

Review Article

CAR-NK cells for acute myeloid leukemia immunotherapy: past, present and future

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Abstract: Acute myeloid leukemia (AML) is a deadly disease and the most common leukemia in adult with clonal heterogeneity and abnormality in myeloid lineages, which has been recognized with high morbidity and mortality attributes to the recurrence and resistance to chemotherapy. Numerous literatures have indicated the encouraging progress in allogeneic hematopoietic stem cell transplantation (allo-HSCT) and chimeric antigen receptor-transduced T (CAR-T) cells. However, the outcomes of recurrent and refractory AML (r/rAML) patients with current strategies are still unsatisfactory, which largely due to the matching restriction as well as adverse reactions, including graft-versus-host disease (GvHD), neurotoxicity and cytokine release syndrome (CRS). State-of-the-art literatures have indicated CAR-transduced NK (CAR-NK) cells for the management of diverse hematologic malignancies including AML, which are recognized as novel weapons for reinforcing the specificity and cytotoxicity of autogenous and allogeneic "off-the-shelf" NK cells dispense with prior sensitization. Therefore, in this review, we mainly focus on the latest updates of alternative cell sources, therapeutic targets, CAR-modification and delivery strategies, standardization and productization, together with prospective and challenges of CAR-NK cell-based cytotherapy, which will collectively benefit the further development of novel treatment paradigms for combating AML via both CAR-dependent and NK cell receptor-dependent signaling cascades in future.

Keywords: CAR-NK cells, cancer immunotherapy, acute myeloid leukemia, target modification, clinical trials

Introduction

AML is a rare but intractable malignancy with multifaceted alterations in the precursors of myeloid lineage, which is largely attributed to genetic variations and the resultant clonal proliferation and neoplastic changes [1, 2]. AML has been reported with an incidence of over 20,000 cases per year in the United States, while the relapse rate of childhood AML takes

up around 30% [3, 4]. As a clinically heterogeneous and biologically complex disorder, AML with recurrent genetic alterations in genomic landscape has provided novel insights into the pathogenesis, clinical manifestations and the overall survival as well [5, 6]. For example, AML with myelodysplasia-related changes (AML-MRC) is a well-established subtype of AML, which represents a proportion of 25-34% of all AML diagnoses and relates with worse out-

comes compared to the non-MRC AML [7]. We also conducted systematic and detailed dissection of the pathogenesis of AMLs from the perspectives of platelet generation and bone marrow-derived mesenchymal stem/stromal cells (BM-MSCs) in the hematopoietic microenvironment [8, 9].

Currently, the conventional treatment options for AML patients are largely dependent on hypomethylating agents (e.g., Homoharringtonine, Gilteritinib) and intensive chemotherapy [10-13]. In recent years, noteworthy breakthroughs in stem cell transplantation, including hematopoietic stem cells (HSCs) and mesenchymal stem/stromal cells (MSCs), have been demonstrated beneficially for improving the survival and outcomes of AML patients [10]. However, the deficiency in matched marrow donors, *in vitro* preparation, graft rejection and relapse after HSC transplantation (HSCT) further hinders the extensive application for AML administration [9, 14].

Differ from the aforementioned regimens (e.g., HSCT, MSC infusion), immunotherapy of diverse categories has been continuously developed aiming to accomplish effective cancer administration, such as monoclonal antibodies (e.g., anti-PD-L1, anti-HER-2), cancer vaccines (e.g., mRNA vaccine), non-gene-edited immune cells (e.g., natural killer cells, macrophages, dendritic cells), and gene-edited immune cells (e.g., CAR-T cells, T cell receptor-engineered T (TCR-T) cells, CAR-macrophage (CAR-M), and CAR-neutrophil) [15-20]. For instance, Chen *et al* emphasized the superior anti-tumor response of HER2 and CD47 CAR-M therapy against ovarian cancer via macrophage phagocytosis and the consequent adaptive immune cross-priming (e.g., enhancing CD8⁺ T cell activation, and affecting tumor-associated macrophage (TAM) phenotype) [21]. Very recently, Chang and the colleagues recently reported the application of CAR-neutrophils for the transportation of tumor-microenvironment responsive nano-drugs for glioblastoma chemo-immunotherapy [20]. Interestingly, Yu *et al* took advantage of the lentiviral-mediated cell entry by engineered receptor-ligand interaction (ENTER) and RNA sequencing-based single-cell readout to deliver genetic payloads to the indicated antigen-specific T or B cells (e.g., TCR-T cells) [22]. As to CAR-T therapy, Sauer *et al* introduced the novel

