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Review Article

Natural Killer Cell Cellular-based Therapeutic Options to Manage Acute Myeloid Leukemia: Prospects and Challenges

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Abstract

Over the past decade, significant progress has been made in managing acute myeloid leukemia (AML). However, refractory disease and relapse continue to pose major challenges. These issues highlight the need for innovative therapeutic options to achieve deeper remission and effectively treat refractory and relapsed diseases, thereby improving survival rates. Natural killer (NK) cell-based therapies have emerged as a promising option. NK cells, a specialized population of innate lymphoid cells, exhibit inherent anti-viral and anti-cancer capabilities. Unlike T cells, NK cells do not require prior antigen sensitization to eliminate their target cells, enhancing their potential as immunotherapeutic agents. However, NK cells often exhibit dysfunction in patients with hematological malignancies. Revitalizing these cells represent another immunotherapeutic strategy. Various NK cell-based therapies have been explored in recent decades, particularly in managing AML. These therapies include chimeric antigen receptor-NK cell therapy, bispecific and trispecific NK cell engagers (bi-specific killer cell engager (BiKEs) and tri-specific killer cell engager (TriKEs), and cytokine-induced memory-like NK cells. These therapies are also associated with fewer adverse events, such as neurotoxicity. Despite their potential for clinical cancer management, challenges such as the *in vivo* expansion of NK cells remain unresolved. This review summarizes the biology of NK cells and the diverse NK cell-based therapies being developed for the potential management of AML, as evidenced in preclinical studies and clinical trials.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous, clonal malignancy of myelogenous cells characterized by the accumulation of myeloid blast cells, primarily in the bone marrow, resulting in impaired production of normal blood cells. Until recently, the primary treatment option for AML was often cytotoxic chemotherapy. However, in 2017, midostaurin became the first targeted therapy approved for AML treatment in nearly four decades,[1] followed by subsequent new approvals.[2] With an increasing lifespan worldwide, the incidence of AML is also rising. According to Surveillance, Epidemiology, and End Results (SEER) statistics, in the United States alone, in 2022, there were an estimated 20,050 new cases of AML, representing 1% of all new cancer cases, and 11,540 deaths, representing 1.9% of all cancer deaths.[3] This represents approximately a 6% increase in deaths compared to SEER data from 2019. Despite advancements in drug therapy, relapse remains a significant issue. Currently, hematopoietic stem cell transplant (HSCT) may be the only curative therapy available for AML, albeit with considerable risks and side effects. Therefore, there is a critical need for newer therapeutic options that can achieve greater complete remission (CR) with negative minimal residual disease. Immunotherapy represents one such promising option (Fig. 1).[4,5]

Immunotherapy in cancer is a type of treatment that harnesses the specificity and killing mechanisms of the immune system to target and eradicate malignant cells. Immunotherapy has been noted as a viable treatment strategy in managing various cancers and was voted "breakthrough of the year" by Science in 2013.[6] Immune cells and biochemicals of the immune system are constitutively made to fight disease-causing microbes and their infected cells, as well as cells with the potential for neoplastic transformation. However, malignant cells have devised various means of evading immune cells through the loss of immunogenicity, upregulation of negative regulatory pathways, or creating an immunosuppressive microenvironment, thereby making immune cells less potent in destroying cancer cells.[7,8] Extensive research in cancer immunotherapy and the dynamic interactions between cancer cells and host immune cells have brought up innovative ways of boosting the host immune cells or initiating novel ways to elicit immune responses in fighting cancer, which has led to the approval of new

Keywords: Natural killer cells; Acute myeloid leukemia; Cellular therapy; Cytokines; Chimeric antigen receptors natural killer (CAR-NK) cell therapy; Innate lymphoid cell.

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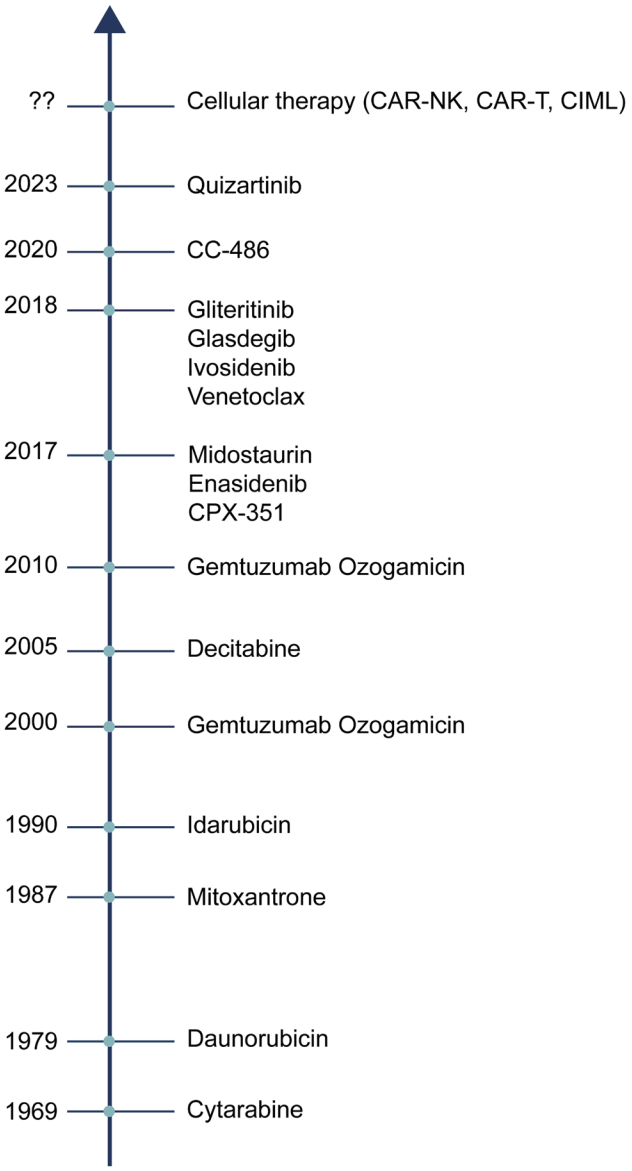

Fig. 1. Timeline of FDA-approved medicines for acute myeloid leukemia (AML). CAR-NK, chimeric antigen receptors natural killer; CAR-T, chimeric antigen receptors T; CC-486, oral azacitidine; CIML, cytokine-induced memory-like; CPX-351, liposomal duanorubicin and cytarabine; FDA, Food and Drug Administration.

therapies against both solid and hematological cancers.[9,10] Recently, Natural killer (NK) cell cellular-based therapies have been muted as one of the novel strategies in fighting cancers, especially hematological cancers. It has been observed that there is a quantitative and qualitative dysfunction in NK cells in hematological cancers. There is further impairment in their numbers and function as a result of chemotherapy and radiation used during treatment.[11,12] Restoration of these immune impairments can improve therapeutic outcomes. Over the years, a better understanding of NK cell immunobiology coupled with improvements in molecular biology techniques have led to increased development in the field of NK cell cellular-based therapy in hematological cancers, including chimeric antigen receptor (CAR)-modified NK cells,[13–17] adoptive cell transfer,[18–20] cytokines,[21,22] bispecific natural killer cell engager (BiKE),[23–25] drug treatment, etc.[26–28] Despite the major developments in NK cell-based therapies, especially in AML, it is yet to make inroads into the clinics. This is a result of different factors, the chief one being that clinical trials are still in progress in many of them. In this work, I look into the biology of NK cells, the various NK cell-based therapies being developed in preclinical and clinical trials, and the challenges faced getting them to the clinic.

NK cell biology

NK cells are a distinct group of innate lymphoid cells capable of identifying and destroying virally infected and tumor cells. They can be classified based on CD56 (neural cell adhesion molecule) and CD16 expression. NK cells constitute 5–20% of circulating lymphocytes in humans.[29] There are two primary subsets of NK cellsCD56^{bright} or CD56^{dim}. Approximately 90% of circulating NK cells are CD56^{dim}, representing the final stage of NK cell maturation. This subset expresses killer cell immunoglobulin-like receptors (KIRs), which are inhibitory receptors and cytotoxic effector proteins such as perforin and granzyme B at rest. There is also an increased expression of CD16 (FcγRIIIa), which plays a role in targeting antibody-opsonized cells. The remaining 10% of the NK cell population are CD56^{bright} and express lower levels of cytotoxic effector proteins at rest. CD16 is also expressed at lower levels in this subset. Unlike the CD56^{dim} subset, which expresses KIRs as an inhibitory molecule, CD56^{bright} NK cells express CD94/NKG2A, CD94/NKG2C, and NKG2D receptors. Compared to the CD56^{dim} subset, bright NK cells possess specialized chemokine and homing receptors such as CCR7.[30] Additionally, the CD56 bright subsets can produce immunoregulatory cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α/β, and interleukin (IL)-10 upon combined cytokine receptor stimulation.[31–33] Traditionally, CD56^{bright} NK cells exhibit low antitumor activity at rest, unlike the CD56^{dim} subset known for its robust cytolytic activity. However, CD56^{bright} NK cells from multiple myeloma (MM) patients have enhanced *ex vivo* functional responses when primed with IL-15.[34,35] NK cells mature in the bone marrow (BM) and other secondary lymphoid tissues; however, the BM is the primary site for NK precursor cells, and unlike T cells,[36–38] other secondary sites such as the spleen and thymus do not hinder NK cell growth and function.[36,37,39] Further stages of NK cell ontogeny occur in secondary lymphoid tissues such as the liver, lymph nodes, and tonsils.[40,41] Specifically, in the parafollicular T cell region of the lymph node, which is rich in CD56^{bright} NK cells, differentiation into mature CD56^{dim} NK cells occurs after stimulation by IL-2.[42]

