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Reciprocal Crosstalk between Dendritic Cells and Natural Killer T Cells: Mechanisms and Therapeutic Potential

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Natural killer T cells carrying a highly conserved, semi-invariant T cell receptor (TCR) [invariant natural killer T (iNKT) cells] are a subset of unconventional T lymphocytes that recognize glycolipids presented by CD1d molecules. Although CD1d is expressed on a variety of hematopoietic and non-hematopoietic cells, dendritic cells (DCs) are key presenters of glycolipid antigen *in vivo*. When stimulated through their TCR, iNKT cells rapidly secrete copious amounts of cytokines and induce maturation of DCs, thereby facilitating coordinated stimulation of innate and adaptive immune responses. The bidirectional crosstalk between DCs and iNKT cells determines the functional outcome of iNKT cell-targeted responses and iNKT cell agonists are used and currently being evaluated as adjuvants to enhance the efficacy of antitumor immunotherapy. This review illustrates mechanistic underpinnings of reciprocal DCs and iNKT cell interactions and discusses how those can be harnessed for cancer therapy.

Keywords: dendritic cells, natural killer T cells, CD1d, glycolipids, immunotherapy

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NATURAL KILLER T CELLS (NKT CELLS)

Natural killer T cells belong to the group of innate-like T lymphocytes and represent an important link between the innate and the adaptive immune response. They can be activated in both antigen-dependent and independent manners, secrete large amounts of cytokines upon activation, and exhibit remarkable functional plasticity with both pro-inflammatory and immunoregulatory characteristics (1, 2). Depending on their T cell receptor (TCR), CD1d-restricted NKT cells are subdivided into type I or invariant NKT (iNKT) cells, and type II or diverse NKT (dNKT) cells. Herein, we will focus on the unique iNKT cell subset, which expresses a semi-invariant TCR and we refer the reader to excellent reviews on type II NKT cells elsewhere (3, 4).

In 1986 and 1987, respectively, three key discoveries facilitated the identification of this innate-like T cell subset. Two groups independently described a Vβ8-overexpressing, double-negative thymocyte subset in mice, while a third research team cloned an invariant TCR Vα14-Jα18 rearrangement from a set of murine suppressor T cell hybridomas (5–7). It was not until 10 years later that the ligands, which these peculiar cells recognize, were identified (8).

Type I NKT cells are characterized by the expression of a semi-invariant TCR (Vα14Jα18 paired with Vβ8, Vβ7, or Vβ2 in mice and Vα24Jα18/Vβ11 in humans) (3, 9). Interestingly, the Vα14

TCR is exclusively used by iNKT cells but not by conventional T cells (10). Furthermore, iNKT cell subsets bear morphological markers on their surface that were believed to be characteristic for natural killer (NK) cells like NKG2D (11), KLRG (12), IL-12 receptor (13), or NK1.1 (CD161) (8, 14–16). However, although expression of these molecules may characterize some NKT cell subsets, other subsets do not share these NK cell markers. Therefore, the more stringent characteristic of NKT cells appears to be their CD1d restriction (1).

Unlike conventional $CD4^+$ or $CD8^+$ T cells, iNKT cells recognize antigenic glycolipids presented *via* the monomorphic MHC class I-like molecule CD1d (17, 18). iNKT cell responses have proven to be highly conserved between humans and mice. They enhance the activation of innate immune cells, such as dendritic cells (DCs) and NK cells, and shape immune responses in concert with other lymphocytes, such as B cells. Thereby, iNKT cells not only act as an amplification relay but bridge innate and adaptive immunity (19–22). The frequencies of iNKT cells among total lymphocytes differ greatly between tissues and the possibility of detecting these unconventional T cells has greatly improved by the introduction of lipid-loaded CD1d-tetramers (15, 16). In mice, iNKT cells are most abundant in the liver (10–30%) and the spleen (0.5–1.5%) with lower frequencies found in thymus, blood, bone marrow (all 0.2–0.5%), and lymph nodes (0.1–0.2%). In humans, substantial interindividual variability is observed. However, high iNKT cell frequencies are detected in the liver (1%), omentum (10%), the adipose tissue (in which iNKT cell frequencies vary between 0.5 and 1% of total $CD3^+$ cells) (23), and in healthy donors iNKT cells represent 0.01–0.5% of PBMCs (24, 25). The iNKT cell subset develops in the thymus, emerges from the same progenitor pool as conventional T cells, and undergoes somatic recombination and thymic selection. Rather than *via* thymic epithelial cells, iNKT cells are positively selected through interaction with double-positive thymocytes that CD1d-present endogenous ligands, leading to an unusually strong TCR signal. The directing of iNKT cell precursors toward a particular subset lineage may involve specific endogenous selecting lipid antigens (19, 26, 27). The majority of human thymic iNKT cells egresses during early fetal development and $CD4^+CD8^-$ iNKT cells are already present at birth, whereas murine iNKT cells only emerge during the first postnatal week (25, 28, 29). Distinct human iNKT cell subsets include $CD4^+/CD8^-$, $CD4^-/CD8^-$, and $CD4^-/CD8^+$ whereas in mouse $CD4^+/CD8^-$ and $CD4^-/CD8^-$ subsets prevail (25).

iNKT CELL HETEROGENEITY AND EFFECTOR FUNCTIONS

Initially believed to be a rather rigid and homogenous cell population that merely acts upon TCR stimulation, it became recently clear that based on their respective transcriptional programs, distinct iNKT cell subsets with designated functional properties exist and that iNKT cells may balance immune homeostasis *via* their steady-state activity. TCR-induced transcription factors Egr2 and Egr1 lead to transcription of PLZF, the key transcriptional factor during the development of iNKT cells (30). In fact, although only a subset of fully matured iNKT cells are positive for PLZF, the majority of iNKT cells expresses this transcription factor at one point during development (31–34). Depending on the subsequent transcriptional program, thymic $CD24^{hi}/CD69^+$ iNKT cell precursors diverge into distinct sublineages (35). T_H1 iNKT cells (NKT1) express T-bet and Bhlhe40 and mainly release IFNγ upon TCR ligation. T_H2 iNKT cells (NKT2) predominantly express GATA3 and PLZF and release IL-4 and IL-13 already in steady state. IL-17-producing iNKT17 express RORγt, a subset of Bcl-6-dependent, CXCR5- and PD1-expressing iNKT follicular helper cells secrete IL-21, thereby shaping B cell responses. IL-10-producing immunoregulatory NKT10 are FOXP3-negative but positive for the transcription factor E4BP4 (20, 27, 36–38). Recently, a KLRG-expressing subset of iNKT cells has been described, which shows an effector-memory-like phenotype and is able to mount stronger secondary responses to cognate antigen (12).

