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Review

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Killers 2.0: NK cell therapies at the forefront of cancer control

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Natural killer (NK) cells are innate cytotoxic lymphocytes involved in the surveillance and elimination of cancer. As we have learned more and more about the mechanisms NK cells employ to recognize and eliminate tumor cells, and how, in turn, cancer evades NK cell responses, we have gained a clear appreciation that NK cells can be harnessed in cancer immunotherapy. Here, we review the evidence for NK cells' critical role in combating transformed and malignant cells, and how cancer immunotherapies potentiate NK cell responses for therapeutic purposes. We highlight cutting-edge immunotherapeutic strategies in preclinical and clinical development such as adoptive NK cell transfer, chimeric antigen receptor–expressing NK cells (CAR-NKs), bispecific and trispecific killer cell engagers (BiKEs and TriKEs), checkpoint blockade, and oncolytic virotherapy. Further, we describe the challenges that NK cells face (e.g., postsurgical dysfunction) that must be overcome by these therapeutic modalities to achieve cancer clearance.

NK cells: sentinels against cancer

The existence of immune cells that mediate cellular cytotoxicity without prior activation was determined by multiple groups who reported the spontaneous killing of tumor cells by lymphocytes from unimmunized mice (1–3). We now know that these cells with natural cytotoxicity, or natural killer (NK) cells, are important mediators of cancer immunosurveillance. NK cells are a heterogeneous population, and in humans they have been historically divided into IFN-γ–producing $CD56^{hi}CD16^{+}$ and cytotoxic $CD56^{lo}CD16^{hi}$ (4), whereas in mice they are grouped according to their expression of CD27 and CD11b (5), although it is now clear that the complexity is much higher. Distinct NK cell subsets play different roles in tumor immunity and cancer immunotherapy, as reviewed in Stabile et al. (6).

NK cells are equipped with many receptors that tightly regulate their activation and allow them to discriminate between "normal" and "dangerous" cells (7). In addition to regulating NK cell activation, signals coming from activating and inhibitory receptors also tune the steady-state responsiveness of NK cells to future stimuli, in a process called NK cell education (reviewed in refs. 8, 9). Inhibitory receptors, such as killer-cell immunoglobulin-like receptors (KIRs), deliver negative signals that prevent NK cell autoreactivity. KIRs and other inhibitory receptors recognize MHC I molecules, whose absence may result in NK activation, the so-called "missing-self recognition" (10, 11). Later studies showed that lack of MHC expression was not sufficient or necessary to induce NK activation; rather, signaling from activating receptors was required. Broadly speaking, activating receptors, including NKG2D, provide activating signals upon binding to stress-induced ligands on target cells, which is referred to as "induced-self recognition" (12, 13). Ultimately, NK activation depends on the balance between activating and inhibitory signals triggered by these receptors binding their ligands. When activating signals prevail, NK cells respond, whereas when inhibitory signaling is stronger, NK cells do not respond. Healthy cells, with some exceptions (14–16), express low levels of activating ligands and an abundance of inhibitory ligands and therefore are not attacked by NK cells. On the other hand, tumor cells often acquire expression of NK cell–activating ligands and/or lose expression of MHC molecules. NK cells sense and respond to changes in the repertoire of molecules expressed on the surface of healthy cells during cellular transformation. This positions NK cells as important sentinels against cancer and as prime targets for cancer immunotherapy (17).

NK cells in cancer immunosurveillance

Despite their potent antitumor activity, NK cells face substantial challenges that hinder their efficacy. Several studies have shown that tumor-infiltrating human NK cells have altered expression of inhibitory and activating receptors and impaired functions (18–20). Many mechanisms mediate NK cell suppression in the tumor microenvironment, several of which also contribute to dampening of T cell responses. Reviewing these mechanisms is beyond the scope of this work, and has been done elsewhere (17). However, one NK cell–regulating process that has attracted much attention is the release of soluble NKG2D ligands. NKG2D ligand release occurs either by shedding, which is mediated by extracellular proteases, or by exosomal secretion (21, 22). Soluble NKG2D ligands engage NKG2D on NK cells, preventing their interaction with membrane-bound ligands on tumor cells that would produce a cytotoxic response (22). Therapeutic targeting of NKG2D-ligand

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shedding proved successful in preclinical studies (23). However, soluble NKG2D ligands have also been shown to promote NK cell antitumor activity, as in the case of soluble MULT1, which prevented NK cell desensitization in mouse models of cancer (24). These results suggest a context-dependent function of these soluble molecules and warrant more investigation.

The tumor microenvironment contains large amounts of immunosuppressive cytokines and other soluble factors that affect NK cell functionality, with one of the most prominent being TGF-β (25). In addition to inducing downregulation of surface NKG2D, resulting in decreased cytotoxicity (26), TGF-β has been shown to be able to alter cytotoxicity, cytokine production, metabolism, and mitochondrial function in NK cells (27–29). Recent studies proposed that TGF-β also converts NK cells into noncytotoxic group 1 innate lymphoid cells (ILCs), allowing for tumor growth and metastasis in mice (30, 31).