CD70-specific CAR-T cells containing a common single-chain variable fragment (scFv) for targeting most leukemic blasts and virus-specific T cells (VSTs) in AML patients but still required monitoring of VST responses [23]. Meanwhile, during the past decades, we and the collaborators also reported the clinical application of CAR-T cells for conquering numerous hematologic malignancies, including CD7, CD22, CD19, CD64, CD32b CAR-T cells for acute lymphoblastic leukemia (ALL) and relapsed B ALL, acute myeloid leukemia (AML), and chronic lymphocytic leukemia (CLL), respectively [24-28]. Of note, Kim *et al* put forward the proof-of-concept assumptions for enabling CAR-T-based AML immunotherapy via genetic inactivation of CD33 in HSCs, which would help conquer the major impediment of CAR-T application [29]. To overcome the restricted efficacy of CAR-T cells against solid tumors, Ma *et al* designed amphiphile CAR-T ligands and enhanced CAR-T activity by vaccine boosting through the chimeric receptor [30]. Even though, the inherent defects of CAR-T-based regimens are still challenging and further restrict the extensive application in clinical practice, including donor limitation, variations in cellular vitality, uncertain molecular heterogeneity of AML, the adverse reactions (e.g., CRS, GvHD, and neurotoxicity), off-target effects, and the possibility of long-term hematopoiesis inhibition [16, 31-33].

Natural killer (NK) cells are innate immunocytes with unique cytotoxicity for the elimination of tumor cells and pathogenic microorganisms dispense with recognition of peptide antigens or prior sensitization [34-38]. NK cells function via orchestrating diverse modes of action such as direct cytolytic effect, paracrine effects (e.g., GM-CSF, IFN- γ), antibody-dependent cell-mediated cytotoxicity (ADCC), and manipulating other immune contexts [39-42]. Considering the low immunogenicity and the multiple cytotoxic effect, more and more investigators have turned to develop novel targeted immunotherapy by delivering CAR-construction into NK cells for the preparation of allogeneic CAR-NK cells for AML treatment [43-45]. Distinguish from the aforementioned "immune enhancement" strategies, CAR-NK cell-based immunotherapy exempts from the immune-related adverse events (irAEs) but displays more beneficial tumor response-to-toxicity profile via orchestrat-

CAR-NKs for AML immunotherapy

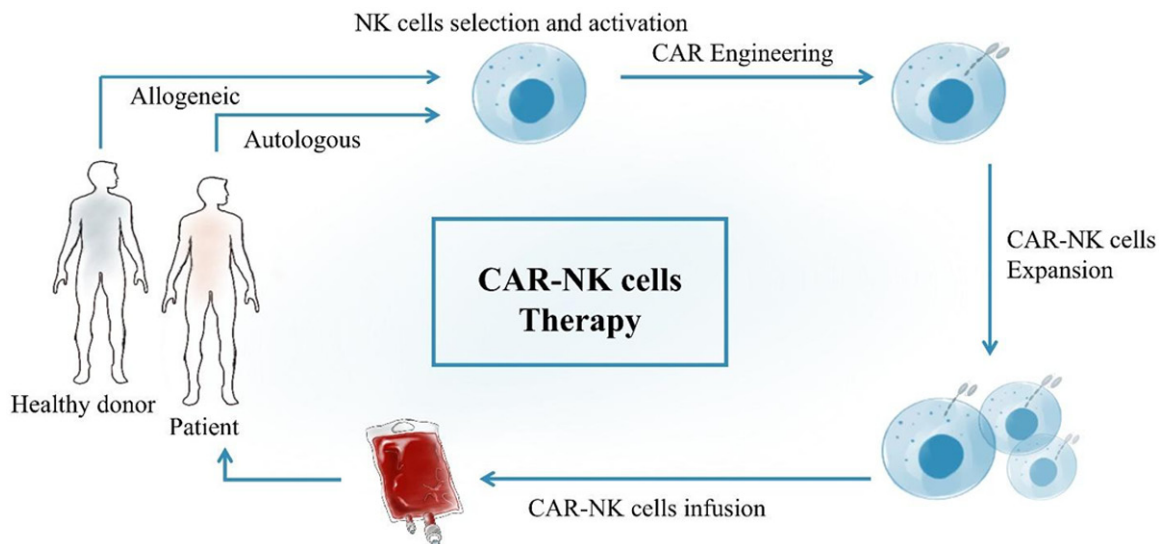


Figure 1. CAR-NK-mediated cellular therapy. Allogeneic NK cells and autologous NK cells are isolated from healthy donors and AML patients for ex vivo NK cell selection and activation. After CAR engineering, the CAR-NK cells are turned to further expansion in vitro and systemic infusion into AML patients.