Invariant NKT cells can be activated either upon stimulation of their TCR by CD1d-presented glycolipid antigens, or in a TCR-independent manner (e.g., by cytokines) (39, 40). Upon activation, iNKT cells readily proliferate and undergo significant remodeling of their surface expression patterns with regards to several markers, such as NK1.1 and the semi-invariant TCR (41).

Although iNKT cells have adaptive characteristics, they exist in a preactivated memory-like effector state primed to release large amounts of immunomodulatory cytokines (including IFNγ, IL-4, IL-13, IL-17, GM-CSF, and TNF-α) not only upon engagement of their TCR but also in response to innate signals (13). One of their key features is the cytokine-mediated transactivation of other innate and innate-like immune cell subsets, thereby amplifying initial responses (19, 42–45). In addition, iNKT cells may also provide both antigen-specific cognate and non-cognate help for B cells (20, 46, 47) and in turn can be activated by B cells (48, 49). Interestingly, unlike the non-cognate iNKT cell–B cell interactions, antigen-specific iNKT cell help induces a more innate-biased B cell response, which is characterized by a discontinuous germinal center B cell expansion and rapid initial proliferation of IL-10-producing B cells, but fails to induce humoral memory (50).

A key difference between iNKT cells and conventional T cells are the kinetics of their responses, which in case of iNKT cells occur already within hours after engagement, as opposed to several days in the case of conventional T cells (1, 51). In line with this, iNKT cells have been reported to carry preformed mRNA of cytokines in their cytoplasm, which enables them to rapidly release large quantities of these effector molecules upon TCR ligation (52, 53). The translational regulation of preformed cytokine mRNA has been shown to be dependent on p38 MAPK (54).

Aside from rapidly releasing numerous immunomodulatory cytokines, iNKT cells also have immediate cytotoxic capacity, which correlates with the amount of surface CD1d on the target cell (55). While reports in patients suffering from acute myeloid leukemia (AML) and juvenile myelomonocytic leukemia describe (analogous to NK cell-mediated cytotoxicity) predominant usage of the perforin/granzyme B pathway in executing cytotoxicity, other reports in C57BL/6 mice ascribe a higher importance to Fas/FasL interaction (55). In addition to exerting direct effector functions, it becomes more and more apparent that iNKT may

shape immune responses indirectly through crosstalk with other immune subsets.

Myeloid-derived suppressor cells (MDSCs) are a unique $Gr1^+$ population of activated myeloid cells that retain an immature phenotype and are functionally able to dampen adaptive immune responses during malignancies and infection (56). De Santo et al. described an intriguing mechanism through which iNKT cells reverse the suppressive properties of MDSCs during influenza A virus (IAV) infection in a CD1d- and CD40:CD40L-dependent manner (57). While infection of both $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice with the IAV strain A/Puerto Rico/8/34 (PR8) lead to a more severe phenotype and a greater expansion of CD1d- and CD40-expressing MDSCs in the lungs of $J\alpha18^{-/-}$ and $CD1d^{-/-}$ mice as compared to PR8-infected wild-type mice, only adoptive transfer of iNKT cells into $J\alpha18^{-/-}$ mice ameliorated the disease course and reduced MDSC numbers whereas $CD1d^{-/-}$ mice remained hypersusceptible and depicted unchanged numbers of MDSCs. MDSCs isolated from the lungs of PR8-infected $J\alpha18^{-/-}$ mice depicted a stronger suppressive activity as those from wild-type mice. Pulsing MDSCs with αGalCer or TLR agonists (for TLRs 3, 7/8, and 9) in the presence of iNKT cells reduced suppressive activity of MDSCs. The results of this study suggest that TLR-mediated upregulation of (yet to be defined) endogenous iNKT cell ligands contribute to the iNKT cell-mediated modulation of MDSC suppressive activity during IAV infection. Accordingly, immunosuppressive properties of MDSCs isolated from IAV-infected patients could be reversed by iNKT cells (57). Likewise, it was shown that MDSCs pulsed with tumor-associated antigens and the prototypic iNKT cell agonist αGalCer fail to suppress cytotoxic T lymphocytes (CTLs) and do not induce generation of $FOXP3^+$ T regulatory cells (TREGs), thus leading to longer survival of mice in a murine metastatic tumor model. Activated iNKT cells are able to modify MDSCs, transforming them back to a more immunogenic APC phenotype (58). MDSCs do not only include macrophages but also neutrophils, which acquire immunosuppressive properties such as IL-10-secretion, resulting in damping of antigen-specific T cell responses (59). It was shown that the acute-phase protein serum amyloid A 1 fosters iNKT-mediated conversion of suppressive activity of neutrophils. This immunomodulatory crosstalk between iNKT cells and neutrophils is highly dependent on CD1d:TCR interaction (60). All-*trans*-retinoic acid (ATRA) is known to promote MDSC differentiation (61, 62). Exposure of αGalCer-loaded MDSCs with ATRA has shown to restore immunogenicity of this immune subset in an iNKT cell-dependent way (63). These findings extend the previously reported arsenal of iNKT cells to execute their immunomodulatory functions.