Despite the immunosuppressive environment of solid tumors, NK cell activity/infiltration has been correlated with improved prognoses in humans. Rate of local recurrence following surgical tumor resection of colorectal cancer correlated with lower NK cell levels (32). Correlations between reduced NK cytotoxicity and incidence of metastasis have been established in head and neck as well as pharyngeal cancer (33–35). In gastrointestinal sarcoma, NK cell infiltration negatively correlates with metastases (20). Additionally, improved survival has been correlated with NK infiltration in lung metastases of renal cell carcinoma patients (36). These examples highlight the potential for NK cell immunotherapies to improve patient outcomes.

NK cells in conventional cancer treatments

The longstanding anticancer strategies chemotherapy and radiotherapy are now known to mediate their effects, at least partially, via the immune system. Both chemo- and radiotherapy induce cellular stress in tumor cells, leading to upregulation of NK-activating ligands, release of damage-associated molecular patterns (DAMPs), and induction of immunogenic cell death (37–39).

Through different mechanisms, genotoxic agents, HSP90 inhibitors, histone deacetylase (HDAC) inhibitors, glycogen synthase kinase 3 (GSK-3) inhibitors, and proteasome inhibitors can all increase tumor surface expression of NK-activating ligands (40–43). Several chemotherapeutics downregulate the NK inhibitory ligands MHC I and Clr-b on tumors to promote missing self recognition (44, 45). Effects on NK recruitment and activation were also observed with several chemotherapeutics. For example, in mouse models, successful tumor clearance following treatment with DNA-alkylating agents required recruitment of neutrophils and NK cells (46). Recently, MAPK and CDK4/6 inhibitors were shown to promote NK-mediated tumor clearance (47). DNA damage induced by ionizing radiation has effects that are similar to those of chemotherapeutics. DNA damage from high-dose radiation and chemotherapy both led to increased expression of NKG2D ligands through an ATM- and ATR-dependent pathway (48). The exact mechanisms of action induced by each chemo- and radiotherapeutic agent discussed here are unique, and newer pathways are constantly being targeted to enhance responses, which led to a renewed interest in exploiting chemotherapy and radiotherapy as immune-modulating modalities.

Surgical resection is still the predominant curative treatment modality for many solid malignancies, but, surprisingly, the immune-modulatory effects of surgery have been understudied. Increased metastatic disease or recurrence following surgery has been widely observed in humans and recapitulated in animal models (49). In addition to unintentional mechanical dissemination and altered proliferation and signaling in tumor cells, it is now clear that surgery compromises NK cell functions, providing an opportunity for tumor spread and growth (49–54). Several mechanisms contribute to NK cell dysfunction following surgery, including soluble inflammatory mediators and immunomodulatory cells such as myeloid-derived suppressor cells (MDSCs), which arise by emergency myelopoiesis following surgery (49–51).

In conclusion, NK cells play a fundamental role in traditional cancer treatments, and further research is needed to ameliorate their efficacy following chemo/radiotherapy as well as surgery.

Adoptive NK cell therapy

Allogeneic NK cells. One of the most striking examples of the anticancer functions of NK cells comes from missing self recognition. Hematopoietic stem cell transplantation (HSCT) is an effective and curative treatment option for acute leukemia patients. Allogeneic HSCT relies on HLA matching between donor and recipient to avoid graft-versus-host disease (GVHD). In the absence of an HLA-compatible donor for allogeneic HSCT, HLA-haploidentical HSCT, whereby the recipient shares only one HLA haplotype with the donor (often a parent), is performed. A series of pioneering studies showed that in haplo-HSCT, recipients whose HLA molecules were mismatched with donor KIRs had less relapse after transplant, indicating a potent NK-mediated graft-versus-leukemia (GvL) effect, whereas KIR mismatch was not found to cause GVHD (55–57). The contribution of NK cells in HSCT has been comprehensively reviewed previously (58, 59), with discussions on how to select recipient-donor pairs in order to enhance NK cell alloreactivity and transplant outcomes that are currently ongoing (9, 60). While T cells play a critical role in the efficacy of HSCT, this example highlights the often-overlooked contribution of NK cells to antitumor immunity.

The impressive GvL effect generated by KIR-mismatched NK cells in haplo-HSCT spurred hematologists to explore infusions of highly purified haplo-identical NK cells to increase GvL. Clinical trials reported complete remissions in elderly acute myeloid leukemia (AML) patients (61, 62), as well as 100% event-free survival in a pediatric AML cohort with 18 months follow-up (63). In multiple myeloma (MM), encouraging results from a phase I trial where patients received cord blood–derived KIR-mismatched NK cells prior to HSCT (64) led to an ongoing phase II study (NCT01729091).

Autologous NK cells. Autologous NK cells have also been explored for cancer immunotherapy, although this field is less advanced than for autologous T cell transfer. While NK cells can be isolated and ex vivo expanded from the peripheral blood of patients, NK expansion has proven more troublesome than T cell expansion. Clinical trials have not observed clinical responses with autologous NK cell infusion, despite successful NK engraftment and persistence in peripheral blood (65, 66). However, the functional status and expansion of the autologous NK cells is

often poor (67). This could be due to the treatments received by the patients before NK isolation, which may also explain their poor clinical efficacy. Multiple approaches are being investigated to overcome this issue, including different combinations of activating cytokines (IL-2, IL-12, IL-15, IL-18) and the use of feeder cells to supply important factors during ex vivo expansion (17). To this end, a phase I trial in MM using autologous NK cells activated by a feeder cell line expressing membrane-bound IL-15 and 4-1BBL resulted in modest clinical activity (68), which suggested it may be possible to optimize feeder cells to improve NK activation before adoptive transfer (69). Additionally, studies have shown that autologous NK cells are more effective when tumor cells lack at least one HLA ligand for the KIR expressed by the transferred NK cells ("missing ligand" hypothesis) (70, 71).

