

Emerging NK cell therapies for cancer and the promise of next generation engineering of iPSC-derived NK cells

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ABSTRACT

Adoptive cell therapy is a rapidly advancing approach to cancer immunotherapy that seeks to facilitate antitumor responses by introducing potent effector cells into the tumor microenvironment. Expanded autologous T cells, particularly T cells with engineered T cell receptors (TCR) and chimeric antigen receptor-T cells have had success in various hematologic malignancies but have faced challenges when applied to solid tumors. As a result, other immune subpopulations may provide valuable and orthogonal options for treatment. Natural killer (NK) cells offer the possibility of significant tumor clearance and recruitment of additional immune subpopulations without the need for prior antigen presentation like in T or B cells that could require removal of endogenous antigen specificity mediated via the T cell receptor (TCR and/or the B cell receptor (BCR). In recent years, NK cells have been demonstrated to be increasingly important players in the immune response against cancer. Here, we review multiple avenues for allogeneic NK cell therapy, including derivation of NK cells from peripheral blood or umbilical cord blood, the NK-92 immortalized cell line, and induced pluripotent stem cells (iPSCs). We also describe the potential of engineering iPSC-derived NK cells and the utility of this platform. Finally, we consider the benefits and drawbacks of each approach and discuss recent developments in the manufacturing and genetic or metabolic engineering of NK cells to have robust and prolonged antitumor responses in preclinical and clinical settings.

ligands that are associated with cancer.⁵ Notably, tumor cells can often downregulate expression of MHC class I and thus escape surveillance by CD8⁺ T cells, but because MHC I molecules are cognate ligands for NK cell inhibitory receptors, NK cells kill such MHC-deficient cells.⁷ Furthermore, NK cells express a variety of other activating and inhibitory receptors central to antitumor innate immunity. NKG2D is an important activating receptor that recognizes MHC class I polypeptide-related sequences A and B (MICA and MICB) as well as UL16 binding proteins, which can be expressed on rapidly dividing tumor cells.^{4,6} Additionally, CD16 is a human IgG1 Fc receptor on NK cells that provides a mechanism for antibody-dependent cell-mediated cytotoxicity (ADCC) of tumor cells.⁶ NK cells can exert direct cytotoxicity via granzyme and perforin-mediated lysis and also via death-inducing ligands like FasL.⁶ Additionally, the LFA1-ICAM1 axis normally involved in immune cell trafficking has been implicated in optimizing NK cell recognition of tumor cells.⁴

In addition to their direct cytotoxicity, NK cells are also capable of driving tumor inflammation and making the tumor microenvironment (TME) more immunologically 'hot' by producing chemokines like XCL1 or XCL2 or cytokines like IFN- γ and recruiting and maturing additional immune cells.^{4,8,9} NK cells themselves can effectively home to tumor sites via chemokine signaling, and CXCR3 in particular is important for NK cell localization to tumor.^{10,11} Furthermore, NK cells in circulation have been shown to mitigate progression of disease by inhibiting tumor metastasis.^{4,12,13} From a clinical perspective, many cancer types—including melanoma, head and neck squamous cell carcinoma (HNSCC), non-small-cell lung cancer (NSCLC), leukemia, and gastrointestinal cancer—show improved outcomes with greater NK cell infiltration or involvement,

NATURAL KILLER CELLS IN CANCER

Natural killer (NK) cells are an essential component of the innate lymphoid cell (ILC) compartment. NK cells contrast from other lymphoid populations like T or B cells, which drive adaptive immune responses through specificity of individual cells for distinct antigens.^{1,2} Instead, NK cells can exert their effector responses without prior antigen exposure and have an analog signaling mechanism in which the integration of numerous activating and inhibitory signals ultimately determine the cytotoxic response of the NK cell.^{3–6}

NK cells have significant roles in anti-tumor responses. They can detect transformed tumor cells with expression or absence of



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confirming the importance of this distinct cell type in the antitumor response.^{4 14–17}

Despite these antitumor roles for NK cells, cancer can persist and progress due to various malfunctions in immunosurveillance, including the NK cell response. The TME can be highly immunosuppressive via secretion of negative chemical modulators, nutrient deficiency (including hypoxia), expression of inhibitory checkpoint molecules, and other factors that continue to be investigated.¹⁸ Moreover, NK cell activating receptors are downregulated and inhibitory receptors like NKG2A/CD94 heterodimer are upregulated to inhibit NK activity.¹¹ NK cell recruitment, metabolism, and cytotoxicity can all become dysfunctional in the disruptive TME, posing a need for novel therapeutics that can potentially overcome the inhibitions of the TME.

