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Natural Killer Cell Recognition and Control of Epithelial Cancers

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Abstract: Natural killer (NK) cells possess an innate ability to recognize cancer and are key mediators of cytotoxic efficacy for anticancer antibodies. Recent advances in the ability to generate, qualify, and safely infuse NK cells have led to a wide variety of clinical trials in oncology. Although their efficacy is best established for liquid cancers, their potential application in solid cancers has received increased attention. Here, we provide general background across a disparate group of exemplary solid tumors for which there is evidence for an NK cell role, discuss NK cell recognition motifs specific to each and murine and human studies of each that are supportive of NK cell adoptive immunotherapy, and end with special considerations relevant to the solid tumor microenvironment.

Key Words: Carcinoma, natural killer cells, neuroepithelial cancer

(*Cancer J* 2022;28: 263–269)

Natural killer (NK) cells are large granular lymphocytes that make up a small proportion of peripheral blood leukocytes but serve an important role in immune recognition of cancers and viral infections. The anticancer role of NK cells has been most studied in hematologic malignancies, particularly in the setting of allogeneic hematopoietic stem cell transplantation, but interest has recently increased in adapting them to the solid tumor setting. The solid tumor setting, however, presents unique challenges for cellular therapies with respect to migration and homing to tumor sites, targeting of tumor cells, and survival in harsh solid tumor micro-environments.

Nonmelanoma Skin Cancers

Natural killer cell dysregulation has been implicated in the pathogenesis of nonmelanoma skin cancers, including basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (CSCC). Basal cell carcinoma is the most common malignancy worldwide, affecting approximately 2 million individuals in the United States each year.[1] Basal cell carcinoma has a low risk of metastasis but can be associated with significant tissue destruction in advanced cases; these outcomes are most common in high-risk histologic subtypes such as micronodular, morpheaform, and infiltrative BCC and in tumors with perineural invasion.[2] Although surgical treatment with excision or Mohs micrographic surgery is the mainstay of treatment, topical immunotherapy agents such as imiquimod and ingenol mebutate are useful in select cases.[3] The activity of NK cells can be suppressed through HLA binding to inhibitory killer-cell immunoglobulin-like receptors (KIRs), and activating KIR haplotypes have been associated with the development of multiple BCCs.[4,5] Furthermore, p53 is necessary for NK ligand upregulation, and p53 mutations have been associated with this activating KIR haplotype, as well as BCC pathogenesis.[4] Although isolated NK cell–based therapies have not yet been studied in the treatment of BCC, innate NK cells have been implicated in the local immune response that drives the effectiveness of the topical immunotherapy agents ingenol mebutate and imiquimod.[6,7]

The incidence of CSCC is also rising.[8] Although most CSCCs are curable by surgical intervention alone, a subset of tumors harbors high-risk features and is at an increased risk of adverse outcomes including local recurrence, regional and distant metastasis, and disease-specific death.[9] The immunogenicity of CSCC has been well established. First, immunosuppression imparts a significantly increased risk of CSCC development, with solid organ transplant recipients having up to 250 times the incidence compared with the general population; the CSCCs seen in this patient population are also more likely to display aggressive behavior, with a significantly higher risk of poor outcomes.[10] In addition, immune checkpoint inhibitors such as cemiplimab (anti–programmed cell death 1) have shown great promise in the treatment of advanced tumors, further evidence of the critical role of the immune system in CSCC pathogenesis.[11] Impaired NK function in particular has been shown to play a pivotal role in the development and progression of CSCC. Patients with functional NK T cell deficiency have higher rates of CSCC, independent of human papillomavirus infection.[12,13] Low levels of NK cells in the peripheral blood of renal transplant recipients have also been shown to be predictive of CSCC development.[14] Furthermore, cetuximab, an epidermal growth factor receptor inhibitor that is effective in the treatment of advanced CSCC cases, exerts its effect in part due to NK cell activation.[13,15] Given this collective evidence, it is hypothesized that NK cell activation may be a novel therapeutic target for CSCC.[16] Although NK cell therapy for CSCC has not yet been studied in humans, in mouse models, induced NK deficiency resulted in increased CSCC tumor growth, and adoptive transfer of purified NK cells decreased tumor size.[17,18]

Breast Carcinoma

Breast cancer has historically been considered a cold tumor and evades the immune system through a variety of strategies that decrease NK cell activation.[19,20] Evasion tactics used by breast tumors include downregulating the NK cell activating receptor

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The Cancer Journal, special theme issue on "Adoptive Cell Therapies for Solid Epithelial Cancers."