Off-the-shelf NK cells. Given the difficulties of sourcing abundant numbers of cytotoxic NK cells from peripheral blood, additional strategies have been investigated to provide readily available banks of NK cells for patients. The human cell line NK92, widely used for preclinical applications, has been clinically investigated as an allogeneic NK therapeutic. One clinical trial involving 15 advanced lung cancer patients observed encouraging responses (72), but clearly much research is needed to carefully validate the safety profile of NK92 cells as a cancer therapeutic.

NK cells can be differentiated from stem cells, both induced pluripotent stem cells (iPSCs) and those obtained from umbilical cord blood. iPSC-derived NK cells have been shown to have high cytotoxicity against tumors of various origin, both in vitro and in vivo (73–75) and clinical trials have commenced using expanded cord blood–derived NK cells. More recently, an effort to derive NK cells from iPSCs generated from peripheral blood cells has been made. NK cells derived from peripheral blood iPSCs show low KIR expression and a promising capacity to perform both cellular cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) against cancer cell lines in vitro (76). While these findings await in vivo corroboration, peripheral blood represents an attractive alternative source of iPSCs, as cord blood is still rare and difficult to recover.

CAR-NK cells. A promising avenue in adoptive NK therapy is the use of chimeric antigen receptors (CARs). A CAR, usually encoded in a lentiviral construct, consists of three main domains: an extracellular antigen-targeting domain (ectodomain), a transmembrane region, and one or more intracellular signaling domains. Specificity for targets is conferred by the ectodomain, which is reactive against a tumor-specific or tumor-associated antigen (e.g., CD19, CD20, CD22, Her2, ROR1) (77). CAR-T cells have shown remarkable responses particularly in B cell malignancies, which led to FDA approval in non-Hodgkin lymphoma and diffuse large B cell lymphoma (78, 79).

Given the success of CAR-T cells, CARs are now being used to potentiate NK antitumor activity. Currently, there are 14 listed trials for CAR-NKs on ClinicalTrials.gov (as of July 10, 2019) (Table 1), with the majority of the trials focused on leukemia. One of these trials showed that CD33-targeting CAR-NKs were safely administered to patients with relapsed or refractory AML, albeit with limited clinical efficacy (80). The authors speculated that reduced longevity and cytotoxicity of irradiated (for safety) CAR-NKs were potential pitfalls and noted that efficacy might only be achievable when used to treat malignancies with a slower disease progression.

Preclinical studies continue to explore strategies to enhance CAR-NK efficacy, such as changing the CAR intracellular domains. Historically, the CD3ζ chain has been used alone or in combination with CD28, 4-1BB, or OX40 signaling domains (77). Although these costimulatory domains were designed to promote T cell responses, they also activate NK cells (81). More recently, the signaling domains of adaptor molecules associated with activating NK receptors were used to mimic physiological NK signaling and, remarkably, a CAR-NK with the DAP12 intracellular domain exhibited enhanced cytotoxicity compared with a CAR-NK relying on a CD3ζ domain (82). However, a CAR based on DAP10 motifs performed poorly when used as the sole signaling domain (83). More encouraging results were obtained using an NKG2D-DAP10-CD3ζ construct (84).

While most of these studies explored a few closely related CAR-NK constructs, Li et al. conducted a comprehensive screen and found that a 2B4 costimulatory plus CD3ζ intracellular signaling domain mediated better specific cytotoxicity than other combinations of CD3ζ, DAP10, DAP12, CD28, 2B4, and CD137 domains (85). As the field continues to expand, a better understanding of what dictates efficacy of different CAR constructs in various situations will likely follow.

Adoptive NK cell transfer, with or without a CAR, may provide a safer and more feasible alternative or, at the very least, an addition to T cell–based approaches. In fact, whereas allogeneic CAR-T cells are currently not an option due to the risk of GVHD, allogeneic NK cells are safe in this regard. This allows for the use of more readily accessible NK sources to engineer using CARs, such as cell lines (NK92, KyHG1) or allogeneic NKs derived from cord blood or iPSCs (81, 85–87). Use of allogeneic sources can pave the path for CAR-NKs to eventually become off-the-shelf therapies, whose safety can be substantially increased by the possibility of including suicide genes (88). Finally, whereas CAR-T cells become ineffective if tumor cells downregulate the CAR antigen (89), CAR-NKs would still recognize tumors through their germline-encoded receptors, reducing the chances of tumor escape through antigen modulation. Overall, CAR-NKs have the potential to become a safe and practical addition to the immunotherapy arsenal.

In conclusion, the numerous ongoing clinical trials employing autologous or allogeneic NK cells for a variety of indications hold great promise. NK cell transfer could complement, and in some scenarios substitute for, T cell–based adoptive transfer therapies to maximize antitumor effects and reduce treatment toxicity.

Cytokine therapy: mobilizing NK cells in cancer

One major disadvantage of an adoptive transfer approach is the high costs and expertise required to manufacture large amounts of clinical-grade immune cells (81, 90). For this reason, off-the-shelf therapies have attracted much research and investments.