ing the modes of action, including paracrine (e.g., granzyme, perforin, IFN- γ , TNF, GM-CSF), antibody-dependent cell mediated cytotoxicity (ADCC), pro-apoptotic approaches (e.g., Fas-FasL, TRAI-TRAIL), direct cytolytic effect, and CAR-mediated targeting [46, 47]. In this review, we mainly summarized the current advances of CAR-NK cells and the immunotherapy for AML management from the aspects of cell sources, target selection, CAR-modification and delivery, standardization and productization, and prospective and challenges. Overall, our data would benefit our understanding towards novel CAR-NK cell-based innovative immunotherapy and the concomitant cell product development for AML administration.

Cell sources for CAR-NK cell manufacturing

Considerable literatures have indicated the important role of NK cells in clearing the residual AML cells after HSCT, together with enhancing the effect of graft-versus-leukemia (GVL) without aggravating GvHD [34, 48]. Longitudinal studies have indicated the application of diverse candidate sources for the preparation of non-gene-edited NK cells and the gene-edited CAR-NK cells for eliminating pathogenic microorganisms, malignant tumors and aging cells, such as the peripheral blood, perinatal tissue (e.g., umbilical cord blood, placenta blood, decidual tissue, uterine tissue), stem

cells (e.g., HSCs, human pluripotent stem cells), and NK cell lines (e.g., NK-92, YT) (Figure 1) [37, 49, 50]. For example, Chiossone and the colleagues reported the *in vivo* generation of mature decidual NK cells from resident hematopoietic progenitors, which were crucial for placental development, the maternal-fetal vasculature during pregnancy, and preeclampsia [51-54].

Proverbially, NK cells only occupy a low frequency in peripheral blood-derived mononuclear cells (PBMCs) compared with relative fractions (e.g., T lymphocytes, B lymphocytes, and NKT cells), which approximately account for 5-20% of leukocytes including the CD56^{bright}CD16^{low/neg} subpopulation and the CD56^{dim}CD16^{high} counterpart [17, 55]. To fulfill the allogeneic NK cell-based immunotherapy, a variety of methodologies have been developed to gain sufficient number of activated NK cells *in vitro*, including enrichment by magnetic activated cell sorting (MACS), co-stimulation with feeder cells (e.g., K562 cell lines without/with IL-2 and/or IL-15 transfection), monolayer stimulation by cytokine cocktails (e.g., IL-2, IL-7, IL-12, IL-15, IL-18, IL-21), antibody coating (e.g., anti-CD3), and physicochemical irritations (three-dimensional rotation, bioreactor, hypoxia, nanomaterial stimulation) [16, 17, 55]. For example, we took advantage of diverse cytokine cocktails for standardized and convenient NK cell cultiva-

tion, and eventually obtained 30-fold amplification activated NK (aNK) cells and a proportion of cytokine-induced memory-like NK cells (CIML-NKs) endowed with prolonged effector function and splendid longevity from PBMCs [56]. Instead, Somanchi and the colleagues compared the efficacy of a commercial kit (Miltenyi Biotec, Auburn, CA), EBV-LCL, and K562-41BBL-mIL15 aAPCs (artificial antigen-presenting cells) for *ex vivo* NK cell expansion, and eventually obtained 100-fold, 490-fold, and 21,000-fold expansion within 21 days, respectively [55]. Fallaciously, due to the small amount and the individual variations, the inability to enrich resident NK (rNK) cells and propagate activated NK (aNK) cells *in vitro* has hindered the large-scale preparation of CAR-NK cells with high cytotoxicity and cellular vitality for optimal clinical outcome [57].

