Enhancing Neuroblastoma Immunotherapies by Engaging iNKT and NK Cells

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Neuroblastoma (NB) is the most common extracranial solid tumor in children and, in the high-risk group, has a 5-year mortality rate of ~50%. The high mortality rate and significant treatment-related morbidities associated with current standard of care therapies belie the critical need for more tolerable and effective treatments for this disease. While the monoclonal antibody dinutuximab has demonstrated the potential for immunotherapy to improve overall NB outcomes, the 5-year overall survival of high-risk patients has not yet substantially changed. The frequency and type of invariant natural killer T cells (iNKTs) and natural killer cells (NKs) has been associated with improved outcomes in several solid and liquid malignancies, including NB. Indeed, iNKTs and NKs inhibit tumor associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs), kill cancer stem cells (CSCs) and neuroblasts, and robustly secrete cytokines to recruit additional immune effectors. These capabilities, and promising pre-clinical and early clinical data suggest that iNKT- and NK-based therapies may hold promise as both stand-alone and combination treatments for NB. In this review we will summarize the biologic features of iNKTs and NKs that confer advantages for NB immunotherapy, discuss the barriers imposed by the NB tumor microenvironment, and examine the current state of such therapies in pre-clinical models and clinical trials.

Keywords: invariant natural killer T cells, natural killer cells, cancer immunotherapy, immunotherapy, neuroblastoma, tumor microenvironment

INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumor in children and accounts for ~15% of childhood cancer deaths (1). NB is stratified into risk groups on the basis of clinical and molecular features using the International Neuroblastoma Risk Group (INRG) classification with the high-risk group being the most prevalent (2). High risk neuroblastoma (HRNB) is treated with a combination of conventional chemotherapy, surgical resection, autologous stem-cell transplant, radiation, and immunotherapy. Despite the extensive treatment regimen, HRNB still carries a 5-year overall survival (OS) of ~50%, and these treatments have significant late adverse effects including hearing loss, cognitive deficits, endocrinopathies, and ovarian failure (1, 3–5). As such, there is a critical need for more tolerable and effective treatments in this group.
Immunotherapy with dinutuximab, a monoclonal antibody against GD2 (a disialoganglioside that is highly expressed on neuroblasts), has been incorporated into the recommended treatment regimen for HRNB after FDA-approval in 2015, and significantly improved 2-year event-free-survival (EFS) (6). Additionally, the addition of dinutuximab to irinotecan and temozolomide in the relapsed or refractory NB setting demonstrated dramatic clinical activity. In those treated with the dinutuximab-containing regimen, 9 out of 17 patients (53%) had a disease response vs. 1 out of 18 patients (6%) in a comparator arm that included the same chemotherapy but without dinutuximab (7). These studies demonstrate that dinutuximab immunotherapy has significant clinical utility in both minimal and high disease burden contexts. However, despite the improved 2-year event-free-survival (EFS) in the minimal residual disease setting and substantial response in the bulk disease setting, 5-year overall survival (OS) in HRNB remains ~50%. Improving the long-term efficacy of these promising immunotherapies in HRNB therefore remains an important unsolved challenge.

While the use of chimeric antigen receptor (CAR) T cells and checkpoint inhibitors have shown success in the treatment of other malignancies, these immunotherapies have not yet demonstrated similar efficacy in HRNB or other pediatric solid tumors (8–10). The lack of response in HRNB is thought to be due to multiple factors including an immunosuppressive tumor microenvironment (TME), low expression of MHC-I, and MHC-II antigens on neuroblasts leading to immune evasion, low mutational burden in NB with a paucity of neoantigens, and diminished T cell persistence (for CAR T cell therapies) (11, 12). Alternative immunotherapies that can overcome these barriers are therefore being sought.

Interestingly, the presence of invariant natural killer T cells (iNKTs), and natural killer cells (NKs) is associated with improved prognosis of patients with NB as well as other malignancies (12–15). iNKTs and NKs inhibit tumor-associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs), kill cancer stem cells (CSCs), and tumor cells that have downregulated their MHC antigens, and robustly secrete cytokines to recruit additional immune effectors. These features allow NKs and iNKTs to decrease tumor immunosuppression and overcome immune evasion in the HRNB TME (16). Thus, NK and iNKT therapies may prove useful in both stand-alone and combination treatments for NB and other pediatric solid tumors.

**BIOLOGY OF INKT CELLS**

**Overview and Relevance in Human Cancer**

Natural Killer T cells (NKTs) are innate-like lymphocytes that make up about 1% of total lymphocytes in the human liver, and also have residence in the spleen and bone marrow. They bridge the innate and adaptive immune systems, helping to coordinate robust responses to malignant or infected cells, and have demonstrated importance in tumor immunosurveillance (17–19). NKTs share features with both NKs and T cells, and are divided into type I, or invariant NKTs (iNKTs), that express a conserved T cell receptor (TCR) made up of an α chain composed of Vα24 and Jα18 segments paired with a β chain composed of the Vβ11 segment. Conversely, type II NKTs express polyclonal TCRs, similar to conventional CD4+ and CD8+ T cells. These invariant TCRs allow for the recognition of glycolipid antigens (GAgs) presented by a non-polymorphic and conserved MHC class 1-like protein called CD1d. Due to the availability of CD1d tetramers specific for the invariant TCR, the majority of the literature on NKTs has focused on the role of iNKTs in antitumor responses; we will similarly do so in this review.

