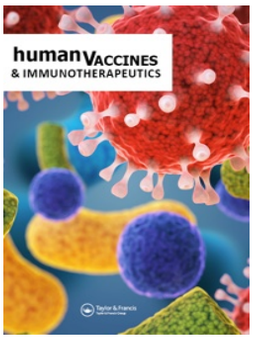

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REVIEW

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NK cell-based immunotherapy

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ABSTRACT

NK cells have rapidly become a leading platform for cancer immunotherapy. Unlike T cells, NK cells recognize transformed cells through integrated signals from inhibitory and activating receptors, enabling allogeneic "off-the-shelf" therapies without risk of graft-versus-host disease and low rates of cytokine release syndrome and neurotoxicity. Refinement in sourcing of allogenic NK cells, especially umbilical cord blood and induced pluripotent stem cells, *ex vivo* activation and expansion, CAR engineering, and combinations with novel engagers have resulted in clinically applicable products with encouraging activity and excellent safety. However, barriers remain, including limited *in vivo* persistence, host rejection of allogeneic cells, and inefficient trafficking into solid tumors. Venues of progress include multiantigen and logic-gated CAR platforms, inducible safety switches, cytokine armoring, and metabolic and transcriptional rewiring to improve NK fitness. As NK cell therapies progress into the clinic, exploration of rational combinations with checkpoint inhibitors, small-molecule kinase inhibitors, chemotherapy, and radiotherapy warrant study.

PLAIN LANGUAGE SUMMARY

NK cells are part of the innate immune system. They share their cytotoxic mechanisms with T cells, but in contrast to these, NK cells don't require a previous exposure to antigen and their cytotoxicity ultimately depends on the balance of an array of activating and inhibitory receptors that is unique to each NK cell. This review will examine the sources of the NK cells for therapeutic use and how NK cells have been modified to effectively kill cancer cells. The challenges to developing effective allogeneic NK cell-based therapy will be discussed. We will review advanced genetic engineering of the NK cells to address these problems with a particular focus on CAR-NK cells, and future directions of progress.

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Introduction

Cancer immunotherapy has its roots in the late nineteenth century, when clinicians began noticing the immune system's capacity to influence tumor behavior. Busch first reported striking tumor regressions following erysipela infections, observations that were soon echoed and expanded by William Bradley Coley (now widely regarded as the Father of Immunotherapy), who in 1891 attempted to stimulate antitumor immunity in patients with bone sarcomas.[1] Although Coley's work remained largely unrecognized for decades, subsequent landmark immunological discoveries, including the identification of T cells and their central role in adaptive immunity, and of natural killer (NK) cells and their unique biology,[2] laid the foundation for the development of contemporary cancer immunotherapies.

Today, immunotherapy represents one of the most transformative advances in oncology, spanning cancer vaccines, immune checkpoint blockade, and engineered adoptive cell therapies.[3–5] The impressive success of CAR-T cells in hematologic malignancies has catalyzed an intense interest in harnessing additional immune cell platforms to overcome limitations inherent to autologous T-cell–based approaches.[6] Unlike T cells, NK cells do not rely on an αβ T-cell receptor (TCR) and HLA-restricted antigen recognition, which makes them naturally compatible with allogeneic "off-the-shelf" use without the risk of graft-versus-host disease (GVHD).[7] This eliminates the need for gene editing to remove an endogenous TCR expression and avoids the logistical constraints of harvesting heavily pretreated patients'

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own immune cells, often a rate-limiting step in autologous CAR-T manufacturing. NK cells can be obtained from a variety of allogeneic sources, including haploidentical donors, umbilical cord blood (UCB), induced pluripotent stem cells (IPSCs), and established cell lines such as NK-92, enabling scalable, standardized production at a significantly lower cost. CAR-NK cells also elicit a markedly safer cytokine release profile.[8] This likely reflects NK biology, which does not involve a massive IL-1β/IL-6 release, in contrast to when triggered by T-cell activation.

However, NK cell therapy is not without challenges. Early NK cell infusions were safe but offered limited clinical benefit because of poor persistence, susceptibility to host-versus-graft rejection, inadequate tumor infiltration, and strong suppression by the tumor microenvironment (TME).[9] Nonetheless, those issues are being addressed, and advances in cell sourcing, expansion, cytokine support, and genetic engineering have improved outcomes.[10] Furthermore, combination of NK cells with other immunotherapies, such as monoclonal antibodies or checkpoint inhibitors, add synergistic benefit and help counteract the suppressive effect of the TME. These emerging developments are positioning engineered NK cells as a promising next-generation platform in cancer immunotherapy. In this review we will explore the underlying biology and sourcing of NK cells, the current use of NK based immunotherapies, their limitations, and a perspective on future directions in the field.

NK cell biology

NK cells are central cytotoxic lymphocytes of the innate immune system, included in group 1 innate lymphoid cells (ILCs), and share the same cytotoxic mechanisms as $CD8^+$ T cells.[11] NK cells secrete IFN-γ, but not type 2 cytokines such as IL-4, IL-13, IL-17, or IL-22. Human NK cells classically express CD56 but not CD3, differentiating them from NK-like T (NKT) cells and antigen-experienced T cells, which express both.[12]

Human NK cells comprise several distinct subsets defined by differential CD56 and CD16 expression, each contributing uniquely to immune defense and tissue homeostasis. The major circulating population, $CD56^{dim}$ $CD16^+$ NK cells, constitutes 90–95% of peripheral blood NK cells and specialize in rapid cytotoxic responses. In contrast, $CD56^{bright}$ $CD16^{-/low}$ NK cells represent only 5–10% of circulating NK cells and produce cytokines such as IFN-γ, TNF-α, and GM-CSF.[13–15] These cytokine-rich cells preferentially home to secondary lymphoid tissues (and decidua during early pregnancy), where they comprise up to 70% of local lymphocytes and stimulate the adaptive immune response.[16] The cytokines IL-2 and IL-15 are key drivers of maturation of the immunomodulatory $CD56^{bright}$ NK cells into the cytotoxic $CD56^{dim}$ phenotype.[17]

NK cells present a variable expression of germline-encoded inhibitory receptors (e.g., several killer immunoglobulin-like receptors (KIR) and NKG2A), activating receptors (e.g., CD16a, NKG2C, NKG2D, or NKp46), cytokine receptors (e.g., high-affinity IL-2 R), and growth factor receptors (e.g., CD117) (Figure 1).[18] Each array of receptors is unique to each individual NK cell, and the final balance of signaling from activating and inhibitory receptors against a potential target cell will result in the decision to kill or not to kill that cell (Figure 2).[19,20]

Together, these developmental pathways and receptor repertoires illustrate the multifaceted nature of human NK-cell biology. Depending on the source and expansion as well as exposure to stimulatory cytokines the population of NK cells can be distinctly altered.