NON-INDUCED PLURIPOTENT STEM CELL ALLOGENEIC NK CELL THERAPIES

Overview

Allogeneic NK cell therapies have been increasingly evaluated for the treatment of numerous malignancies, and many have even progressed to clinical testing. This treatment modality presents copious opportunities for next-generation cell therapy, although much remains to be discovered and understood about allogeneic NK cells. Typically, adoptive NK cell therapy is administered after lymphodepletion via fludarabine and cyclophosphamide in order to minimize NK graft rejection.¹⁹ NK cell graft rejection is thought to be mediated via recipient T cells, particularly CD8⁺ T cells that activate and proliferate following IL-15 supplementation intended to boost NK cell persistence and expansion. Thus, suppression of recipient T cell activity may help promote NK graft persistence.^{20 21}

Considering their alloreactivity, allogeneic NK cells are often mismatched such that donor NK inhibitory killer Ig-like receptors (KIRs) fail to recognize recipient human leucocyte antigen (HLA) molecules. This mismatch can induce a protective effect that promotes survival and reduces risk of relapse, as observed in acute myeloid leukemia (AML) patients with haploidentical transplants.²² Unfortunately, not all KIR-HLA mismatches are robust, and some may not convey any therapeutic benefits for patients undergoing haploidentical transplants or HLA-matched, related, or unrelated transplants.²³

Moreover, allogeneic NK cell transfers do not show an increased risk of graft-versus-host-disease (GVHD) and actually may even decrease risk of GVHD, a stark contrast from the increased risk of GVHD observed in adoptive T cell therapies.^{23 24} Donor NK cells can possibly eliminate recipient dendritic cells from priming reactive T cells, or these NK cells can directly eliminate alloreactive T cells triggering GVHD. Furthermore, KIR-HLA mismatch can promote the anti-GVHD effects of donor NK cells.²⁵ The lack of GVHD is a significant advantage of allogeneic NK cell therapies that paves the path for off-the-shelf

administration of these treatments, allowing for scaling of manufacturing and ultimately treatment of patients.

Additionally, allogeneic NK cell transfers can be combined with orthogonal therapies. Radiation and chemotherapy are classic adjuvants that can improve overall and/or progression-free survival from hematologic malignancies. Some clinical studies found that pretreatment with NK cells or multiple infusions over time of NK cells could improve efficacy of hematopoietic stem cell treatment.²⁶ One significant strategy for allogeneic NK cell combination immunotherapy involves co-infusion of monoclonal antibodies with donor NK cells in hopes of achieving a synergistic, therapeutic effect. One study of patients with advanced NSCLC found combination allogeneic NK cells and pembrolizumab (anti-PD-1) improved overall survival more than pembrolizumab alone in a trial of 109 enrolled patients (15.5 months vs 13.3 months). Patients receiving both NK and pembrolizumab dosed with multiple courses of NK therapy also had longer survival (18.5 months vs 13.5 months for single course).²⁷ Additionally, a phase I study of patients with refractory or relapsed non-Hodgkin's lymphoma found the combination of allogeneic NK cells with rituximab (anti-CD20) to have an objective response rate of 55.6% in 9 patients with no GVHD.²⁸ Similarly, weekly infusions of NK cells via hepatic artery combined well with cetuximab (anti-EGFR) and showed therapeutic benefit in 9 patients with liver metastases of colorectal or pancreatic cancer. Objective response was observed in three patients, and two of these patients had HLA-KIR mismatch.^{29 30} Finally, trastuzumab (anti-HER2) shows synergy with allogeneic NK cells in HER2-positive patient tumors, and engineering of a modified NK cell line to be conjugated to trastuzumab had significant preclinical potency in vitro and in vivo.^{31 32} Taken together, allogeneic NK cells can be enhanced through a multitude of combinations with monoclonal antibodies to improve clinical efficacy.

Peripheral blood-derived and umbilical cord-derived NK cell therapies

Traditional approaches to adoptive NK cell therapy involve allogeneic transfusions of NK cells derived from the peripheral blood (PB-NK) or umbilical cord blood (UB-NK). PB-NK cells comprise approximately 10% of circulating lymphocytes.^{24 33} In UB, NK cells are relatively more frequent, comprising up to 30% of lymphocytes.³³

Both PB-NK and UB-NK have shown successes in preclinical and clinical studies. PB-NK cells derived from healthy donors that were activated by coculture with a K562 cell line expressing membrane-bound IL-15 and 4-1BB ligand were found to show potent cytotoxicity against multiple hepatocellular carcinoma cell lines in vitro and in vivo in immunodeficient mice.³⁴ When this coculture stimulus system was applied to previously treated patients with high-risk relapsing myeloma, NK-cell infusion resulted in a partial response for one out of seven evaluable patients with no severe adverse events, and fresh (not cryopreserved) cells were found to be key

to significant *in vivo* NK cell proliferation.³⁵ Furthermore, Kottaridis *et al* showed in a phase I trial that allogeneic NK cell infusions from haploidentical donors in patients with high risk AML had mild efficacy with one patient having a complete remission out of seven total treated patients at 1-year post-treatment.³⁶