The frequency of iNKTs within a tumor, or in circulation, has been associated with improved survival and reduced progression in various malignancies including prostate cancer, medulloblastoma, melanoma, multiple myeloma, colon cancer, lung cancer, breast cancer, head and neck squamous cell carcinomas (HNSCC), and NB (12, 20, 21). Additionally, lack of function in iNKTs is associated with advanced cancers and worse prognosis in patients with multiple myeloma, myelodysplastic syndrome, and prostate cancer (22–25). In xenograft models for NB, mice lacking iNKTs had more metastases and shortened survival in comparison to their iNKT-replete counterparts (26). Additionally, in mice lacking one allele of the tumor suppressor p53, absence of iNKTs predisposed to earlier development of a variety of cancers and decreased survival (27). Finally, for NB patients at the time of diagnosis, a high frequency of iNKTs in NB tumors was found to be associated with improved survival and lower stage NB (28). Given the apparent importance of iNKTs in tumor immunology, elucidating the various modes of iNKT activation, and activity is of great interest.

**iNKT Activation and Activity**

iNKTs can be activated in CD1d-dependent and -independent manners (Figure 1). As mentioned above, the iNKT TCR binds to GAg presented by CD1d proteins, which are expressed by most cells of hematopoietic origin including TAMs and MDSCs. Cells that are transformed or infected present immunogenic GAg s, or have changes in their actin cytoskeleton that create CD1d nanoclusters of high avidity, leading to iNKT activation (29–31). Independent of their CD1d-driven activation, iNKTs can also be activated through exposure to the cytokines IL-12 and IL-18 (32–34). Additionally, co-stimulatory signals from CD28, 4-1BB (CD137), NKG2D, CD40L, and ICOS (CD278) mediate robust iNKT activation (35–39). Dendritic cells (DCs) use cytokine release (IL-12) and cell-cell signaling with iNKTs through GAg-CD1d/TCR and CD40L/CD40 binding, and are of particular importance for iNKT activation. Notably, the production of cytokines IFN-γ and IL-12 by the activated iNKTs and DCs results in enhanced NK- and T cell mediated antitumor responses (12, 40–42).

When iNKTs are activated, they release large amounts of cytokines that mature, recruit, and activate other immune effector cells. Similar to conventional CD4+ T cells, iNKTs produce Th1-, Th2-, and Th17-like cytokines. Th-1 cytokines are associated with pro-inflammatory responses and enhanced antitumor cytotoxicity, whereas Th-2 cytokines promote immune tolerance and are useful in limiting autoimmunity (43). The avidity and stability of the CD1d-GAg complex
Influence the ratio of Th1:Th2 cytokines released (43–45). Furthermore, CD1d-GAg complexes with high affinity for the iNKT TCR have been shown to limit accumulation of immunosuppressive MDSCs in tumors when compared with lower affinity CD1d-GAg complexes. Higher affinity CD1d-GAg complexes also induce less iNKT anergy, which is defined by a lack of activation on repeated stimulation (45, 46). These observations illustrate the importance of the GAg in mediating iNKT stimulation (45, 46).

In addition to iNKT cytokine production, iNKTs can also recognize, kill, and reprogram CD1d+ cells including immunosuppressive TAMs and MDSCs. They mediate cytotoxic responses through release of perforin/granzyme B, or through upregulation of Fas ligand (FasL; CD178) and TNF-related apoptosis inducing ligand (TRAIL; CD253) (17, 42). iNKTs also produce granulocyte-macrophage colony stimulating factor (GM-CSF), which reprograms TAMs to display a pro-inflammatory (M1) phenotype (26, 47, 48). The ability to kill or reprogram TAMs is crucial, as expression of TAM-specific genes in the NB TME is associated with poor 5-year EFS (48). TAMs are not only immunosuppressive, but also promote neuroblast growth and increased osteoclastic activity associated with bone metastases through release of IL-6 (48, 49). Notably, iNKTs may be the only known effector cells that recognize and negatively regulate TAMs (50), iNKTs are also capable of culling IL-10 expression from immunosuppressive MDSCs, which can limit suppression of cytotoxic T cells, resulting in improved tumor control (51).

Anergy and Tumor Microenvironment

Immunosuppression of iNKT Cells

Despite their many favorable features, certain barriers face the use of iNKTs as cancer immunotherapeutics. One such limitation is that of anergy, a state in which iNKTs fail to produce cytokines, or proliferate after stimulation. Indeed, murine iNKTs have been shown to become anergic for periods of up to 6 months after a single exposure to α-GalactosylCeramide (α-GalCer), the highly potent canonical GAg used to stimulate iNKTs in numerous studies (46). iNKT anergy not only limited anti-tumor activity, but further stimulation of anergic iNKTs actually worsened tumor metastasis in a mouse model of melanoma (46).