The Lin−CD34+CD133+CD244+ HSCs are known to differentiate into CD45RA+ lymphoid-primed multipotential progenitor (LMPP) in the early stages of development, which are also found to be CD38- and CD10- but have CD62L.[43,44] The LMPPs can differentiate into multiple lymphoid lineages and some residual myeloid lineage but lack erythroid and megakaryocytoid potentials and no self-renewal capacity.[45] The LMPP transit into the common lymphoid progenitor (CLP), which lacks the potential for myeloid differentiation but can make lineage commitment into all subsets of lymphocytes, i.e., Pro-B, Pre-T, NK progenitors (NKPs), or other innate lymphoid cells (ILCs).[46] CLP were earlier thought to be Lin− cKitlowSca-1lowCD127hi (IL-7Rαhi) but were later refined to include high expression of Fms-related tyrosine kinase 3 (Flt3).[47] CLPs have also been discovered to be associated with the expression of Ly6D. This surface marker divides CLPs into two distinct populations. The Ly6d− subset of CLP, called all lymphoid

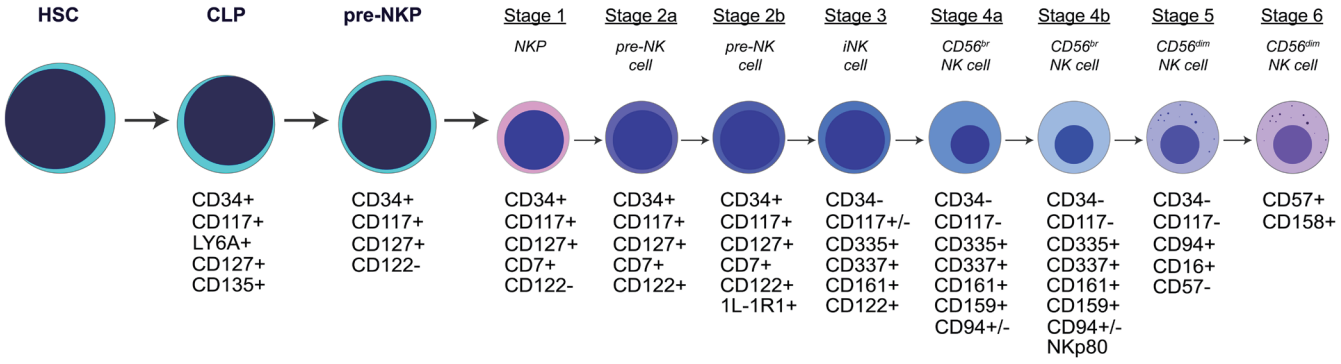

Fig. 2. Schematic diagram of NK cell development from the hematopoietic stem cell (HSC) to the terminal stage 6. CLP, common lymphoid progenitor; NK, natural killer; NKP, NK progenitor.

progenitor, has T and NK potentials, whereas the Ly6d+ subset, called BLP (B-cell-biased lymphoid progenitor), up-regulates the B-cell-specifying factors *Ebf1* and *Pax5*, thus acting as B cell progenitors.[48] It should be noted that NK cells were for some time the only known ILCs - innate lymphocytes that cannot express *RAG*-dependent rearranged antigen-specific cell surface receptors until another innate lymphoid cell known as the lymphoid tissue-inducer cell was discovered in the 1990s and subsequently helper-like innate lymphoid lineages from 2008. ILCs are classified into five groups, and this is based on their developmental course and cytokine profile. They include the cytotoxic NK cell, lymphoid tissue-inducer cells which express the integrin α4β7, lymphotoxin (LT) α1β2, and lymphoid cytokine receptors, and helper-like ILCs (ILC1, ILC2, and ILC3) with their distinct functional expression like CD4+ T helper (Th) type 1, Th2 and Th17 cells.[49–52] The families of innate lymphocytes share a common progenitor known as the early innate lymphoid progenitor; the cytokine-producing ILCs also have a more restricted progenitor known as common helper-like innate lymphoid cell progenitor.[53–55] All ILCs except NK cells require *GATA-3* for their differentiation.[56] In addition, NK cells and ILC1 cells depend on two evolutionary-related T-box transcription factors (TFs): *eomesodermin* (EOMES). T-box expressed in T cells (T-bet) for their development. However, EOMES is strictly required to develop NK cells, while ILC cells do not develop without T-bet in conjunction with *Aiolos* and *Bcl6*.[55,57–59] *GATA-3*, *B-cell lymphoma/leukaemia 11B* (BCL11B) and *RAR-related orphan receptor alpha* (RORα) are required for the development of ILC2 cells as well as the control of the production of type 2 effector cytokines, IL-5, IL-13, and IL-4.[60,61] The group 3 ILC cells depend on *GATA-3*, *RAR-related orphan nuclear receptor γt* (RORγt), and *Hypoxia-Inducible Factor* (HIF-1) to develop and produce cytokines IL-17 and IL-22.[59,62] In all these, mature ILCs can be generated from CLPs.[55]

While the ontogeny of NK cells is not fully understood, their development has been classified into six stages. Stage 1 begins with CLPs transitioning into NKPs characterized by the expression of CD7+, CD127+ (IL-7Rα+), CD122+ (IL-2Rβ+), CD117+ (c-Kit+), and IL-1R1low. The acquisition of CD122 indicates a commitment to the NK lineage, promoting NK cell differentiation, functional maturation, and survival.[63–65] Stage 2a of pre-NK cells is defined by CD3ε−CD7+CD127+ cells, while the transition from stage 2a to 2b is marked by the expression of IL-1R, a receptor for IL-1β at stage 2b.[66] The progression from stage 2b pre-NK cells to stage 3 immature NK cells is indicated by the expression of activating receptors CD335 (natural cytotoxicity receptor, NCR1, NKp46), CD337 (NCR3, NKp30), and CD161.[67] NKG2D, which uses the DAP10 adaptor protein, along with NCR1 and NCR3, which utilize CD3ζ and FcεRγ, respectively, characterize this development.

Stage 4 of NK cell development is subdivided into stages 4a and 4b, with stage 4a being NKp80 negative and characterized by increased levels of NKG2D, CD335, CD337, inhibitory NKG2A (CD159a, containing two immunoreceptor-based tyrosine inhibitory motifs (ITIMs) and CD161 (NK1.1, KLRB1, NKR-P1A) which are $CD56^{bright}$. The Stage 4b shows positivity for NKp80 while maintaining their $CD56^{bright}$ status. Stage 5 is characterized by the transition from $CD56^{bright}$ to $CD56^{dim}$, which are mature NK cells. This stage involves the gradual up-regulation of CD94/NKG2C and CD16 (FcγRIII), and down-regulation of CD56, c-Kit (CD117), and CD94/NKG2A.[68] $CD56^{bright}$ NK cells, considered less mature, are primarily found in secondary lymphoid tissues, unlike the $CD56^{dim}$ that form the majority of NK cells in circulation.[12,69]

Ultimately, the terminal maturation of $CD56^{dim}$ NK cells is marked by the expression of CD57 (HNK-1, Leu-7) and killer cell immunoglobulin-like receptors (KIR+/CD158+), which constitutes stage 6 of NK cell development (Fig. 2, Table 1).[70–72]

NK cells recognition of self from non-self

The process of NK recognition of self from non-self is still being debated. This education process results from NK cells interaction with self-major histocompatibility complex (MHC)-I.[73] Several studies examining the basis of tolerance in NK cells have linked it to MHC-I surface expression. The KIR family of receptors is the NK group of receptors that primarily associate with MHC-1. Through them, MHC-I regulates NK cell function. In trying to explain the NK cell education process, Yokoyama and colleagues brought up a theory called the NK cell licensing hypothesis, which states that for NK cells to respond to subsequent stimuli received by inhibitory receptors, they must first engage in self-MHC class I. This is termed "licensing". Conversely, the NK cells that could not engage in self-MHC class I were considered "unlicensed".[74,75] Thus, this process gives rise to two kinds of self-tolerant NK cells which are: (a) the licensed NK cells, which are capable of maintaining self-tolerance by direct inhibition through binding to self-MHC and (b) the unlicensed NK cells, which cannot engage self-MHC but are self-tolerant due to their inherent resistance to stimulation received through activating receptors.