NK activation and cell killing

NK cells are potent innate lymphocytes capable of rapidly eliminating abnormal cells (particularly virus-infected and malignant cells) without prior sensitization, in contrast to their counterparts in the adaptive immune system, the CD8+ T cells.[21] Unlike T cells, which rely on somatically mutated antigen-specific TCRs to engage peptides presented within HLA (human leukocyte antigen) molecules, NK cells integrate signals from their repertoire of germline-encoded activating and inhibitory receptors.[22–24] Key inhibitory receptors, such as certain KIRs and NKG2A, survey HLA class I expression to maintain self-tolerance. When HLA-I is reduced or lost, as it often occurs in virally infected or malignant cells to avoid recognition by CD8+ T cells, the inhibitory no-kill signal is absent ("missing-self" recognition).[25] Additionally, a second

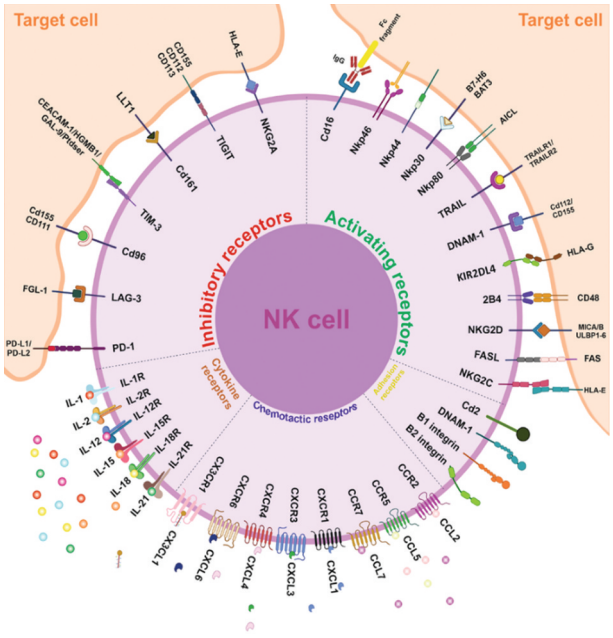

Figure 1. NK cell receptors. NK cells on their surface have activating receptors, inhibitory receptors, and cytokine receptors. Activating receptors give a positive signal for NK cells to kill, whereas inhibitory receptors give a negative signal for NK cells NOT to kill. Inhibitory receptors recognize either HLA class I or HLA class I-like molecules. Activating receptors, at least for those receptors whose ligands have been described, recognize stress molecules or DNA stress molecules, which are upregulated on the surface of abnormal cells.

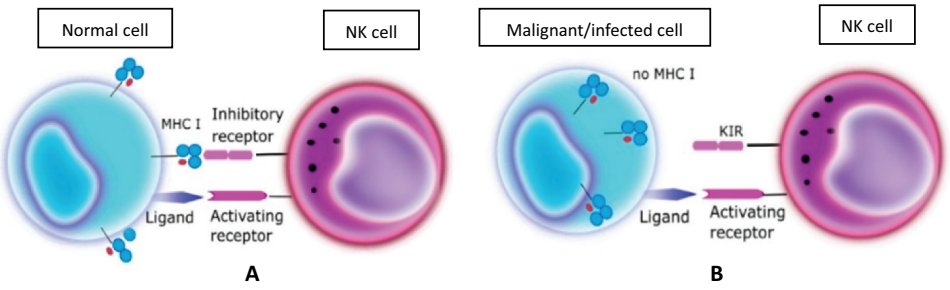

Figure 2. How licensed NK cells distinguish between normal and abnormal cells. (A). When the NK cell comes into contact with a normal cell that expresses HLA class I on the surface it will get a signal through its inhibitory receptor not to kill. And the strength of this negative signal from an inhibitory receptor always trumps that of the activating receptor. (B). Cancer cells or virally infected cells often downregulate HLA class I expression on their surface in order to escape CD8 T-cell recognition. So, since there is no longer an HLA class I expressed, the inhibitory NK receptor doesn't have an HLA class I or class I-like molecule to engage with and does not send an inhibitory signal. The NK cells activating receptor will recognize its activating ligand, which, in many cases, are stress molecules and will mediate killing of the cancer cell.

signal from an activating receptor is needed (e.g., stress-induced ligands on the target cells binding NKG2D, or the Fc of a tumor cell-targeting antibody that binds CD16a).[26,27] This complex equilibrium between inhibitory and activating receptors enables the identification and elimination of distressed cells independent of antigen specificity.

Upon an overall transduction favoring a kill signal, NK cells deploy several effector mechanisms. They can directly induce apoptosis through perforin- and granzyme-mediated cytotoxicity or through death-receptor pathways such as TRAIL and FasL.[28–30] They also secrete a range of cytokines and chemokines (including IFN-γ, TNF-α, GM-CSF, XCL1, CCL5, and MIP-1α/β) that shape downstream adaptive immune responses.[31,32] Therefore, in addition to the cytolytic activity of the $CD56^{dim}$ subset, $CD56^{bright}$ NK cells participate in immunoregulatory processes and engage dendritic cells (DC) in reciprocal activation. NK cells promote DC maturation – increasing

Table 1. Sources of allogeneic NK cells.