Similar to conventional T cells, an additional barrier can be imposed by proteins expressed on neuroblasts, TAMs, MDSCs, and regulatory T cells (TREG). Proteins such as programmed cell death ligand-1 (PD-L1) binds to PD1 on iNKTs and other immune effectors to inhibit their cytotoxic function (52–57). Aside from expression of checkpoint ligands such as PD-L1, neuroblasts, and co-opted immune cells also release TGF-β1, IL-4, IL-6, IL-10, IL-13, adenosine, and prostaglandin E-2 to suppress infiltrating immune cells (48, 49). This immunosuppressive milieu can bias iNKTs toward Th2 cytokine release, thereby skewing the TME in an immunosuppressive direction.

These barriers have been targeted using strategies to reduce anergy and block checkpoint pathway signaling. Anergy has been shown to be inhibited by pulsing DCs with GAg, or loading soluble CD1d with synthetic GAgS modified for
greater CD1d/TCR affinity. Indeed, the C-glycoside analog of α-GalCer and phenylated GAggs activate and skew iNKTs toward Th1-cytokine secretion, thereby counteracting the immunosuppressive cytokines of the TME (45). Phenylated GAggs also limit the anergy experienced by iNKTs on repeated stimulation and result in less accumulation of MDSCs in the TME than α-GalCer, suggesting the advantage of their use for iNKT stimulation (45). Finally, the immunosuppressive effects of checkpoint receptor expression have been targeted by antibody-mediated blockade of PD-1/PD-L1 interactions; this blockade restored IFN-γ release and augmented anti-tumor activity of iNKTs (37, 54, 56–58).

In summary, iNKTs are associated with favorable prognosis in various human malignancies, likely due to their ability to secrete pro-inflammatory cytokines and culling and/or reprogramming of immunosuppressive and tumor-growth promoting cells in the TME. As such, therapies that minimize iNKT anergy and promote the release of Th1 over Th2 cytokines are likely to have greater antitumor efficacy. We will now discuss iNKT-based treatments for NB that have been studied to date.

**INKT CELL-BASED TREATMENTS OF NEOUROBLASTOMA**

There has been an accumulation of pre-clinical and early phase clinical data indicating that iNKT-based immunotherapies have promise in the treatment of NB. The approaches employing iNKTs for immunotherapy of NB include GAg stimulation of iNKTs, adoptive transfer of iNKTs, and CAR-iNKTs (Figure 2).

**GAg Stimulation of iNKT Cells**

GAg stimulation of iNKTs for antitumor therapy involves providing GAg alone or in the context of antigen-presenting cells (APCs) for promotion of antitumor immunity by endogenous

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**FIGURE 2** | INKT-based NB treatments. NB therapies using INKTs may employ administration of glycolipid antigens (GAg) or GAg-pulsed dendritic cells (A), adoptive transfer of previously stimulated INKTs (B), or CAR-modified INKTs (C), and combined with PD-1/PD-L1 inhibition (D).
iNKTs. This strategy has been used in patients with various solid tumors with modest, but encouraging results. For example, in a Phase I trial evaluating the effects of iNKT stimulation with α-GalCer in patients with advanced solid tumors, serum GM-CSF, and TNF-α were increased in 5 of 24 patients treated, albeit without disease response. Significantly, the patients that had a cytokine bump from the α-GalCer had higher levels of pre-treatment iNKTs, and there was no dose-limiting toxicity (59). Another strategy for GAg-stimulation of iNKTs involves pulsing DCs with α-GalCer, and administering these pulsed DCs intravenously. In various trials of patients with non-NB solid tumors, this strategy led to decreases in tumor markers, iNKT infiltration into tumors, and in some patients, tumor necrosis (60–62). In NB, iNKTs stimulated with CD1d-loaded with α-GalCer were shown to enhance NK-mediated killing of NB cell lines, but could not directly induce such killing when cell lines were CD1d−, as is characteristic of NB (63). However, in elegant co-culture studies, α-GalCer-activated iNKTs have been shown to enhance the NK antibody-dependent cellular cytotoxicity (ADCC) of NB cell lines when anti-GD2 antibodies were provided (64). In addition to enhancing NK-cell killing, iNKTs can reinvigorate exhausted CD8+ T cells. For instance, in melanoma, α-GalCer-pulsed APCs were administered to humanized mice xenografted with PD-1 inhibitor resistant melanoma. This restored cytotoxic activity of exhausted CD8+ T lymphocytes via IL-2 and IL-12 production, and resulted in reduced tumor progression and improved survival (65). iNKTs are therefore shown to be capable of enhancing an immune response to CD1d− tumors, such as NB, when activated with GAg or GAg-loaded APCs. However, given that many metastatic solid tumors including NB are associated with lower levels of iNKTs (limiting the potential benefits of mere iNKT activation for these patients), options for iNKT therapies that boost iNKT numbers and activation have been sought.