The use of PB-NK subsets with ‘adaptive’ memory-like features may also have clinical utility. Preactivation of PB-NK with IL-12, IL-15, and IL-18 has been demonstrated to result in NK cells with a lower threshold for activation by cytokine or activating receptor restimulation.^{37–39} These ‘cytokine-induced memory-like’ NK cells have shown efficacy in treatment-refractory AML and may be an important strategy for enhancing chimeric antigen receptor (CAR)-NK cell immunotherapy approaches.^{37,40} Another emerging strategy is the *ex vivo* expansion and adoptive transfer of a subset of PB-NK with antibody-dependent adaptive features. These cells have epigenetically silenced the expression of the FcεR1γ adaptor protein and exhibit robust effector responsiveness to tumor cells when directed by target-specific antibodies.^{41–44} The FcεR1γ-deficient (‘G[−]’) NK cells are found in approximately one third of the population, and their presence in individuals is associated with prior infection by human cytomegalovirus, a virus that has also been observed to be associated with expansion of NKG2C⁺ adaptive NK cells *in vivo*.^{45,46} The *ex vivo* expansion and use of healthy donor-derived G[−] NK cells as an adoptive cell therapy approach, in combination with target-specific antibodies, has been proposed to be another possible therapeutic strategy.⁴⁷

UB-NK cells have also been evaluated in clinical treatment of patients with various hematologic cancers, including AML, acute lymphocytic leukemia, and chronic lymphocytic leukemia (CLL).⁴⁸ In particular, UB-NK cell engineering efforts have advanced clinically. A CD19 CAR-NK therapy administered after lymphodepleting chemotherapy showed significant efficacy with 8 out of 11 patients treated having a response at a median follow-up of 13.8 months, with 7 of those 8 having complete responses. None of the patients had above a grade 3 adverse event. However, a small number of contaminating CAR-T cells were detected in infusions, although this was a small fraction that likely did not contribute to any adverse reaction or drive the host anticancer response.⁴⁹ Other UB-NK therapeutic approaches include precomplexing the allogeneic cells with engineered cell engagers. For instance, Kerbaux *et al* precomplexed AFM13, a bispecific CD30/CD16 antibody, with UB-NK cells for treatment of CD30⁺ leukemias or lymphomas in order to engage NK cells more robustly with tumor cells for enhanced cytotoxicity. This technology preclinically showed significant *in vitro* cytotoxicity and *in vivo* efficacy.⁵⁰ This platform has been advanced to clinical trials for which press reports have been written but no formal data published yet, and an objective response rate of 100% with a complete response rate of 42% was observed in 12 patients after 1 cycle of treatment in a Phase I-II study with no major adverse reactions noted (NCT04074746).⁵¹

Although PB-NK and UB-NK therapies have shown success in multiple contexts, they have certain limitations. PB-NK cells have poor efficacy if cryopreserved and worse bone marrow homing relative to UB-NK cells. However, UB-NK cells have weaker cytotoxicity compared with PB-NK cells, showing an immature phenotype with higher expression of inhibitory NKG2A with decreased expression of activating receptors.^{48,52} However, IL-2 and/or IL-15 stimulation has been noted to boost UB-NK activity and differentiation into more functional effector cells.⁴⁸ Importantly, PB-NK and UB-NK cell infusions fundamentally are heterogeneous. First, other leucocytes, especially lymphocytes like T cells, can potentially contaminate NK cell infusions and pose an increased risk for GVHD or passenger lymphocyte syndrome. To avoid such complications, robust purification protocols to isolate solely NK cells would be necessary, possibly complicating efforts to genetically modify NK populations. Additionally, within the NK compartment, cells can have variable effector capabilities, with some cells having greater proliferation and cytotoxic potential than others. The intrinsic heterogeneity associated with blood-derived NK treatments poses a challenge for efforts to deliver consistent and homogeneous treatment to patients, although applications of PB-NK and UB-NK therapies thus far have not faced significant drawback from this issue.^{49,53} Importantly, both UB-NK and PB-NK cells require *ex vivo* expansion from their donor sources in order to obtain sufficient quantity of NK cells for therapeutic effects. Specifically, membrane-bound (mb) IL-2, IL-15, or IL-21 with K562 feeder cells that lack HLA but express costimulatory molecules are used for consistent, effective *ex vivo* expansion, and K562-mb15-4-1BBL cells are a common generative basis for numerous clinical trials involving allogeneic NK cell therapy.^{48,52,54–56} This complex manufacturing process poses obstacles to scaling of NK cell therapies and can be a contributory factor to higher cost of treatment.

Overall, PB-NK and UB-NK adoptive cell therapies have seen numerous successes in the treatment of various hematologic cancers. However, they are limited by donor supply and require non-trivial investment of time and resources to expand the limited numbers of NK cells in blood and purify the NK population to obtain a homogeneous infusion.

NK-92 cell line-derived therapies

In addition to the isolation and expansion of NK cells from blood, clonal NK-cell lines have also been investigated for adoptive NK cell therapy. NK-92 is an immortalized cell line derived from a patient with lymphoma that has shown good cytotoxicity in various cancers.⁵⁷ NK-92 expresses numerous activating receptors while lacking expression of some inhibitory ones. The immortalized NK-92 cell line provides a path for clinical translation by allowing for easy *in vitro* culturing. The homogeneity of the cell line also confers ease of engineering and genetic modification that produces a consistent product.^{53,57} Notably,