In recent years, considerable attention has been paid to assess the feasibility of the “discarded” perinatal blood for allogeneic HSCs and CAR-NK cell preparation, including umbilical cord blood and placental blood. Generally, NK cells in umbilical cord blood and placental blood occupy approximately 5% and 1% of total mononuclear cells (MNCs), respectively [16, 17]. Interestingly, both the PBMC-enriched resident NK (rPB-NK) cells and the umbilical cord blood-enriched resident NK (rUC-NK) cells are composed of the CD3⁺CD56⁺CD16⁺ subset (>60%) and the CD3⁺CD56⁺CD16⁻ subset (<40%), whereas less than 40% are CD3⁺CD56⁺CD16⁺ cells and more than 60% are CD3⁺CD56⁺CD16⁻ cells in placental blood-enriched resident NK (rP-NK) cells instead [17]. Despite the inferior baseline cytotoxicity of rNKs compared with relative sources (e.g., peripheral blood, bone marrow), yet this phenomenon in perinatal blood-enriched rNKs can be largely overcome after *in vitro* amplification and activation [58]. For instance, we recently decoded the multidimensional biological and transcriptomic signatures of PB-NK cells and UC-NK cells, resident NK (rNK) cells and activated NK (aNK) cells, respectively [59, 60]. With the “3IL” cocktail-based stimulation (rhIL-2, rhIL-15, rhIL-18) for 14 days, we eventually obtained over 200-fold total cell (CD3⁺ subset and CD3⁺ subset) expansion, 1800-fold total NK cell (CD3⁺CD56⁺) expansion, and 4000-fold total activated NK cell (CD3⁺CD56⁺CD16⁺) expansion from perinatal blood-derived MNCs or MACS-enriched

rNKs, respectively [59, 60]. Compared with aPB-NKs, both aP-NKs and aUC-NKs revealed multifaceted superiorities, including juvenility, robust *ex vivo* proliferation, equivalent activation, and better oncolytic and killing ability [60]. For example, Herrera *et al* compared the efficacy of CAR-NK cells prepared from different sources upon CD19 positive B-cell lymphoma, and found that the percentage of CD56⁺ transduced cells from umbilical cord blood (UCB) was higher than that in peripheral blood at diverse time points (day 14, 21, 28) after post-transfection, which indicated the preferable lifespan of UCB CAR-NK cells [61]. Moreover, considering the large number of placenta perfusate and umbilical cord blood, together with robust amplification and activation *in vitro*, placental blood possesses promising prospects for large-scale clinical-grade NK cell and CAR-NK cell preparation for cancer immunotherapy [16, 43, 57]. Additionally, a certain number of investigators also employed NK cell lines (e.g., NK-92MI cells, YT cells) as cost-effective strategies for exploiting the CAR-NK cell-based immunotherapy in both subcutaneous tumor models or 3D organoids and first-in-man clinical trials [62-67].

Of note, current prospective advances have also highlighted the alternative options of stable NK cell generation from stem cells including hematopoietic stem cells (HSCs) and pluripotent stem cells (PSCs) for CAR-NK cell preparation [68-70]. For instance, Li *et al* elaborated the historical overview of allogeneic HSC-engineered invariant natural killer T (alloHSC-iNKT) cells with high safety and low immunogenicity for “off-the-shelf” cancer immunotherapy, which collectively demonstrated the feasibility of stem cell-based solutions for large-scale CAR-NK cell preparation and provided the foundation for translational and clinical development [71]. Furthermore, Arias *et al* and Zhu *et al* highlighted the latest renewal of HSCs- and engineered human embryonic stem cells (hESCs)- or induced pluripotent stem cells (iPSCs)-based procedures as reliable “off-the-shelf” CAR-NK cell therapeutics for tackling seemingly incurable oncological malignancies and avoiding GvHD and CRS, respectively [68, 72]. Differ from the aforementioned peripheral blood or perinatal blood with donor-dependent variability, the homogenous stem cells are adequate for genetic modification at a clonal level