Adoptive Transfer of iNKT Cells

Adoptive transfer of iNKTs has been attempted in numerous pre-clinical and clinical studies in NB and other solid tumors. The importance of iNKTs in tumor immunity in NB was demonstrated in iNKT-deficient and iNKT-replete mice xenografted with NB, with the iNKT-replete mice developing significantly fewer metastases and having longer survival than iNKT-deficient mice (26). When iNKTs were adaptively transferred to humanized NSG mice with NB xenografts, TAMs were reprogrammed from M2 to the M1 phenotype. Despite this reprogramming, NB tumors progressed, and adoptive transfer of iNKTs resulted in increased PD-L1 expression on M1 and M2 TAMs (66). Given that iNKTs increase their PD1 expression on activation, there is reason to hypothesize that adjunctive use of PD1/PD-L1 inhibitors could prove useful in improving efficacy of iNKTs responses against NB. In addition to the data on adoptive transfer of iNKTs in NB, iNKT adoptive transfer has been shown to reduce liver metastases of melanoma in a mouse model and has also demonstrated disease responses in patients with HNSCC (67, 68). Taken together, these pre-clinical NB studies and clinical studies in other solid tumor patients suggest that the adoptive cell transfer of iNKTs may offer a therapeutic and complementary role in NB by targeting TAMs and enhancing or restoring NK- and T-cell cytotoxicity. However, clinical trials of adoptive transfer of unmodified iNKTs have not yet been performed in patients with NB.

CAR-iNKT Cells

CAR-modified iNKTs offer another area of great promise in the treatment of NB. GD2-specific CAR-iNKTs reduced the tumor volumes of xenografted CD1d− NB tumors in lymphocyte-deficient mice and prolonged survival (69). Additionally, in contrast to a comparison group in which these mice were treated with GD2-CAR T cells, CAR-iNKTs had significantly greater trafficking to NB tumors, and resulted in no graft vs. host disease (GVHD), while the CAR T cells showed liver and lung edema and lymphocytic infiltration consistent with GVHD (69). Although the reason for differences in GVHD between the CAR-iNKTs and CAR T cells is unknown, it is postulated that it may be due to the release of Th2-like cytokines by CD4+ CAR-iNKTs. Importantly, CAR-iNKTs retain both their ability to recognize CD1d/GAg complexes as well their cytotoxic activity against immunosuppressive TAMs (69). In a separate study, a subset of CAR-iNKTs that express CD62L were found to have five-fold longer persistence in host mice than CD662L− CAR-iNKTs (70). Artificial antigen presenting cells (aAPCs) were then created and used to enrich for CD62L+ iNKTs that were subsequently modified by CARs specific for GD2 and CD19 antigens. The CAR-iNKTs generated from CD62L+ enriched iNKTs were used in mice with NB and lymphoma, and demonstrated significantly longer in vivo persistence and therapeutic efficacy when compared with CAR-iNKTs generated without CD62L+ cell enrichment (70). These data provide an exciting new method for iNKT-CAR development that has not yet been tested clinically.

However, CAR-iNKTs are now being explored in a Phase I clinical trial (GINAKIT2 trial at Baylor) for patients with relapsed or refractory NB. This study aims to identify the maximum tolerated dose of CAR-iNKTs and involves the use of expanded autologous iNKTs modified with a GD2-CAR containing the IL-15 gene. This trial is currently recruiting and early results from two patients treated at the lowest dose level show that one patient’s disease was stabilized, while the other had a significant partial response without dose-limiting toxicity (71). The iNKTs used in this clinical study are derived from expanded human peripheral blood mononuclear cells that have not been enriched using the aAPCs discussed above (70).

The potential advantages of CAR-iNKTs over CAR-Ts in NB include their relatively higher NB tumor penetration and reduced incidence of GVHD in pre-clinical models. Additionally, retention of the TCR function on CAR-iNKTs allows for clearance of immunosuppressive TAMs that contribute to tumor growth and refractoriness to immunotherapies. Given that GD2 CAR T cells have been limited due to decreased persistence and tumor penetration in NB, CAR-iNKTs offer an exciting potential solution for overcoming this barrier (10).
BIOLOGY OF NK CELLS