Cell source	Advantages	Disadvantages
Peripheral blood	• Mature phenotype • Highly functional and cytotoxic	• Only 5–10% of peripheral blood lymphocytes are NK cells • Heterogeneous product • Not readily available, need donors • Low proliferative capacity
NK cell line (NK-92)	• High proliferative capacity • Easy to manipulate and engineer • Homogeneous product • Reduced sensitivity to freeze/thaw cycles	• Derived from a patient with NK lymphoma → require irradiation • Limited *in vivo* persistence following irradiation • Low/absent CD16 expression → low ADCC
iPSC	• High proliferative capacity • Homogeneous product	• Immature phenotype • Low CD16 expression → low ADCC • Long culture condition
Umbilical cord blood	• Readily available from UCB banks • 15–30% of UCB lymphocytes are NK cells • High proliferative capacity • High CD16 expression	• Low number – require *ex vivo* expansion • Heterogeneous product

Note: iPSC: induced pluripotent stem cells. ADCC: Antibody-dependent cellular cytotoxicity.

expression of CD83, CD86, and HLA-DR – via cell-to-cell contact and cytokine release (TNF-α, IFN-γ). This interaction is heavily mediated by the NKp30 receptor and plays a crucial role in bridging innate and adaptive immunity.[33,34] Furthermore, NK cells drive antitumor immunity by promoting intratumoral stimulating DC expansion via FLT3L and recruiting conventional type 1 DCs through CCL5/XCL1. This NK–DC axis correlates with better anti–PD-1 responses and survival, but is disrupted by tumor-derived PGE2, enabling immune evasion and highlighting a key therapeutic target.[35,36]

The immune system continuously monitors for and eliminates transformed malignant cells. NK cells contribute directly to this surveillance but also collaborate with other immune components, most notably through antibody-dependent cellular cytotoxicity (ADCC), in which CD16a engages IgG-coated tumor cells. These biological properties have positioned NK cells as compelling candidates for cancer immunotherapy.

Lastly, although NK cells are part of the innate system, they can exhibit traits of immunological memory with enhanced recall responses, after exposure to CMV, other viruses, or certain inflammatory cytokines.[37,38]

Adoptive NK cell immunotherapy

The source of NK cells for adoptive immunotherapy can be autologous or allogeneic. Allogeneic sources include immortalized cell lines, donor peripheral blood, umbilical cord blood (UCB) and induced pluripotent stem cells (iPSCs) (Table 1).

Autologous NK cells

Autologous NK cells are harvested from the patient's peripheral blood. Since NK cells make up only 10–20% of peripheral blood mononuclear cells, it is necessary to isolate and expand them for therapeutic use. Additionally, early efforts used high-dose IL-2 to boost NK-cell activity, but its toxicity and the resulting activation of regulatory T cells (Tregs) limited its clinical benefit. Although IL-2 can drive NK-cell proliferation *in vitro* (and low-dose IL-2 can selectively expand NK cells *in vivo*), cytokine stimulation alone appears insufficient for a sustained, large-scale expansion. Additionally, autologous NK cells may be dysfunctional and are often metabolically exhausted, even if their cytotoxicity can be boosted by cytokine stimulation with IL-2, IL-12, IL-15, IL-18, or type I interferons.[39–41] Unlike allogeneic therapies, autologous NK cells can be infused without lymphodepletion, which reduces treatment toxicity. Autologous NK cells can persist *in vivo* for weeks to months, but they rapidly lose cytotoxic potential, likely due to inhibitory receptor engagement by self-HLA molecules, an intrinsic barrier to effective autologous NK-cell tumor surveillance.

Clinical trials utilizing expanded and co-stimulated autologous NK have demonstrated safety but low efficacy.[42–45] As a result, the field has largely shifted toward allogeneic NK-cell platforms.

Allogeneic nonengineered NK cells

Allogeneic NK therapy addresses the limitations of autologous NK cells, which are often dysfunctional in cancer patients and difficult to engineer efficiently. Nontargeted allogeneic NK cell therapy builds on the principle that donor NK cells encountering HLA-mismatched recipient cells lack inhibitory engagement through self-HLA, enabling potent NK cell alloreactivity.[46] Thus, haploidentical NK cells can exert anti-tumor effects in patients. Allogeneic NK cells are usually expanded *ex vivo* using cytokines like IL-2 or IL-15 in combination with irradiated feeder systems (most commonly K562 cells engineered to express 4–1BBL and membrane-bound IL-15 or IL-21).[47,48]

Immortalized cell lines

The only immortalized NK cell line tested clinically is NK-92.[49] NK-92 is a continuously growing, IL-2–dependent human NK cell line originally established from the peripheral blood of a patient with an aggressive NK lymphoma. The cells require exogenous IL-2 for survival and cytotoxic function as its deprivation leads to rapid loss of activity and cell death. NK-92 cells highly express CD56 and lack CD16,[50] although a "NK-92 CD16" cell line was generated via transduction with the CD16 gene (often the high-affinity V158 variant) to enable ADCC.[51] Consistent with their immature and unlicensed $CD56^{bright}$-like profile, NK-92 NK cells express few KIRs, retaining only the atypical KIR2DL4, which possesses both inhibitory and activating potential.[52]

One advantage of NK-92 cells is their ease of large-scale expansion.[51] Their manufacturing simplicity allows NK-92 to function as an "off-the-shelf" product that can be prepared from a cryopreserved master cell bank. In contrast, they present important disadvantages. Besides being susceptible to recognition and destruction by a patient's own immune system (as any allogeneic NK cells), their malignant origin imposes a critical safety requirement for irradiation prior to infusion to prevent uncontrolled proliferation. This irradiation step compromises their longevity and functional durability inside the patient, leading to curtailed *in vivo* survival and reduced cytolytic capacity.[53] NK-92 cells often exhibit limited persistence, especially within the cytokine-poor TME. Their activity declines rapidly under such conditions, restricting their clinical utility.