Jochems *et al* engineered the NK-92 cell line to express a high-affinity variant of CD16, which is normally absent on the NK-92 cell line despite being an important driver of ADCC in circulating NK cells. This high-affinity CD16 (haNK) cell line was also genetically modified to produce endogenous IL-2 for enhanced survival and propagation in culture. In *in vitro* studies, gamma-irradiated haNK cells showed potent cytotoxicity against multiple tumor types, including lung and breast.⁵⁸ The haNK system was further modified to express a PD-L1-targeting CAR (t-haNK), and this system had good efficacy *in vitro* and *in vivo*, with PD-L1-dependent tumor control achieved in murine MOC1 HNSCC.^{59 60}

Clinically, NK-92 cells that are irradiated by gamma rays have been proposed to be safe for allogeneic adoptive cell therapy.^{57 61} Un-engineered NK-92 cells did not induce any serious adverse effects in trials for renal, lung, and other advanced cancers. Furthermore, NK-92 shows therapeutic potential in aggressive, metastatic tumor models.⁵⁷ 4 of 11 patients with metastatic renal cell carcinoma had stable disease after NK-92 infusions, and the 1 patient with metastatic melanoma treated had a minor response. Unfortunately, 10 of the 12 patients treated died within 4 years of follow-up due to progressed disease, as these tumors were advanced. NK-92 infusions were moderately safe, and of the 12 patients, one patient had a grade 3 fever and one patient had a grade 4 hypoglycemic episode.⁶² In a separate phase I trial, three out of four patients with advanced lung cancer had a mixed response or stable disease after NK-92 infusion, with no major toxicities.⁶¹ More recently, the QUILT 3.064 trial of the PD-L1 t-haNK system has shown no dose-limiting toxicities in preliminary data (not yet peer-reviewed) from six patients with locally advanced or metastatic solid tumors (NCT04050709).⁶³

While the NK-92 cell line addresses the heterogeneity and difficulties with isolation and expansion of NK cells seen in the blood-sourced NK cell therapies, it does carry its own set of drawbacks. Mainly, NK-92 cell lines have poor *in vivo* expansion to drive strong and durable effector responses. This lack of expansion decreases the maximum efficacy that NK-92 cell therapies can achieve.^{53 64} NK-92 cells have failed to provide significant benefit to patients with refractory or relapsed AML, and infusion of irradiated NK-92 cells were found to only have transient effects on a patient's cytokine profile. Gamma-irradiation of NK-92 cells is necessary to control proliferation of the cells prior to infusion, but the irradiation protocol prevents persistence of the infused cells.⁶⁵ Additionally, healthy PBMCs, particularly NK cells, have been found to target and lyse NK-92 cells, further diminishing the potency and survival of this cell-line in systemic circulation.⁶⁶ However, coadministration of IL-15 could mitigate this rejection of NK-92 cells *in vitro*, possibly through alterations of KIR and KAR profiles.⁶⁷ All together, these data suggest that the longevity of NK-92 cells in systemic circulation is likely a key obstacle that must be addressed to advance this class of allogeneic NK cell therapy forward clinically to a greater degree.

Induced pluripotent stem cell-derived NK cell therapies

Induced pluripotent stem cells (iPSCs) are derived by reversing the developmental program of somatic cells. They were first generated from mouse fibroblasts via expression of four key transcription factors: Oct3/4, Sox2, c-Myc, and Klf4.⁶⁸ iPSCs present vast opportunities in regenerative medicine and were first applied to treat sickle cell anemia in humanized mice.⁶⁹ iPSCs initially faced a major obstacle with translation because the induced stem cells could spontaneously differentiate and needed continuous expression of genes conferring pluripotency, yet integration of these genes into human cell genomes proved risky because of poor control over the locus of integration. However, Valamehr *et al* reported a transgene-free culture system that allows for stable iPSCs in culture, further enabling clinical translation.⁷⁰

NK cells derived from iPSCs (iPSC-NK) present another avenue for adoptive cell therapy. Like NK-92 cells, iPSC-NK cells are homogeneous, as they are derived from a clonal population, and can be engineered.⁵³ For instance, CAR-NK cells derived from iPSCs targeting mesothelin showed strong *in vivo* responses in human A1847 ovarian cancer and outperformed PB-NK cell therapy.⁷¹ Compared with primary NK cells derived from blood, iPSC-NK cells are significantly easier to genetically engineer in a reliable and efficient manner. Primary NK cells comprise a small subpopulation in blood, and viral integration is a poorly controlled and inefficient process that involves the death of numerous cells. However, the clonal iPSC population is well suited to these viral integration methods, making iPSC-NK products simpler to use for engineering pipelines by allowing for straightforward knock-in or knock-out of genes.^{71 72} An example of how iPSCs can enable the engineering of tolerogenic allogeneic NK cells is the forced expression of HLA-E and the knockout of the $\beta 2M$ gene in iPSCs prior to differentiation into NK cells. The NK cells from these iPSCs would have the ability to avoid GVHD by the downregulated surface expression of MHC I (thus avoiding host T cell recognition) and the overexpression of HLA-E, which is the cognate ligand for the inhibitory receptor NKG2A (thus avoiding activation of host NK cells).⁷³ Furthermore, the clonal iPSC-NK populations can overcome the donor-to-donor variability seen in PB-NK or UB-NK therapies. This variability in NK cell function and expansion potential results in an added step of validating PB-NK or UB-NK batches prior to adoptive transfer, complicating manufacturing.⁵³ iPSC-NK cells can streamline manufacturing by overcoming this challenge and provide more consistent batches of cells for therapy.