Later, Raulet and Vance introduced their NK cell self-tolerance model, termed the arming and disarming model. According to the

Table 1. Surface antigens expression at different stages of NK cell development

Surface markers	Stage 1	Stage 2a	Stage 2b	Stage 3	Stage 4a	Stage 4b	Stage 5	Stage 6
CD34	+	+	+	–	–	–	–	–
CD10	+	+/–	+/–	–	–	–	–	–
HLA-DR	+	+	+	–	–	–	–	–
CD117	–	+	+	+	+	–	–	–
CD127	+	+	+	+	–	–	–	–
CD122	–	–	+	+	+	+	+	+
CD161	–	–	–	–/+	+	+	+	+
CD56	–	–	–	–	++	++	+	+
CD94	–	–	–	–	+	+	+/–	+/–
NKG2A	–	–	–	–	+	+	–	–
NKG2D	–	–	–	–	+	+	+	+
NKp30	–	–	–	–	+	+	+	+
NKp46	–	–	–	–	++	++	+	+
NKp80	–	–	–	–	–	+	+	+
NKG2C	–	–	–	–	–	–	+	+
CD16	–	–	–	–	–	–	+	+
KIRs	–	–	–	–	–	–	+	+
CD57	–	–	–	–	–	–	–	+

NK, natural killer.

arming model of NK cell education, the KIR inhibitory receptor interaction with MHC class I molecules gives rise to inhibitory signals that promote functional maturation of human precursor NK cells but not mature NK cells. This hypothesis appears counterintuitive in that these receptors are essentially inhibitory. However, signaling through these receptors may seem more complicated than previously thought. On the other hand, the disarming model proposes that precursor and mature NK cells that lack self-MHC-I inhibitory receptors are rendered hyporesponsive upon receiving sustained positive signaling via activating receptors.[76] Thus, these models show that increased expression of inhibitory receptor signaling in comparison to activating signaling invariably leads to a heightened response of NK cells; therefore, NK cells with functional copies of *KIR* genes are functionally more competent than those without in their education process.[77] As a result of the alterations in the expression of inhibitory receptors during NK cell development, various combinations of inhibitory receptors can be expressed on distinct NK cells, particularly in a disease state, thus making it function like a rheostat to set a quantitative threshold of NK cell responsiveness during the education process.[78] This is the rheostat model, which incorporates concepts from the licensing and disarming model that different inhibitory receptors can bind MHC ligands with varying affinities, and the interactions between the various inhibitory receptors and the expressed MHC molecules will result in varying degrees of inhibition between distinct NK cells which allows for a range of NK cells responses.[79,80] Just like the diversity of the MHC molecules, the *KIR* displays a high level of polymorphism. The *KIR* haplotypes are grouped into two primary sets: "A" and "B".[81] The *KIR A* haplotypes mainly contain inhibitory *KIR* genes and only one activating *KIR* gene, *KIR2DS4*. On the other hand, *KIR B* haplotypes have different numbers and combinations of activating *KIR* genes besides inhibitory *KIR* genes.

NK cell signaling and effector functions

Unlike T cells, NK cells do not express clonotypic receptors. Nevertheless, they can still generate significant anti-tumor cytotoxicity and produce inflammatory cytokines. These functions are regulated by an array of germline-encoded activating and inhibitory receptors, including NKG2D, NCR1, NCR2, NCR3, NKG2C, CD244, Ly49D, Ly49H, KIRs, CD94/NKG2A, and leukocyte Ig-like receptor 1 (LIR1). These receptors are transmembrane proteins with an extracellular ligand-binding portion and an intracellular cytoplasmic tail. The cytoplasmic tail of inhibitory receptors contains immunoreceptor tyrosine-based inhibitory motifs, which can directly activate their protein phosphatases. Conversely, activating receptors, which lack signaling domains in their cytoplasmic tails, indirectly stimulate protein kinases by recruiting adaptor proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs). The adaptor molecules propagating activation receptor signaling include FcεRIγ, CD3ζ, and DAP12.

NKG2D and Ly49H can also propagate signals through the Tyr-Ile-Asn-Met (YINM) motif present within the adaptor, DAP10. The activating receptor NKG2D is a type II transmembrane and C-type lectin-like type II homodimeric receptor that is involved in NK cell lysis (just like other activating receptors, (NCR) NKp46 (NCR1), NKp30 (NCR3), and NKp44 (NCR2)). It is constitutively expressed on NK cells and mediates signaling through the adapter proteins DAP10 and DAP12 via YINM and ITAM tyrosine-based signaling motifs- DAP10 is involved in the recruitment and activation of the p85α subunit of PI(3)K and Grb2, while DAP12 is involved in the recruitment of ZAP70 and Syk to initiate NKG2D-

mediated NK cell activation.

In addition to these activating receptors, co-receptors such as 2B4, NTB-A, DNAM-1, CD59, and NKp80 play complementary and synergistic roles in NK cell activation. 2B4 and NTB-A, part of the signaling lymphocyte activation molecule (SLAM) family, enhance the potentiation and cytotoxic activity of NK cells triggered by primary receptors.[82] These two co-receptors, associated with the SLAM-associated protein (SAP), a molecule involved in X-linked lymphoproliferative syndrome type 1 (XLP-1) — a severe form of immunodeficiency[83,84] — have been noted to deliver inhibitory signals in the absence of SAP, rather than activating signals.[84–86]

CD59, a glycosylphosphatidylinositol (GPI)-linked protein, and a paroxysmal nocturnal hemoglobinuria marker depends on the simultaneous engagement of NKp46 and NKp30 receptors via the tyrosine phosphorylation of CD3zeta chains to enhance NK cell-mediated cytotoxic activity.[87] Low CD59 is associated with increased proliferation and abnormal coagulation function in AML.[88] An adhesion molecule, DNAX Accessory Molecule (DNAM-1 or CD226), is involved in NK cell activation. DNAM-1 has two ligands, poliovirus receptor (PVR) and Nectin-2, widely expressed in hematological cancers.[89] The dual interaction of the ligands with the activating coreceptor, DNAM-1, and the inhibitory receptors CD96 and T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) (DNAM-1 enhances NK cell-mediated cytotoxicity via PVR and Nectin-2, whereas TIGIT interaction with these ligands leads to a reduction in IFN-γ production by NK cells, as well as a diminished NK cell-mediated cytotoxicity) makes them an ideal target for immunomodulation in cancer.[90–93] Studies have shown a reduced expression of DNAM-1 in AML while the inhibitory receptors TIGIT and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) are increased.[94] Consequently, loss of DNAM-1 and reduced expression of PVR is a primary NK cell escape mechanism in AML.[95]

Human leukocyte antigens (HLA) class I and non-classical MHC class Ib molecules such as *HLA-E* are recognized by the inhibitory receptors KIRs and CD94/NKG2A. KIRs are clonally distributed; only a fraction of NK cells express a given KIR, making them highly polymorphic. The HLA class I molecules that express either the Bw4, C1, or C2 motifs are the ideal ligands for KIR. On the other hand, *HLA-C* alleles that are characterized by Lys at position 80 (HLA-CLys80) are recognized by KIR2DL1, while KIR2DL2/3 recognize *HLA-C* alleles characterized by Asn at position 80 (HLA-CAsn80). Likewise, KIR3DL1 is specific for *HLA-B* alleles sharing the Bw4 supertypes specificity (HLA-BBw4), and KIR3DL2 recognizes *HLA-A3* and *-A11* alleles.[89] The KIR system acts through specific interactions and varying degrees of signal strength to diversify NK cell stimulation. Thus, weakly inhibitory KIR/HLA combinations permit a lower threshold for cell activation and vice versa. Therefore, target cells are susceptible to NK-mediated killing when there are no effective inhibitory interactions. A study by Dai *et al.*[96] showed that increased KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, and KIR3DL2 mRNA levels were significantly related to poor prognosis and overall survival (OS) in AML patients (Fig. 2).

In performing their effector functions, i.e., cytotoxic death of the target, NK cells are reported to use various mechanisms. This requires specific processes. In target destruction, NK cells first recognize their target through specific molecular mechanisms. These inhibitory and activating receptors recognize surface molecules expressed at steady state and stress-induced molecules, respectively. Once a target cell is recognized, there is a direct interaction of the NK cell with the target through the formation of an immunological synapse, which facilitates target cell death through some mechanisms. Human NK cells kill their target primarily by releasing lytic granules in a process called 'degranulation'.[97] These lytic granules are delivered to the target cell through membrane fusion at the immunological synapse. This process involves cytoskeletal rearrangement, which includes actin polymerization and polarization of the cytoskeletal rearrangement-assisted microtubule-organizing center towards the target cell.[98–101] Once polarized, These lytic granules move along the microtubules and at the immunological synapse fuse with the target cell membrane and release their lytic enzymes, which cause the activation of an apoptotic process within the target cell.[102] The major components of the lytic granules in the "degranulation" process are Granzyme B and perforin. Perforin, a 60–70-kDa pore-forming glycoprotein, forms pores in target cells, leading to osmotic lysis. A partial deficiency in perforin production causes increased susceptibility to hematological cancers.[103] On the other hand, Granzyme B, a class of serine proteases, can induce apoptotic cell death through caspase-dependent and independent mechanisms.[104]

Another mechanism through which NK cells eliminate their targets involves the engagement of death receptors on target cells via their cognate ligands, which are present on the NK cells.[105] The TNF-related apoptosis-inducing ligand-receptor (TRAIL-R) and Fas (CD95) are two such death receptors activated by their respective ligands, Fas ligand (FasL, CD95L) and TRAIL. The binding of these receptors by their ligands induces a conformational change through receptor oligomerization and the recruitment of adapter proteins, initiating apoptosis either directly through effector caspases or indirectly via the intrinsic mitochondrial pathway.[106,107]