Peripheral blood haploidentical NK cells

Pioneering work by Miller and his group at the University of Minnesota first showed the safety of haploidentical donor peripheral blood NK cells without triggering GVHD. These investigators treated 43 patients with various malignancies, including acute myeloid leukemia (AML), melanoma, renal cell carcinoma, and refractory Hodgkin lymphoma, with lymphodepletion followed by haploidentical NK cell infusions and IL-2. Notably, 5 out of 19 AML patients (26%) achieved a complete remission (CR).[54] Subsequent trials in lymphoma patients yielded a 33% response rate, further demonstrating the therapy's feasibility despite limited duration of efficacy.[55]

Those studies established the critical requirement of preinfusion lymphodepletion to prevent host rejection of the allogeneic NK cells, as well as to favor their expansion by increasing exposure to homeostatic cytokines (IL-7 and IL-15), and to eliminate competing elements of the immune system (cytokine sinks like the Tregs). However, it also eliminates cells that produce the cytokines necessary for NK cell survival and function, such as macrophages, dendritic cells or CD4+ T cells. To compensate for this, exogenous cytokine support was added, first with IL-2.[54,56] While this stimulates NK cell proliferation, it can also be counter-productive due the pleiotropic nature of IL-2, which activates Tregs, inhibitors of NK cell activity. Likewise, exogenous systemic IL-15 support, including superagonists, has been shown to increase the rejection of allogeneic NK cells by boosting the recipient's CD8+ T cells.[57]

Umbilical cord blood (UCB)-derived NK cells

To avoid the burden of harvesting NK cells from peripheral blood, streamlining their production, and creating a truly "off-the-shelf" treatment, our group has focused on UCB as source of NK cells. In an early

study, we treated 12 patients with multiple myeloma with high-dose chemotherapy (HDC) and with autologous stem-cell transplant (ASCT), lenalidomide, and UCB-derived NK cells at escalating doses.[58] To optimize their antitumor effects, the UCB units were selected based on HLA/KIR mismatch. We saw no GVHD or dose-related toxicities. After 21 months, 10 patients achieved at least a partial response (PR). This small study first first showed the safety of UCB-derived NK cells, although outcomes were comparable to standard HDC/AHSCT.

In a second trial we treated 20 patients with relapsed B-NHL with UCB-derived NK cells and rituximab, lenalidomide, and HDC/ASCT.[59] In this study we did not prioritize HLA-KIR matching. We saw no treatment-related mortality within the first 30 d. At a 4-y follow-up, the relapse-free survival (RFS) and overall survival (OS) rates were 53% and 74%, respectively. Although donor NK cells remained detectable for less than two weeks, we observed that UCB cells expressing a high-affinity CD16 variant were linked to better outcomes, which highlights the importance of ADCC in the effectiveness of UCB-derived NK therapy.

Taken together, both trials clearly showed the safety of nontargeted UCB-derived NK cells, but also that their activity appeared limited by their lack of specific tumor recognition.

Cytokine-induced memory-like NK cells

Adaptive-like NKG2C^{+} NK cells from CMV-seropositive donors exhibit long-lived, cytotoxic behavior with a characteristic activating receptor profile and potent activity against AML, ALL, and multiple myeloma.[60,61] Tumor-primed NK cells also acquire memory-like properties and have produced encouraging responses in early AML studies.[62] Researchers at Washington University generated cytokine-induced memory-like (CIML) NK cells through brief exposure to inflammatory cytokines (IL-12, IL-15, and IL-18).[15] These CIML NK cells are reminiscent of virally induced memory T and NK cells in that they represent a functionally enhanced state characterized by greater persistence, heightened proliferation, and more durable effector function than conventional NK cells.[63] They upregulate activating receptors such as NKG2D, NKp46, and DNAM-1, with reduced expression of inhibitory receptors like killer-cell immunoglobulin-like receptors (KIR), enabling broader tumor recognition and stronger cytokine production. CIML have demonstrated safety and long-term expansion in early clinical trials.[64,65]

In our early UCB-derived NK cell trials, it became clear that cell persistence was a significant issue.[58,59] To address this obstacle, we generated UCB-derived CIML NK cells after preactivation with inflammatory cytokines, which were then expanded in the presence of universal antigen presenting cells (uAPCs)[66] and precomplexed with AFM13 (acimtamig), a bispecific antibody construct that binds CD16a on the NK cells with CD30 on the lymphoma cells.[67] We treated 42 patients with heavily pretreated CD30+ lymphoma, refractory to both brentuximab vedotin and checkpoint inhibitors, with lymphodepletion followed by

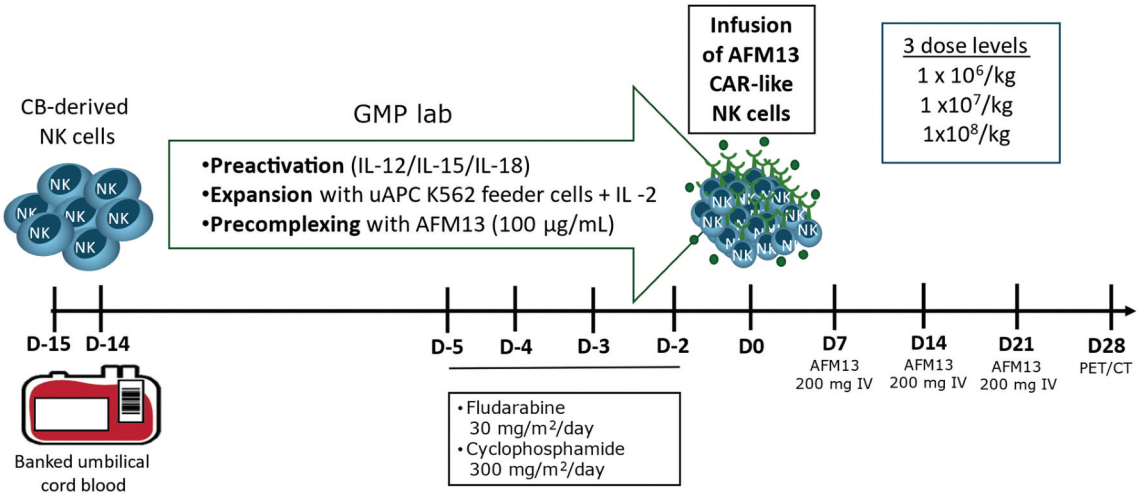

Figure 3. Treatment schema of the MD Anderson trial of AFM13-precomplexed preactivated and expanded umbilical cord blood-derived NK cells for CD30+ lymphomas.