NK Activation and Activity in Neuroblastoma

NK cells are innate lymphoid cells that represent 5–15% of circulating lymphocytes and are important effectors against virus-infected, foreign, and cancerous cells. Human NKs are divided into a predominantly cytotoxic group found mainly in the peripheral blood (CD56<sup>dim</sup>CD16<sup>+</sup>) and a predominantly immunomodulatory group found mainly in lymph nodes (CD56<sup>bright</sup>CD16<sup>-</sup>) (72–74). The rapid response of NKs to their targets is mediated by a network of activating and inhibitory cell surface receptors that allow NKs to distinguish healthy “self” cells from cancerous, infected, or foreign cells (13). There are several groups of NK surface receptors including killer cell immunoglobulin-like receptors (KIRs); the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46; natural killer group 2D receptors (NKG2D); the Nectin family of receptors including DNAM-1 and CD96; the SLAM family; and FcyRIIIa (CD16). Cytokines also play a role in NK activation, and, as will be described below, are used clinically for NK-based therapies in NB. When activated, NKs utilize perforin, granzyme B, TRAIL (CD253), and FasL (CD178) to mediate cellular cytotoxicity. NKs also release cytokines and chemokines including IFN-γ, TNF-α, GM-CSF, MIP1-α, RANTES, IL-6, and CCL5 thus attracting and activating monocytes, macrophage, T cells, DCs, and neutrophils to promote an anti-tumor response (13, 75). We will first focus on the influence of NK surface proteins on outcomes in NB, and then discuss specific NK-based NB therapies.

NK Receptors and Influence on Anti-tumor Activity

First, KIRs are a family of NK surface receptors that are largely inhibitory, and important in helping NKs to distinguish their targets from healthy cells. They are encoded by highly polymorphic germline genes, and recognize MHC-I as their ligand. When inhibitory KIRs are bound to matched MHC-I, they typically send inhibitory intracellular signals, thereby avoiding auto-reactivity. Importantly, when MHC-I is absent or downregulated (e.g., in cancerous, virus-infected, or transformed cells), NKs no longer receive inhibitory signaling from KIR-MHC-I binding, and are then positioned to become cytotoxic (13). Significantly, in NB, MHC-I downregulation is very common, resulting in a lack of KIR-mediated NK inhibition that allows NKs to recognize and kill these cancerous cells.

Interestingly, the type of KIRs expressed on NKs have been shown to affect clinical outcomes and predict response to immunotherapy with dinutuximab. A Children’s Oncology Group (COG) study, ANBL0032, evaluated the influence of KIR and KIR-ligand genotype on the response of NB to immunotherapy. This study found that patients with genes for inhibitory KIR and KIR ligands (KIR-L) had the greatest improvement in EFS and OS when treated with dinutuximab, IL-2, and GM-CSF, and that these patients were responsible for most of the benefit from immunotherapy seen in the trial (76). In fact, those without inhibitory KIR/KIR-L genes did not have a significant difference in EFS or OS between the dinutuximab-containing arm and the non-dinutuximab-containing comparator arm. This counterintuitive finding suggests that perhaps the inhibitory KIR/KIR-L interaction that would have led to reduced NK anti-tumor cytotoxicity was mitigated by the immunostimulation with dinutuximab, IL-2, and GM-CSF. Supporting this hypothesis, in the group not treated with dinutuximab, those with inhibitory KIR/KIR-L expression trended toward worsened survival compared with those without inhibitory KIR/KIR-L expression, suggesting that immunotherapy with dinutuximab and cytokines IL-2 and GM-CSF may have restored the antitumor activity of otherwise inhibited NKs (76). Interestingly, this same effect was not seen in prior studies that used 3F8 (a murine-derived GD2-directed monoclonal antibody) but did not consistently utilize IL-2 (77–79). The difference in cytokine exposure between these studies may be relevant, as IL-2 does increase expression of activating NK receptors NKp30, NKp44, and DNAM-1 on NKs, potentially shifting the balance toward NK activation (80). The baseline expression of activating NK receptors has been shown to be relevant in NB as well. In another study that compared progression-free-survival of HRNB patients with greater expression of an activating NKp30 isoform, NKp30B, with patients expressing an inhibitory NKp30 isoform, NKp30C, it was found that those with greater NKp30B relative to NKp30C expression had improved progression-free survival (81).

Another important factor in NK activation in the NB TME is the presence of NK-activating ligands on the NB cell surface (Figure 3). As previously mentioned, neuroblasts downregulate MHC-I allowing for NK recognition and activation. However, in NB, NKs also recognize activation signals from proteins on neuroblasts including polio-virus receptor (PVR) and NCR-ligands via their DNAM-1 and NCRs (82). Additionally, cancer stem cells (CSCs), which are often refractory to radiation and chemotherapy, express stress ligands recognizable by NKG2D with resultant NK-mediated cytotoxicity (83). Thus, for NKs in NB, the combination of downregulated MHC-I proteins and expression of NK receptor ligands provides an environment conducive to NK activation and killing of neuroblasts and CSCs. Furthermore, the influence of NK receptors on NB outcomes demonstrates the potential of NKs as therapeutic tools, and also represent an area for personalized medicine given the differential response to immunotherapies in the case of dinutuximab. The influence of NK receptors on clinical outcome has previously been shown in other cancer types as well (84–87).