In addition to their cytotoxic capabilities, NK cells are potent producers of pro-inflammatory and immunosuppressive cytokines, primarily mediated by CD56bright NK cells, which are less cytolytic.[31,32] The primary cytokines produced include IFN-γ and TNF-α, and, depending on the inflammatory environment, IL-5, IL-10, IL-13, and some growth factors like IL-3, G-CSF, and GM-CSF. NK cells also secrete chemokines such as CCL1, CCL2/MCP-1, CCL3 (MIP-1α), CCl4 (MIP-1β), CCL5 (RANTES), XCL1 (lymphotoxin), CXCL10/IP-10, and CXCL8 (IL-8), which attract effector lymphocytes and myeloid cells to inflamed tissues.[108–110]

Several transcriptional regulators are involved in the production of these cytokines, including the *nuclear factor kappa-light-chain-enhancer of activated B cells* (NF-κB), the *c-Fos* and *c-Jun* heterodimer of the *AP-1* TF genes, and the nuclear factor of activated T cells.[111–114] While NK cell cytokine secretion can be both beneficial and deleterious, NK cells have been observed to display "split anergy," a phenomenon characterized by increased cytokine secretion but reduced cytotoxicity, particularly following interactions with cancer stem cells.[109] This process is mediated by IFN-γ. However, some cytokines mediate beneficial immunoregulatory functions: IFN-γ facilitates dendritic cell maturation and indirectly promotes adaptive T-cell responses, activating helper T cells to a Th1 phenotype.[115] Similarly, TNF-α is involved in B-cell proliferation and exhibits anti-proliferative effects on tumor cells. TNF-α also mediates endothelial activation, leading to increased production of adhesion molecules and inflammatory cytokines.

In addition to its MHC-I targeting of diseased cells, NK cell is also involved in antibody-dependent cellular cytotoxicity. NK cells possess Fc receptors that can bind antibodies in their Fc region. They bind to the Fc portion of immunoglobulins through their own FcγRIIC/CD32c and FcγRIIIA/CD16a.[116,117] FcγRIIC

has an ITAM in its cytoplasmic tail just as FcεRI-γ chains or CD3-ζ chains within the cell membrane equally have, though the primary activating receptor is the low-affinity FcγRIIIA/CD16a that binds the Fc domain of IgG. However, upon binding to FcγR there is phosphorylation of the ITAMs, which initiates a signal cascade, i.e., there is binding to tyrosine kinases ZAP-70 and Syk, with subsequent activation of the PI3K, NF-κb, and extra-cellular signal regulated kinases (ERK) pathways that cause NK cell degranulation, cytokine release, and tumor cell lysis.[118–120]

Although NK cells do not have clonotypic receptors like T cells, studies have shown that a relatively small population can elicit memory-like responses.[121,122] Memory NK cells were first described in mice deficient in T & B cells. Following secondary exposure to specific haptens such as 2,4-dinitrofluorobenzene and oxazolone, there was a hypersensitivity response against the haptens mediated by NK cells on contact with these haptens. The sensitized NK cells that were adoptively transferred persisted for about four weeks.[123] The development of memory NK cells has been studied in mice infected with murine cytomegalovirus (MCMV). The C57BL/6 mice were an ideal choice to study memory NK cells. This is because of their expression of the activating receptor Ly49H, which is specific for the viral glycoprotein m157 expressed on virally infected cells. Following MCMV infection, a small population of NK cells persists despite NK cell contraction. When isolated and adoptively transferred to naïve neonate mice, these memory NK cells that lack effective MCMV defense were better able to protect and prevent MCMV-mediated death compared to NK cells isolated from naïve hosts.[121,124] More research has shown that cytokine-mediated activation, particularly IL12 and IL18, can induce NK cells with such memory traits, and when adoptively transferred back into mice, led to heightened IFN-γ secretion for some weeks along with cytotoxicity usually observed in resting NK cells.[125,126] Similarly, Jin *et al.*[126] showed that the in vivo pre-activation and re-stimulation of NK cells with interleukins (IL-12, IL-15, and IL-18) led to enhanced IFN-γ secretion which could be transferred to the next generation of NK cells and was associated with prolonged survival. The increased IFN-γ secretion was suggested to be likely NKG2D-dependent. Also, Brillantes and Beaulieu have shown that NK cells can produce memory and memory-like responses towards different microbial pathogens.[127] The ability of these memory NK cells to produce enhanced levels of IFN-γ with cytotoxic granules and their ability to persist for a long time, these cells are being muted as potential cancer chemotherapies.[128–130] It remains to see how they can be harnessed.

NK cell dysfunctions in AML

From its biology, NK cells can eliminate malignant cells by exerting both direct and indirect anti-neoplastic effects through their cytotoxic and immunoregulatory functions, which are essential for directing an enhanced immune response against cancer cells. However, studies have shown that in AML, the immune microenvironment is impaired, including myeloid and erythroid differentiation, macrophages and T-cell functions, osteogenesis, and NK cell immune surveillance.[131–136] Using single-cell RNA sequencing, Guo *et al.*[137] observed significant differences between normal and AML BM immune cells. Kutznesova *et al.*[138] also reported impaired degranulation of NK cells in ex vivo AML models with increased transcriptional signatures observed in IL-6-STAT3 and IL-1β/TNFα. Thus, impaired immune function, particularly in NK cells, is one of the means that AML escapes immune surveillance. AML evade NK cell immune surveillance through different ways, including 1) the reduction in the number of NCRs on NK cell surface, 2) the overexpression of the inhibitory receptors KIRs and NKG2A with the resultant increase in inhibition of cytotoxicity, 3) interference with the maturation of NK cells with the majority of cells expressing $CD56^{bright/dim}$ KIRs- CD57- 4) the expression of checkpoint inhibitors like PD-1 and TIGIT resulting in NK cells with reduced ability to proliferate and lower cytotoxic and cytokine-producing capabilities.

One of the features of AML progression is the reduction in the number of functionally active NK cells. There is an inverse correlation between the anti-leukemic activity of NK cells and disease progression in AML with the observed suppression of NK cell number during active disease, increase in number in remission, and suppression again in the event of a relapse.[139–141] Conversely, NK cell fusion post-HSCT was associated with reduced relapse and without an increased incidence of graft-versus-host-disease; in a study, the 1-year OS, CR rate, ORR, relapse rate (RR) of acute and chronic graft-versus-host disease (GvHD) rates were 69%, 42%, 77%, 28%, 24.9% and 3.7%, respectively.[142–144] In addition, NK cells significantly correlate to OS and risk stratification in AML patients.[145]

The NCRs are surface receptors that are important in NK cell cytotoxicity. Blocking of these receptors inhibits NK cell cytotoxicity. NCR expression on NK cells is either bright (NCR^{bright}) or dull (NCR^{dull}), and most healthy individuals express the NCR^{bright} phenotype. Studies have shown a correlation between NCR expression and NK cell-mediated cytotoxicity.[146,147] In AML, these receptors are also under-expressed, affecting NK cell cytotoxicity and cytokine production.[148,149] While there is a low expression of NCRs by NK cells in AML, the expression of ligands for NK cell activating/inhibitory receptors is also defective.[137,150,151] This lack of expression of NCR ligands on their cell surface makes it difficult for NK cells to target them via NCR engagement, allowing them to escape immune surveillance. For example, the activating receptor found on NK cells, NKG2D interacts with its ligands (NKG2D-L), which comprise two members of the MHC class I-related chain (MIC) family (MICA, MICB) and six members of the UL16-binding protein (ULBP) family of proteins (ULBP1–6) and are generally not found on healthy cells but are induced on the surface of malignant cells. The NKG2D/NKG2D-L system has been observed as an important player in tumor development generally in cancer patients. The expression of some of these NKG2D-L is regulated by c-myc and DNA methylation, making them therapeutic targets for NK cell therapy.[152–155] Furthermore, tumor cells cause proteolytic shedding by metalloproteases and release of soluble NKG2D-L, causing downregulation of NKG2D and blocking receptor activation.[156] Likewise, the expression of NKG2D/NKG2D-L has been observed to decrease in the later stage of AML development, thus impairing NK cells destruction of AML cells. The absence of NKG2D-L in AML cells has also been noted to be responsible for disease relapse; in addition, increased DNA methylation for NKG2D-L is found in AML cells, which can be reversed with demethylating agents. In their investigation of soluble NKG2DL in 205 leukemia patients, Hilpert *et al.*[156] discovered that about 75% expressed at least one NKG2DL at the surface. All investigated patients had elevated soluble NKG2DL levels in their sera. They also demonstrated that soluble NKG2DL in their sera reduced NKG2D expression in NK cells, which impaired antileukemic activity.[157] Thus, AML cells escape NK cells' target and elimination by reducing the levels of NKG2D-L expression. The clinical importance of the NKG2D/NKG2D-L system is also highlighted in a study that shows that the blocking of MICA/MICB shedding prevented