Conflicts of Interest and Source of Funding: The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

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ISSN: 1528-9117

NKG2D through increased release of its ligand, major histocompatibility complex (MHC) class 1–related MICA molecule from tumors,[21] overexpression of self-signals HLA-E and HLA-G that inhibit activation through binding to NKG2A,[22] and increased anti-inflammatory cytokines (transforming growth factor β [TGF-β], interleukin 10 [IL-10]) that lead to NK cell dysfunction.[19,20] Although NK cells play a role in all 3 main breast cancer subtypes (hormone receptor positive or HER2 positive or triple negative), the impact of NK-targeting approaches has been most commonly evaluated in HER2-positive breast cancer, where NK cell activation promotes antibody-dependent cellular cytotoxicity (ADCC)–induced tumor killing by monoclonal antibodies (such as trastuzumab, pertuzumab, or T-DM1). Antibody-dependent cellular cytotoxicity, in turn, results in the release of cytokines and chemokines and the direction of dendritic cells, macrophages, and T cells to the HER2-positive tumor.[19,23] Given that HER2-positive breast cancers account for approximately 20% of all breast tumors and the aggressiveness conferred by the expression of the HER2 oncogene,[24] there is a strong rationale for evaluating NK cell augmentation of these commonly used HER2-targeting antibodies.[25]

The KIR functional genotype has been suggested as a predictive biomarker for pathologic complete response in patients undergoing neoadjuvant HER2-based chemotherapy.[26] Several *in vitro* preclinical studies have demonstrated promising results with chimeric antigen receptor (CAR) HER2-CAR NK in HER2-expressing tumors,[27] as well as improved lysis of erbB2-expressing (HER2 gene), triple-negative breast cancer cell lines.[27–29] In addition, *in vitro* studies with epidermal growth factor receptor targeting CAR NK cells plus oncolytic herpes simplex virus in a triple-negative cell line have shown promise.[30]

Several cytokines that naturally stimulate NK cell activity including IL-2, IL-12, and IL-15 and TGF-β have been evaluated in the preclinical and clinical settings, and although several preclinical studies have been promising, clinical data have yet to pan out.[31,32] Interleukin 12 has been evaluated in combination with trastuzumab (Herceptin) (NCT00004074, trial completed; NCT00028535, trial completed), but data have yet to be published. There is specific interest in targeting TGF-β given its immunosuppressive role within the breast cancer tumor microenvironment (TME), including its impact on NK function.[33,34]

Several clinical trials have evaluated donor NK cells plus IL-2 in advanced breast cancer, but there has been limited activity to date (NCT00376805, trial terminated early because of toxicity; NCT01105650, trial completed, no published results). Because HER2 is also expressed on other solid tumors including ovarian, esophageal, bladder cancer, and gastric cancers,[35] autologous NK cells combined with trastuzumab in HER2-expressing solid tumors have been evaluated in the clinical setting and produced preliminary signs of efficacy.[36] Another promising agent is DF1001, a small molecule that targets NK cells and T-cell activation signals in HER2-expressing tumors. DF1001 is being evaluated in HER2-expressing tumors in a phase I/II clinical trial (NCT04142711, ongoing clinical trial) as part of the Dragonfly Therapeutics TriNKET platform.