Like MDSCs, tumor-associated monocytes/macrophages (TAMs) are part of the tumor microenvironment but unlike MDSCs, TAMs are $Gr1^-$ (64). Primary human neuroblastoma cells are $CD1d^-$, however, the tumor neuroblastoma microenvironment is highly enriched for $CD68^+/CD1d^+$ TAMs, which aliment tumor growth mainly through secretion of IL-6. CD1d-dependent killing of growth-promoting TAMs *via* iNKT cells decelerated tumor growth in a NOD/SCID human neuroblastoma xenograft model (65). Tumor necrosis factor related apoptosis inducing ligand (TRAIL)-expressing autologous or allogeneic $CD4^+$ iNKT cells induce apoptosis in myeloid leukemia cells derived from AML patients. However, TCR:CD1d interaction was not required for this effector function (66). Further TCR-independent effector functions include NKG2D-dependent cytotoxic degranulation (11), differential cytokine expression pattern upon stimulation with IL-2, IL-12, IL-18 (67) and potentiating NK-cell mediated cytotoxicity in an IL-2-dependent manner (68).

iNKT CELL ACTIVATION BY DCs

The CD1 family is comprised of five isoforms that can be partitioned in two groups. Group 1 consists of CD1a, CD1b, CD1c, and CD1e and group 2 only includes CD1d. While all isoforms can be found in humans, only CD1d is expressed in mice (69).

Dendritic cells constitutively express CD1d and may activate iNKT cells by presenting antigenic glycolipids. CD1d is a highly conserved non-polymorphic MHC class I-like transmembrane molecule; its expression is regulated by cytokines as well as through engagement of innate receptors (70). Similar to the structurally related MHC class I molecules, CD1d represents a heterodimer comprised of the CD1d heavy chain non-covalently coupled to β2-microglobulin. Many hematopoietic and non-hematopoietic cell types express CD1d on their surface either constitutively or upon activation (71–75). However, in mice, constitutively CD1d-expressing DCs appear to be the most potent APCs for exogenous glycolipids (76–78). The interaction between iNKT cells and DCs is not unidirectional but characterized by reciprocal feedback loops depending on the chemical structure of the CD1d ligand as well as the nature of the APC (**Figure 1**) (9, 76). DCs acquire and CD1d present exogenous lipid antigens for the direct recognition by iNKT cells but may also transduce innate signals toward to the induction of iNKT cell responses (39, 40, 79). In many cases, the activation of iNKT cells results from a combination of TCR-mediated recognition of cognate lipid antigen and TCR-independent signals. For example, pattern recognition receptor (PRR)-bearing DCs will CD1d-present endogenous glycolipids in response to stimulation with pathogen-associated molecular patterns (39, 79–81). Recent studies suggest that PRR activation may specifically modulate the lysosomal processing of glycolipids in APCs to increase the abundance of endogenous iNKT cell agonists (80, 81). In concert with signals provided by pro-inflammatory cytokines secreted by PRR-activated DCs, the weaker TCR recognition of endogenous antigens is sufficient for iNKT cell activation (39, 40). To which extent similar cytokine signals are required for iNKT activation by microbes expressing stimulatory lipid antigens remains debated (13, 82). iNKT cells constitutively express the IL-12 receptor and PRR-mediated secretion of IL-12 by DCs triggers Stat4 phosphorylation and consecutive IFNγ secretion in iNKT cells (13, 83). Furthermore, direct cellular contact between DCs and iNKT cells in a CD40:CD40L-dependent manner provides a strong feed-forward signal, resulting in additional IL-12 production by DCs and consecutive further upregulation of the IL-12 receptor on iNKT cells. CD40/CD40L as well as CD28:CD80/CD86 interactions are required for subsequent iNKT cell-mediated-IFNγ secretion whereas IL-4-secretion was described to be solely dependent on CD28:CD80/CD86 interaction (83, 84). Co-administration of