AFM13-NK cells and three subsequent weekly AFM13 infusions (Figure 3). The therapy was well tolerated, with no reports of cytokine release syndrome (CRS), neurotoxicity, or GVHD. Donor NK cells peaked in blood at one day postinfusion, persisted for up to three weeks, and trafficked to tumor sites. The antitumor activity was outstanding, with an overall response rate (ORR) of 93% with 67% CRs, and 11 patients maintained a CR for 14 to 40 months. The 2-y RFS and OS rates were 26% and 76, respectively. This study highlighted the significant potential of using bispecific antibodies to direct NK cell activity against treatment-resistant cancers with minimal toxic side effects.

Allogeneic engineered NK cells

CAR-NK cells

CAR T-cell therapy has reshaped modern cancer treatment, demonstrating potent clinical activity across multiple malignancies. However, individualized manufacturing, high costs, the risk of GVHD with allogeneic T cells (initiated through their αβ T-cell receptor), and toxicities like CRS and neurotoxicity that can be life threatening, limit its broader application. These challenges have accelerated the interest in engineering CARs into immune effector cells different from αβ T lymphocytes, including γδ T cells, invariant NKT cells, macrophages, and, particularly, NK cells.[68] The purpose is to facilitate targeted tumor recognition with a more favorable safety profile. The properties of NK cells, i.e., rapid and HLA-unrestricted cytotoxicity without prior antigen priming, recognition of "missing-self" and "induced-self" signals, and mediation of ADCC, make them inherently resistant to GVHD and uniquely suited for allogeneic, off-the-shelf adoptive therapy. Banked UCB or iPSCs are clinically scalable and donor-independent sources of NK cells that can be engineered with viral transduction of CAR.[69]

The CAR engineered into NK cells shares the same basic structure as in CAR-T cells, with an scFv for antigen recognition, a transmembrane region, and intracellular costimulatory domains, and, as in CAR-T cells, their engineering has evolved from simple first-generation constructs containing only CD3ζ to second- and third-generation designs incorporating costimulatory domains such as CD28, 2B4, 4–1BB, OX-40, DAP10, and DAP12.[70–72]

Tang et al. reported the first-in-human experience using CD33-targeted CAR-NK-92 cells in three patients with refractory AML, demonstrating that the therapy was well tolerated with no dose-limiting toxicities or severe adverse events. Although no CRs were achieved, one patient showed transient reduction of leukemic blasts.[73]

Our group investigated the use of UCB-derived NK cells engineered with an anti-CD19 CAR including a CD28 costimulatory domain, "armored" through transduction with the *IL15* gene to secrete stimulatory IL-15, and including an inducible Caspase 9 (iCaspase 9) "suicide switch" for enhanced safety.[72] Eleven patients with relapsed or refractory CD19-positive B-cell malignancies were treated with lymphodepleting chemotherapy and CAR- NK cells. As in our prior trials of nontargeted UCB-derived NK cells,[58,59] treatment was well tolerated, with no cases of GVHD despite large HLA mismatches, or any instances of CRS or neurotoxicity. The ORR and CR rates were 73% and 64%, respectively, with 1-y PFS and OS rates of 32% and 68%, respectively. Responders exhibited higher expansion and longer persistence of CAR-NK cells than nonresponders, supporting the relevance of *in vivo* durability. We subsequently reported the dose expansion phase of that study, with 26 additional patients.[74] The therapy was most effective in low-grade B-cell non-Hodgkin lymphoma (B-NHL) (100% ORR; 83% CR at 1 y), followed by chronic lymphocytic leukemia (67% ORR; 50% CR at 1 y), and diffuse large B-cell lymphoma (DLBCL) (41% ORR; 29% CR at 1 y). We identified two critical UCB unit factors for better clinical results: a low nucleated red blood cell (nRBC) count ($\leq 8 \times 10^7$ cells per cord blood unit) and a cryopreservation time of 24 hours or less. NK cells from optimal UCB units were highly functional and enriched in effector-related genes. In contrast, NK cells from suboptimal units showed upregulation of inflammation, hypoxia and cellular stress programs. Ultimately, these findings highlight that UCB-derived CAR NK cells provide a safer alternative to CAR T-cell therapy with comparable efficacy for CD19+ malignancies.

Lei et al., subsequently reported on UCB-derived anti-CD19 CAR NK including a different costimulatory domain (4–1BB) in eight patients with relapsed B-NHL. Treatment was again well tolerated and active, with a 62.5% ORR and 50% CR rate.[75] It remains to be determined whether the distinct effects of different costimulatory domains on CAR-T cells (CD28 promoting early T-cell activation and a T effector memory

phenotype with rapid tumor cell killing, and 4–1BB favoring a longer-lived central memory phenotype) also applies to CAR NK cells and, if so, whether it translates into different clinical results. Acharya et al. evaluated the preclinical impact of various costimulatory domains on CAR-NK cell activity, using a CD70-targeting CAR, and found that CD28, a costimulatory molecule not inherently present in mature NK cells, significantly enhanced the antitumor efficacy and long-term cytotoxicity of CAR-NK cells, as compared to other costimulatory molecules naturally associated with NK cells (DAP12, DAP10) or with both T and NK cells (4–1BB).[76] CD28 linked to CD3ζ creates a platform that recruits critical kinases, such as lymphocyte-specific protein tyrosine kinase (LCK) and zeta-chain-associated protein kinase 70 (ZAP70), initiating a signaling cascade that enhances CAR-NK cell function.

Preclinical studies continue to broaden allogenic derived CAR-NK applications to solid tumors and diverse antigens, including CS1 and CD70 in multiple myeloma,[77] CD5 for T-cell malignancies,[78] EGFR and EGFRvIII for glioblastoma and brain metastases,[79] prostate stem cell antigen (PSCA) for pancreatic cancer,[80] and FLT3 for AML.[81]

Altogether, CAR-NK therapy combiness the specific tumor-targeting capabilities of CARs with the inherent safety, allogeneic compatibility, and off-the-shelf manufacturability of NK cells.

