer	Once, 2 days prior to B16 inoculation	Intravenous	$1–3 \times 10e^{6}$	B16 melanoma/hCD1d-KI	Inhibition of B16 metastasis at lower iNKT cell abundance	(21)
B16 loaded α-GalCer	Once, 2 to 4 weeks prior to B16 inoculation	Intravenous	$5 \times 10e^{5}$	B16 melanoma	Long-term inhibition of lung metastasis	(32)
B16 loaded α-GalCer	Once, 3 hours post B16 inoculation	Intravenous	$3 \times 10e^{5}$	B16 melanoma	Prevention of lung metastasis	(18)

(Continued)

TABLE 1 | Continued

Treatment agent	Treatment regime	Administration	α-GalCer amount/Cell number per mouse	Cancer type/mouse model	Outcome	References
DC-derived exosomes loaded with α-GalCer/OVA	Once or twice, 4 or 4 and 11 days post B16 inoculation	Intravenous	40 μg exosomes	B16.OVA melanoma	Effective suppression of tumor growth, no anergy induction	(19)
Cationic liposomes loaded with α-GalCer	Once, 6 days post B16 inoculation	Intravenous	200 ng liposomes	B16.OVA melanoma	Prolonged survival time	(33)
PLGA nanoparticle encapsulated with α-GalCer/Trp2/gp100	Multiple, days 14 and 7prior to B16 inoculation	Intravenous	5 ng nanoparticle	B16 melanoma	Slowed tumor growth	(34)
PLGA nanoparticle encapsulated with α-GalCer/Trp2/gp100	Multiple, days 5 and 12 post B16 inoculation	Intravenous	5 ng nanoparticle	B16 melanoma	Slowed tumor growth	(34)
α-GalCer loaded to soluble CD1d fused to anti-HER2-svFv	Multiple, every 3-4 days from day 2 post B16 inoculation	Intravenous	40 μg fused sCD1d	B16.HER2 melanoma	Potent anti-tumor effect	(35)
DC-loaded with α-GalCer and B16 cells plus pre-treatment with anti-CD25 Ab	Once, day 7 and anti-CD25 treatment on day 9 prior to B16 inoculation	Intravenous or intraperitoneal	$5 \times 10e^5$	B16.OVA melanoma	Slowed tumor growth, prolonged survival, depleting Tregs	(36)

above, DC-vehicled α-GalCer can immediately induce anti-tumor activity. Free α-GalCer takes 2 days to induce an anti-tumor response in mice (20), suggesting that these two approaches boosted different downstream effectors. It is particularly important to note that NK cells are only responsible for approximately half of the anti-tumor effect for DC-vehicled α-GalCer (17), while they account for almost all of free α-GalCer mediated anti-tumor function (26, 39, 40). Given the fact that DC-loaded α-GalCer has been widely used in anti-tumor clinical trials (31, 41–43), it is important to further delineate the exact anti-tumor mechanism of DC-vehicled α-GalCer.

The second reported approach to suppress NKT cell anergy is to use exosomes loaded with α-GalCer (19). While in early clinical trials, exosomes loaded with tumor antigens have mostly been tolerated and had little immunostimulatory effects (44, 45), exosomes loaded with α-GalCer as an immune-stimulatory adjuvant led to an effective anti-tumor responses in mice (19). Using a subcutaneous B16 melanoma model, Gehrmann et al. (19) demonstrated that dendritic cells-derived exosomes loaded with α-GalCer administered 4 days after tumor inoculation could effectively suppress tumor growth and extend mouse survival. More importantly, a second injection of loaded exosomes 1 week after the first one can further inhibit tumor growth, suggesting that the first injection with α-GalCer-loaded exosomes did not induce anergy.

Rejuvenating anergic NKT cells at molecular levels is the third approach for suppressing NKT cell anergy. Expression of inhibitory co-stimulatory molecules including PD-1 and PD-L1/L2 is partially responsible for the anergy of NKT cells (28). Three injections of anti-PD-L1/L2 or anti-PD-1 antibodies post α-GalCer activation of iNKT cells could maintain the iNKT cells response for at least 30 days after the α-GalCer treatment (28). This allowed the recovery of iNKT cells to a responsive state and repeated activation of iNKT cells with α-GalCer extended the anti-B16 metastatic function (28). Considering the recent success of anti-PD-L1/2 and anti-PD-1 antibodies in rejuvenating tumor-specific T cells in clinics, future combination treatment with these antibodies and α-GalCer may synergize their anti-tumor functions.