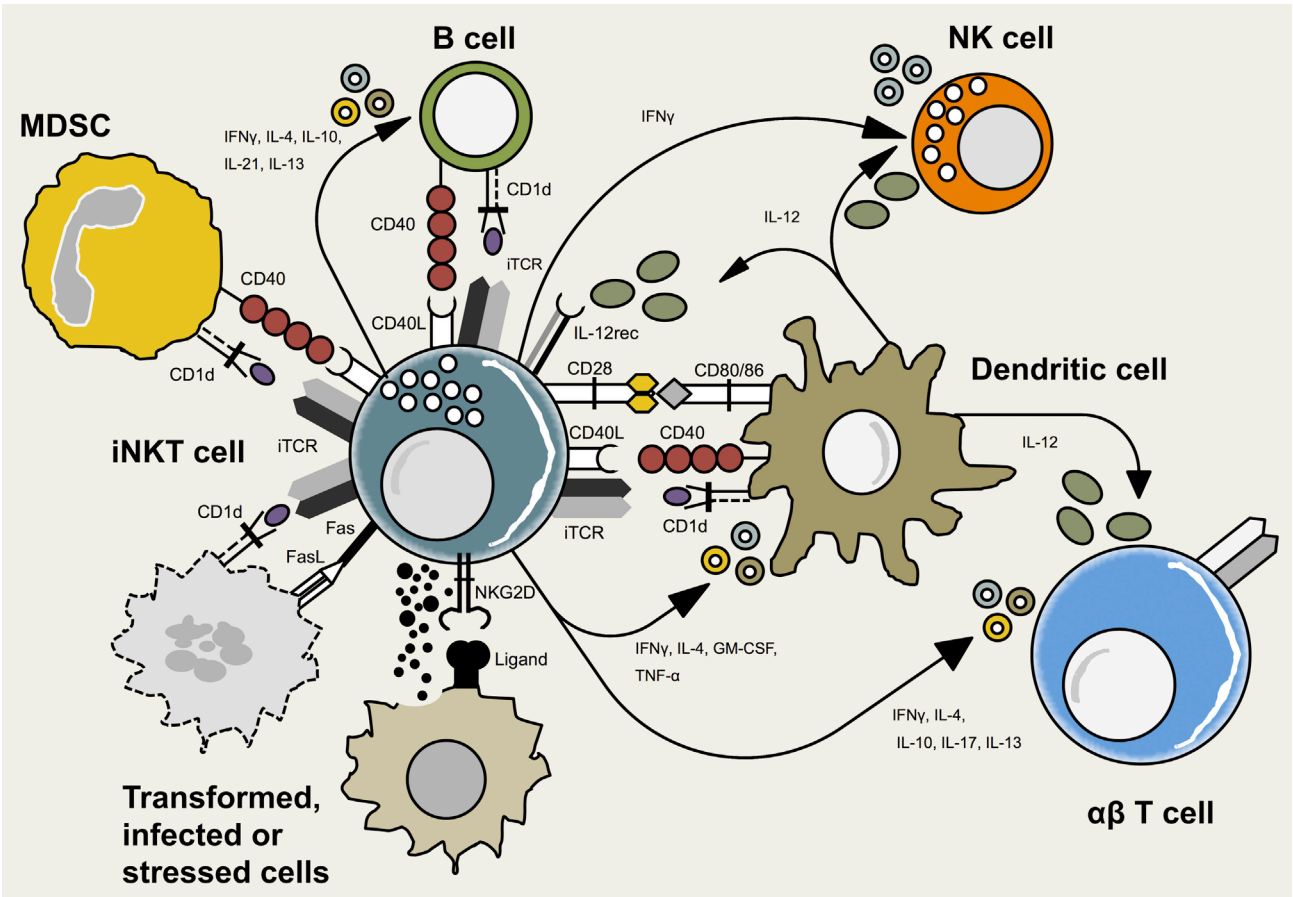

FIGURE 1 | **Interactions between invariant natural killer T (iNKT) cells and other immune cells**. iNKT cells crosstalk with adaptive and innate immune cells including dendritic cells (DCs), B cells, natural killer (NK) cells, myeloid-derived suppressor cells (MDSCs), and conventional T cells. This reciprocal interaction is mediated *via* T cell receptor (TCR)-CD1d-recognition, interaction of co-stimulatory molecules or cytokines. Additionally, iNKT cells may interact with non-hematopoietic cells that expose signs of infection, transformation, or cell stress.

iNKT cell agonist αGalCer and OVA in CD40$^{-/-}$ and CD40L$^{-/-}$ mice leads to abrogation of CD4^{+} and CD8^{+} T cell responses, while DCs are not affected in their ability to present antigen on MHC class I or II and are capable of upregulating CD80/86 (77). Recent reports show that artificial APCs loaded with iNKT cell agonists can activate and expand human iNKT cells *in vitro* as potently as autologous immature DCs. Engineering artificial APCs with differential association to co-stimulatory factors will help to obtain valuable insights into the crosstalk between iNKT cells and DCs and will foster our understanding of how to harness their therapeutic potential (85–87).

CD1d TRAFFICKING IN MOUSE PROFESSIONAL APCs

Similar to MHC class I molecules, CD1d molecules are synthesized, folded, and equipped with β2-microglobulin in the ER (**Figure 2**) (88). Analogous to the placeholder function of the pseudopeptide CLIP in MHC class II, CD1d most likely leaves the ER with an endogenous lipid in its antigen-binding groove in order to maintain stability. The biochemical nature of these lipids and the exact mechanisms underlying the respective transfer processes are not fully elucidated yet. However, the ER chaperone protein microsomal triglyceride transfer protein (MTP) has been suggested to load phospholipids onto nascent CD1d (89). CD1d, after having passed several protein quality control checkpoints, follows the secretory pathway and is being guided to the Golgi apparatus and subsequently reaches the cell surface (88, 90). From there, CD1d is being internalized in clathrin-coated pits *via* the interaction of the adaptor protein complex 2 (AP2) and adaptor protein 3 (AP3) through tyrosine-based sorting motifs present in the cytoplasmic tail of CD1d, and subsequently delivered to endosomal compartments (91–93). The autophagic machinery assists in the recruitment of AP2 to CD1d molecules. Loss of the essential autophagy protein ATG5 in DCs impaired clathrin-dependent internalization of CD1d molecules *via* AP2 and, thus, increased surface expression of stimulatory CD1d:glycolipid complexes, which resulted in enhanced iNKT activation (94).

Having generated a knock-in mouse by homologous recombination in which all CD1d is expressed as CD1d-EYFP, Sillé and colleagues described that in order to activate iNKT cells, endosomal sorting of CD1d is dependent on both its tyrosine-based