iPSC-NK cells can also be selected for good cytotoxic profiles conducive for strong antitumor responses. iPSC-NK cells showed effector cytotoxic responses *in vitro* against a variety of hematologic and solid tumor cell lines, including lung cancer, hepatocellular cancer, ovarian cancer, myeloid leukemia, and melanoma. These iPSC-NK cells also successfully delayed tumor progression in a xenograft ovarian cancer model. Notably, iPSC-NK

Table 1 Ongoing clinical trials with unengineered iPSC-derived NK cells

Trial identifier	Intervention(s)	Phase	Cancer subtypes	Date initiated
NCT03841110	1. iPSC-NK monotherapy 2. iPSC-NK with nivolumab, pembrolizumab, or atezolizumab 3. iPSC-NK with IL-2 and nivolumab, pembrolizumab, or atezolizumab	I	Various advanced solid tumors	February 2019
NCT04023071	High-affinity, non-cleavable CD16 iPSC-NK with or without rituximab or obinutuzumab	I	AML (monotherapy) or B-cell lymphoma (combination)	July 2019
NCT04630769	High-affinity, non-cleavable CD16 iPSC-NK at various doses with or without enoblituzumab	I	Ovarian, fallopian tube, or primary peritoneal cancer	November 2020

AML, acute myeloid leukemia; iPSC, induced pluripotent stem cell; NK, natural killer.

cells can also recruit PB T cells and synergize with anti-PD-1 therapy to elicit a robust antitumor response in ovarian cancer.⁷⁴ iPSC-NK cells are able to generate an inflammatory environment to elicit antitumor responses that are coordinated with other immune subpopulations.

In clinical contexts, iPSC-NK cell therapy has shown significant promise, although there are no therapies yet that have received FDA-approval. One clinical trial is in place for iPSC-NK cell monotherapy or combination with immune checkpoint inhibitors (NCT03841110). Another trial is evaluating iPSC-NK engineered to express high-affinity, non-cleavable CD16 (NCT04023071) as a monotherapy and combination therapy. Although no formal data has been published, reports indicate that this high-affinity CD16 iPSC-NK system has good safety and efficacy in patients with relapsed and refractory AML, with four of nine patients having an objective response.⁷⁵ This same therapy has also been reported to be safe and effective in combination with rituximab in B cell lymphoma with 6 of 11 patients having a complete response.⁷⁶ Although these trial data are promising, they are still in phase I and formal peer-review of data is still necessary before further conclusions can be made. Table 1 highlights key ongoing clinical trials involving iPSC-derived NK cell therapies.

Manufacturing challenges of iPSC-NK cells and potential solutions

While iPSC-NK cells present the opportunity for effective adoptive NK cell therapy at a clinical scale, they do have limitations. The process of differentiating iPSCs into NK cells and maintaining a stable iPSC-NK cell culture presents a major challenge in manufacturing clinical scale therapies. Differentiation of iPSCs must occur in a controlled manner and can be disturbed by numerous factors, including components of culture media and oxygen supply.⁷⁷

In the case of NK cells, traditional methods involved a single cell adaptation step in which iPSCs survive in culture as a single cell and differentiated cells are completely removed from culture. This process takes roughly 12–15 passages and can require weeks to months of culturing before the iPSC single-cell suspension is ready for differentiation.^{78, 79} This rate-limiting step impedes efforts to

translate iPSC-NK cells to patients in an efficient manner for reliable adoptive cell therapy.

However, Zhu and Kaufman reported a novel, method that bypasses the single-cell adaptation process. Instead, iPSCs are grown in feeder cell-free media and then formed into embryoid bodies directly via addition of Rho-associated protein kinase inhibitor (ROCKi) to the mixture of SCF, VEGF, and BMP4 in APEL that was used in traditional methods. The use of ROCKi and feeder-free media allows one to skip the single cell adaptation process and expedites differentiation of iPSCs into NK cells significantly.⁷⁸ Furthermore, this pipeline can work well with genetic engineering of iPSCs, and iPSCs engineered to express high-affinity non-cleavable CD16 could successfully differentiate into iPSC-NK cells expressing the same construct and maintained their cytotoxic profile with enhanced ADCC.⁸⁰ Additionally, Angelos *et al* report that the aryl hydrocarbon receptor antagonist, StemRegenin-1, could enhance differentiation of embryonic stem cells to CD34 +CD45+hematopoietic progenitor cells. Moreover, StemRegenin-1 treatment increased hematopoietic differentiation into conventional NK cells, pointing to aryl hydrocarbon receptor antagonism as a possible tool to enhance iPSC-NK manufacturing.⁸¹ Thus, while iPSC differentiation does present a manufacturing challenge, significant advances have already been made to expedite the process, and additional developments may arise in the future.

Metabolic reprogramming of NK cell activity

One challenge that all NK-based cell therapies face is the relatively short lifespan of these infused cells when compared with other adoptive cell therapies like CAR-T. Autologous CAR-T cells are capable of surviving for up to years after transplantation, while allogeneic NK cells do not survive beyond the scope of weeks.⁸² There are numerous elements that contribute to the metabolic function and persistence of NK cells, and several of them have been targeted for further engineering.