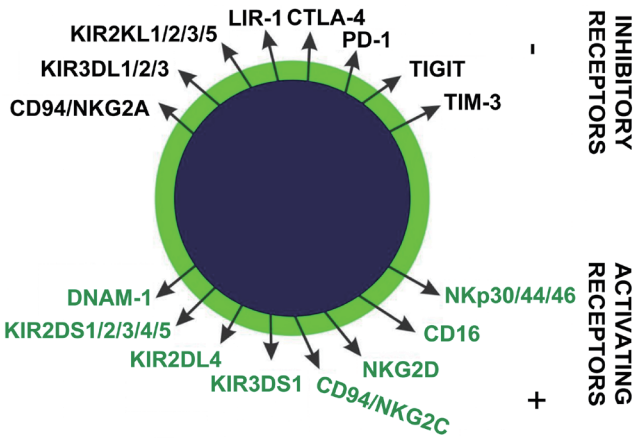

Fig. 3. Schematic representation of NK cell activation and inhibitory receptors. CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; DNAM-1, DNAX accessory molecule-1; KIR2DL1, killer cell immunoglobulin-like receptors two Ig domains and long cytoplasmic tail 1; KIR3DL1, killer cell immunoglobulin-like receptors three Ig domains and long cytoplasmic tail 1; LIR-1, leukocyte Ig-like receptor 1; NK, natural killer; NKG2D, natural killer group 2 member D; PD-1, programmed death-1; TIGIT, T cell immunoreceptor with Ig and ITIM domain; TIM-3, T-cell immunoglobulin and mucin-containing domain;

cancer cell growth in immunocompetent mouse models and with the reduction of melanoma metastasis in a humanized model.[158] In contrast, DNAM-1 and its ligands (CD112, 155) are frequently expressed in leukemic blasts, and its expression is associated with a favorable prognosis.[149,159,160] However, under-expression of DNAM-1 has also been reported in AML, and it correlates with poor NK cell lysis.[151,161,162]

Alterations in the expression of inhibitory receptors have also been described in AML. In their research, Sandoval-Barrego *et al.*[163] reported that patients with all FAB types of AML had overexpression of inhibitory receptors CD158b and NKG2A and decreased expression of the activating receptor NKp46. The CD94/NK group 2 member A (NKG2A) heterodimeric receptor binds to the non-classical HLA-E on cancer cells. It is one of the most prominent NK inhibitory receptors. NKG2A levels have been higher in the peripheral blood NK cells of patients with AML compared to NK cells of age-matched controls164. Its ligand, HLA-E, is known to be overexpressed in several cancer types, and it is also associated with poorer prognosis.[164,165] The administration of a novel anti-human NKG2A antibody was able to impede tumor cell growth in leukemic cells, suggesting that HLA-E could be a therapeutic target.[166] The KIR inhibitory receptors have also been studied. Shen *et al.*[167] reported that inhibitory KIR ligands were present in significantly higher frequencies in the prognostically poor risk group than in those with favorable risk. Ghasemimehr *et al.*[168] in their research of gene expression of activating and inhibitory receptors of NK cells in patients with newly diagnosed AML before and after induction therapy, reported a 6-fold increase in KIR2DL1 expression compared to healthy controls and a significant decrease in mRNA expressions of KIR2DL1 and NKG2A after induction therapy. Yang *et al.*[169] also reported that the levels of other inhibitory receptors like TIM-3, ILT-4, ILT-5, and PD-1 were increased in NK cells from patients with AML (Fig. 3).

Defective maturation of NK cells

The NK cell development process involves different stages regulated by cytokines and transcription factors. The earlier described process moves them from the precursor stage through different maturation phases until they acquire full maturation with the expression of a host of receptors, especially the NKG2A or KIRs. This process transforms the NK cell into a cell with a high cytotoxic capacity that can recognize and eliminate cancer cells and viruses. However, this process can be hijacked by AML. Mundy-Bosse *et al.*,[170] in their study on AML cells evasion of NK cells using specific murine maturation markers, showed that there was the selective loss of the intermediate (CD27+CD11b+) phenotype with the upregulation of the immature phenotype (CD27+CD11b−). The NK cells in AML also had lower levels of *T-bet* and *EOMES* along with the upregulation of microRNA *miR-29b*, a regulator of *T-bet* and *EOMES*, indicating a block in NK cell differentiation by AML. In their study on AML patients, Chretien *et al.* delineated three groups based on their NK cell maturation profile.[171,172] This include: the hypomaturation (CD56bright/dim KIRs− CD57−), intermediate (CD56dim KIR−/+ CD57−/+) and hypermaturation (CD56dim KIRs+ CD57+) groups.[171,172] They equally reported that patients in the hypomaturation group showed a poor 3-year overall survival and relapse-free survival, suggesting that maturation profiles of NK cells in AML may play an important role in prognostication and clinical course of the disease.[171,172] In their most recent work (NCT02320656), Chretien *et al.*[173] were able to demonstrate the presence of a moderate to increased number of CD56−CD16+ unconventional NK cells that showed a lower expression of NKG2A, as well as the activating receptors NKp30 and NKp46 in about a quarter of AML patients studied. These NK cells had a significantly decreased OS and event free survival (EFS) and a poor clinical outcome. Liu *et al.*,[174] on their part, showed the expression characteristics of antigens and functional markers of NK cells in AML patients; NK cells were divided into two groups: CD3-CD56^{high}CD16- (CD56^{high}) and CD3-CD56^{dim}CD16+ (CD56^{dim}). The expression of CD56^{high} NK cells was higher in AML patients than in healthy controls, and DNAM-1 expression was significantly low in CD56^{high} NK cells, while NKG2D, DNAM-1, and perforin were significantly low in CD56^{dim} NK cells.[174] Single-cell profiling also revealed three subsets of NK cells in the bone marrow of AML patients, which also showed stress-induced repression of NK cell effector functions. This also showed the role AML plays in NK maturation and how it affects the course of the disease.

Immune checkpoint inhibitor expression

Immune checkpoint molecules are part of the arsenals of the immune system that play an important role in self-tolerance and the prevention of lysis of self-cells. Immune checkpoint molecules are expressed on many immune cells.[175,176] Some immune checkpoint molecules expressed in NK cells include PD-1, TIM-3, LAG-3, TIGIT, and SIGLEC-7. Mature NK cells are known to express PD-1 when stimulated by MHC class I-deficient tumor cells or infected cells. These cells display reduced proliferative and cytolytic abilities and lowered cytokine production. Targeting immune checkpoints has been clinically proven and approved for managing some cancers, and the inhibition of PD-1 interaction with its ligand PDL-1 has been shown to restore NK cytolytic activity in some cancers.[177–179] PDL-1 expression is elevated in AML patients, though its clinical significance to NK cell function is not well understood.[180] Elevated PD-L1 expression in AML is associated with poor OS rate.[181–183]

Another immune checkpoint protein, the TIM-3 originally described on T-cells, is known to be expressed on the surface of NK cells, while its ligand Galectin-9 is also expressed in AML blasts.

TIM-3 is reported to be associated with disease progression in cancer. In AML, TIM-3 is reported to be associated with poor prognosis. However, there is contradictory evidence to this. Darwish *et al.*[184] and Kamal *et al.*[185] reported TIM-3 as a poor prognostic marker in AML, while Xu *et al.*[186] and Rakova *et al.*[187] reported it as an excellent prognostic marker. High levels of soluble Galectin-9 have been demonstrated in the serum of AML patients. Its interaction with TIM-3 on leukemic stem cells activates the NF-kB and β-catenin pathways, which play a role in leukemic cells' self-renewal.[188]

NK cell-based immunotherapy in AML

Adoptive NK cell transfer

While T-cell immunotherapy has gained prominence and approval in managing hematological cancers, NK cells have shown great promise; moreover, alloreactivity of NK cells in the allo-HSCT setting, which is triggered by a mismatch between the inhibitory receptors on the donor NK cells and the HLA class I molecules on recipient cells has been observed and muted as a therapeutic strategy, especially in the management of leukaemia.[189,190] This alloreactivity of NK cells in leukemia is known to be mediated through the graft-vs-leukemia effect. It is also beneficial in preventing GvHD by destroying the recipient's antigen presenting cells and fighting some infections. Ruggeri *et al.*,[191] in their study of the impact of donor-versus-recipient NK cell alloreactivity on survival in acute leukemia patients, reported EFS at five years of 60% in those with the KIR ligand incompatibility versus 5% in those without the KIR ligand incompatibility. The KIR ligand incompatibility was the only independent predictor of survival in AML.[191] In a related study, Mancusi *et al.*[192] demonstrated the effect of the KIR ligand–mismatched NK cell donors on acute leukemia. They showed that in 69 patients that underwent HSCT with donor-vs-recipient NK-cell alloreactivity, there was a reduced risk of non-relapse mortality, superior EFS, and a 50% reduction in infection rate when the transplant was from donors with KIR2DL1 and/or KIR3DL1.[192] Taken together, the adoptive transfer of NK cells is a viable option in managing leukemia.

Adoptive NK cell transfer can be done in the HSCT or non-HSCT setting, and at the same time, it can be either autologous or allogeneic.[193,194] As a therapeutic strategy, autologous NK cell adoptive transfer is based on the extraction of the patient's own NK cell from the peripheral blood, which is then expanded ex vivo and transduced back to the patient. This has its advantages in terms of convenience of source of NK cells, independence from immunosuppressants, and low likelihood of GvHD.[195] To generate sufficient and high-quality NK cells, cytokines such as IL-2, IL-12, IL-15, and IL-18 stimulate NK cells to enhance their effector functions and proliferative capabilities. However, the increased proliferative capacity does not necessarily lead to a significant therapeutic outcome, and this is due to the inhibitory effect of the patient's HLA ligands.