Induced pluripotent stem cells derived NK cells

Induced pluripotent stem cells (iPSC) constitute another important source of allogeneic NK cells. Since iPSC-derived NK cells express low levels of CD16 (Fc receptor), these cells are transduced with high-affinity noncleavable CD16 (hnCD16). Strati et al. treated 55 patients with CD20-positive B-NHL with multiple infusions of nontargeted iPSC-derived NK cells (FT516) with IL-2, preceded by lymphodepleting chemotherapy and combined with an anti-CD20 antibody (rituximab or obinutuzumab).[82] There was greater activity in indolent lymphomas (100% ORR and 92% CR) than in aggressive lymphomas (49% ORR, 30% CR), or in lymphomas that had been previously treated with CD19.CAR-T (42% ORR, 26% CR). Ghobadi et al. treated next 86 patients with CD19+ B-NHL with iPSC-derived CD19.CAR NK cells transduced with hnCD16 and membrane-bound IL-15/IL-15 receptor fusion (FT596). As in the preceding study, they saw greater activity in follicular lymphoma (100% ORR, 85% CR) than in DLBCL (38% ORR, 25% CR), or after prior CAR-T (45% ORR, 30% CR).[83]

NK cell engagers

Immune cell engagers redirect the immune cells to tumor-associated antigens (TAA) while simultaneously delivering activating signals. Bispecific CD3-based T-cell engagers (BITE) are an effective and established class of immunotherapeutics for a variety of tumors, including B-NHL, acute lymphoblastic leukemia, myeloma, or small-cell lung cancer. In an earlier stage of development, the emerging class of NK cell engagers (NKCE) also mediate potent antigen-dependent cytotoxicity but with important mechanistic differences: While BITE require activation and clonal expansion of the engaged T cells, NKCEs exploit preformed cytotoxic machinery for rapid killing, retain activity against HLA-I-deficient tumors, and induce lower IL-6/TNF-α release, reducing CRS risk.[84]

Bispecific and multispecific NKCEs typically engage activating NK receptors, including CD16a, NKG2D, or NKp46, to promote immune synapse formation and trigger antigen-dependent cytotoxicity. Advances in protein engineering have further enabled trispecific and tetraspecific formats that combine dual tumor antigen recognition with coordinated activation of multiple NK receptors and cytokine support – most commonly IL-15— to enhance NK-cell expansion, persistence, and functional fitness (Figure 4).

NKCEs in monotherapy are generally well tolerated but have limited activity in advanced disease due to their dependence on endogenous NK cells, which are often numerically and functionally impaired. Thus, combination with adoptively transferred NK cells provides metabolically fit effectors and enhances anti-tumor activity. TAA targets for NKCE include CD30 in lymphoma cells (for AFM13 or acimtamig, the most clinically advanced NKCE),[85] BCMA in multiple myeloma,[86] CD33 and CD123 in myeloid malignancies,[87] and CD19 in B-cell malignancies.[88] CIML, UCB-derived, and iPSC-derived NK platforms are compatible with NKCE strategies, which can also modularly augment CAR-NK approaches by enabling simultaneous targeting of non-overlapping antigens. Preclinical data show synergistic antileukemic activity when IL-15-

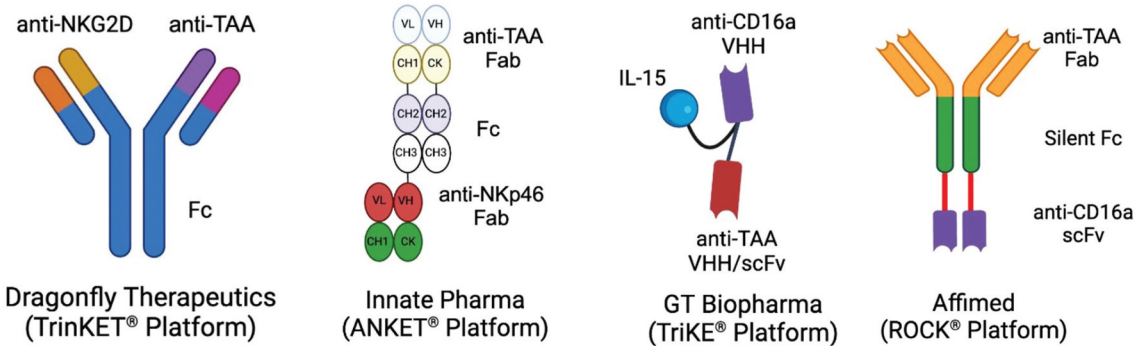

Figure 4. Platforms of bispecific and trispecific NKCE in current clinical testing.

expressing CD16 × CD33 TriKEs are combined with CAR-NK cells targeting MICA/B, reducing immune escape.[89,90]

Other trispecific NKCEs extend bispecific platforms through dual activating receptor engagement in addition to tumor targeting. These molecules typically bind a TAA while simultaneously engaging two activating NK receptors, most commonly CD16a together with NKp46 or NKG2D.[91–93] This design addresses the limitations of CD16a-only engagement, which include receptor shedding and functional impairment within the TME.

In solid tumors, NKCE activity is constrained by limited tissue penetration due to physical barriers, such as the extracellular matrix, abnormal vasculature, or elevated interstitial pressure, which impair infiltration and create spatially heterogeneous drug distribution.[94–96] Thus, NK-cell trafficking represents a critical limiting step in solid tumor immunity, with chemokine receptor programming shown to regulate tumor infiltration and tissue homing.[97] To address these barriers, engineering strategies have focused on optimizing molecular format, effector support, and NK-cell fitness.[98] Reduced-size antibody scaffolds improve tumor penetration and spatial distribution within dense stromal architectures, enhancing target engagement in solid tumor models.[99] Consistent with these engineering principles, NKCE platforms such as EGFR × CD16a bispecific antibodies demonstrate NK-dependent cytotoxicity in solid tumor models but also highlight the need for additional optimization to sustain activity within suppressive TME.[100] Once within the TME, NK cell function is further constrained by hypoxia and nutrient deprivation, which suppress NK-cell metabolism by impairing mitochondrial and glycolytic metabolism and ATP production, ultimately reducing their effector function.[101,102] Incorporation of cytokine signaling domains – particularly, *IL15*—promotes NK-cell expansion, survival, and functional persistence.[103] Although IL-15–armed constructs partially restore fitness, durable activity likely requires combination approaches targeting tumor metabolism and hypoxia.[104] Finally, immunosuppressive signaling from the TME via PD-L1 or the HLA-E-NKG2A axis remains a major barrier, supporting combination with checkpoint blockade or multifunctional engagers that simultaneously target tumor antigens and inhibitory pathways.[105,106] Collectively, these findings parallel hematologic NKCE strategies and support a unified framework in which molecular format optimization, trafficking enhancement, cytokine support, metabolic reprogramming, and combinatorial approaches are jointly leveraged to improve NKCE efficacy across different solid tumor contexts.