CAR-NKs for AML immunotherapy

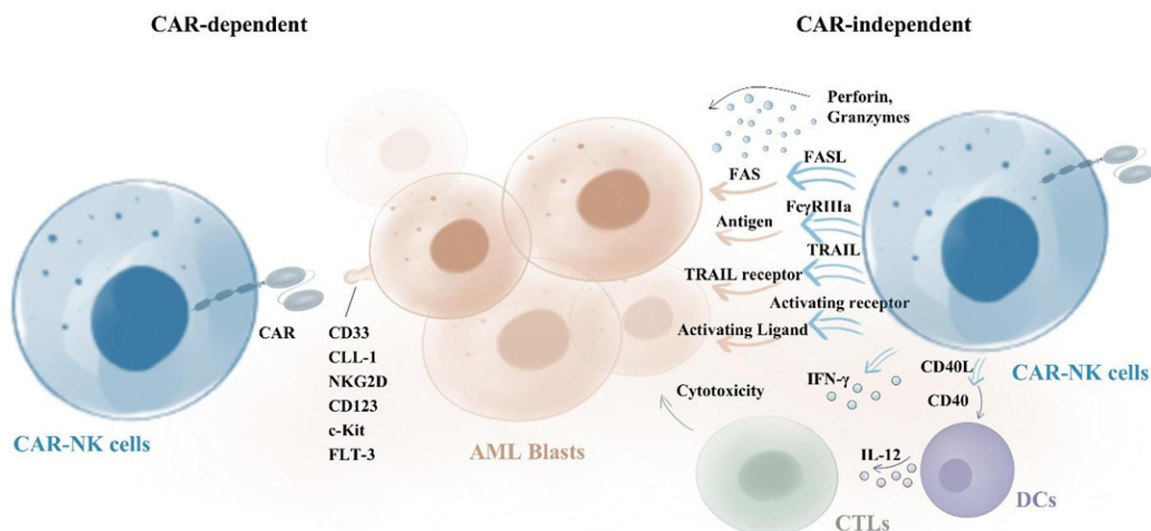


Figure 2. CAR-NK cell-based cytotherapy for AML. CAR-NK cells are adequate to conquer AML blasts via both CAR-dependent and CAR-independent manners.

and thus ideal for developing standardized, large-scale, cutting-edge CAR-NK cell products and novel therapeutic modalities with the proof-of-concept safety and efficiency for AML management [16, 72, 73].

Therapeutic targets for CAR-NK cells

Autogenous and allogeneic NK cells are advantaged options for cancer immunotherapy dispense with prior sensitization or manipulation of other immune contexts, whereas the “tumor escape” and the resultant exemption from immunological surveillance attributes to the heterogeneity of cancer cells with genetic or epigenetic variations largely impact the cytotoxicity of NK cells via interdicting the corresponding activating receptors (**Figure 2**) [17, 39-42, 74, 75]. To conquer the boundedness of NK cells, pioneering investigators have considered the costimulatory molecules and tumor-associated antigens (TAAs) as the first-line decision for cancer immunotherapy [76, 77].

Therewith, a variety of CAR structures in CAR-T cells have been attempted for direct transfer into CAR-NK cells, including CD19, CD20, CD22, BCMA, CD28, and CD33 [25, 78, 79]. Of them, CD33, a myeloid differentiation antigen, is broadly expressed on AML blasts and during all stages of physiological myeloid differentiation, and in particular, strongly expressed in nucleophosphmin-1 (NPM1)-mutated AML cells [80, 81]. For instance, Dong and the colleagues

reported the delivery of a neoepitope-specific CAR into cytokine-induced memory-like (CIML) NK cells, which revealed potent activity against NPM1 mutated AML both *in vitro* and *in vivo* [82]. Christodoulou *et al* verified that CD123 CAR-NKs with interleukin (IL)-15 expression revealed enhanced persistence and a highly activated and proliferative signature of anti-AML activity *in vitro* and *in vivo* over the non-transduced NK cells and 4-1BB.ζ CAR-NK cells [43]. Instead, Du *et al* demonstrated that CAR-NK cells with ectopic NKG2D and IL-15 co-expression revealed enhanced tumor control *in vivo* and prolonged survival of xenograft KG-1 AML mice [83]. Interestingly, our group for the first time reported the preclinical feasibility of CD64 as a novel potential target for AML management dispense with ablation of HSCs [27]. Overall, as shown in **Table 1**, a variety of CAR-transduced therapeutic targets have been extensively explored in both preclinical and clinical investigations, which will facilitate the robust development of the complementary and potentially “off-the-shelf” CAR-NK cell-based immunotherapy for AML treatment.