In some cases, the quality of NK cells may be below par because of prior heavy pretreatment of patients, giving rise to poor effector functions. While this may be so in AML, autologous adoptive NK cell transfer has shown efficacy in solid tumors and some hematological cancers.[196–198] Various strategies are being developed to restore NK cell function. Wang *et al.*,[199] in their study, noted that increased levels of TGF-β1 impaired bone marrow NK cells, and the use of TGF-β1 inhibitors like galunisertib or anti-TGF-β1 antibodies could restore NK cell effector functions. Furthermore, Lirilumab, an anti-KIR antibody that potentiates NK cells, has been shown to enhance therapeutic response as a combination therapy *in vitro* and *in vivo*. However, the EFFIKIR randomized, double-blind 3-arm placebo-controlled trial (NCT01687387) failed to improve leukaemia-free survival in elderly AML patients.[200–202] These findings have caused a shift from autologous NK cells to allogeneic NK cell transfer by researchers.

For allogeneic NK cell transfer, NK cells obtained from healthy, HLA-matched, or haploidentical donors are prepared and expanded under standard conditions (Fig. 3). The NK cells are derived from different sources like autologous transfer, including peripheral blood NK cells, umbilical cord blood NK cells, NK cell lines, and stem cell-derived NK.[203] In their study, Ruggeri *et al.*[190] showed that allogeneic NK cell transfer in AML patients induced a significant EFS. An increase in donor chimerism was observed, while a decrease in chimerism and relapse was noted in one AML patient in another study.[204] Different clinical studies of allogeneic NK cell transfer in the HSCT setting have shown tolerability and good efficacy.[205–208] Several patients may not be eligible for HSCT, but this has not hindered the development of allogeneic NK transfer outside the HSCT setting. Miller *et al.*[209] performed allogeneic NK cell transfer outside the HSCT setting, and 5 out of 19 achieved complete remission; this was significantly higher in those with KIR–ligand mismatched donors. Modifications to their approach have been replicated in other studies.[210,211] These methods equally have their challenges, which include low clinical-grade activation, lack of in vivo persistence, and problems with ex vivo expansion. In all, adoptive NK cell transfer appears to be a sound therapeutic strategy for AML for induction remission and CR maintenance.

CAR-NK cell therapy

Following the success of CAR-T cell therapy in managing B-cell precursor acute lymphoblastic leukaemia (ALL) and B-cell lymphoma, cellular therapy has shown much optimism in managing other neoplasms, including AML. Despite such optimism, CAR-T cell therapy is yet to become a reality in the management of AML due to adverse events like cytokine release syndrome (CRS).[212–214] Other obstacles encountered with CAR-T cells include inefficiencies of T cell isolation, modification and expansion, and high costs.[215] There is much enthusiasm that CAR-NK cells can prove a better alternative to CAR-T cells due to their shorter lifespan, favorable toxicity profile, and lower manufacturing costs.[216] Though it has some advantages, it has yet to be translated into a treatment option. Some challenges are still faced, including a loss of targeted antigen, hostile tumor microenvironment, and tumor heterogeneity. However, with progress made in NK cell engineering and target design, it is expected to prove efficacious in future trials. A CAR-NK cell product created from universal cord blood (UCB) NK cells by Liu and his colleagues was transduced with a retroviral vector that expressed genes that encoded anti-CD19 CAR, interleukin-15, and inducible caspase 9 as a safety switch. These were infused into 11 patients with B cell lymphoma and chronic lymphocytic leukaemia (CLL) in a phase 1/2 trial. Out of the 11 patients in the study, 7 had a CR, and a remission of the Richter's transformation was reported in one, but with persistence of the CLL. These clinical responses were seen within 30 days.

The CAR-NK cells persisted in the patients for about 12 months, and there were no reported adverse events like cytokine release syndrome, neurotoxicity, or GvHD.[217] This reflects some optimism in AML management. In preclinical studies, allogeneic CAR.CD123-NK cells induced significant anti-leukemic activity in vitro against CD123+ AML cell lines and CD123+ primary blasts and ex vivo in animal models.[218] Another study using CD33/

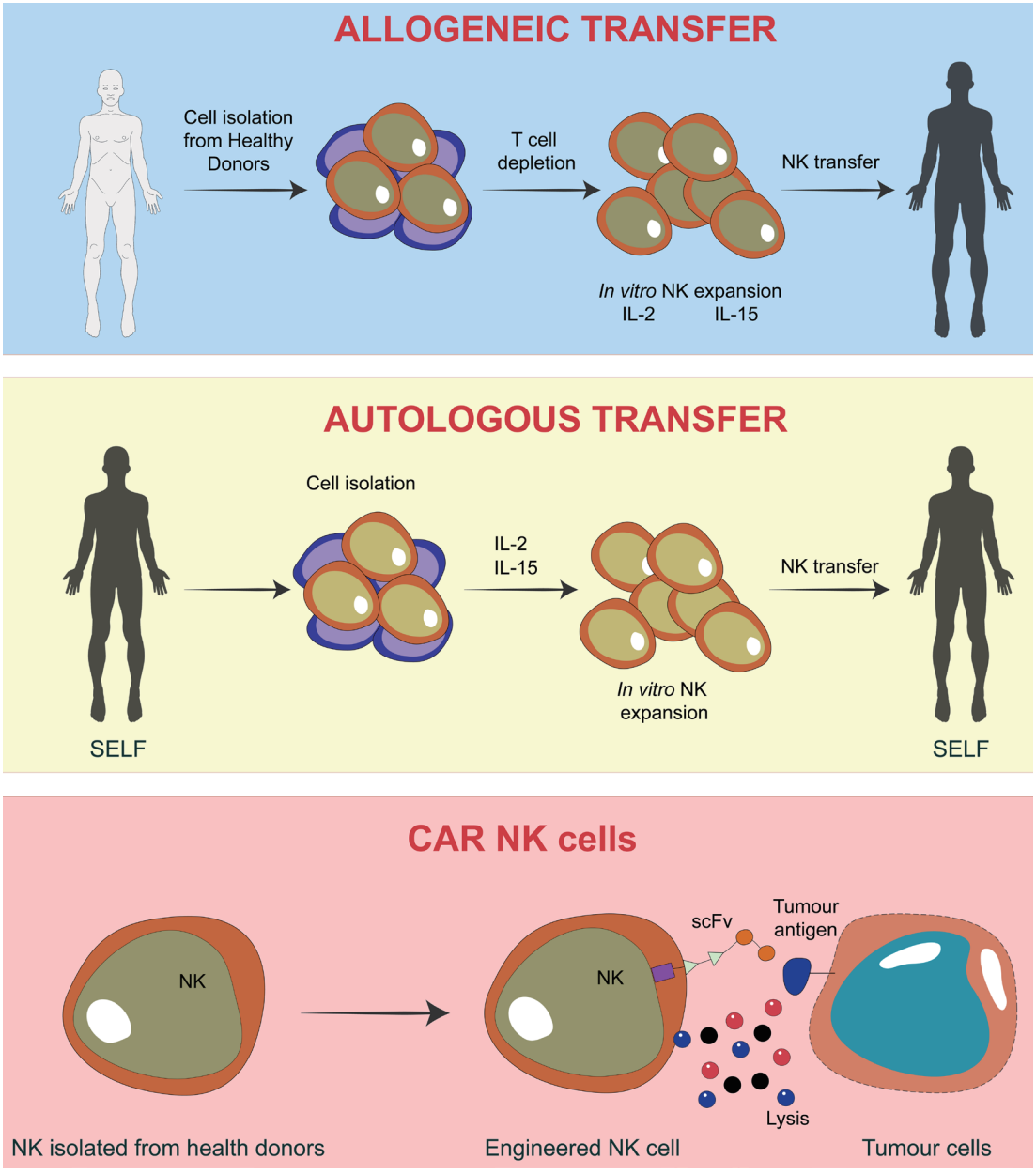

Fig. 4. Schematic representation of adoptive NK cell transfer for AML patient. For the allogeneic NK cell transfer, NK cells are isolated from a healthy HLA-matched or haploidentical donor. After T cell depletion and in vitro expansion, it is infused into the AML patient. For the autologous NK cell transfer, after NK cells are isolated from the peripheral blood of the AML patient, there shall be T cell depletion, followed by in vitro expansion before it is infused back into the patient. In CAR-NK cell therapy, NK cells can be harvested from different sources and engineered to express specific receptors that recognize ligands on the AML cells, leading to their destruction. AML, acute myeloid leukemia; CAR, chimeric antigen receptors; HLA, human leukocyte antigen; IL, interleukins; NK, natural killer.

FLT3 CAR-NK cells showed antileukemic activity against primary AML blasts and LSC-enriched target cell populations and demonstrated improved survival in an MV4-11 xenograft AML mouse model.[219] The C-type lectin-like molecule 1 (CLL-1), which is a widespread expression in AML blasts, has also been seen as an ideal target for CAR-NK cells. A phase 1 clinical trial recruits patients for CAR-NK cell targeting of CLL-1 in AML (NCT06027853).