Pediatric Mesenchymal Tumors

A majority of the non–central nervous system, nonhematologic solid tumors in pediatrics are of mesenchymal origin, so tumors of epithelial origin are relatively rare in this age group. However, atypical solid tumors of neuroepithelial, neuroectodermal, embryonal, or undifferentiated origin (e.g., melanoma, neuroblastoma, primitive neuroectodermal tumors (PNETs), germ-cell tumors, Ewing sarcoma [EWS]) are relatively frequent. These tumors share epithelial-like features and frequently show sensitivity to NK cell recognition and lysis. Treatment for these pediatric tumors is typically multimodal, combining cytotoxic chemotherapy, radiation, and surgery. The adverse effects of these treatments frequently persist into adulthood with long-term negative health outcomes, so the addition of effective cell therapy holds the promise of maximizing targeted cytotoxicity while minimizing adverse effects, particularly for relapsed disease. Natural killer cells are an attractive treatment option for this group for several reasons.

Low MHC-I expression has been reported for many of these tumors (particularly neuroblastoma, EWS,[37] and poorly differentiated rhabdomyosarcomas [RMSs][38]) and is associated with high-risk patients and disease progression. Although this corresponds to escape from adaptive immune recognition, pediatric tumors with low MHC-I are an ideal target for NK cell adoptive therapies because these target cells cannot deliver an inhibitory signal through KIR or NKG2A and are therefore highly susceptible to NK cell lysis as defined in the "missing-self" hypothesis.[39] The NKG2 family, CD16/FcγRIIIA, DNAM-1, and natural cytotoxicity receptors NKp30, NKp44, and NKp46 are crucial receptors for NK cell–mediated targeting of low MHC-I tumors, and these receptors have been targeted in preclinical studies of pediatric solid tumors. NKp30 and NKp46 have specifically been implicated in NK cell–mediated cell lysis of neuroblastoma cell lines.[40] Importantly, NK cells have been shown to eliminate metastases via NKp46.[41]

However, NK cells have been shown to be largely reduced or have impaired activity in many newly diagnosed oncology patients.[42,43] In osteosarcoma, for example, NK cells show intact functionality and interferon γ production but low numbers,[44] and early recovery of lymphocytes after chemotherapy is associated with improved survival.[45] These findings suggest that NK cells are critical to survival in solid tumor patients and suggest that NK cell infusions may be a viable treatment option to restore number and function of endogenous NK cells.

Whereas autologous hematopoietic stem cell transplantation is commonly used in neuroblastoma, allogeneic hematopoietic stem cell transplantation has the added potential benefit of the graft-versus-tumor (GVT) effect. Specifically, haploidentical transplants offer a unique opportunity for intentional mismatch of donor NK cells (with respect to HLA licensing of inhibitory KIR) against HLA molecules present on the tumor, enhancing the GVT effect.[46] As GVHD is T-cell mediated, NK cells present a viable treatment option that minimizes GVHD while maintaining GVT effect.[47,48] Several studies utilizing manipulated haploidentical transplant have been performed in patients with relapsed solid tumors, including with desmoplastic small round cell tumor, neuroblastoma, RMS, EWS, and PNET. Survival in this heavily pretreated population was 6 to 14 months[49,50] and demonstrated feasibility of NK cell–enriched haploidentical transplants, but also showed the need for further immunomodulation and refinement with combination trials such as those as outlined in Table 1.

As mentioned previously, the graft-versus-tumor effect of allogeneic NK cells may be maximized by selecting for KIR mismatch.[51] Although the benefit of KIR-mismatched NK cells in leukemias is relatively well documented,[52–56] more clinical and preclinical studies are needed to demonstrate the benefit of KIR mismatch in pediatric solid tumors. Delgado et al.[57] found that osteosarcoma cell lines were most sensitive to allogeneic NK cell lysis when KIR receptor-ligand incompatibility was maximized. In addition, activating KIR gene content has been associated with increased NK potency against a variety of pediatric tumors including EWS, RMS, neuroblastoma, lymphoma, leukemia, and brain tumors.[58]

Preclinical models *in vitro* and in mouse models have consistently demonstrated the importance of IL-2, IL-15, and IL-21 for NK cell activation.[59–61] We developed an NK cell expansion system utilizing membrane-bound IL21 that resolves some of the obstacles faced with NK cell clinical trials, enabling sufficient cell

TABLE 1. Clinical Trials of NK Cell Adoptive Immunotherapy Listed on ClinicalTrials.gov