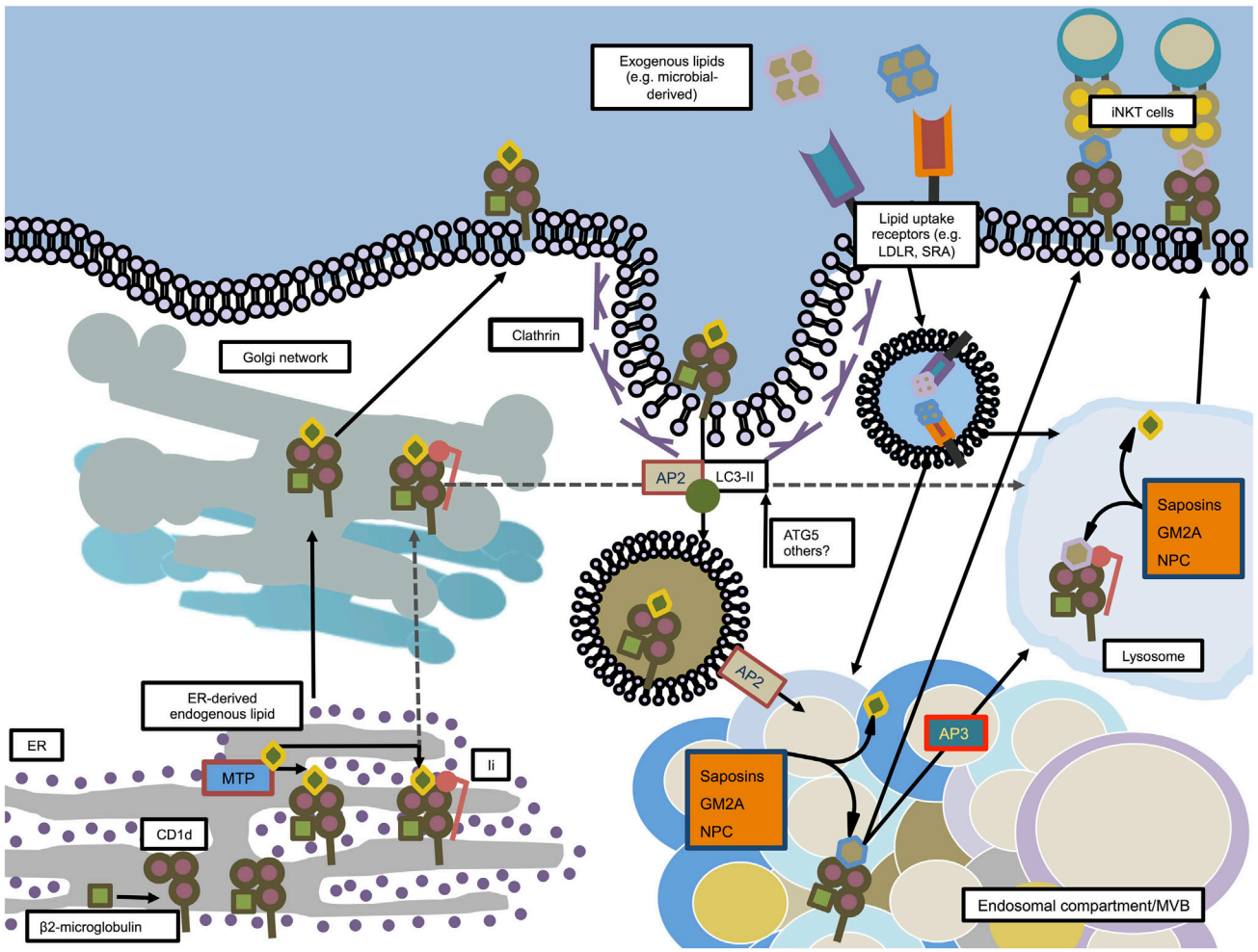

FIGURE 2 | CD1d trafficking and loading in mouse dendritic cells. CD1d molecules are synthesized in the ER where they associate with β2-microglobulin. Microsomal triglyceride transfer protein (MTP) facilitates loading of ER-derived endogenous lipids onto CD1d in order to stabilize the molecule for further transport. In an independent pathway, some CD1d molecules associate in the ER with invariant chain (Ii). The Ii/CD1d complexes, after traveling through the *trans*-Golgi network, are directly guided to the lysosome. The non-Ii-associated CD1d molecules also pass the *trans*-Golgi network on their way to the plasma membrane. From there, facilitated by AP2 and members of the autophagy machinery, CD1d is internalized by clathrin-mediated endocytosis and guided toward endosomal compartments, where saposins, GM2 ganglioside activator (GM2A), and Niemann–Pick type C1 and C2 proteins (NPC1, NPC2) help exchanging endogenous lipids with exogenous or other endogenous lipids. From there, loaded CD1d may either be transported to the lysosome in an adaptor protein 3 (AP3)-dependent manner, or directly transported to the plasma membrane in order to interact with iNKT cells.

sorting motif and on the association with the invariant chain (Ii) in peripheral DCs (95).

CD1d itself is unable to extract and acquire lipids from membranes and, therefore, is in need of lipid transfer proteins (LTPs) that, in analogy to the MHC class II/H2-DM interaction, facilitate loading of antigens within the endosomal/lysosomal compartment. Low molecular-weight proteins called saposins (A, B, C, and D), GM2 ganglioside activator (GM2A), and the Niemann–Pick type C1 and C2 proteins (NPC1 and NPC2, respectively) have so far been identified to mediate the loading of lipids on CD1d molecules (96–99). Additionally, in the cases of saposins and GM2A, it was reported that these molecules also aid in the unloading of lipids from the CD1d antigen-binding groove (93). Following sampling of antigens in the endo/lysosomal system, loaded CD1d molecules are being recycled to the cell surface, similar to MHC class II molecules, and present bound antigens for iNKT cell activation.

ENDOGENOUS AND EXOGENOUS iNKT CELL ANTIGENS

Different from MHC class I or II-restricted antigen presentation during which processing of proteins or larger peptides results in smaller antigenic peptides presented by polymorphic antigen-presenting molecules, monomorphic CD1d presents mainly unprocessed lipids of varying size and biochemical structure (100). Lipid antigens that stimulate CD1d-restricted iNKT cells comprise endogenous (also called self-lipids) and exogenous (e.g., microbial-derived) non-peptidic molecules. Within the mammalian class, self-lipids predominantly consist of

glycosphingolipids (GSLs) and phosphoglycerolipids (69). Endogenous self-lipids have been described to be crucial for thymic selection of iNKT cells *via* $CD4^+CD8^+$ thymocytes (28, 98) but might also be involved in modulating antiviral and antineoplastic iNKT activity in the periphery (57, 79, 101). With regards to MHC molecules, methodological advances in proteomics allowed for progressive elucidation of the MHC-binding immunopeptidome in recent decades (102). In stark contrast, little is known about the endogenous lipid repertoire bound on CD1d *in vivo*, which is largely due to the fact that current techniques to extract CD1d molecules from cell membranes irretrievably entail the dissociation of CD1d-associated ligands. Although generation of secreted human CD1d molecules (sCD1d) by truncating the transmembrane and cytoplasmic domains shed some light on which lipid antigens are associated with CD1d (103), this approach implicates obvious shortcomings. Since the intracellular cytoplasmic tail of CD1d is required for trafficking of CD1d through endolysosomal compartments in which lipid exchange and transfer occurs (28, 104), the detected lipidom is unlikely to reflect the *in vivo* setting. Therefore, the development of more refined techniques is required for the unequivocal identification of *in vivo*-relevant endogenous CD1d ligands.

The lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3), a moderate activator of iNKT cells, has been proposed to function as a self-lipid (105). However, the biological relevance of this finding warrants further investigation (106, 107). Other candidate self-lipids to be involved in iNKT cell development are the peroxisomal-derived ether-bonded phospholipids 1-*O*-1′-(Z)-hexadecenyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine and 1-*O*-1′, 9′-(Z,Z)-octadecadienyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine. Not only depicted the synthetic plasmalogen C16-lysophosphatidylethanolamine (pLPE) similar iNKT cell stimulatory capacities as the prototypical agonist αGalCer but mice deficient in glyceronephosphate O-acyltransferase (GNPAT), the peroxisomal enzyme essential for synthesis of ether lipids, showed impaired iNKT cell development. However, $GNPAT^{-/-}$ mice still harbored around 50% of iNKT cells found in GNPAT-competent mice (108). Other endogenous lipid antigens might, therefore, be involved in thymic selection of iNKT cells as well and the understanding of the relative contribution and distinct functions of a given endogenous CD1d ligand to iNKT cell biology will need further clarification.

Most exogenous CD1d ligands identified to date are of bacterial origin. iNKT cell-activating lipid antigens have been found in *Borrelia burgdorferi* [α-galactosyldiacylglycerols (αGalDAGs)] (109), *Sphingomonas* spp. (α-glucuronosylceramides and α–galacturonosylceramides) (40, 110, 111), *Streptococcus pneumoniae*, and group B *Streptococcus* [α-glucosyldiacylglycerols (αGlcDAGs)] (112), *Mycobacterium tuberculosis* (phosphatidylinnositol mannosides) (113), *Helicobacter pylori* (cholesteryl α-glucoside) (114), and *Bacteroides fragilis* (α-galactosylceramides) (115). But also the porifera *Agelas mauritianus* (α-galactosylceramides) (8, 116) and the ascomycete *Aspergillus fumigatus* (asperamide B) (117) have been reported to contain antigenic lipids that activate iNKT cells.

The α-linked monoglycosylceramide αGalCer, initially isolated from *Agelas mauritianus* was the first glycolipid identified to activate iNKT cells. Its synthetic derivative KRN7000 has become a commonly used experimental tool in iNKT cell research and to this day remains to be the most potent iNKT cell agonist (8, 116). Until recently, it was believed that mammalian cells are incapable of generating α-anomeric GSLs such as αGalCer. Making use of high-sensitivity biological assays, lipid immunopurification, and multiple reaction monitoring-mass spectrometry, Kain et al. reported that trace amounts of α-linked GSLs (both, αGluCer and αGalCer) are produced in mammalian cells and most likely function as endogenous ligands during thymic selection of iNKT cells (81).

iNKT CELL-MEDIATED MATURATION AND LICENSING OF DCs

As a feedback loop, αGalCer-activated iNKT cells contribute to maturation of DCs *in vivo* resulting in increased cell surface expression of MHC class II, the co-stimulatory molecules CD40, CD80, CD86, and the endocytic receptor DEC-205. iNKT cell-matured DCs elicit specific $CD4^+$ and $CD8^+$ T cell responses against a co-administered peptide. The observed DC maturation is highly dependent on iNKT cells since administration of αGalCer fails to induce DC maturation in $J\alpha18^{-/-}$ mice lacking iNKT cells (118). Challenge with OVA-expressing tumors demonstrated significant tumor resistance in animals that had been previously immunized with OVA in combination with the iNKT cell agonist αGalCer (119). In mice, the unique subset of $CD8\alpha^+$ DCs is able to cross-present extracellular antigens *via* MHC class I to evoke CTL responses (120). Both mouse (121) and human (122) studies have shown that cross-presentation is $CD4^+$ T helper (T_H) cell dependent. The interaction between mouse $CD4^+$ T_H cells and DCs leads to release of CCL3 and CCL4 attracting CCR5-expressing CTLs to the site of cross-presentation (123). However, the CCR4-CCL17-dependent licensing of DCs by iNKT cells for cross-presentation has been described as an alternative pathway. iNKT cell-mediated upregulation of CCL17 in DCs required CD1d and spatial interaction between iNKT cells and DCs (124). Interestingly, Arora et al. reported that despite numerous cell types expressing high levels of CD1d, the $CD8\alpha^+$ DCs are the most competent presenters of lipid antigens *in vivo* (125). Whether and to which extent these mechanisms are translated to humans, remains to be addressed.

FUNCTIONAL OUTCOMES OF iNKT CELL ACTIVATION

Recognition of CD1d:glycolipid complexes *via* the iNKT cell TCR can result in either pro-inflammatory T_H1-biased or T_H2-biased cytokine production by iNKT cells (2, 18). Mechanisms that mediate such potentially opposing functional outcomes need to be taken into account in designing iNKT cell-targeting therapies. Differential expression of co-stimulatory signals on distinct APC subsets (126, 127) and the chemical structure of iNKT cell agonists, which target iNKT cell ligands to distinct APC populations (128, 129) contribute to the functional outcome of iNKT cell activation. In serum, soluble iNKT cell agonists associate with lipoprotein