CAR-NK cell therapy has also shown efficacy in human clinical trials. In a first-in-human phase 1 clinical trial, Huang *et al.*[220] infused anti-CD33 CAR-NK cells into 10 R/R AML patients after preconditioning with fludarabine and cyclophosphamide. 60% of the patients had a complete response 28 days after the infusion of the CAR-NK cells, and only one patient developed grade 2 CRS, which was alleviated with dexamethasone.[220] There were no reported incidences of neurotoxicity or any other adverse events. Some CAR-NK preclinical and clinical studies are underway across different cancers.[221–223] Unfortunately, the phase 1 NKG2D CAR-NK cell therapy for R/R AML (NCT05247957) patients was prematurely terminated.[224] CAR-NK cell therapy is a prospective option for managing AML (Fig. 4).

Antibodies

The receptor-ligand interaction and antibody-dependent cellular cytotoxicity are two pivotal mechanisms for activating NK cells. Leveraging antibody mediation in these processes has emerged as a viable therapeutic strategy in AML. This can be achieved by targeting tumor-associated antigens or inhibiting NK cell receptors using specific antibodies.

Antibodies targeting tumor-associated antigens

The primary mechanism involves the induction of antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells. While unconjugated antibodies alone have shown limited efficacy, the engineering of antibodies to enhance their Fc regions can significantly improve their affinity for CD16, a receptor on NK cells that mediates ADCC. Preclinical studies have demonstrated the potential of these antibody-mediated actions. For example, Riegg *et al.*[225] developed an anti-tumor antibody targeting CD133, a protein commonly found on the surface of B-ALL cells. This antibody specifically activated NK cells to lyse B-ALL cells. Similarly, Koerner *et al.*[226] applied this molecule in studies involving AML cells and xenotransplanted mice, achieving cell lysis of CD133-expressing AML cells. Steinbacher *et al.*[227] used Fc-optimized NKG2D-immunoglobulin G fusion proteins to activate NK cells against leukemia cell lines, including AML and primary AML cells, showing significant activity.

These studies underscore the effectiveness of using Fc-optimized antibodies against specific antigens expressed on AML cells as a promising therapeutic option. This approach enhances the innate immune response and offers a targeted method to combat leukemia cells by harnessing the natural cytotoxic functions of NK cells.

The antibody-drug conjugates and antibody-radio conjugate are promising therapeutic strategies for enhancing antibody potency. Gemtuzumab ozogamicin, a calicheamicin conjugate of anti-CD33 antibody, is approved to manage AML.[228,229] Some other antibodies with conjugates such as CD13, FLT3, and CLL-1 and antibodies combined with NK cell transfer have shown promise for the management of AML.[230–232]

Antibodies targeting NK cell inhibitory receptors

NK cell's inhibitory receptors are essential in immune cell recognition and tumor escape.[233] These inhibitory receptors, including the MHC-I-specific inhibitory receptors (KIRs, LIRs) and immune checkpoints (PD-1, CTLA-4, TIGIT, Siglec-7, TIM-3), are known to cause NK cell dysfunction, as earlier discussed. While anti-KIRs have not proved to be efficacious in clinical trials, checkpoint inhibitors approved for some solid tumors have shown some efficacy in AML.[201,202,234] In a phase 2 trial of nivolumab and azacitidine in pre-treated AML patients, the ORR was 33% with a CR of 22%.[235] Another phase 2 trial of nivolumab in combination with cyclophosphamide in R/R AML patients has recently been concluded (NCT03417154). As for the anti-TIM-3 antibody sabatolimab (MBG453) in the phase 1 clinical study (with decitabine or azacitidine), it was safe and well tolerated in higher-risk myelodysplastic syndrome and AML.[236,237] Of 11 clinical studies involving TIM-3 inhibitors in AML/MDS, three have completed recruitment (NCT03066648, NCT03946670, and NCT04266301).

BiKE and TriKE

BiKE and TriKE represent an innovative class of potential immunotherapeutic agents. These agents function as the NK cell counterparts to the bispecific T cell engager (BiTE), serving as immunological synapses between NK cells and cancer cells, similarly to how T cell engagers operate. T cell engagers activate T cells, leading to proliferation, cytokine release, and cancer cell death by bypassing the T cell receptor and MHC contact. While BiTE has been highly effective in managing hematological malignancies, it is associated with severe adverse events such as CRS and Immune effector cell-associated neurotoxicity syndrome (ICANS), which can cause significant morbidity and mortality.

Conversely, NK cell engagers primarily activate NK cells through cell surface receptors such as CD16, NKp46, or NKG2D.[238–241] BiKEs and TriKEs have demonstrated promising activity against several cancers. Preclinical studies of a CD16xCD33 BiKE have shown that it can adequately activate NK cells, destroying AML cell lines and primary AML cells.[242] A TriKE incorporating a modified human IL-15 into the CD16x CD33 BiKE has been shown to induce significant NK cell cytotoxicity, degranulation, and cytokine production against CD33+ HL-60 cells.[241] Further studies on second-generation TriKEs have indicated that they are more potent than the first-generation and can induce cell death in patient-derived xenograft AML tumor models, as well as in both AML cell lines and primary patient-derived AML blasts.[243–246]

Additionally, Reusing *et al.*[247] demonstrated that primary cells from pediatric AML and biphenotypic ALL responded positively to BiKE treatment. A phase 1/2 clinical trial (NCT03214666) using a designed TriKE reported significant reductions in bone marrow blast levels in patients with AML and MDS) without the need for costly progenitor-derived or autologous/allogeneic cell therapies.[248] These findings suggest that BiKEs and TriKEs, like their cousin BiTE, hold substantial potential in the management of AML, offering a targeted and effective approach to cancer immunotherapy.

Cytokines

In the developmental spectrum of NK cells, IL-2, IL-12, IL-15, IL-18, and IL-21 are critical players in the proliferation, activation, and effector functions of NK cells. IL-2 was the first cytokine shown to enhance NK cell activity, and to date, is the only Food and Drug Administration approved cytokine for the treatment of cancer patients. However, IL-15 is another very promising cytokine for activating NK cells. It is reported that in the ex vivo stimulation of NK cells in AML patients, 50 ng/mL of IL-15 or 10 ng/mL of IL-2 was optimal for the recovery of its function through the upregulation of activating receptors NKp30, NKp46, NKG2C, and NKG2D.[249–251] Though it can expand and activate NK cells, studies have shown that 1L-2 may not have adequate clinical efficacy as a monotherapy in AML patients.[252,253] However, IL-2, in conjunction with other therapies, has shown clinical efficacy in AML, especially as a maintenance therapy.[254–257] Clinical studies have shown that IL-15 expands NK cells in cancer; however, high expression of IL-15 is reportedly linked with CNS disease and neurocognitive impairment in ALL.[258–263] IL-15 has also been shown to increase the cytotoxicity of NK cells in patients with AML.[264] In a phase 1 clinical trial, the IL-15 superagonist complex ALT-803 given as a monotherapy to AML patients who relapsed after allogeneic HSCT was observed to be safe and well-tolerated and with one CR (NCT01885897).[265] A phase 1 trial of ALT-803 in solid tumors also produced a significant rise in NK cell numbers (NCT01727076).[266] However, a recent clinical study by Berrien-Elliott *et al.*[267] reported that systemic IL-15 can promote allogeneic cell rejection in R/R AML patients treated with natural killer cell adoptive therapy (NCT03050216 and NCT01898793). Recently, CAR-NK cells that co-expressed transgenes for the NKG2D CAR and IL-15 were developed, and it shows enhanced

in vitro and in vivo activity in an AML mouse model.[268] Regarding IL-21, a membrane-bound 1L-21 adoptive NK product was shown to reduce AML burden in vivo and had better OS in human subjects with AML.[269] IL-21 was also found to inhibit primary AML stem cells in vitro with the enhancement of cytarabine treatment.[270] Currently, two studies are recruiting for IL-21 trial in AML (NCT04220684) (NCT02809092).[271,272]

NK cells pre-activated with a cocktail of cytokines (IL-12, IL-15, and IL-18) have demonstrated sustained anti-leukemia responses to restimulation, maintaining effectiveness for weeks to months, regardless of inhibitory KIR-KIR ligand interactions. These cytokine-induced memory-like NK cells are reported to possess significant antineoplastic potential. A clinical trial involving the adoptive transfer of cytokine-induced memory-like (CIML) NK cells in R/R AML patients has shown that this approach can induce remission without serious adverse events.[273] Further, an ongoing clinical trial involving donor transfer of CIML NK cells (NCT03068819) targeting R/R pediatric and young adult AML patients has provided encouraging data, reporting sustained CR.[274,275] These findings highlight cytokine-induced NK cell products as promising therapeutic candidates for AML management. Details of these clinical trials are summarized in Table 2.