Clinical Trial No.	Phase	Title	Sponsor Institution	Status	Condition Treated
NCT00582816	1	Haploidentical Transplant With NK Cell Infusion for Pediatric Acute Leukemia and Solid Tumors	University of Wisconsin, Madison	Terminated (toxicity)	Relapsed/refractory leukemia or solid tumors
NCT01875601	1	NK White Blood Cells and Interleukin in Children and Young Adults With Advanced Solid Tumors	National Cancer Institute	Completed	Relapsed/refractory solid tumors
NCT00640796	1	Pilot Study of Expanded, Donor Natural Killer Cell Infusions for Refractory Non-B Lineage Hematologic Malignancies and Solid Tumors	St. Jude Children's Research Hospital	Completed	Relapsed/refractory hematologic malignancies, EWS family of tumors (ESFT) and RMS
NCT02130869	1	A Pilot Study of Immunotherapy Including Haploidentical NK Cell Infusion Following $CD133^{+}$ Positively-Selected Autologous Hematopoietic Stem Cells in Children With High Risk Solid Tumors or Lymphomas	St. Jude Children's Research Hospital	Completed	Relapsed/refractory neuroblastoma, lymphoma, high-risk solid tumor
NCT02100891	2	Phase 2 STIR Trial: Haploidentical Transplant and Donor Natural Killer Cells for Solid Tumors (STIR)	Medical College of Wisconsin	Active, not recruiting	Relapsed/refractory neuroblastoma, EWS, RMS, osteosarcoma, central nervous system tumors
NCT02508038	1	Alpha/Beta $CD19^{+}$ Depleted Haploidentical Transplantation + Zometa for Pediatric Hematologic Malignancies and Solid Tumors	University of Wisconsin, Madison	Recruiting	Relapsed/refractory leukemia, lymphoma, neuroblastoma, EWS, RMS, osteosarcoma, PNET
NCT01576692	1	Combination Chemotherapy, Monoclonal Antibody, and Natural Killer Cells in Treating Young Patients With Recurrent or Refractory Neuroblastoma	St. Jude Children's Research Hospital	Completed	Relapsed/refractory neuroblastoma
NCT03209869	1	Treatment of Relapsed or Refractory Neuroblastoma and Osteosarcoma With Expanded Haploidentical NK Cells and Hu14.18-IL2	University of Wisconsin, Madison	Suspended (COVID)	Relapsed/refractory neuroblastoma and osteosarcoma
NCT00877110	1	Anti-GD2 3F8 Antibody and Allogeneic Natural Killer Cells for High-Risk Neuroblastoma	Memorial Sloan Kettering Cancer Center	Completed	Relapsed/refractory neuroblastoma
NCT02650648	1	Humanized Anti-GD2 Antibody Hu3F8 and Allogeneic Natural Killer Cells for High-Risk Neuroblastoma	Memorial Sloan Kettering Cancer Center	Active, not recruiting	Relapsed/refractory neuroblastoma
NCT02573896	1	Immunotherapy of Relapsed Refractory Neuroblastoma With Expanded NK Cells	New Approaches to Neuroblastoma Therapy Consortium	Recruiting	Relapsed/refractory neuroblastoma
NCT00698009	1	Haploidentical Natural Killer (NK) Cells in Patients With Relapsed or Refractory Neuroblastoma	MD Anderson Cancer Center	Terminated (slow accrual)	Relapsed/refractory neuroblastoma
NCT03294954	1	GD2 Specific CAR and Interleukin-15 Expressing Autologous NKT Cells to Treat Children With Neuroblastoma (GINAKIT2)	Texas Children's Hospital	Recruiting	Relapsed/refractory neuroblastoma
NCT01287104	1	A Phase I Study of NK Cell Infusion Following Allogeneic Peripheral Blood Stem Cell Transplantation From Related or Matched Unrelated Donors in Pediatric Patients With Solid Tumors and Leukemias	National Cancer Institute	Completed	Relapsed/refractory leukemia or solid tumors