IL-2 has shown anti-anergy function to iNKT cells. In light of its function in breaking anergy of conventional T cells (46), Parekh et al. (25) demonstrated that IL-2, but not IL-12, IFN-γ or IL-4 could re-stimulate the anergic iNKT cells to proliferate both *in vitro* and *in vivo*.

Additional Approaches to Enhance the Anti-tumor Efficacy by α-GalCer

Vaccination With Tumor Cells or Tumor Antigens Complexed With α-GalCer

One major innovation in the field pioneered by the Fujii group is to load α-GalCer to the tumor cells for immunization (18, 32). Even for low immunogenicity tumor cells including B16 melanoma cells, one single vaccination with α-GalCer-loaded tumor cells could stimulate potent tumor-specific $CD8^+$ T cell responses. Memory CD4 and CD8 T cells could protect the immunized mice from tumor re-challenge for as long as 6–12 months (32). It was also demonstrated that CD1d expression significantly improved the efficacy of iNKT cell-based therapies, presumably due to increased efficiency of direct killing by iNKT cells. Therefore, CD1d expression on tumor cells can be a positive

biomarker for future iNKT cell therapies in clinics, as suggested by another report (47). Importantly, the tumor protection from vaccination in this study is tumor-specific. The mice were only immune to the specific tumor that was used for vaccination (32). On the other hand, α-GalCer-loaded dendritic cells induce short-term tumor resistance against different types of tumors, including melanoma (29), multiple myeloma (48), pancreatic cancer (31) and B cell lymphoma (49). These studies suggested that dendritic cells loaded with α-GalCer induce mostly innate immunity-based non-specific anti-tumor responses including activated NK cells, whereas tumor cells loaded with α-GalCer induce more specific long-term adaptive immunity-based anti-tumor responses.

Several other groups have explored delivering tumor cells or specific tumor antigens with α-GalCer using vehicles such as dendritic cells (36, 50, 51), dendritic cells-derived exosomes (19), dendritic cells loaded with tumor-derived exosomes (52), PLGA nanoparticles (34), cationic liposomes (33), chemically conjugated α-GalCer-tumor peptide antigen compound vaccine (53), or α-GalCer-loaded recombinant soluble CD1d protein fused with single chain antibodies against neoantigen (35, 54). *In vivo*, the tumor antigens are either directly or cross-presented by endogenous dendritic cells to $CD8^+$ T cells while the co-delivered α-GalCer is presented to iNKT cells. As expected, all of these approaches have shown enhanced tumor antigen-specific CTL responses and increased IFN-γ secretion in these T cells. These approaches have demonstrated both prophylactic (36), or therapeutic effects (19, 34, 50–53) to challenges by vaccinated tumors. One Phase I trial has been completed using dendritic cells loaded with α-GalCer and the well-established neoantigen NY-ESO-1 (51). It is encouraging that there were increases in NKT cell proliferation, NKT cell-associated cytokine secretion and more importantly, the circulating NY-ESO-1-specific T cells in most (7 out 8) patients (51).

More Approaches to Enhance the Anti-tumor Function of α-GalCer

It has been well-established that $CD4^+CD25^+$ T_{reg} cells suppress anti-tumor immunity (55, 56). On the other hand, several reports showed that α-GalCer-activated NKT cells secret IL-2 leading to the expansion of T_{reg} cells (57, 58). Pre-administration of depleting anti-CD25 monoclonal antibody (PC61) 2 days prior to α-GalCer vaccination increased the α-GalCer-induced prophylactic anti-tumor function in a subcutaneous challenge model with B16 melanoma cells (36). However, pre-administration with the same PC61 antibody prior to α-GalCer treatment did not enhance the anti-tumor function of α-GalCer in a therapeutic tumor challenge model with a lung tumor cell line TC1 (59). Interestingly, in the Petersen report (36), α-GalCer challenge and NKT cell activation did not induce an expansion of T_{reg} cells as previously reported (57). This difference is likely due to the different routes of α-GalCer delivery. While in the previous report, delivery of free α-GalCer led to T_{reg} expansion (57), the α-GalCer delivered in dendritic cell-vehicled form in the later study did not (36). More studies are needed to delineate the interaction between iNKT cells and T_{regs} in order to manipulate T_{regs} for the benefit of iNKT cell-mediated cancer therapies.