IL-15 is a key cytokine in the immune synapse that promotes innate and adaptive immune cell development and survival, including for CD8 +T cells and NK cells. Blocking IL-15 receptor α (IL-15R α) can lead to

NK apoptosis and diminished survival.^{83–84} However, in clinical applications, IL-15 has a short in vivo half-life and is not reliable for eliciting strong, durable immune responses, even though IL-15R α +cells can serve as a sustained source of IL-15.^{83–85} Protein engineering of IL-15 or IL-15R α has improved on the wild-type variant of this cytokine. Soluble expression of the N-terminal sushi domain of IL-15R α enhances IL-15 binding to IL-2R β and γ .⁸⁶ Additionally, fusion of a IL-15 superagonist with IL-15R α could successfully inhibit TGF- β 1 and maintain NK cell cytotoxicity in immunosuppressive environments like the TME.⁸⁷ Thus, maintaining and stimulating the IL-15 signaling axis appears to be a crucial driver of NK cell survival in adoptive transfers.

Considering regulators of IL-15, cytokine-inducible SH2-containing protein (CIS) is an inhibitor of IL-15 signaling for NK cells. CIS is expressed in response to IL-15 signaling and deletion of *CISH*, the gene encoding CIS, confers IL-15 hypersensitivity to NK cells, allowing them to have enhanced survival, proliferation, and effector functions.⁸⁸ Notably, CIS-ablation does not drive spontaneous hyperactivity of NK cells; rather, CIS-ablation reduces the threshold for NK cell activation.⁸⁹ Thus, *CISH*-knockout can pave the path for more robust NK cell responses.

IL-15 metabolic control has been harnessed in preclinical NK cell therapies. A *CISH*-knockout (*CISH*-KO) iPSC-NK cell platform showed improved proliferation and strong cytotoxicity in vivo in murine AML. Further characterization found that these *CISH*-KO iPSC-NK cells had better metabolic fitness, as measured by glycolytic and oxidative phosphorylation rates.⁹⁰ Additionally, application of this *CISH*-KO to UB-CAR-NK cells was also successful, and NK cell effector functions were enhanced with increased mTORC1 and c-Myc signaling.⁹¹ These

data suggest that numerous avenues for allogeneic NK cell therapy could benefit from metabolic reprogramming via *CISH*-KO. In particular, iPSCs are an easy system in which to execute targeted genetic modification, making iPSC-NK cells a well-suited model for enhanced effector functions via *CISH* modulation. However, other forms of allogeneic NK cell therapy are by no means excluded from the possible benefits of *CISH*-KO.

Besides the IL-15 signaling axis, other metabolic modulators of NK cells can affect survival and function. CD38 catalyzes hydrolysis of cyclic ADP-ribose (cADPR), a NAD⁺ metabolite.⁹² CD38+cells have reduced persistence and are more susceptible to oxidative stress.⁹³ CD38 expression can interfere with antitumor responses, and blockade of CD38 improved T cell tumor control, providing an avenue for enhancing cell therapies.⁹⁴ In cells derived from patients with multiple myeloma (which commonly expresses CD38), genetic ablation of CD38 in PB-NK cells via CRISPR/Cas9 synergized with daratumumab, an anti-CD38 monoclonal antibody, and improved in vivo persistence and enhanced ADCC. These CD38-KO PB-NK cells had metabolic changes as well, demonstrating increased mitochondrial respiratory capacity.⁹⁵ Moreover, a CD38 knockdown NK cell platform expressing a CD38 CAR showed significant efficacy against AML in vitro.⁹⁶ Thus, CD38-KO presents an orthogonal option to IL-15 signaling enhancement as a tool to promote NK cell survival, proliferation, and function and could be easily adaptable to iPSC-NK and other allogeneic NK platforms.

While it is unlikely that NK cell persistence via metabolic engineering will ever surpass autologous T cell persistence, these efforts collectively point to novel ways of boosting NK cell therapy. Enhanced in vivo survival and effector function encourages prolonged NK cell