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TABLE 1. *(Continued)*

Clinical Trial No.	Phase	Title	Sponsor Institution	Status	Condition Treated
NCT03420963	1	Donor Natural Killer Cells, Cyclophosphamide, and Etoposide in Treating Children and Young Adults With Relapsed or Refractory Solid Tumors	MD Anderson	Recruiting	Relapsed/refractory solid tumors
NCT04211675	1 and 2	A Phase I–II Study of *Ex-Vivo* Expanded Autologous NK Cells Infusions in Combination With Irinotecan, Temozolomide, and Dinutuximab in Patients With Relapsed or Refractory Neuroblastoma: The STING Trial	Nationwide Children's Hospital	Not yet recruiting	Relapsed/refractory neuroblastoma
NCT01857934	2	Therapy for Children With Advanced Stage Neuroblastoma	St. Jude Children's Research Hospital	Active, not recruiting	Newly diagnosed high-risk neuroblastoma

numbers for adoptive transfer of multiple doses, functional activation/priming, and successful cryopreservation.[62,63]

Among pediatric tumors, neuroblastoma has been the most studied target for NK cell immunotherapy. GD2 is a ganglioside expressed throughout the central nervous system and is found on human neural stem cells, mesenchymal stem cells, peripheral nerve cells, and melanocytes, and expression is increased in many solid tumors, including neuroblastoma, osteosarcoma, soft tissue sarcomas, and desmoplastic small round cell tumor.[64–66] Anti-GD2 monoclonal antibodies, including dinutuximab and naxitamab, kill tumor cells in an NK cell–dependent fashion by ADCC and NK cell–independent fashion by complement-mediated cytotoxicity. These antitumor effects are augmented by coadministration with cytokines such as granulocyte-macrophage colony-stimulating factor and IL-2.[67] Combination therapy with NK cells may be a strategy to augment ADCC and is being explored in several clinical trials.

Combination therapy with NK cells and anti-GD2 antibodies has been used in several clinical trials for neuroblastoma. In a pilot study, NK cells derived from parents were administered with the Fc-modified anti-GD2 antibody hu14.18K322A after cycles 2, 4, and 6 of chemotherapy in 13 heavily treated patients with relapsed/refractory neuroblastoma. The overall response rate was 61.5% (4 complete responses, 1 very good partial response, and 3 partial responses), and 5 had a stable disease, with 77% overall survival at 1 year.[65] A similar phase I trial combined the anti-GD2 antibody 3F8 with haploidentical NK cells administered after lymphodepleting chemotherapy in 35 patients.[6] Improved event-free survival was seen in patients receiving more than 10^7 NK cells.[29] These suggest that NK cells can be safely combined with anti-GD2 antibodies with preliminary evidence of antineuroblastoma effects, especially at higher NK cell doses. A clinical trial administering expanded autologous NK cells (to increase the cell dose) with dinutuximab (NCT02573896) and another delivering haploidentical NK cells along with an antibody-cytokine fusion protein (Hu14.18-IL-2) to increase *in vivo* NK cell proliferation are underway for pediatric patients with relapsed neuroblastoma (NCT03209869).

Clinical trials are underway administering NK cells (NCT02839954) or NK cell lines (NCT03656705 and NCT03383978) expressing CARs targeting solid tumor antigens on pediatric cancers, but the trials have so far been limited to adult participants, and results have not yet been reported.

Natural killer cells have been explored for treatment in osteosarcoma, both in human and in canine models.[68] Canine osteosarcoma has long been used as a model for pediatric osteosarcoma, given the similar biology, response to therapy, and even size of the patients.[69] Preliminary human *in vitro* studies demonstrated that NK cells lyse osteosarcoma cell lines in a manner dependent on NKG2D receptor-ligand interaction; this process also demonstrated effectiveness against multiple subpopulations of osteosarcoma cells, including those most responsible for recurrence.[70] Autologous expanded NK cells were given via intralesional injection in dogs not undergoing amputation (standard of care), supplemented with intravenous IL-2 treatment and palliative radiation. Four dogs were killed for progressive disease, and 2 died of unrelated causes. Four dogs (40%) remained alive at 18 months.[71,72] This use of such outbred comparative oncology models of NK cell immunotherapy is useful for translation into pediatric clinical trials.