Neuroblastoma TME and NK Cells

While there are numerous advantageous features of NKs in the NB TME, in this environment NKs can also be downregulated by T<sup>REG</sup>, MDSCs, TAMs, immature DCs, and neuroblasts (88–90). MYCN, a gene whose amplification is associated with HRNB, downregulates NB expression of ligands for NKG2D with resultant NK-mediated cytotoxicity (83). Thus, for NKs in NB, the combination of downregulated MHC-I proteins and expression of NK receptor ligands provides an environment conducive to NK activation and killing of neuroblasts and CSCs. Furthermore, the influence of NK receptors on NB outcomes demonstrates the potential of NKs as therapeutic tools, and also represent an area for personalized medicine given the differential response to immunotherapies in the case of dinutuximab. The influence of NK receptors on clinical outcome has previously been shown in other cancer types as well (84–87).
**Interactions of iNKTs, NKs, and the TME.**

iNKTs and NKs are inhibited through the release of TGF-β, IL-4, IL-6, IL-10, IL-13, adenosine, and PGE-2. IL-6 also stimulates the growth of neuroblasts and increases osteoclastic activity associated with metastatic potential. TGF-β reduces expression of the activating NK receptor, NKG2D, limiting NK activation. MYCN expressed in neuroblasts downregulates MHC-I expression and ligands for NK-activating receptors including DNAM-1 and NKG2D ligands, thereby promoting immune evasion. Galectin-3, HLA-G, and PD-L1 expression also contribute to immune evasion or downregulation in the TME.

Galectin-3 and HLA-G allow NB avoidance of immune surveillance. MYCN downregulates MHC-I, DNAM-1 ligands and NKG2D ligands.

*FIGURE 3* | Interactions of iNKTs, NKs, and the TME. iNKTs and NKs are inhibited through the release of TGF-β, IL-4, IL-6, IL-10, IL-13, adenosine, and PGE-2. IL-6 also stimulates the growth of neuroblasts and increases osteoclastic activity associated with metastatic potential. TGF-β reduces expression of the activating NK receptor, NKG2D, limiting NK activation. MYCN expressed in neuroblasts downregulates MHC-I expression and ligands for NK-activating receptors including DNAM-1 and NKG2D ligands, thereby promoting immune evasion. Galectin-3, HLA-G, and PD-L1 expression also contribute to immune evasion or downregulation in the TME.

Associated with increased NB cell proliferation and Schwannian stroma-poor tumors (93, 94). Neuroblasts themselves express B7-H3 which binds to an inhibitory NK receptor, limiting NK killing (82). NKs counteract the immunosuppressive solid TME via cytokine release, activation and/or maturation of other immune cells, and release of exosomal microRNAs (miRs). For example, miR-186 limits NB expression of MYCN, AURKA, TGF-βRI and TGF-βR2, to reduce TGF-β-mediated immune escape, restrict tumorigenesis, and enhance NB cytotoxicity (95).

Taken together, NKs are associated with an improved prognosis in NB and have advantageous features for use in NB immunotherapy. Since particular NK KIR genotypes and activating cell surface receptor expression patterns appear to confer improved prognosis and predict response to immunotherapy treatment, it will be vital to identify treatment options that favor activation and avoid inhibition triggered by known NK surface receptor pathways. We will now review available data regarding the use of NK-based therapies in NB.

**NK CELLS IN THE TREATMENT OF NEUROBLASTOMA**

**Soluble Factors Activating NK Cells in Neuroblastoma**

The most prominent example of immunotherapy for HRNB is dinutuximab, the first ever FDA-approved immunotherapy for NB (6) (Figure 4). Dinutuximab is a monoclonal antibody specific for GD2, and is believed to exert its effects at least in part via the binding of its Fc domain to NK Fc receptors (FcγRIIIa; CD16). Indeed, combination therapy with dinutuximab, IL-2, and GM-CSF resulted in augmented activation of NKs and...
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FIGURE 4 | NK-based treatments of neuroblastoma. Endogenous NKs can be activated against NB via anti-GD2 monoclonal antibodies (A). Conversely, CAR-modified NKs (B) or those expanded/stimulated with cytokines (C) can be adoptively transferred to disease bearing hosts. TGF-β inhibits NK anti-tumor function, and blockade helps to promote an anti-tumor response (D). TGF-β blockade can thus be used to reduce the inhibition of endogenous or adoptively-transferred NKs. The downregulation of MHC expression on neuroblasts allows for NK activation, as inhibitory killer immunoglobulin receptors (KIRs) are not ligated.

led to increased ADCC of GD2-expressing NB cells (96). The use of anti-GD2 antibody in HRNB improved 2-year event-free-survival and this therapy is now standard of care for HRNB patients (6). In the setting of relapsed/refractory disease, dinutuximab used with irinotecan and temozolomide resulted in disease response in >50% vs. 6% in a comparator arm with irinotecan and temozolomide, but without dinutuximab (7). Another method of activating NKs is the development of bi- and tri-specific killer cell engagers (BiKEs and TriKEs, respectively), which promote the simultaneous engagement of tumor associated antigens (TAAs) and activating receptors on NKs (97–99). Several BiKEs and TriKEs have been created using CD16 or NKG2D as the primary mediators to increase NK cytotoxicity in in vitro and in vivo models of multiple myeloma, Hodgkin lymphoma and AML (100–102). Recently, Gauthier et al. showed that TriKEs targeting both CD16 and NKP46, and a TAA showed augmented killing potency in both in vitro and in vivo models of solid and liquid tumors when compared to monoclonal antibodies against TAAs (103). However, the role of BiKEs and TriKEs in NB is yet to be delineated.