IFN-γ is one of the major cytokine effectors after α-GalCer administration (1). The high amount of IFN-γ induces immunosuppressive factors including the iNOS enzyme, which produces nitric oxide and inhibits anti-tumor immunity (60). In iNOS-knockout mice or wild-type mice treated with an iNOS inhibitor, L-NAME, the B16 metastasis was more efficiently suppressed by a suboptimal treatment of α-GalCer (one single treatment seven days after B16 melanoma inoculation) (24). Another study demonstrated that lung metastasis of B16 melanoma was also significantly inhibited by a suboptimal treatment of α-GalCer when the mice were simultaneously treated with all-trans-retinoic acid (ATRA) (30). ATRA, a derivative of vitamin A, can induce the differentiation of $CD11b^+Gr\text{-}1^+$ immature myeloid cells and reduce this major nitric oxide-producing population (30).

There are more innovative approaches of enhancing anti-tumor activity of α-GalCer, such as adoptive iNKT cell transfer (61, 62), using artificial antigen-presenting cells to expand iNKT cells *in vitro* (63), co-administration of NK cell activator, IL-18 (64). Altogether, all the reported approaches could increase α-GalCer function. Clearly more research is required to realize their therapeutic potential and achieve the optimal therapeutic efficacy by combining these novel approaches.

BUILDING BETTER MOUSE MODELS FOR DEVELOPING α-GALCER-BASED ANTI-TUMOR THERAPIES

The sharp difference between mouse and human immune systems, including the difference in the CD1d/iNKT cell system, urgently demand better mouse models with improved predictive powers for clinics. In addition to the significantly lower affinities of the human CD1d and iNKT TCR to α-GalCer compared to that of mice (65, 66), human iNKT cells are present at a much lower abundance with very different subset compositions (21, 67, 68). The journey from the original discovery of α-GalCer's anti-tumor function in mice to current clinical trials also suggests that preclinical modeling with more relevant mouse strains is warranted before translating α-GalCer and its analogs into clinics.

One attractive direction to improve the preclinical modeling of α-GalCer-based immunotherapies is to develop mouse models with a human-like CD1d/iNKT TCR system. The first mouse model with a humanized CD1d/NKT cell system is from the Wang group in which human CD1d is expressed under a mouse MHC class I (K^b) promoter (69). Human CD1d is highly expressed in all nucleated cells as a MHC class I expression pattern. It is not clear how NKT cells are developed in this strain. However, it was clear that the exogenous human CD1d can function as a strong transplantation antigen (69). The second mouse model generated by the Casorati group expressed human CD1d using Lck or CD11c promoters to direct specific human CD1d expression in thymocytes or dendritic cells, respectively (70). By breeding to CD1d-knockout mice,

the authors demonstrated that thymocyte-specific expression of human CD1d alone is sufficient to support iNKT cell development (70). However, because of no human CD1d expression on dendritic cells in these pLck-hCD1dTg mice, *in vivo* α-GalCer treatment is not feasible to test the α-GalCer-based cancer immunotherapy. For a human-like iNKT cell population, the Casorati group generated a pre-arranged human invariant Vα24-Jα18 TCRα chain and expressed it as a transgene under the human CD2 promoter (71). In the Jα18-knockout background, which eliminates the expression of mouse iNKT TCRα chain (Vα14), the human Vα24-Jα18 TCRα chain could support the development of human-like Vα24 iNKT cells. The Gumperz group has utilized the humanized SCID mice to generate mice with a humanized CD1d/iNKT cell system. Immune-deficient mice were engrafted with human fetal thymus, liver and $CD34^+$ hematopoietic cells. Four surface CD1 gene family members, CD1a, CD1b, CD1c, and CD1d were all expressed *in vivo*. Furthermore, T cell responses have been detected for all the CD1 family members. In addition, α-GalCer can stimulate IFN-γ secretion in the mouse serum, suggesting the NKT cells are developed and functional *in vivo* (72). Nevertheless, more investigation on the immune cell development and adaptive immune responses may be needed before this engrafted system can be widely used for modeling NKT cell-based cancer immunotherapies.