Cytokines, as critical regulators of NK cells, are an appealing choice for cancer immunotherapies, particularly in light of results showing that NK cells strongly rely on type I IFN to initiate an anticancer response (91). However, considerable toxicity and morbidity are associated with direct injection of type I IFN into patients (92), and the focus has moved to strategies that elicit IFN production in the tumor microenvironment using agonists of TLR or the cGAS/STING pathway.

IL-2 treatment was FDA approved but has also displayed limited clinical efficacy with alarming toxicity, and more recent work focuses on using engineered cytokines and combination therapies (92). For example, treating NK cells with IL-12, IL-18, or the engineered IL-2 cytokine "super-2" (93) increased NK antitumor activity in a mouse model of cancer (94). Additionally, the engineered IL-15 cytokine ALT-803 has shown impressive preclinical results, in part due to its activation of NK cells (95–97). A more comprehensive discussion on cytokine therapy in cancer can be found in a recent review (92).

BiKEs/TriKEs: directing NK cells against cancer

Antibody therapy also has the appealing advantage of being an off-the-shelf approach to activating NK cells in vivo. In addition to traditional approaches that rely on tumor-binding monoclonal antibodies to activate NK cells via ADCC (17), more recently, bispecific killer cell engagers (BiKEs) have generated great promise. BiKEs are small molecules consisting of two scFvs with different specificity complexed together through flexible linkers (98). One scFv targets a tumor antigen (e.g., CD19, CD20, CD33), while the other is specific for an NK cell receptor (CD16). This effectively brings the cancer and NK cells together, facilitating the formation of an immunological synapse and allowing NK cells to specifically and effectively execute their cytolytic functions (98).

BiKEs' primary target has been CD16, as it potently induces NK activation without additional costimulation (99, 100). Preclinically, CD16 BiKEs have been effectively used to target CD19-, CD20-, CD33-, CD133-, and EpCAM-expressing tumor cells (100–103). NK cells from myelodysplastic syndrome (MDS) patients could be effectively activated with a CD16-CD33 BiKE targeting not only $CD33^+$ MDS cells but also the immunosuppressive $CD33^+$ MDSC population (103). In this and other studies, BiKEs were able to redirect autologous NK cells against tumor cells and overcome the immunosuppression prevalent in these conditions (98).

Additional scFvs, such as tri- and tetra-specific killer cell engagers (TriKEs and TetraKEs), can further potentiate therapeutic benefits by targeting more tumor antigens or adding IL-15 into the engager construct. Using an IL-15 cross-linker, Vallera et al. showed that a TriKE targeting CD16 and CD33, namely 161533, induced tumor cell killing more effectively than a CD16-CD33 BiKE in a xenograft model (104). Although the BiKE mediated some early responses, low NK cell proliferation and persistence attributed to lack of the IL-15 linker resulted in relapse, which was not observed with the 161533 TriKE (104). A phase I/II clinical trial of 161533 TriKE for hematologic malignancies will start recruiting in 2020 (NCT03214666). In a recent study, a multifunctional engager targeting CD16 and NKp46 on NK cells and antigens on the tumor cells has shown promising in vitro and in vivo activity (105).

In conclusion, BiKEs and TriKEs provide a non–cell-based immunotherapeutic approach that can harness the patients' own NK cells against cancer. Clinical trials will determine their safety and effectiveness in patients.

Checkpoint receptors on NK cells: breaking barriers

Immune checkpoint receptors are a group of inhibitory receptors that dampen the effector functions of immune cells. Physiologically, immune checkpoint receptors are essential to prevent autoimmunity and immunopathology, but cancer often exploits them to subvert antitumor immunity (106). Notably, NK cells express many checkpoint receptors, some of which have been targeted by cancer immunotherapy (107).

KIRs and CD94/NKG2A. The majority of KIRs are inhibitory and recognize HLA molecules (108). To replicate missing self recognition, the humanized antagonistic antibody lirilumab (IPH2102) targeting inhibitory KIRs (KIR2DL1-3 and KIR2DS1-2) is in clinical development. In preclinical studies, lirilumab enhances NK-mediated cytotoxicity towards lymphoma, leukemia, and MM (109–111). A phase I trial of lirilumab in MM showed acceptable safety (112), but a phase II trial was halted due to lack of efficacy (113). Interestingly, lack of efficacy was associated with loss of NK cell responsiveness and loss of surface KIR2D expression via trogocytosis (114). Furthermore, while lirilumab treatment was well tolerated, it did not show efficacy in AML in a phase I trial (115), although careful analysis of trends in this trial hinted that optimized dosing may be required (116).

CD94/NKG2A is a heterodimeric inhibitory receptor expressed on NK and T cells that recognizes peptide-bound HLA-E. In both solid tumors and hematological malignancies, HLA-E is upregulated to evade recognition by NK and T cells (117–122), and its expression is associated with poor prognosis (123–125). Two recent preclinical studies blocking NKG2A showed enhanced antitumor immunity by both T and NK cells in various tumor models (121, 122). A recent preclinical study used protein expression blockers (PEBLs), engineered protein constructs consisting of an scFv against a target protein that is linked to an ER/Golgi retention peptide to prevent the transport of NKG2A to the cell surface. In this study, PEBLs enhanced NK cell cytotoxicity and antitumor functions (126). Interestingly, preventing NKG2A expression via PEBLs enhanced NK cell in vitro cytotoxicity more than NKG2A antibody blockade. The antagonistic NKG2A antibody monalizumab (IPH2201) is currently under investigation both as a single agent and in combination with cetuximab (anti-EGFR) or durvalumab (anti–PD-L1). Interim results from both combination trials report encouraging safety profiles and signs of efficacy (121, 127).