NK Adoptive Cellular Therapy

Adoptive NK therapy involves obtaining autologous or allogeneic peripheral blood or bone marrow-derived NKs and re-infusing these cells into a patient to mediate antitumor effects. Despite promising in vitro results for autologous NK therapy, only limited antitumor efficacy was observed in patients with metastatic solid tumors (104, 105). The lack of success in autologous NK therapy is thought to be due to a lack of KIR-mismatch. On the other hand, allogeneic adoptive transfer of NKs has demonstrated promising cytotoxic activity against several malignancies (106). In allogeneic adoptive NK transfer, peripheral blood or bone marrow of an HLA-matched or haploidentical donor is collected, depleted of T cells, and NKs are isolated, expanded and activated before adoptive transfer to a patient. In one study of adoptive transfer of allogeneic NKs
TABLE 1 | Ongoing clinical trials using NK cell-based therapies for neuroblastoma.

| Title                                                                 | Status                        | Phase | NCT number       | Sponsor                      | Study start date | Anticipated completion date |
|----------------------------------------------------------------------|-------------------------------|-------|------------------|------------------------------|------------------|-------------------------------|
| Haploidentical stem cell transplantation and NK cell therapy in patients with high-risk solid tumors | Active, not recruiting       | 2     | 01807466         | Samsung medical center        | 05/2013          | 6/2019                        |
| Phase II STIR trial: haploidentical transplant and donor NK cells for solid tumors | Recruiting                    | 2     | 02100891         | Monica thakar medical college of wisconsin | 03/2014          | 12/2021                       |
| Immunotherapy of neuroblastoma patients using a combination of anti-GD2 and NK cells | Recruiting                    | 1,2   | 03242603         | National university hospital, singapore | 10/2017          | 8/2020                        |
| NK cell infusions with irinotecan, temozolomide, and dinutuximab | Not yet recruiting            | 1,2   | 04211675         | Nationwide children’s hospital | 4/2020           | 12/2023                       |
| Immunotherapy of relapsed refractory neuroblastoma with expanded NK cells | Recruiting                    | 1     | 02573896         | New approaches to neuroblastoma therapy consortium | 11/2018          | 8/2022                        |
| Humanized anti-GD2 antibody Hu3F8 and allogeneic NK cells for high-risk neuroblastoma | Recruiting                    | 1     | 02650648         | Memorial sloan kettering cancer center | 01/2016          | 1/2021                        |
| Treatment of relapsed or refractory neuroblastoma with expanded haploidentical NK cells and Hu14.18-IL2 | Recruiting                    | 1     | 03209869         | University of wisconsin       | 3/2018           | 9/2021                        |

In AML patients, infusion of KIR-HLA-mismatched NKs to 10 AML patients with minimal residual disease (MRD) negative status, resulted in 100% EFS at 2 years (107). Additionally, in adult AML patients receiving allogeneic SCT, improved prognosis was observed when there was KIR-HLA mismatch (84, 86, 87).

In a mouse model of NB, transfer of activated NKs in combination with anti-GD2 antibody led to an increase in survival (108). Moreover, the combination of a TGF-β1 inhibitor (galunisertib), NK allogeneic infusion, and dinutuximab showed enhanced NK-mediated cytotoxicity in mice xenografted with NB cell lines or patient-derived NB (109). This effect was mediated by decreasing the immunosuppressive effect of TGF-β1 on NKs, which reversed the decreased expression of TRAIL, DNAM-1, NKG2D, and Nkp30 (109). Similar strategies have been employed in clinical trials (7). In one study, 35 patients with refractory or progressive HRNB were treated with allogeneic NKs and 3F8, a murine-derived antibody with higher binding affinity for GD2 than dinutuximab; this treatment resulted in an overall response in 28% of patients, stable disease in 49%, and progressive disease in 23% (110). The same group is currently conducting a clinical trial, NCT 02650648, evaluating the use of allogeneic NKs with cyclophosphamide conditioning, humanized 3F8 antibody, and IL-2 administration. A separate trial used parental NK infusions (with maximal KIR-recipient KIR-ligand mismatches), in combination with humanized ch14.18K322A, an anti-GD2 antibody with the same binding affinity as dinutuximab, along with GM-CSF, IL-2, and various chemotherapeutic agents in 13 patients with HRNB. This study demonstrated impressive results with 61.5% (8 out of 13 patients) having a complete or partial response, and 38.5% (5 out of 13 patients) having a stable disease (111). Finally, another ongoing trial, NCT03209869, is employing four cycles of chemotherapy followed by adoptive transfer of haploidentical NKs grown ex vivo, and subsequent administration of anti-GD2 antibody fused with IL-2. Results from these ongoing clinical trials should be very illuminating (Table 1).