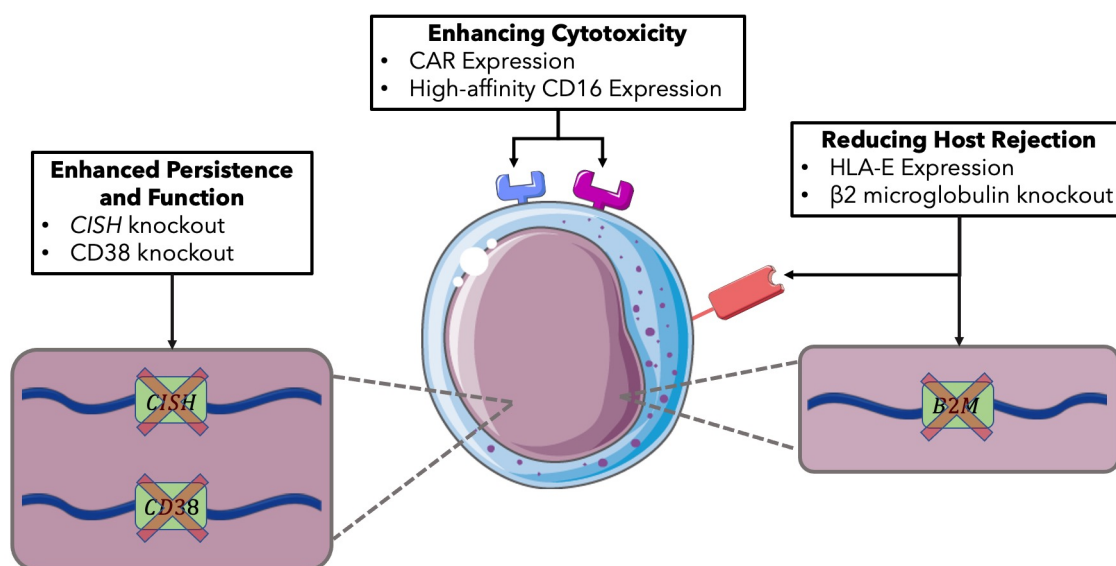


Figure 1 Engineering of NK cells for allogeneic therapy. NK cells can be genetically modified via knock-in or knockout of genes to enhance efficacy, reduce host rejection, or increase in vivo persistence of allogeneic NK therapies. Figure uses adaptations of cartoon images from Servier Medical Art, licensed under CC BY 3.0. CAR, chimeric antigen receptor; HLA, human leucocyte antigen; NK, natural killer.

Table 2 Summary and comparison of distinct allogeneic NK cell donor sources

Donor source	Peripheral blood NK (PB-NK)	Umbilical cord blood NK (UB-NK)	NK-92 cell line	iPSC-derived NK (iPSC-NK)
Safety	Well tolerated with no severe adverse events commonly reported in clinical data. Contamination with other lymphocytes (T cells) can occur during ex vivo processing and potentially affect toxicity.	Well tolerated with no severe adverse events commonly reported in clinical data. Contamination with other lymphocytes (T cells) can occur during ex vivo processing and potentially affect toxicity.	Well tolerated with no severe adverse events commonly reported in clinical data. Clonal population reduces risk of potential toxicity caused by heterogeneity of product.	Generally well tolerated, although some adverse events were occasionally reported. Clonal population reduces risk of potential toxicity caused by heterogeneity of product.
Cytotoxicity	Activation via ex vivo coculture with HLA-deficient K562 cells and IL-2, IL-15, or IL-21 stimulation. Clinical data shows good efficacy in liquid tumors. PB-NK has better cytotoxicity on average than UB-NK cells. However, poor efficacy was seen if cells were cryopreserved.	Activation via ex vivo coculture with HLA-deficient K562 cells and IL-2, IL-15, or IL-21 stimulation. Clinical data show good efficacy in liquid tumors, and UB-NK has better bone marrow homing than PB-NK cells but poorer cytotoxicity.	Poor in vivo expansion due to gamma irradiation, resulting in limited cytotoxicity. Clinical data shows some efficacy, but more limited compared with other NK cell sources. NK-92 cells thus have poor longevity in systemic circulation that prevents a durable response.	Do not require gamma irradiation or a coculture system for expansion. Significant in vivo cytotoxicity has been observed in hematologic and solid tumor models. Clinical data shows good efficacy in hematologic tumors. Ease of engineering makes metabolic reprogramming to boost cytotoxicity and persistence more facile.
Manufacturing	NK cells are a small subset of total lymphocyte population that must be expanded ex vivo.	NK cells are more prevalent than in PB but ex vivo expansion still needed.	NK-92 cell line can be easily expanded via culture before dosing.	Differentiating iPSCs to NK cells can be complex, but new methods to bypass single cell adaptation are simplifying manufacturing.
Engineering	Difficult to extract a clonal population, and genetic engineering can kill many of the NK cells available.	Difficult to extract a clonal population, and genetic engineering can kill many NK cells present, making the process inefficient.	NK-92 is a clonal population, allowing for homogeneous engineering and genetic manipulation.	iPSC cells provide a clonal population with well-established methods of viral integration to knock in or knock out genetic constructs.

iPSC, induced pluripotent stem cell; NK, natural killer.

antitumor responses that can result in stronger and more durable patient responses.

CAR-NK cell therapy

CAR-NK cells present another avenue for engineering allogeneic NK cells that is rapidly advancing. Although CAR-T cells can achieve durable responses as they persist longer and have the potential to differentiate into memory T subsets, CAR-NK cells retain the additional ability to clear tumor cells independent of CAR-function.^{19,97} Moreover, CAR-T cells can have significant associated toxicities, such as cytokine release syndrome and neurotoxicity. Due to their limited lifespan in circulation as well as the lack of HLA-recognition necessary for activation, CAR-NK cells generally have less toxicities, especially in allogeneic contexts.¹⁹ Moreover, given the more facile and reliable methods of engineering iPSC-NK cells, CAR-NK may be easier to manufacture than CAR-T cells. Thus, CAR-NK cells are an avenue for off-the-shelf

CAR therapy and can potentially bypass the leukapheresis and subsequent T cell processing that complicates CAR-T manufacturing.