CTLA-4 and PD-1. The first checkpoint receptor targeted for cancer therapy was CTLA-4 (128, 129), owing to its important role in suppressing T cell activation (130). Interestingly, very little research has focused on the role of CTLA-4 on NK cells. In murine models, CTLA-4 engagement suppressed effector functions of NK cells (131), but the importance of NK cells in mediating the effects of CTLA-4 blockade is still unclear.

PD-1 is the second checkpoint receptor successfully targeted for cancer treatment. PD-1 is an inhibitory receptor with two known ligands: PD-L1 and PD-L2 (132). Multiple approved antibodies target the PD-1/PD-L1 axis in cancer, and their efficacy is attributed to reinvigoration of tumor-targeting T cells. However, multiple lines of evidence indicate that NK cells play a role in the therapeutic efficacy of PD-1/PD-L1 blockade. Probably the most striking example is Hodgkin lymphoma, which is highly responsive to PD-1 blockade yet exhibits frequent defects in MHC class I presentation, suggesting a T cell–independent mechanism of action (133–135). Human NK cells from healthy donors and cancer patients express PD-1 (136–139). We and others have found that $PD\text{-}1^+$ NK cells

Table 1. List of CAR-NK trials on ClinicalTrials.gov as of July 10, 2019

NCT ID	CAR-NK target	Study title	Status	Conditions[A]	Phase	(Expected) completion	Location
NCT03692767	CD22	Study of Anti-CD22 CAR NK Cells in Relapsed and Refractory BCL	Not yet recruiting	Refractory BCL	Early Phase 1	November 2021	
NCT03690310	CD19	Study of Anti-CD19 CAR NK Cells in Relapsed and Refractory BCL	Not yet recruiting	Refractory BCL	Early Phase 1	November 2021	
NCT03692663	PSMA	Study of Anti-PSMA CAR NK Cell in Castration-Resistant Prostate Cancer	Not yet recruiting	Castration-resistant prostate cancer	Early Phase 1	December 2021	
NCT03692637	Mesothelin	Study of Anti-Mesothelin Car NK Cells in Epithelial Ovarian Cancer	Not yet recruiting	Epithelial ovarian cancer	Early Phase 1	November 2021	
NCT03415100	NKG2D ligands	Pilot Study of NKG2D-Ligand Targeted CAR-NK Cells in Patients With Metastatic Solid Tumors	Recruiting	Solid tumors	Phase 1	December 2019	China
NCT03824964	CD19/22	Study of Anti-CD19/CD22 CAR NK Cells in Relapsed and Refractory BCL	Not yet recruiting	Refractory BCL	Early Phase 1	January 1, 2021	
NCT02944162	CD33	CAR-pNK Cell Immunotherapy for Relapsed/Refractory CD33$^+$ AML	Unknown status	Acute myelogenous leukemia, AML, ANLL	Phase 1/2	September 2018	China
NCT02892695	CD19	PCAR-119 Bridge Immunotherapy Prior to Stem Cell Transplant in Treating Patients With CD19 Positive Leukemia and Lymphoma	Recruiting	ALL, chronic lymphocytic leukemia, follicular lymphoma	Phase 1/2	September 2019	China
NCT03579927	CD19	CAR.CD19-CD28-zeta-2A-iCasp9-IL15-Transduced Cord Blood NK Cells, High-Dose Chemotherapy, and Stem Cell Transplant in Treating Participants With BCL	Not yet recruiting	Mantle cell lymphoma, recurrent diffuse large BCL, recurrent follicular lymphoma	Phase 1/2	January 1, 2020	United States
NCT03056339	CD19	Umbilical & Cord Blood (CB) Derived CAR-Engineered NK Cells for B Lymphoid Malignancies	Recruiting	B-lymphoid malignancies, ALL, chronic lymphocytic leukemia	Phase 1/2	June 2022	United States
NCT03383978	Her2	Intracranial Injection of NK-92/5.28.z (HER2.taNK) Cells in Patients With Recurrent HER2-positive Glioblastoma (Quilt 3.C001)	Recruiting	Glioblastoma	Phase 1	June 2020	Germany
NCT02742727	CD7	CAR-pNK Cell Immunotherapy in CD7 Positive Leukemia and Lymphoma	Unknown status	AML, precursor T cell lymphoblastic leukemia-lymphoma, T cell prolymphocytic leukemia	Phase 1/2	March 2018	China
NCT02839954	MUC1	CAR-pNK Cell Immunotherapy in MUC1 Positive Relapsed or Refractory Solid Tumor	Unknown status	Hepatocellular carcinoma, non–small cell lung cancer, pancreatic carcinoma	Phase 1/2	July 2018	China
NCT00995137	CD19	Genetically Modified Haploidentical Natural Killer Cell Infusions for B-Lineage ALL	Completed	ALL	Phase 1	May 2014	United States
NCT03941457	ROBO1	Clinical Research of ROBO1 Specific BiCAR-NK Cells on Patients With Pancreatic Cancer	Recruiting	Pancreatic cancer	Phase 1/2	May 2022	China
NCT03931720	ROBO1	Clinical Research of ROBO1 Specific BiCAR-NK/T Cells on Patients With Malignant Tumor	Recruiting	Malignant tumor	Phase 1/2	May 2022	China
NCT03940820	ROBO1	Clinical Research of ROBO1 Specific CAR-NK Cells on Patients With Solid Tumors	Recruiting	Solid tumor	Phase 1/2	May 2022	China
NCT03940833	BCMA	Clinical Research of Adoptive BCMA CAR-NK Cells on Relapse/Refractory MM	Recruiting	Multiple myeloma	Phase 1/2	May 2022	China