Our group has been working on yet another approach to humanize the CD1d/iNKT cell system. By homologous recombination, we generated a human CD1d knock-in mouse, in which human CD1d is under the endogenous mouse CD1d promoter (21). Consistent with the previous report (70), thymic expression of human CD1d supports NKT cell development. Importantly, this new human CD1d-knock in mouse possesses an iNKT cell population with human-like abundance and similar subset composition in terms of co-receptor expression pattern (21), making this strain a particularly useful tool for modeling *in vivo* human iNKT cell responses to α-GalCer or its analogs. By expressing the pre-arranged human Vα24/Jα18 TCRα chain (23), this further improved mouse strain can be particularly instrumental to test and optimize the glycolipid ligands for anti-tumor therapies. However, since the human Vα24/Jα18 TCRα is a transgene, the current mouse strain is not optimal for investigating the antigen-specific T cell responses during anti-tumor immunotherapies. Nevertheless, since the NK cells and other innate immune cells are not affected by the transgene, this strain can still be used to investigate the innate immunity-mediated anti-tumor function of α-GalCer. To further improve this model, future "knock-in" of human Vα24/Jα18 and Vβ11 genomic regions will be necessary. The continuous improvement of current gene-editing techniques, including CRISPR-Cas9 (73), may make the knock-in more feasible. For preclinical modeling of α-GalCer-mediated anti-tumor therapy, we have demonstrated that prophylactic treatment with α-GalCer in the two CD1d-humanized mouse strains can suppress B16 metastasis (21, 23). Nevertheless, it will be most interesting to investigate whether α-GalCer can suppress B16 melanoma in these humanized mice under therapeutic settings, and if not, how the treatment regimes can be improved for an optimal anti-tumor effect.

In summary, joint efforts from researchers in chemistry, pharmaceutics and immunology fields will bring about more potent α-GalCer analogs, optimized delivery and treatment schemes and much-improved preclinical models. We envision that the α-GalCer-based cancer immunotherapy will be reaching its full potential in clinics in the near future.

AUTHOR CONTRIBUTIONS

WY, YZ, RS, and SC participated in conceptualization and drafting of the article as well as critical revision of the article for important intellectual content. All authors participated in writing and revision of the manuscript and gave final approval of the submitted publication.

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REFERENCES

1. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol.* (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711
2. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8^- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med.* (1993) 178:1–16.
3. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific $CD4^+$ and CD4-8^- T cells in mice and humans. *J Exp Med.* (1994) 180:1097–106.
4. Porcelli S, Gerdes D, Fertig AM, Balk SP. Human T cells expressing an invariant V alpha 24-J alpha Q TCR alpha are $CD4^-$ and heterogeneous with respect to TCR beta expression. *Hum Immunol.* (1996) 48:63–7.
5. Bedard M, Salio M, Cerundolo V. Harnessing the power of invariant natural killer T cells in cancer immunotherapy. *Front Immunol.* (2017) 8:1829. doi: 10.3389/fimmu.2017.01829
6. Nair S, Dhodapkar MV. Natural killer T cells in cancer immunotherapy. *Front Immunol.* (2017) 8:1178. doi: 10.3389/fimmu.2017.01178
7. Natori T, Morita M, Akimoto K, Koezuka Y. Agelasphins, novel antitumor and immunostimulatory cerebrosides from the marine sponge agelas-mauritianus. *Tetrahedron.* (1994) 50:2771–84. doi: 10.1016/S0040-4020(01)86991-X
8. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, et al. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem.* (1995) 38:2176–87.
9. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, et al. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci USA.* (1998) 95:5690–3.

10. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, et al. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med.* (1998) 188:1521–8.
11. Wolf BJ, Choi JE, Exley MA. Novel approaches to exploiting invariant NKT cells in cancer immunotherapy. *Front Immunol.* (2018) 9:384. doi: 10.3389/fimmu.2018.00384
12. Teyton L. New directions for natural killer T cells in the immunotherapy of cancer. *Front Immunol.* (2017) 8:1480. doi: 10.3389/fimmu.2017.01480
13. Shissler SC, Lee MS, Webb TJ. Mixed signals: co-stimulation in invariant natural killer T cell-mediated cancer immunotherapy. *Front Immunol.* (2017) 8:1447. doi: 10.3389/fimmu.2017.01447
14. King LA, Lameris R, de Gruijl TD, van der Vliet HJ. CD1d-invariant natural killer T cell-based cancer immunotherapy: α-galactosylceramide and beyond. *Front Immunol.* (2018) 9:1519. doi: 10.3389/fimmu.2018.01519
15. Fujii S, Shimizu K, Okamoto Y, Kunii N, Nakayama T, Motohashi S, et al. NKT cells as an ideal anti-tumor immunotherapeutic. *Front Immunol.* (2013) 4:409. doi: 10.3389/fimmu.2013.00409
16. Overwijk WW, Restifo NP. B16 as a mouse model for human melanoma. *Curr Protoc Immunol.* (2001) Chapter 20:Unit 20.21. doi: 10.1002/0471142735.im2001s39
17. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol.* (2002) 3:867–74. doi: 10.1038/ni827
18. Shimizu K, Goto A, Fukui M, Taniguchi M, Fujii S. Tumor cells loaded with alpha-galactosylceramide induce innate NKT and NK cell-dependent resistance to tumor implantation in mice. *J Immunol.* (2007) 178:2853–61. doi: 10.4049/jimmunol.178.5.2853
19. Gehrmann U, Hiltbrunner S, Georgoudaki AM, Karlsson MC, Näslund TI, Gabrielsson S. Synergistic induction of adaptive antitumor immunity by codelivery of antigen with alpha-galactosylceramide on exosomes. *Cancer Res.* (2013) 73:3865–76. doi: 10.1158/0008-5472.can-12-3918
20. Schmieg J, Yang G, Franck RW, Tsuji M. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med.* (2003) 198:1631–41. doi: 10.1084/jem.20031192 jem.20031192
21. Wen X, Rao P, Carreño LJ, Kim S, Lawrenczyk A, Porcelli SA, et al. Human CD1d knock-in mouse model demonstrates potent antitumor potential of human CD1d-restricted invariant natural killer T cells. *Proc Natl Acad Sci USA.* (2013) 110:2963–8. doi: 10.1073/pnas.1300200110
22. Chennamadhavuni D, Saavedra-Avila NA, Carreño LJ, Guberman-Pfeffer MJ, Arora P, Yongqing T, et al. Dual modifications of alpha-galactosylceramide synergize to promote activation of human invariant natural killer T cells and stimulate anti-tumor immunity. *Cell Chem Biol.* (2018) 25:571–84.e578. doi: 10.1016/j.chembiol.2018.02.009
23. Wen X, Kim S, Xiong R, Li M, Lawrenczyk A, Huang X, et al. A subset of CD8alphabeta$^+$ invariant NKT cells in a humanized mouse model. *J Immunol.* (2015) 195:1459–69. doi: 10.4049/jimmunol.1500574
24. Ito H, Ando T, Seishima M. Inhibition of iNOS activity enhances the anti-tumor effects of alpha-galactosylceramide in established murine cancer model. *Oncotarget.* (2015) 6:41863–74. doi: 10.18632/oncotarget.6172
25. Parekh VV, Wilson MT, Olivares-Villagómez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest.* (2005) 115:2572–83. doi: 10.1172/JCI24762
26. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, et al. Sequential production of interferon-gamma by NK1.1$^+$ T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood.* (2002) 99:1259–66. doi: 10.1182/blood.v99.4.1259
27. Okajo J, Kaneko Y, Murata Y, Tomizawa T, Okuzawa C, Saito Y, et al. Regulation by Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 of alpha-galactosylceramide-induced antimetastatic activity and Th1 and Th2 responses of NKT cells. *J Immunol.* (2007) 178:6164–72. doi: 10.4049/jimmunol.178.10.6164
28. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol.* (2009) 182:2816–26. doi: 10.4049/jimmunol.0803648
29. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol.* (1999) 163:2387–91.
30. Yanagisawa K, Exley MA, Jiang X, Ohkochi N, Taniguchi M, Seino K. Hyporesponsiveness to natural killer T-cell ligand alpha-galactosylceramide in cancer-bearing state mediated by CD11b$^+$ Gr-1$^+$ cells producing nitric oxide. *Cancer Res.* (2006) 66:11441–6. doi: 10.1158/0008-5472.can-06-0944
31. Nagaraj S, Ziske C, Strehl J, Messmer D, Sauerbruch T, Schmidt-Wolf IG. Dendritic cells pulsed with alpha-galactosylceramide induce anti-tumor immunity against pancreatic cancer *in vivo*. *Int Immunol.* (2006) 18:1279–83. doi: 10.1093/intimm/dxl059
32. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S. Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *J Exp Med.* (2007) 204:2641–53. doi: 10.1084/jem.20070458
33. Neumann S, Young K, Compton B, Anderson R, Painter G, Hook S. Synthetic TRP2 long-peptide and alpha-galactosylceramide formulated into cationic liposomes elicit CD8$^+$ T-cell responses and prevent tumour progression. *Vaccine.* (2015) 33:5838–44. doi: 10.1016/j.vaccine.2015.08.083
34. Ghinnagow R, De Meester J, Cruz LJ, Aspord C, Corgnac S, Macho-Fernandez E, et al. Co-delivery of the NKT agonist α-galactosylceramide and tumor antigens to cross-priming dendritic cells breaks tolerance to self-antigens and promotes antitumor responses. *Oncoimmunology.* (2017) 6:e1339855. doi: 10.1080/2162402X.2017.1339855
35. Corgnac S, Perret R, Derré L, Zhang L, Stirnemann K, Zauderer M, et al. CD1d-antibody fusion proteins target iNKT cells to the tumor and trigger long-term therapeutic responses. *Cancer Immunol Immunother.* (2013) 62:747–60. doi: 10.1007/s00262-012-1381-7
36. Petersen TR, Sika-Paotonu D, Knight DA, Dickgreber N, Farrand KJ, Ronchese F, et al. Potent anti-tumor responses to immunization with dendritic cells loaded with tumor tissue and an NKT cell ligand. *Immunol Cell Biol.* (2010) 88:596–604. doi: 10.1038/icb.2010.9
37. Aspeslagh S, Li Y, Yu ED, Pauwels N, Trappeniers M, Girardi E, et al. Galactose-modified iNKT cell agonists stabilized by an induced fit of CD1d prevent tumour metastasis. *EMBO J.* (2011) 30:2294–305. doi: 10.1038/emboj.2011.145
38. Banchet-Cadeddu A, Hénon E, Dauchez M, Renault JH, Monneaux F, Haudrechy A. The stimulating adventure of KRN 7000. *Org Biomol Chem.* (2011) 9:3080–104. doi: 10.1039/c0ob00975j
39. Hayakawa Y, Takeda K, Yagita H, Kakuta S, Iwakura Y, Van Kaer L, et al. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol.* (2001) 31:1720–7. doi: 10.1002/1521-4141(200106)31:6<1720::aid-immu1720>3.3.co;2-l
40. Nakagawa R, Nagafune I, Tazunoki Y, Ehara H, Tomura H, Iijima R, et al. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by alpha-galactosylceramide in mice. *J Immunol.* (2001) 166:6578–84. doi: 10.4049/jimmunol.166.11.6578
41. Richter J, Neparidze N, Zhang L, Nair S, Monesmith T, Sundaram R, et al. Clinical regressions and broad immune activation following combination therapy targeting human NKT cells in myeloma. *Blood.* (2013) 121:423–30. doi: 10.1182/blood-2012-06-435503
42. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med.* (2005) 201:1503–17. doi: 10.1084/jem.20042592
43. Exley MA, Nakayama T. NKT-cell-based immunotherapies in clinical trials. *Clin Immunol.* (2011) 140:117–8. doi: 10.1016/j.clim.2011.04.015
44. Escudier B, Dorval T, Chaput N, André F, Caby MP, Novault S, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J Transl Med.* (2005) 3:10. doi: 10.1186/1479-5876-3-10
45. Morse MA, Garst J, Osada T, Khan S, Hobeika A, Clay TM, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med.* (2005) 3:9. doi: 10.1186/1479-5876-3-9