As of March 2022, there are over 20 clinical trials testing CAR-NK constructs in various hematologic and solid tumor malignancies, according to ClinicalTrials.gov. As discussed in the section on UB-NK therapies, a CD19-targeting CAR-NK construct showed significant efficacy in an initial phase I study with no serious toxicities.⁴⁹ Moreover, Bachanova *et al* presented initial clinical data on FT596, a CD19-targeting, off-the-shelf, iPSC-derived CAR-NK therapy, in B-cell lymphoma at the 62nd American Society of Hematology Annual Meeting. Although formal peer-reviewed publication of the data is still pending, initial data of FT596 in a single patient showed a partial response with over 50% reduction in tumor volume. However, several adverse events, including leukopenia, neutropenia, anemia, urinary tract

infection, and hypertension were observed. Although FT596 shows promise, more data and peer-review are still necessary before any conclusions can be made about this therapy.⁹⁸ Clinical trials of other CAR-NK constructs in different hematologic malignancies and solid tumors are currently recruiting, and we expect to see further clinical validation of CAR-NK treatments in the coming years.⁹⁹ CAR-NK therapy is merely one key example of the benefits of genetical manipulation of NK cells, and [figure 1](#) highlights different ways to genetically modify NK cells that have been discussed in this review.

FUTURE DIRECTIONS FOR NK CELL THERAPY

Allogeneic NK cell therapy continues to be a promising avenue for adoptive cell immunotherapy in a variety of hematologic and solid cancers. NK cells can be produced at a clinical scale with a variety of methods, including isolation and expansion from blood, generation of clonal, immortalized NK cell lines, and more recently, the use of iPSCs. Preclinical data supporting all these technologies exist, and clinical data demonstrating the safety of these technologies have been well documented for blood-derived NK cells and NK-92 cells. iPSC-NK cells are a newer approach to clinical-scale generation of NK cells, but the preliminary safety and efficacy data from clinical trials are very promising. The results and formal publication of these clinical trials will confirm the safety and efficacy profiles seen thus far in phase I data. [Table 2](#) compares the different types of allogeneic NK cell sources with each other.

Additionally, a deeper understanding of how other metabolic markers and signaling axes can influence NK cell persistence and effector functions is needed. CIS and CD38 have been important negative regulators of NK cell activity, and knockout of these proteins contribute to more robust NK cell function. Investigation of the role of other metabolic regulators on NK cell biology can pave potential future directions for adoptive NK cell therapy.

As NK cell therapies advance in clinical efficacy, efforts will need to focus on streamlining manufacturing in all allogeneic approaches. Reliable, homogenous infusions are necessary to ensure standard of care as NK cells are administered to patients. This means PB-NK and UB-NK approaches require methods to reduce donor variability and effectively extract and expand the NK cell population. iPSC-NK cells represent an emerging technology in which clonal populations can adequately maintain effector function. Manufacturing challenges for iPSCs focus on their production and differentiation, and solutions are already emerging. In coming years, iPSC-NK cell production may be streamlined. At the level of clinical translation, efforts should be made to identify new ways to maintain NK cell persistence in systemic circulation without lymphodepletion. Such immunosuppression often results from chemotherapy or radiation administered alongside allogeneic NK cells. Avoiding patient lymphodepletion can potentially unlock the full spectrum of NK cell functionality

to trigger a larger and possibly more robust network of immune interactions and create strong, durable anti-tumor responses.

As problems in manufacturing and engineering of allogeneic NK cell therapies are addressed, new technologies that can modulate NK cell activity and mitigate toxicities can offer new paths for the field. While genetic modification has already been successfully executed in NK cells, more complex genetic circuits can be established in the future to improve longevity and functionality of infused cells. Furthermore, genetic modulation of cytotoxicity can mitigate off-target toxicities and adverse events in patients. For instance, a CAR construct was developed whose surface expression is controlled by the small molecule asunaprevir, which blocks CAR expression when administered.¹⁰⁰ Such technologies can expand in the future to provide options to ensure the safety of patients receiving these therapies.

Additionally, opportunities exist to extend cell therapy to closely related ILC subsets. Other ILC populations besides NK cells can be used and offer the possibility of fine-tuned immune responses that are adapted to the patient's specific tumor profile. Additionally, newly characterized subtypes of NK cells could enhance antitumor responses. Intraepithelial ILC1-like (ieILC1-like) NK cells have recently been documented as a potent antitumor subpopulation in the TME of HNSCC samples, and this novel population could be a source of future NK cell therapies.¹⁰¹

Overall, allogeneic NK cell therapies have been an established yet continuously evolving approach to cancer immunotherapy with immense potential for the treatment of numerous forms of cancer.

Correction notice This article has been corrected since it was first published online. In the section titled 'Induced pluripotent stem cell-derived NK cell therapies', The sentence 'For instance, CAR-NK cells derived from iPSCs targeting mesothelin showed strong in vivo responses in mouse A1847 ovarian cancer and outperformed PB-NK cell therapy' has had the phrase 'mouse A1847 ovarian cancer' updated to 'human A1847 ovarian cancer'.

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