[A]The first three indications listed on ClinicalTrials.gov are included. BCL, B cell lymphoma. ANLL, acute nonlymphocytic leukemia. ALL, acute lymphocytic leukemia.

have impaired responses when PD-1 ligands are present but can be re-activated by PD-1/PD-L1 blockade (140–145). To analyze the contribution of NK cells in PD-1/PD-L1 blockade immunotherapy, we employed several murine models of cancer. In leukemia models where cancer cells express low levels of MHC I, and are therefore poor targets for cytotoxic T cells, expression of PD-L1 accelerated tumor growth. PD-1/PD-L1 blockade provided a therapeutic effect that was completely abolished by NK cell depletion. We also determined the contribution of NK cells to PD-L1 blockade in a cancer model where T cells participated in tumor immune surveillance. Notably, even in this case, NK cells were essential for the full therapeutic effect of PD-L1 blockade (143).

TIGIT. Ligands for the inhibitory receptor TIGIT, CD155 (PVR) and CD112 (PVRL2/Nectin-2), are expressed on many cancer cells (146–148). TIGIT competes for binding to CD155 and CD112 with the receptors DNAM-1 (CD226) and CD96 (Tactile), forming a pathway whereby ligand binding to DNAM-1 delivers an activating signal, while binding to TIGIT or CD96 delivers an inhibitory signal (149). Interestingly, TIGIT blockade results in NK-dependent antitumor immunity in several murine models of cancer (150). TIGIT blockade also enhanced T cell immunity in an NK-dependent manner. Moreover, TIGIT blockade showed synergy with PD-1/PD-L1 blockade, providing rationale for this combination therapy in the clinic. CD96 blockade is less explored, but a recent study targeting this pathway found that this strategy enhanced the antimetastatic properties of NK cells in murine tumor models (151). Further research into combined blockade of TIGIT and CD96 to enhance NK cell antitumor immunity is warranted.

LAG3. LAG3 is an MHC II–binding inhibitory receptor expressed on NK and T cells that has structural homology to CD4 (152, 153). Other ligands of LAG3 are LSECtin (154) and FGL1 (155), both of which can be expressed by tumor cells. While LAG3's functions on T cells have been characterized, its role in NK cells is still unclear. NK cells from LAG3-deficient mice displayed impaired cytotoxicity towards some cancer cells, but retained cytotoxicity against MHC I–mismatched cells (156). However, antibody blockade or soluble LAG3 treatment of human NK cells did not impact their cytotoxicity (157). As antibodies targeting LAG3 are currently in clinical evaluation (158), further work on the consequences of LAG3 engagement on NK cells can be expected.

TIM-3. The inhibitory receptor TIM-3 binds to galectin-9, phosphatidylserine, HMGB1, and CEACAM1 (159–162). TIM-3 is constitutively expressed on human NK cells and is upregulated in response to cytokine stimulation (163, 164). Like PD-1, TIM-3 expression can mark NK cells that produce IFN-γ and release cytotoxic granules as well as NK cells with an exhausted phenotype (164). TIM-3 is upregulated on peripheral NK cells in patients with gastric cancer, lung adenocarcinoma, melanoma, and on tumor-infiltrating NK cells in gastrointestinal stromal tumors (165–168). Importantly, in melanoma and lung adenocarcinoma, TIM-3 blockade enhanced NK cell cytotoxicity and IFN-γ production (165, 167).

Other checkpoint receptors in NK cells and conclusions. In addition to the checkpoint receptors described above, preclinical research has identified additional negative regulators of NK cell functions, including the negative regulator of cytokine signaling, CIS (169, 170), and the high-affinity adenosine receptor A_{2A} (171).

In summary, these preclinical and clinical studies challenge the dogma that T cells are the sole mediators of the anticancer effects of checkpoint blockade immunotherapy and highlight the importance of NK cells, which in some cases work in tandem with cytotoxic T cells and in others play the dominant role. As more targets are discovered, it becomes essential to identify the most effective combination to maximize the therapeutic efficacy of checkpoint blockade on a per-patient basis. Considering the effects of checkpoint blockade on NK cells, they will be of great importance in this process.