Future ways of progress

Improvements in the construct, expansion, novel targets and changes in the stimulatory domains continue to enhance targeting, persistence and cytotoxicity of CAR NK cells. Li et al. showed that conventional CAR-NK cells undergo trogocytosis, acquiring target-cell antigens that then trigger fratricide and rapid functional loss.[107] Engineering KIR-based inhibitory CARs prevented this process and reduced NK-cell death by over 70% in vitro. In multiple xenograft models, these KIR-CAR NK cells maintained durable antitumor activity, achieving complete tumor clearance in 80–100% of treated mice, whereas standard CAR-NK cells rapidly failed due to antigen loss and exhaustion.

Other advances in CAR-NK engineering focus on overcoming tumor antigen heterogeneity and preventing tumor evasion mediated by antigen loss.[108] One such strategy involves targeting multiple antigens simultaneously: for example, NK cells co-expressing CD19 and BCMA CARs show stronger cytotoxicity than single-CAR NK cells, and dual-target CAR constructs may reduce the likelihood of tumor evasion since loss of both antigens is far less common.[109] NK cells also hold the unique advantage of retaining endogenous activating receptors, allowing them to respond to stressed tumor cells even if CAR-targeted antigens are downregulated, an ability that CAR-T cells lack.

Sophisticated CAR signal engineering has introduced logic-gated CAR circuits that integrate activating and inhibitory inputs to maximize tumor recognition while sparing healthy tissue. Examples include CAR-NK cells that activate in response to CD33 or FLT3 but are simultaneously inhibited by recognition of endomucin (codified by the *EMCN* gene), a marker of healthy hematopoietic stem cells, thereby refining specificity for AML blasts.[110] Additional designs use NK-specific inhibitory CARs to block fratricide triggered by trogocytosis, a process where tumor cells transfer target antigens to the CAR NK cells themselves, and these begin attacking one another.[107] Synthetic Notch (SynNotch) systems add further programmability, enabling NK cells to express cytokines or secondary CARs only after recognizing a priming antigen (such as inducing IL-12 production or activating a CD147-targeted CAR following GPC3 detection), thus improving precision and reducing off-tumor effects.[111] SynNotch receptors extend this programmability by replacing the native Notch extracellular region with a tumor-specific scFv and substituting its intracellular domain with transcriptional regulators to activate or repress gene expression. Upon ligand binding, the engineered transcription factor is released, enabling precise control over downstream pathways, thereby supporting complex logic-gated responses. Such systems can execute AND-gate logic, ensuring NK cells kill only when multiple tumor antigens are co-expressed, or OFF-gate logic, where an inhibitory CAR prevents attack on healthy cells by triggering suppressive or apoptotic programs (e.g., leveraging the truncated Bid protein) upon recognition of normal tissue antigens. These concepts are now progressing into clinical testing. The SENTI-202 program illustrates the translational potential of multi-antigen, logic-gated CAR-NK therapy for AML. In the first-in-human phase I trial, SENTI-202 was designed to activate upon the CD33/FLT3 recognition while using an EMCN-specific inhibitory receptor to protect normal stem cells described above.[112] Among nine patients treated across dose levels up to 1.5 billion CAR-NK cells per dose, no dose-limiting toxicities occurred, and four of seven evaluable patients achieved a MRD-negative CR, some of them lasting over eight months. These early results highlight the promise of logic-gated CAR-NK cells in addressing antigen heterogeneity, enhancing specificity, and delivering deep clinical responses, with the potential to extend to solid tumors where precise discrimination between healthy and malignant tissues is clearly needed.

Inhibitory signals and protection against more severe adverse effects, which may become increasingly relevant as more potent NK cell products are developed, are also desirable. To this end, NK cell products have been engineered with built-in 'kill switches.' Rimiducid is a small-molecule chemical inducer of dimerization (CID) that enables precise, drug-controlled regulation of genetically engineered immune cells.[113] Rimiducid engages two distinct switches: iCaspase-9, a safety switch that rapidly induces apoptosis of modified cells to halt severe toxicities, and inducible MyD88/CD40, an activation switch that enhances CAR-cell proliferation, persistence, and resistance to exhaustion within the TME. Because rimiducid is bioinert outside its interaction with engineered cells, its effects are fully restricted to those cells carrying the CID switches, allowing clinicians to turn cell therapies "on" or "off" in real time.[114] This pharmacological control aims to address the pitfall of severe toxicity that may result from more potent CAR NK cells.

Other efforts to improve NK cellular immunotherapy focus on overcoming suppression within the TME. An example of this is the neutralization of TGF-β, a pleiotropic cytokine, secreted in the TME by various cells, including macrophages, dendritic cells, T cells and B cells. While in early stages of tumor development, TGF-β acts as a tumor suppressor, in advanced cases it mediates immunosuppression in the TME and promotes metastasis,[115] and, relevant to NK cell therapy, it decreases the proliferation and cytotoxicity of NK cells.[116] We have developed *TGFBR2* knockout CD70.CAR NK cells with increased antitumor activity in various lymphoma and solid tumor xenograft murine models.[117]

Clinical trials testing these cells in patients with refractory lymphomas and glioblastoma multiforme are in progress.

Thus, enhanced antigen and internal signaling cascades, logic-gated activation, and metabolic reprogramming and controlled cytokine enhancement all can substantially improve effector function and persistence CAR-NK platforms. The future of field and of immunotherapy at large will involve complex changes to not just the surface recognition but the entire NK cell.