particles or are transported bound to serum LTPs, which facilitate glycolipid uptake by APCs and loading onto CD1d molecules (130–132). Several receptors mediate the uptake of glycolipids for CD1d presentation, including the low-density lipoprotein receptor and the scavenger receptors SRA, SRB1, and CD36 (130, 132). Importantly, the specificity of this serum transport and receptor-mediated uptake is largely influenced by minor modifications of the chemical structure of iNKT cell agonists (133), suggesting that specific "targeting-motifs" could be used to direct glycolipid antigens toward distinct uptake pathways in order to modulate the resulting iNKT cell effector response (132). Besides affecting glycolipid uptake, the chemical structure of iNKT cell agonists may also influence the nature of the presenting APC as well as the context in which the antigen is CD1d presented. In general, the CD1d presentation of glycolipid antigens requires their access to the lysosomal loading compartment, which provides multiple glycosidases for antigen processing and lysosomal LTPs to assist in the solubilization and loading of glycolipids into CD1d (96–98, 134–136). There is evidence that lipid antigens eliciting a T_H2-type iNKT cell cytokine response do not require intracellular loading onto CD1d but may directly bind surface CD1d instead. Such T_H2-biased iNKT cell agonists typically possess short or unsaturated acyl chains, which increase their solubility in the aqueous environment but also favor a rapid displacement from CD1d upon recycling to the lysosome (137–140). The surface CD1d loading might bypass inclusion of such CD1d/lipid antigen complexes into lipid microdomains (139). Similarly, differential immune responses have been described for MHC class II molecules when presenting peptides either dependent or independent of lipid rafts (141). In addition, the anatomical context can modulate iNKT cell cytokine responses. In mice, the principle presenters of αGalCer and other T_H1-biased antigens *in vivo* are $CD8\alpha^+$ DEC-205^+ DCs (76), while the presentation of T_H2-biased iNKT cell agonists was found to be more promiscuous, likely due to their ability to directly load onto cell surface CD1d (128). Furthermore, Lee et al. (142) showed that differential routes of lipid antigen application may dramatically alter the iNKT cell activation pattern due to a distinct anatomical distribution of iNKT cell subsets.

THERAPEUTIC IMPLICATIONS

Their ability to mature DCs and to transactivate both CTLs and NK cells for tumor cell eradication (143, 144) reflect the potential of iNKT cells in improving cancer immunotherapy (**Figure 3**). However, in contrast to encouraging studies performed in experimental models (145), clinical trials using direct administration of soluble αGalCer in cancer patients failed to show promising results (146). Aside from high interindividual variability in iNKT cell frequencies and inefficient targeting of particular subsets of lipid presenting cells, direct administration of antigenic glycolipids was shown to induce PD1:PDL1-dependent long-term anergy (147–149) or induction of regulatory IL-10-producing iNKT cells (36, 150), which negatively affect antitumor responses (150). As an alternative to αGalCer administration, DCs can be glycolipid-pulsed *ex vivo* followed by re-infusion. This strategy has proven to induce prolonged activation of iNKT cells rather than a regulatory/anergic phenotype, inhibits metastasis in an experimental melanoma model, and can expand human iNKT cells *in vivo* (78, 151, 152). Additionally, adoptive transfer of αGalCer-pulsed matured DCs expands iNKT cells in advanced stage cancer patients (153). A clinical phase I study in a limited number of individuals with metastatic malignancies reported that transfer of immature monocyte-derived DCs loaded with αGalCer was associated with a stronger recall response (154). Matured DCs as compared to immature DCs increased the observed beneficial effects significantly (153, 154). Another phase I trial during which patients with head and neck squamous cell carcinoma (HNSCC) were treated *via* singular co-administration of autologous *in vitro* expanded iNKT cells (intraarterial) and submucosal application of αGalCer-loaded APCs showed partial clinical response (155). In a small phase II clinical study in HNSCC patients using the same treatment regimen, 50% of the patients depicted tumor regression while 50% showed stable disease (156). Promising results were reported from a phase I-II study in non-small cell lung cancer patients: sequential intravenous administration of αGalCer-pulsed PBMCs increased the frequencies of IFNγ-producing cells in a majority of patients. This iNKT cell-mediated T_H1 skewing in responders was associated with significantly prolonged median survival time (157). In a follow-up study, two candidate genes, *LTB4DH* and *DPYSL3*, were proposed to predict responsiveness to abovementioned treatment regimen (158). Late stage cancer patients often times are immune suppressed and retrieving enough APCs from these individuals for autologous transfer might prove difficult. Therefore, novel artificial APC constructs may help to circumvent lack of appropriate autologous APC numbers (85–87). Moreover, novel glycolipid-antigen delivery systems that systematically target relevant APC populations are currently being investigated. Some of these nanovector systems already show promising results. αGalCer-containing silica microspheres, poly(lactic-co-glycolic acid) (PLGA) polymers, and modified liposomes have already been reported to efficiently elicit iNKT cell responses (159–161). In order to initiate *in situ* responses of DCs, artificial adjuvant vector cell systems have been recently introduced. Herein, allogeneic CD1d-expressing NIH3T3 fibroblasts loaded with αGalCer were transfected with target-antigen mRNA. Injection of NIH3T3 fibroblasts lead to activation of iNKT cells, consecutive maturation of DCs, and activation of NK cells and antigen-specific CTLs. Animals that were immunized with adjuvant vector cells show potent immunity against antigen-bearing tumors. Interestingly, memory CTL responses can still be detected 12 months after initial single injection (143, 162).

Humans, as compared to mice, show high interindividual variability in iNKT cell frequencies. Patients with low steady-state numbers of iNKT cells might not efficiently profit from autologous transfer of lipid-pulsed APCs. Mouse embryonic fibroblast-derived induced pluripotent stem cells (iPSCs) can readily differentiate into functional iNKT cells. These iPSC-derived iNKT cells are able to produce IFNγ and mediate anti-neoplastic effects *in vivo* (163). Therefore, patients with low iNKT cell frequencies may be reconstituted with iPSC-derived iNKT cells as an efficient means to fully harness their immunomodulatory potential (143).