CAR-modification and clinical trials

Genetically modified CAR-NK cells have accelerated the application of cancer immunotherapy for AML management, which largely attributes to the major histocompatibility complex (MHC) mismatch and multitudinous modes of

CAR-NKs for AML immunotherapy

Table 1. Therapeutic targets of CAR-structures for AML

Targets	Cell sources	Study stage	Reference
CD13, TIM3	CAR-Ts	Preclinical	Ref. [127]
CD123	CAR-NKs	Preclinical	Ref. [43]
CD33, CD123	CAR-Ts	Clinical	Ref. [80, 81, 128]
NKG2D	CAR-NKs	Preclinical	Ref. [2, 83]
CD27, CD70	CAR-Ts	Preclinical	Ref. [88]
CD19, CD33, CD123	CAR-NKs, CAR-Ts	Preclinical	Ref. [84, 129]
NPM1c	CAR-Ts	Preclinical	Ref. [77]
CD19, CD20, CD93, PD-1	CAR-Ts	Clinical	Ref. [90]
CD7	CAR-Ts	Preclinical	Ref. [130]
TIM3, CLL-1, CD38	CAR-Ts	Preclinical	Ref. [2]
CD64	CAR-Ts	Preclinical	Ref. [27]

action, including both the CAR-dependent and the CAR-independent patterns [84]. Differ from CAR-T cells with high-efficiency CAR delivery, the preparation of CAR-NK cells with considerable CAR expression is challenging because the standard techniques for efficiently and genetically engineering the parental NK cells are still largely obscure [84]. For the purpose, talented investigators are devoted themselves to developing novel alternative strategies. On the one hand, Soldierer and the colleagues modified the CAR constructs in recognizing target antigens for AML (e.g., CD19, CD33, and CD123) to facilitate efficient detection of CAR NK cell products with high purity (>95%) based on CD34 microbead-assisted selection [84]. On the other hand, diverse novel technologies have been employed for improving the delivery efficiency of CAR-structure into NK cells, including CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9), TALENs (transcription activator-like effector nucleases), virus (e.g., lentivirus, adenovirus, retrovirus), electroporation, PiggyBack system, and lipid nanoparticles (LNPs) [85-87]. For instance, Du and the colleagues took advantage of the PiggyBack system for NKG2D CAR delivery into NK cells for treating relapsed or refractory AML [83]. Very recently, Leick *et al* reported a CD27-based enhanced CAR for targeting CD70 in AML by utilized the orthogonal approaches for the design of transmembrane-modified regions to alleviate cleavage of the extracellular portion [88]. Of note, for generating transient anti-fibrotic CAR-T cells *in vivo* for cardiac injury treatment, Rurik *et al* even delivered the modified mRNA

into T cell-targeted lipid nanoparticles (LNPs), which thus provided novel therapeutic platforms for further developing *in vivo* CAR-NK cells to treat multisystem disorders including the refractory and recurrent AML (r/r AML) [89].

Generally, the CAR-structure is composed of three key components, including the extracellular domain for antigen recognition, the transmembrane domain, and the intracellular domain [17]. To

date, the CAR-structure has developed for five generations, including the first-generation with an intracellular signal component CD3 ζ , the second-generation with a single costimulatory molecule (e.g., 4-1BB, CD28), the third-generation with diverse co-stimulatory domains, the fourth-generation with cytokine production-inducing effect (e.g., IL-12, IL-18), and the fifth-generation with suicide genes for avoiding hyper-cytotoxicity or incorrect insertions of CAR into TRAC gene for inactivation of TCR α and TCR β [16, 90]. Simultaneously, current advances have also suggested the re-designment of CAR-NK cells with enhanced cytotoxicity and INF- γ secretion for facilitating the antitumor efficacy via modifying NK cell signaling-associated domains (e.g., DAP-10, 2B4, DAP-12) [91].

As to CAR-NK cell-based immunotherapy for AML administration, there are only 9 interventional clinical trials with 297 enrolled cases have been registered according to the *ClinicalTrials.gov* (<https://www.clinicaltrials.gov/>) database under the administration of National Institute of Health (NIH), and most of the trials are launched by China with the recruiting status (**Figure 3**, up to January 9th, 2023). The majority of the trials is in the Phase I and Phase I/II stages for evaluating the safety and efficacy of CAR-NK cell-based cytotherapy by targeting CD33/CLL1 (NCT05215015, NCT02944162, NCT05008575), CD123 (NCT05574608), NKG2D (NCT05247957), and CD70 (NCT0509-2451) (**Table 2**). The parameters for evaluating CAR-NK cell-based outcomes mainly including incidence of dose limiting toxicity (DLT), overall survival (OS), progression free survival (PFS),