NK cells in oncolytic virotherapy

An alternative to taking off the brakes using checkpoint immunotherapies is to boost NK activation. One successful approach is the use of viruses that specifically infect and lyse cancer cells, broadly referred to as oncolytic viruses (OVs). OVs exploit tumor-specific receptors or observed impairments in infection control in neoplastic cells to selectively infect and replicate in cancer cells, leaving healthy cells unharmed (172, 173). It is now clear that OVs' ability to induce a systemic antitumor immune response is perhaps even more critical than their ability to induce direct oncolysis (172, 174). One appealing aspect of OV-based therapy is the ability to engineer delivery of immune-modulating cargos to the tumor microenvironment (172, 174). Championing the OV cause, talimogene laherparepvec, an attenuated herpes simplex virus (HSV) expressing GM-CSF, was FDA approved for metastatic melanoma and subsequent studies in combination with checkpoint inhibitors have also indicated remarkable results (174–176). A large number of OVs have now entered clinical trials, and even more are at various stages of preclinical development (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI129338DS1).

NK cells are evolutionarily designed to detect and eliminate virally infected cells, which can be a detriment for the early OV spread that is necessary for therapeutic purposes (177). In some in vivo models, NK depletion enhanced OV efficacy (178, 179), but the majority of studies have illustrated a beneficial or even essential role for NK cells in mediating OV effects (180–184). To explain this dichotomy, an interesting study mathematically modeled the role of NK cells in a treatment regimen of HSV and bortezomib for glioblastoma multiforme. Interestingly, both high and low ratios of NK to cancer cells contributed to enhanced efficacy, while intermediate levels were detrimental. Experimental validation in a glioma PDX model led the authors to speculate that early transient removal of NK cells during viral therapy allows necessary unhindered viral propagation, and subsequent NK adjuvant therapy enhances tumor killing, synergizing with OV therapy (185). This further highlights the importance of considering kinetics in developing combinatorial therapies.

Overall, OV therapy benefits from the innate immune response. In addition to induction of immunogenic cell death, TLR engagement, and release of DAMPs and pathogen-associated molecule patterns (PAMPs) from infected cells (172, 174), studies have shown that modulating NK ligand expression on cancer cells following infection drives NK-mediated clearance (186–188). Other studies have uncovered the role of DC-NK cross talk following OV therapy. We have shown that the Maraba virus directly infects