In addition to neuroblastoma and osteosarcoma, EWS and RMS have shown *in vitro* susceptibility to NK cells. Ray et al.[58] showed the importance of activating KIR content for enhanced NK cell cytolytic activity against pediatric cancer targets including RMS and ES. Natural killer cell function is also enhanced by cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Interleukin 15 is the most commonly studied cytokine for enhancing NK cell propagation, survival, and cytolytic function,[60] and RMS,

EWS, and osteosarcoma cell lines are susceptible, particularly after IL-15, to allogeneic NK cells.[43,59,73,74] Insulinlike growth factor receptor 1 is important for EWS development[75] that can be targeted by antibodies that have activity against EWS,[76] and targeting shows improved activity in combination therapy with NK cells.[77]

Addressing the Solid Tumor Microenvironment

Tumors induce an environment that is favorable to tumor cell growth and resistant to immune responses, known as the TME. In the course of rapid tumor growth that outstrips vascular supply, the TME has progressively restricted access to both glucose and oxygen,[78] creating a nutrient-poor and hypoxic environment. Malignant cells are able to survive and drive metastatic spread under glucose deprivation.[79,80] In soft tissue sarcomas, hypoxia is closely linked to relapse, and the 5-year overall survival is approximately 50% in patients with high-risk disease.[81,82] Hypoxia-inducible factors (HIFs) are the main transcriptional regulators in response to hypoxia, with an oxygen-regulated α subunit (HIF-1α or HIF-2α) that dimerizes with HIF-1β under hypoxia. Hypoxia-inducible factor 1α was previously described as a regulator of TGF-β–SMAD3 signaling in breast cancer patients.[83] In addition to upregulating TGF-β, hypoxia is also closely linked to regulation of other immune-suppressive pathways such as indoleamine-2,3-dioxygenase (IDO), tryptophan-2,3-dioxygenase (TDO).[83–85] Targeting these hypoxic conditions improves immune responses and reduces the risk of lung metastasis in experimental sarcoma models.[86–88]

The TME can also induce metabolic reprograming of the tumor-infiltrating immune cells themselves, in part due to their large energy consumption rate. Cellular metabolism is necessary to provide the energy needed for critical functions of antitumor activity and is necessary to maintain homeostasis such as redox balance.

Transforming growth factor β is a potent immune suppressor of NK cell antitumor function and is an abundant cytokine present in the TME that regulates cell proliferation, carcinogenesis, and angiogenesis[89–92] and is linked to chemotherapy and radiation resistance,[93] in addition to immune suppression. Our group developed a unique platform expanding NK cells in the presence of TGF-β that induces epigenetic changes that result in TGF-β resistance through downregulation of SMAD3. Interestingly, the presence of TGF-β does not impact NK cell proliferation and paradoxically enhances production of the inflammatory cytokines interferon γ, tumor necrosis factor α, and granulocyte-macrophage colony-stimulating factor.[34] We are developing pediatric and adult clinical trials of these TGF-β–resistant NK cells in neuroblastoma, brain tumors, sarcomas, melanoma, and breast carcinoma.

Together, IDO and TDO promote metabolism of tryptophan into kynurenine (Kyn) and kynurenic acid (KA); IDO and TDO are commonly upregulated in tumors, and the resulting Kyn/KA are highly inhibitory to antitumor immunity in several models.[94,95] Kyn/KA exert their action on NK cells through binding to the aryl hydrocarbon receptor (AHR), resulting in immune suppression and tolerance.[96–98] Activation of AHR pathway by these ligands can also promote selective expansion of regulatory T cells. Natural killer cell function is impaired by inducing a stress response that, in turn, perturbs cellular homeostasis.[99,100] For this reason, AHR antagonists have been developed to restore NK cell function.

SUMMARY

Natural killer cells play an important antitumor role across a wide range of epithelial and neuroepithelial cancers but are often suppressed and dysfunctional in patients with these advanced cancers. Recent progress in NK cell manufacturing at clinical grade and scale and numerous trials showing a high safety profile give promise for adoptive immunotherapy for these cancers.

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