Combination therapies

The combination of NK cells with other immunotherapies is an attractive venue of progress. Whether NK cells express PD-1 remains debated, with some studies showing minimal baseline expression,[118] and others reporting inducible PD-1 on NK cells within the myeloma TME.[119] Despite this uncertainty, blocking PD-1/PD-L1 has shown to enhance NK-cell antitumor activity in multiple myeloma models,[120] and in clinical trials combining pembrolizumab with NK-cell infusions.[121] In a randomized study of advanced NSCLC, pembrolizumab plus allogeneic KIR-mismatched NK cells improved the ORR and outcomes as compared to pembrolizumab alone.[117] In addition, human NK cells frequently upregulate PD-L1 in response to tumors (especially those with low HLA-I) and PD-L1^{+} NK cells exhibit enhanced cytotoxicity.[122] Atezolizumab can bind PD-L1 on these NK cells, further increasing their killing and cytokine secretion, functioning both as a checkpoint inhibitor and as an NK cell activator.[122]

Tyrosine kinase inhibitors, such as apatinib, regorafenib, cabozantinib, dasatinib, nilotinib, or ponatinib, can upregulate NK-activating ligands, suppress oncogenic signaling, or reduce inhibitory cues.[123–125]

Another strategy relies on chemotherapy to boost CAR-NK activity through wide-ranging immunologic effects. Chemotherapy can induce immunogenic tumor cell death, making cancer cells more recognizable to immune effectors, cause temporary lymphodepletion followed by a rebound wave of immune reconstitution, reduce tumor-induced immunosuppression by depleting Tregs and myeloid-derived suppressor cells, and directly or indirectly stimulate NK- and T-cell activity.[126] These properties create windows in which CAR-NK cells can expand, persist, and infiltrate tumors more effectively.

NK cell therapy for autoimmune diseases

Adoptive cellular immunotherapies represent a paradigm shift in the treatment of autoimmune diseases by restoring immune balance through the elimination of pathogenic B and T cells. These approaches target a wide spectrum of conditions, ranging from neuroimmunological disorders like multiple sclerosis and myasthenia gravis to systemic diseases like systemic lupus erythematosus (SLE). German investigators reported the first encouraging results using CAR-T cells in refractory cases.[127–129]

Unlike autologous CAR-T cells, NK cells offer a scalable, off-the-shelf therapy with a superior safety profile, specifically characterized by a lower risk of CRS and GVHD. Current strategies include CAR-NK cells and Chimeric Autoantibody Receptor (CAAR) NK cells, which precisely target autoreactive B-cell clones.[130,131] Additionally, cytokines like IL-15 are being evaluated to enhance the ability of endogenous NK cells to destroy dysfunctional cells.[132] The efficacy of these NK-cell therapies relies on both CD56^{dim} cells, which can deplete autoreactive B cells via ADCC, and CD56^{bright} cells, which act as immunomodulators by suppressing activated T cells to prevent the initiation of autoimmunity. While engineering often targets the cytotoxic power of the CD56^{dim} subset, the CD56^{bright} subset offers better survival and expansion potential. During clinical manufacturing, these subsets often converge into a homogenous, highly potent phenotype.

Recent trials have yielded breakthrough results. A CD19.CAR-NK trial reported a 67% remission rate at 12 months for SLE patients,[133] while iPSC-derived CAR-NK cells successfully reversed long-term fibrosis in severe systemic sclerosis cases.[134] Toxicity remains remarkably low, which allows for outpatient administration, significantly increasing patient accessibility compared to CAR-T.

While CAR-T cells offer long-term persistence, NK cells are emerging as a safer and more practical alternative. Through genetic engineering–such as membrane-bound cytokines and CRISPR-mediated cloaking–the naturally short lifespan of NK cells is being extended to provide durable clinical remissions.[135]

Conclusions

NK cells represent a powerful emerging treatment modality for cancer, evolving from a conceptual alternative to T cells into a distinct pillar of cellular immunotherapy with their own biological advantages, engineering strategies, and clinical results. Their lack of HLA-restricted antigen recognition, no risk of GVHD, and favorable cytokine toxicity profile make them inherently well-suited for allogeneic, off-the-shelf products, addressing many of the logistical and safety limitations that constrain autologous CAR-T cells. Clinical experience with CAR-engineered or bispecific engager-bound NK cells has consistently confirmed excellent safety and meaningful and sometimes durable responses in heavily pretreated patients, particularly in B-cell non-Hodgkin and Hodgkin lymphoid malignancies.

At the same time, the field has clearly defined a set of recurring obstacles: limited *in vivo* persistence, vulnerability to host rejection, suboptimal trafficking into solid tumors, and profound functional suppression within the TME

The next few years of NK-cell research will likely be defined by three broad themes. First, convergence on robust, standardized allogeneic platforms, such as UCB- or iPSC-derived NK cells. This will require manufacturing processes that preserve metabolic fitness and cytotoxic potential after cryopreservation. Second, deep integration of systems-level engineering, including multiantigen and logic-gated CARs, which will be necessary to maintain function within hostile tumor niches while preventing off-tumor damage and late toxicities. Third, rational combinations with other immunotherapies warrant evaluation. Novel NK cell engagers, checkpoint inhibitors, small-molecule kinase inhibitors, chemotherapy, and radiotherapy no longer constitute separate treatment silos but tools to reshape antigen density, vasculature, stromal barriers, and immune composition in ways that favor NK cell recruitment and persistence. Going forward, a major challenge will be to define optimal timing, dosing, and biomarker-guided patient selection so that combination strategies enhance the NK cell antitumor effect.

Finally, advances in single-cell omics, spatial profiling, and high-content functional assays will provide the mechanistic framework needed to rationally design and iterate these therapies. As NK-based products move from early-phase trials toward registrational studies, regulatory frameworks will also need to adapt to accommodate logic-gated, switch-controlled, and heavily edited cells.

In summary, NK-cell immunotherapy is entering a pivotal phase: its safety and feasibility are established, its proof-of-concept efficacy has been demonstrated, and the technological toolkit for sophisticated engineering is rapidly maturing. The central task for the coming years is to convert this promise into a consistent and durable benefit across a broader range of cancers, including solid tumors, through standardized manufacturing, smarter circuit design, and rational, biomarker-driven combination strategies. If these challenges can be met, NK cells are poised to become a core, mainstream component of the immuno-oncology armamentarium rather than a mere alternative to T-cell – based approaches.

Author contributions

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