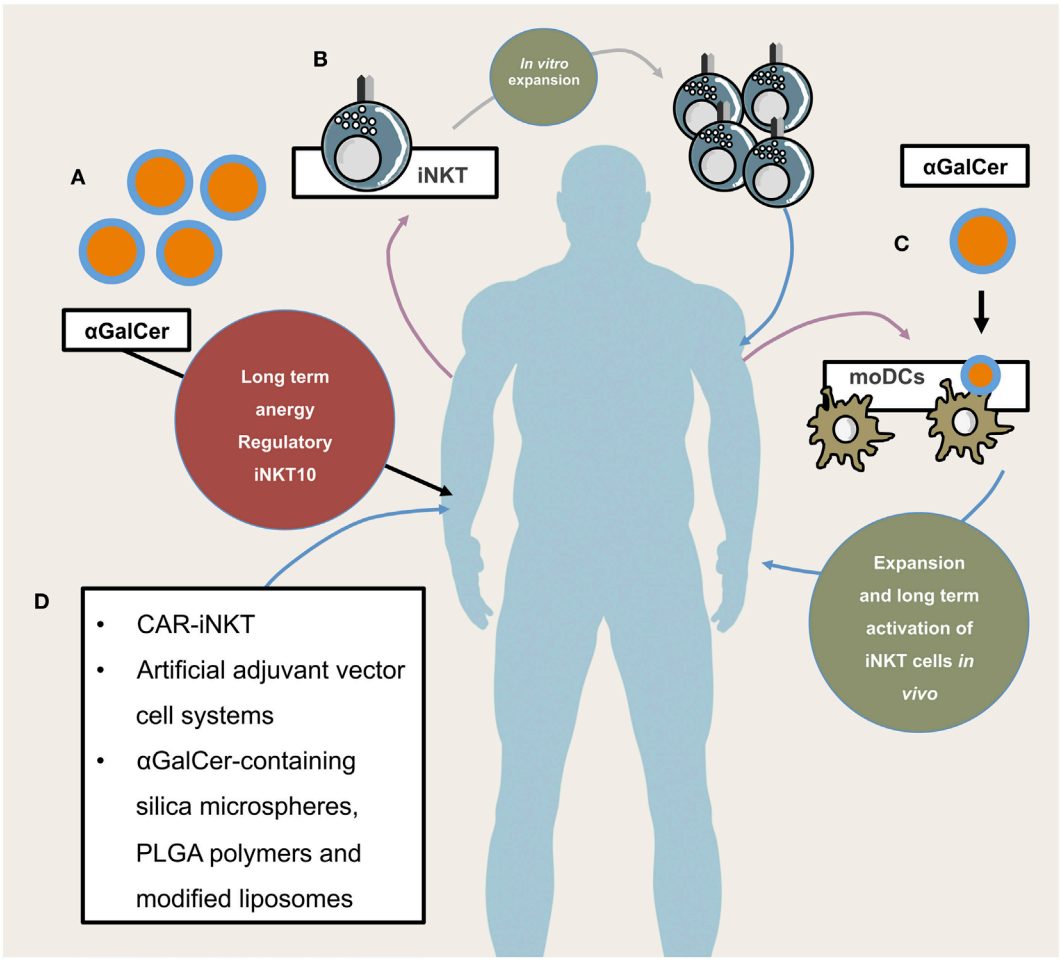

FIGURE 3 | **Potential and already implemented invariant natural killer T cells (iNKT) cellular therapeutic approaches**. Illustrates different application modes of iNKT-based therapies. **(A)** Direct administration of iNKT cell agonist αGalCer. This has so far not proven to be efficacious due to long-term anergy and regulatory phenotype of iNKT cells following αGalCer administration. **(B)** Isolation, purification, *in vitro* expansion, and re-infusion of peripheral iNKT cells, **(C)** isolation of PBMCs and subsequent generation of monocyte-derived DCs which, after lipid-pulsing, are re-infused (either alone or in combination with *in vitro* expanded autologous iNKT cells), **(D)** novel and future approaches include chimeric antigen receptor (CAR)-iNKT cell generation, artificial adjuvant vector cell systems, αGalCer-containing silica microspheres, PLGA polymers, and modified liposomes.

First attempts in using engineered iNKT cells with chimeric antigen receptors (CARs) show promising results. CAR-bearing iNKT cells home to designated tumor sites, eradicate tumor cells, and effectively execute cytotoxicity against TAMs without inducing graft-versus-host disease (164). Additionally, $CD62L^+$ CD19-specific CAR-bearing iNKT cells show potent immunotherapeutic efficacy in a B cell lymphoma model (165).

In conclusion, murine and clinical trials performed to date demonstrate that therapeutic strategies that harness the biology of iNKT cells are generally well tolerated and, in some cases, effective in inducing tumor regression and prolonged survival. All of the tested and currently investigated strategies harness both the powerful cytolytic and adjuvant activity of iNKT cells in order to enhance protective antitumor immune responses. In order to fully exploit their therapeutic potential, it will not only be essential to elucidate the differential effector functions and modes of activation of individual iNKT cell subsets but also the immunological contexts and transcriptional programs that direct $CD24^{hi}/CD69^+$ iNKT cell progenitors into development toward specific iNKT cell subpopulations as well as determinants that gear specific iNKT cell subsets to distinct anatomical sites (31, 142, 166). Profound mechanistic insight into understanding how DCs activate and instruct iNKT cells and which factors regulate iNKT cell responses are prerequisites for improving the efficacy of iNKT cell-targeting therapies. In addition, clinical trials will be instrumental in identifying the optimal ligands and APC populations to induce vigorous iNKT cell activation and in determining the routes and intervals of administration to achieve sustained antitumor immunity.

AUTHOR CONTRIBUTIONS

CWK, SF, and JDL participated in drafting the article and revising it critically for important intellectual content. All authors gave final approval of the submitted manuscript.

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