Nanoparticles in enhancing NK cell therapy

New avenues are being explored for NK cell-based therapies. One such area is nanotechnology and nanomedicine. Nanotechnology is used to see its feasibility in NK cell expansion and activation. This can be done in several ways, including enhancing NK cell activity through nanoparticle-assisted immunomodulation, enhancing NK cell homing by nanoparticles, and activating NKG2D receptor by nanoparticles, etc.[276,277] Several NK cell-based nano-immunotherapies for cancer are actively being developed, and one is currently in phase 2 trial.[276] In a study on NK cells, Sanz-Ortega and her colleagues used magnetic nanoparticles to improve the targeting of adoptively transferred NK cells without altering their function.[278] Selenium-containing nanoparticles were used in a study to enhance NK cell function.[279] Nanoengagers were shown to be more effective in activating NK cells than antibodies. In addition, they could augment both NK-activating agents and chemotherapy to achieve a greater intensity of chemoimmunotherapy.[280] Nanoengagers were also created for T cells against an AML xenograft model, which effectively activated T cells and induced AML cell death *in vitro* and *in vivo*.[281] This shows the potential of nano-immunotherapies in the management of hematological malignancies. Very recently, Zeinabad engineered an NK cell mimic nanoparticle, which was functionalized against an anti-CD38 antibody (Daratumumab). It showed *in vitro* activity against AML cell lines, patient-derived AML cells *ex vivo,* and CD38-positive AML cells *in vivo* in a disseminated AML xenograft model.[282] This same nanocoupling was also successfully used to target some hematological cancer cell lines.[283] NK cell-based nano-immunotherapy is still in its infancy, but it is believed it can be one of the arsenals against AML shortly.

Conclusions and future perspective

NK cell-based therapies have shown potential as viable and strategic therapeutics in managing AML in the future. So far, the various preclinical and clinical studies on NK cells show a challenging but achievable feat. However, it is a priority to get the different NK cell therapeutic forms to do what they are for against a highly heterogeneous enemy like AML. Compared to T cell therapies, NK cells have some advantages. NK cell tumor detection is not strictly based on MHC recognition but can mediate ADCC. NK cells also provide a better safety profile than T cell therapies, including a lower incidence of GvHD, CRS, and ICANS. Though NK cells have a limited lifespan, they are easy to prepare under good manufacturing practice standards, implying an "off the shelf" benefit and a universal administration for managing patients in a short period. However, NK cell cellular-based therapies are still faced with some challenges, including ensuring sustained in vivo expansion and proliferation of NK cells due to their short lifespan in patients, which leads to a short response duration. How can the various immune escape mechanisms used by AML be stopped to evade detection and cell death, especially through the creation of an immunosuppressive tumor microenvironment?

The tumor microenvironment in AML is a complex arena that impairs NK cell function. For example, myeloid-derived suppressive cells, which are found within the tumor microenvironment, can produce immunosuppressive factors such as IL-10, TGF-β, and IL-4, which are capable of inhibiting the expression of NKp30 and NKG2D that are important in NK cell tumor cells recognition and destruction. AML cells also alter glucose utilization, enabling them to survive hypoxic conditions. Glucose is vital for NK cell metabolism and is thus reduced, leading to NK cell dysfunction. Increasing lactic acid production in the microenvironment is a potent inhibitor of NK cell effector function and viability. Thus, this microenvironment hostility has been shown to affect NK cell therapy, especially CAR-NK cells. TGF-β, which plays an inhibitory role in NK cell tumor cell recognition, can be neutralized by engineering CAR-NK cells that lack TGF-β receptor expression. Moreover, catalase can attenuate hypoxia in the microenvironment, reducing the effect of lactic acid and hydrogen peroxide on NK cells, ultimately improving NK cell therapy. In addition, cytokines such as IL-15 and IL-21 can enhance NK cell cytotoxicity in tumor sites, while IL-18 primed NK cells can also engage effector T cells through the help of DCs.

The efficient transduction of CAR-NK cells remains a critical issue that requires further exploration. While the field has seen several phase 1/2 clinical trials, initiating a phase 3 trial is imperative. This next step involves a well-designed, randomized clinical trial with an adequate sample size to determine the optimal dosing and therapeutic efficacy of each NK cell therapy in AML). Such a trial could also elucidate the most efficacious NK cell therapy for AML and address the timing of these therapies—whether AML patients should receive them during induction remission, consolidation, or as part of a maintenance regime, and how many cycles should be administered at each stage.

Considering our current understanding of various NK cellular therapies, there is potential for using them in combination therapies. Such combinations might enhance both the proliferative and effector functions of NK cells and their *in vivo* sustainability to effectively target AML cells. In particular, CIML NK cells could be valuable due to their ability to prolong the duration of NK cells in vivo. Furthermore, combining standard AML therapies with NK cellular therapies could provide synergistic effects that enhance the ability of NK cells to combat AML.

Immunomodulatory drugs, such as lenalidomide and thalidomide, have shown promise in enhancing NK cell functions. They achieve this by stimulating the release of IL-2 and IFN-γ from T cells and dendritic cells in the surrounding environment. Additionally, proteasome inhibitors like bortezomib can increase the sensitivity of AML cells to NK cell-mediated lysis, potentially improving clinical outcomes. Such strategic integration of therapies could

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Table 2. Some clinical trials of NK cells cellular therapies

Identifier	Phase	Condition	NK cell source	Intervention	Status	Outcome
NCT02809092	I/II	R/R AML	Haploidentical NK	Before treatment with chemotherapy	Completed	78.6% overall response; 50.0% CR; CNS responses in 4 patients
NCT01385423	I	Refractory AML	Haploidentical NK	Before treatment with lymphodepleting chemotherapy; After treatment with rhIL-15 intravenously (0.3–1.0 mg/kg)	Completed	Robust NK expansion in 36% of patients at day 14; CR in 32% of patients
NCT00703820	II	Paediatric AML	Haploidentical NK	Before treatment with lymphodepleting chemotherapy and rhIL-2 subcutaneously	Completed	None
NCT02763475	II	Paediatric AML	Haploidentical NK	Before treatment with lymphodepleting chemotherapy and rhIL-2 subcutaneously	Completed	CR in 6 of 7 patients
NCT05247957	I	R/R AML	CAR-NK cell	Pretreated		Not provided
NCT05272293	I/II	Paediatric AML	Haploidentical NK	Pretreated	Recruiting	Not provided
NCT05256277	I	R/R AML adults	CIML NK cells	Pretreated	–	Not provided
NCT02727803	II	AML, MDS, etc	UCB-derived HSPC-NK cell	Treated with Busulfan, Clofarabine, Cyclophosphamide, Fludarabine Phosphate, Melphalan, Rituximab	Recruiting	Not provided
NCT01823198	I/II	AML, MDS, etc	PBMC-derived NK cell	IL-2, Busulfan, Fludarabine	Completed	Not provided
NCT04221971	I	AML adults	PBMC-derived NK cell	Pretreated	Completed	1/3 with MRD negative, low dose group; 3/4 response with 1 case of extramedullary recurrence of AML turned negative, middle dose group.
NCT04310592	I	AML adults	Placental-derived HSPC-NK cell (CYNK-001)	Pretreated	Recruiting	Not provided
NCT04623944	I	AML adults	Car-NK cell (NKX101)	Pretreated	Recruiting	Not provided
NCT04901416	I	AML adults	PBMC-derived NK cell (DVX201)	Pretreated	Recruiting	Not provided
NCT04347616	I/II	AML	UCB-NK cells + 1L-2	Pretreated	Recruiting	Not provided
NCT05008575	I	R/R AML	CAR-NK cell (Anti-CD33)	Pretreated	Recruiting	Not provided
NCT05215015	I	AML	CAR-NK cell (Anti-CD33/CLL1)	Pretreated	Recruiting	Not provided
NCT04220684	I	AML	Haploidentical NK cell (IL-21 expanded)	Pretreated	Recruiting	Not provided
NCT05333705	I	AML	PBMC/ UCB NK cell	–	Recruiting	Not provided
NCT04836390	I	Paediatric AML	Haploidentical NK cell	–	Enrolling by invitation	Not provided
NCT03821519	I/II	AML, MDS etc	CIML NK cells	Pretreated (with allo-HSCT)	Recruiting	Not provided

AML, acute myeloid leukemia; CAR, chimeric antigen receptor; CLL, Chronic lymphocytic leukemia; CNS, central nervous system; CR, complete remission; HSCT, haematopoietic stem cell transplantation; IL, interleukin; MDS, myelodysplastic syndrome; MRD, minimal residual disease; NK, natural killer; PBMC, peripheral blood mononuclear cell; UCB, universal cord blood.

lead to more effective, sustainable treatments for AML, capitalizing on the innate strengths of NK cells in cancer immunotherapy.

In conclusion, the treatment landscape for managing AML has expanded with the potential integration of NK cell cellular-based therapies. These therapies stand out among other cellular treatments due to their off-the-shelf availability, cost-effectiveness, and capability to recognize cancer cells without the constraint of MHC mechanisms. This attribute facilitates broader accessibility and potentially fewer adverse effects for various patients. The efficacy and safety of these therapies are highlighted in the clinical trial (NCT03056339) reported by Marin *et al.*,[284] where CAR-NK cells were used to treat CD19+ B cell malignancies. In this trial, no notable adverse events such as ICANS, CRS, or GVHD were observed. This evidence further solidifies the favorable safety profile of NK cell-based cellular therapies, promising a valuable addition to the arsenal against AML.

NK cell cellular-based therapies have a bright prospect in managing AML, and with more clinical research, it may soon be a reality.

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Conflict of interest

The author declares that he has no competing interests to declare.

Author contributions

OOI is the sole author of the manuscript.

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