46. Schwartz RH. T cell anergy. *Annu Rev Immunol.* (2003) 21:305–34. doi: 10.1146/annurev.immunol.21.120601.141110
47. Haraguchi K, Takahashi T, Nakahara F, Matsumoto A, Kurokawa M, Ogawa S, et al. CD1d expression level in tumor cells is an important determinant for anti-tumor immunity by natural killer T cells. *Leuk Lymphoma.* (2006) 47:2218–23. doi: 10.1080/10428190600682688
48. Nur H, Rao L, Frassanito MA, De Raeve H, Ribatti D, Mfopou JK, et al. Stimulation of invariant natural killer T cells by alpha-Galactosylceramide activates the JAK-STAT pathway in endothelial cells and reduces angiogenesis in the 5T33 multiple myeloma model. *Br J Haematol.* (2014) 167:651–63. doi: 10.1111/bjh.13092
49. Escribà-Garcia L, Alvarez-Fernández C, Tellez-Gabriel M, Sierra J, Briones J. Dendritic cells combined with tumor cells and alpha-galactosylceramide induce a potent, therapeutic and NK-cell dependent antitumor immunity in B cell lymphoma. *J Transl Med.* (2017) 15:115. doi: 10.1186/s12967-017-1219-3
50. Matsuyoshi H, Hirata S, Yoshitake Y, Motomura Y, Fukuma D, Kurisaki A, et al. Therapeutic effect of alpha-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells. *Cancer Sci.* (2005) 96:889–96. doi: 10.1111/j.1349-7006.2005.00123.x
51. Gasser O, Sharples KJ, Barrow C, Williams GM, Bauer E, Wood CE, et al. A phase I vaccination study with dendritic cells loaded with NY-ESO-1 and alpha-galactosylceramide: induction of polyfunctional T cells in high-risk melanoma patients. *Cancer Immunol Immunother.* (2018) 67:285–98. doi: 10.1007/s00262-017-2085-9
52. Liu H, Chen L, Liu J, Meng H, Zhang R, Ma L, et al. Co-delivery of tumor-derived exosomes with alpha-galactosylceramide on dendritic cell-based immunotherapy for glioblastoma. *Cancer Lett.* (2017) 411:182–90. doi: 10.1016/j.canlet.2017.09.022
53. Anderson RJ, Compton BJ, Tang CW, Authier-Hall A, Hayman CM, Swinerd GW, et al. NKT cell-dependent glycolipid-peptide vaccines with potent anti-tumour activity. *Chem Sci.* (2015) 6:5120–7. doi: 10.1039/c4sc03599b
54. Stirnemann K, Romero JF, Baldi L, Robert B, Cesson V, Besra GS, et al. Sustained activation and tumor targeting of NKT cells using a CD1d-anti-HER2-scFv fusion protein induce antitumor effects in mice. *J Clin Invest.* (2008) 118:994–1005. doi: 10.1172/jci33249
55. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res.* (2017) 27:109–18. doi: 10.1038/cr.2016.151
56. Whiteside TL. FOXP3$^+$ Treg as a therapeutic target for promoting anti-tumor immunity. *Expert Opin Ther Targets.* (2018) 22:353–63. doi: 10.1080/14728222.2018.1451514
57. Liu R, La Cava A, Bai XF, Jee Y, Price M, Campagnolo DI, et al. Cooperation of invariant NKT cells and CD4$^+$CD25$^+$ T regulatory cells in the prevention of autoimmune myasthenia. *J Immunol.* (2005) 175:7898–904. doi: 10.4049/jimmunol.175.12.7898
58. Jiang S, Game DS, Davies D, Lombardi G, Lechler RI. Activated CD1d-restricted natural killer T cells secrete IL-2: innate help for CD4$^+$CD25$^+$ regulatory T cells? *Eur J Immunol.* (2005) 35:1193–200. doi: 10.1002/eji.200425899
59. Rosalia RA, Štěpánek I, Polláková V, Šímová J, Bieblová J, Indrová M, et al. Administration of anti-CD25 mAb leads to impaired alpha-galactosylceramide-mediated induction of IFN-gamma production in a murine model. *Immunobiology.* (2013) 218:851–9. doi: 10.1016/j.imbio.2012.10.012