**CAR-NK Cells**

Another promising NB treatment approach involves CAR-modified NKs, which can be manufactured using NKs derived from peripheral blood, umbilical cord blood, bone marrow, embryonic stem cells, induced pluripotent stem cells, or NK lines (NK-92). CAR-modified NKs retain their ability to recognize tumor cells that have down-regulated MHC-I, and induce a more muted cytokine release syndrome (CRS) and GVHD than CAR T cells (112–114). Since the initial report of a CAR-NK in 1995, in which retrovirally transduced human NKs were made to express CD4ζ, several other CAR-NKs have been generated (115, 116). These CAR-NKs target a variety of TAAAs including CD19, CD20, CD7, CD33, MUC-1, mesothelin, and HER-2, and demonstrate the ability to kill TAA-expressing targets. In a recent study, induced pluripotent stem cell-derived NKs transformed with mesothelin-directed CARs containing NK-specific activation domains from NKG2D, 2B4, and the CD3ζ domain, showed improved in vivo cytotoxicity, improved mouse survival, and fewer adverse events than CAR T cells in an ovarian cancer xenograft model (117). Additionally, the NKs transduced with the CAR containing NKG2D, 2B4, and the CD3ζ
activation domains had greater cytotoxicity and IFN-γ release than NKs transduced with CARs comprised of CD28, 4-1BB, and CD3ζ, which are more commonly employed for CAR T cell manufacturing (117). In the clinical domain, IL-15-expressing, CD19-directed CAR-NKs bearing an inducible caspase 9 suicide gene were created using retroviral transduction of NKs derived from human cord blood. These CD19-directed CAR-NKs were used to treat 11 patients with non-Hodgkin lymphoma or chronic lymphocytic leukemia in a Phase I/II clinical trial, with 7 out of 11 (64%) achieving a complete remission (116).

Various CAR-NKs have also been tested in NB. In one study, NK-92 cells were transduced with a CAR containing an anti-GD2 scFv along with CD3ζ domains; these GD2-CAR NKs demonstrated robust specific cytotoxicity against GD2+ neuroblasts and other GD2-expressing tumor cells (118). In another report, NKs were transduced with a CAR containing NKG2D, DAP10, and CD3ζ domains, and when used to against leukemia and sarcoma cell lines, mediated substantial tumor cytotoxicity (119). NKG2D CAR-NKs were also shown to be capable of cytotoxicity against MDSCs in the NB TME, which express high levels of NKG2D ligands (120). The elimination of MDSCs then allowed for increased infiltration and tumor cytotoxicity by GD2 CAR T cells infused after administration of the CAR-NKs (120). These observations illustrate the potential for complementary approaches using CAR-NKs and CAR T cells for solid tumor immunotherapy.

CHALLENGES FOR NK CELL-BASED THERAPIES

Despite the promise of NK-based therapies, several challenges still exist. These include limited in vivo proliferation and persistence of CAR NKs in vivo, and the immunosuppressive TME of solid tumors. Use of different cytokines such as IL-15 and IL-21 have been shown to result in better antitumor activity of NKs while avoiding the simultaneous survival and expansion of TREG that has been seen with the use of IL-2 (113, 121, 122). Moreover, as discussed above, the TME of solid tumors can express appreciable levels of TGFβ1 as well as checkpoint ligands (e.g., PD-L1); as such, the use of TGFβRI or PD-1 inhibitors may enhance NK efficacy (123–125). In addition, several obstacles need to be overcome to allow for the use of CAR-NKs in clinical practice. The optimal composition of CAR-NKs has not been identified yet; focus on this will result in CARs that promote improved effector function and persistence (102). In conclusion, future studies to refine and optimize CAR-NK manufacturing may allow for translation of the promising in vitro and in vivo results into clinically improved outcomes for NB patients, as such optimization and experimental refinement have done for CAR-T therapeutics.

CONCLUSION

iNKTs and NKs have unique and complementary features that hold promise in the treatment of NB and other solid tumors. In particular, the abilities of NKs and iNKTs to kill TAMs and MDSCs, mature dendritic cells, and robustly release proinflammatory cytokines to recruit and activate conventional T cells, make these cells powerful tools in the armamentarium for NB therapy. The ability of these cells to break down the barriers that have previously limited CAR T cell therapies in solid tumors (limited T cell persistence and potency, inability to kill tumors without target TAA or MHC-I expression, and an immunosuppressive TME), and promising pre-clinical data suggest great potential for iNKT and NK therapies in NB. The potential for therapeutic synergy with CAR T cells and checkpoint inhibitors is an exciting area of future study.

AUTHOR CONTRIBUTIONS

KM wrote the initial draft with initial edits and contributions by SK. KM prepared the figures with editorial input from HB. MH edited and oversaw focus of article and provided critical revisions. HB provided oversight of article construction and edited all interim drafts with critical revisions. All authors participated in the editing of the final document.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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