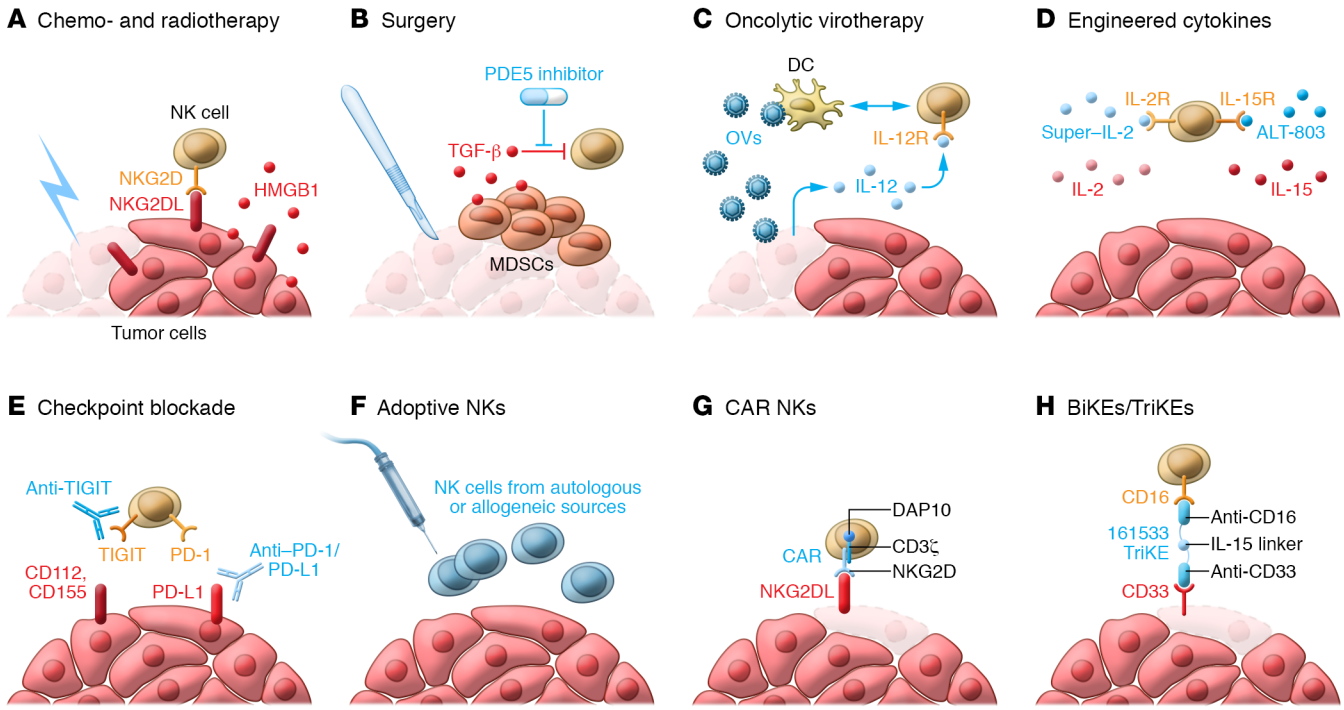

Figure 1. The NK cell armament of cancer immunotherapy: how to harness NK cells against cancer. NK cells kill and eliminate cancer cells, but in the tumor microenvironment they are often insufficiently active or inhibited by immunosuppressive ligands and cytokines. To overcome this, a number of strategies have been developed to enhance NK cell activity against cancer in these settings: (**A**) Chemo- and radiotherapy induce immunogenic cell death of cancers, leading to expression of NKG2D ligands, HMGB1, and other DAMPs that drive NK cell activation. (**B**) Surgery leads to the development of an immunosuppressive microenvironment, in part through the expansion of MDSCs and the release of inhibitory cytokines such as TGF-β. PDE5 inhibitors alongside viral vaccines have proven to be highly effective in reversing this dysfunction. (**C**) Oncolytic viruses (OVs) infect and lyse cancer cells, but can also infect DCs, leading to their maturation and driving DC-NK cross talk and subsequent NK activation. OVs can also be engineered to deliver cytokines and other immune stimulants to the microenvironment to activate the immune system. (**D**) Engineered cytokines such as ALT-803, an alternate form of IL-15, have increased potency compared with conventional cytokines. (**E**) Checkpoint blockers such as anti–PD-1/PD-L1 and anti-TIGIT relinquish NK cells from the immunosuppressive effects exerted by tumors, allowing them to perform their cytolytic functions. (**F**) NK cells from autologous or allogeneic sources can be safely used as adoptive cell therapy. (**G**) The use of CARs enhances the efficacy of adoptive therapy. In particular, CARs expressing NKG2D with the CD3ζ and DAP10 intracellular signaling motifs drive potent antitumor immune responses. (**H**) BiKEs and TriKEs bring NK cells spatially closer to their targets and activate them. The TriKE 161533 contains a CD16-targeting motif for NK cells, a CD33-targeting motif for cancer cells and MDSCs, and an IL-15 linker to activate NK cells.

conventional DCs (cDCs) and promotes their maturation. Mature cDCs then activate NK cells that better control cancer (189). The centrality of DC-mediated NK activation for OV therapy has also been observed using other oncolytic viruses (190, 191).

To further promote antitumor immunity, OVs have been engineered to express NK-stimulating cytokines such as IL-12, IL-15, IL-18, CCL5, and GM-CSF (192–194). Using a similar approach, we developed an NK-activating infected cell vaccine (ICV) based on injection of irradiated autologous tumor cells previously infected ex vivo with a cytokine-expressing OV (195, 196). Using this platform, we showed that an ICV prepared with an IL-12–expressing Maraba virus led to complete regression of established peritoneal tumors in an NK-dependent manner and overcame some of the inherent issues related with in vivo OV infectivity, such as antibody- and complement-mediated neutralization (particularly upon repeated dosing), sequestration by serum proteins, and immune-mediated clearance (196). Additionally, it allows controlled and safe release of potent cytokines at the site of tumor infection. Another cytokine-based approach that has exhibited promising results is the use of a "superagonist" IL-15, i.e., IL-15 complexed with the α subunit of its receptor (IL-15/IL-15Ra). This approach was shown to increase IL-15's in vivo stability and bioavailability, and a Myxoma virus encoding IL-15/IL-15Ra induced robust NK responses leading to improved outcomes in murine melanoma (197).

As discussed above, surgery can have a major impact on antitumor immunity. As OVs have a strong immunomodulatory potential, we reasoned that virotherapy could recover NK cell dysfunction in tumor models. Indeed, we showed that virotherapy following surgery reduced tumor burden by reverting perioperative NK dysfunction (50, 189). We further showed that TLR3 engagement by an inactivated influenza vaccine similarly enhanced NK activity, with additional benefits when MDSC activity was inhibited using a phosphodiesterase 5 (PDE5) inhibitor (54, 198). We are currently enrolling patients following major surgical resection of primary abdominal tumors to test a combination of influenza vaccine and PDE5 inhibitor (tadalafil) on NK cell function (NCT02998736). Clearly, OVs can help mitigate surgery-induced dysfunction, but more research is required to evaluate these therapies and find ideal combinations.

In conclusion, OV-based platforms and combination therapies continue to identify new ways to harness NK cell antitumor activity. Recently, Chen et al. reported promising synergistic results using HSV with an EGFR-targeting CAR-NK to treat breast cancer brain metastasis (199). Following on the heels of bispecific T cell engagers (BiTEs), potential to engineer BiKEs into OVs is also conceivable (200).

Conclusions and future perspectives: understand to cure

NK cells are powerful tools in the armamentarium against cancer (Figure 1). They inherently differentiate self from non-self, gauge danger signals on stressed cells, and rapidly eliminate malignant cells, making them an ideal target for cancer immunotherapy. Increasing understanding of the basic mechanisms underlying NK recognition, activation, and suppression fosters incredible excitement and paves the way to immunotherapeutic strategies that elicit NK cell responses against cancer. Valuable preclinical mechanistic research must continue to elucidate the key processes regulating NK cell biology, which will also facilitate clinical translation. One lesson learned from the cancer immunotherapy revolution is that only by understanding the basic biology can one manipulate complex systems, and the dynamic interplay between the immune system and cancer is exquisitely complicated. So far, NK cells have been somehow overlooked as the field has tried to empower T cell responses against cancer. It is now evident that many immunotherapies thought to elicit T cell responses also activate NK cells, and that NK cells can be effective in scenarios where T cells fail. For this reason, more research is warranted to accurately and effectively harness the full power of the immune system, including NK cells, against cancer.

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