60. Ekmekcioglu S, Grimm EA, Roszik J. Targeting iNOS to increase efficacy of immunotherapies. *Human Vaccines Immunotherapeut.* (2017) 13:1105–8. doi: 10.1080/21645515.2016.1276682
61. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, et al. Adoptive transfer of invariant NKT cells as immunotherapy for advanced melanoma: a phase I clinical trial. *Clin Cancer Res.* (2017) 23:3510–9. doi: 10.1158/1078-0432.ccr-16-0600
62. Smith DJ, Liu S, Ji S, Li B, McLaughlin J, Cheng D, et al. Genetic engineering of hematopoietic stem cells to generate invariant natural killer T cells. *Proc Natl Acad Sci USA.* (2015) 112:1523–8. doi: 10.1073/pnas.1424877112
63. Webb TJ, Bieler JG, Schneck JP, Oelke M. *Ex vivo* induction and expansion of natural killer T cells by CD1d1-Ig coated artificial antigen presenting cells. *J Immunol Methods.* (2009) 346:38–44. doi: 10.1016/j.jim.2009.05.003
64. Nishio S, Yamada N, Ohyama H, Yamanegi K, Nakasho K, Hata M, et al. Enhanced suppression of pulmonary metastasis of malignant melanoma cells by combined administration of alpha-galactosylceramide and interleukin-18. *Cancer Sci.* (2008) 99:113–20. doi: 10.1111/j.1349-7006.2007.00636.x
65. Pellicci DG, Patel O, Kjer-Nielsen L, Pang SS, Sullivan LC, Kyparissoudis K, et al. Differential recognition of CD1d-alpha-galactosyl ceramide by the V beta 8.2 and V beta 7 semi-invariant NKT T cell receptors. *Immunity.* (2009) 31:47–59. doi: 10.1016/j.immuni.2009.04.018
66. Koch M, Stronge VS, Shepherd D, Gadola SD, Mathew B, Ritter G, et al. The crystal structure of human CD1d with and without alpha-galactosylceramide. *Nat Immunol.* (2005) 6:819–26. doi: 10.1038/ni1225
67. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: natural killer T cell defects and human disease. *Nat Rev Immunol.* (2011) 11:131–42. doi: 10.1038/nri2904
68. Wen X, Xiong R, Dai Z, Kim S, Lawrenczyk A, Yuan W. Humanizing mice for the identification of novel anticancer lipids targeting iNKT cells. *Oncoimmunology.* (2013) 2:e25475. doi: 10.4161/onci.25475
69. Wang B, Chun T, Rulifson IC, Exley M, Balk SP, Wang CR. Human CD1d functions as a transplantation antigen and a restriction element in mice. *J Immunol.* (2001) 166:3829–36. doi: 10.4049/jimmunol.166.6.3829
70. Schümann J, Pittoni P, Tonti E, Macdonald HR, Dellabona P, Casorati G. Targeted expression of human CD1d in transgenic mice reveals independent roles for thymocytes and thymic APCs in positive and negative selection of Valpha14i NKT cells. *J Immunol.* (2005) 175:7303–10. doi: 10.4049/jimmunol.175.11.7303
71. Capone M, Cantarella D, Schümann J, Naidenko OV, Garavaglia C, Beermann F, et al. Human invariant V alpha 24-J alpha Q TCR supports the development of CD1d-dependent NK1.1$^+$ and NK1.1$^-$ T cells in transgenic mice. *J Immunol.* (2003) 170:2390–8. doi: 10.4049/jimmunol.170.5.2390
72. Lockridge JL, Chen X, Zhou Y, Rajesh D, Roenneburg DA, Hegde S, et al. Analysis of the CD1 antigen presenting system in humanized SCID mice. *PLoS ONE.* (2011) 6:e21701. doi: 10.1371/journal.pone.0021701
73. Conboy I, Murthy N, Etienne J, Robinson Z. Making gene editing a therapeutic reality. *F1000Res.* (2018) 7:F1000. doi: 10.12688/f1000research.16106.1

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