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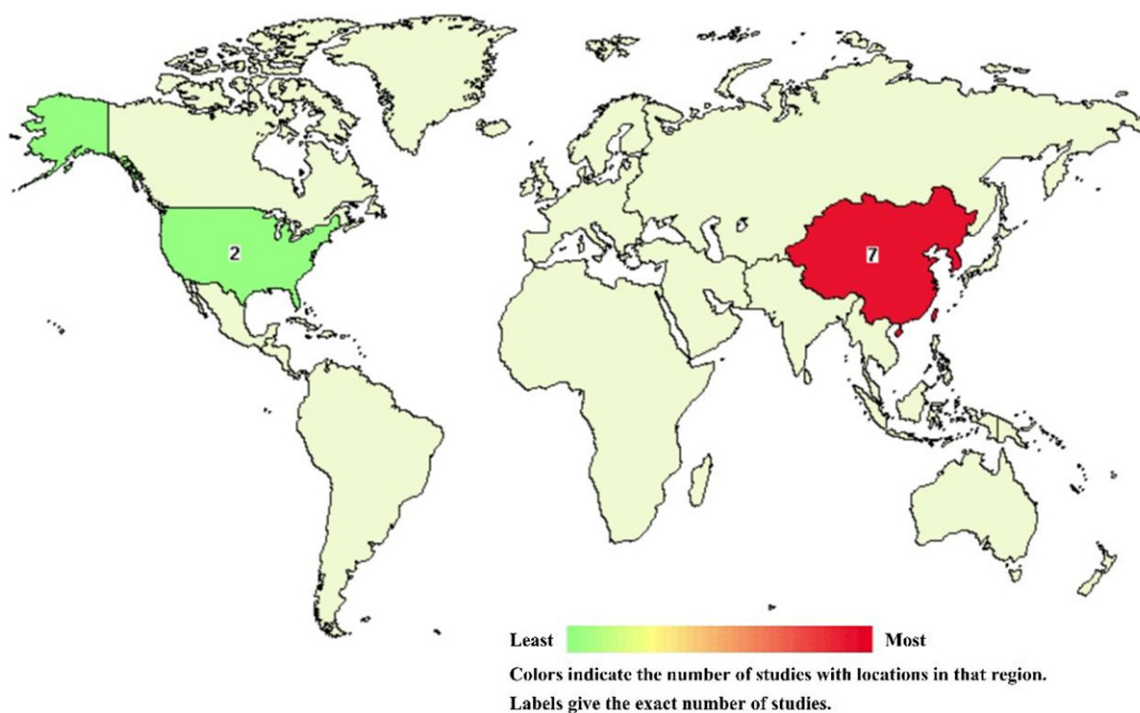


Figure 3. The worldwide distribution of CAR-NK cell-based trials for AML administration. According to the ClinicalTrials.gov website (<https://clinicaltrials.gov/>), a total number of 9 trials has been registered worldwide (up to Feb. 28th, 2023).

Table 2. CAR-NK cell-based clinical trials for AML administration

NCT No.	Status	Targets	Phases	Enrollment	Locations
NCT05215015	Recruiting	CD33/CLL1	Early Phase 1	18	China
NCT05574608	Recruiting	CD123	Early Phase 1	12	China
NCT02944162	Unknown	CD33	Phase 1, 2	10	China
NCT04623944	Recruiting	NKX101	Phase 1	90	USA
NCT05247957	Terminated	NKG2D	Not Applicable	9	China
NCT05008575	Recruiting	CD33	Phase 1	27	China
NCT05601466	Recruiting	QN-023a	Phase 1	18	China
NCT05665075	Recruiting	QN-023a	Phase 1	19	China
NCT05092451	Recruiting	CAR-70/IL15	Phase 1, 2	94	USA

minimal residual disease (MRD), complete response (CR), objective response rate (ORR), duration of overall response (DOR), the pharmacokinetics (PK) and plasma concentration of CAR-NK cells, cytokine release, adverse events (AEs) according to CTCAE (version 5.0), percentage of subjects receiving HSCT, the area under the concentration time-curve (AUC), the immunogenicity features and host immune response. Nevertheless, the rare tumor-specific cell-surface antigens also restricts the broadness and specificity of CAR-NK cell application in clinical trials [21, 46, 92].

CAR-NK cell-mediated immunology in AML

NK cells in AML patients are often dysfunctional compared to the counterparts in healthy donors [93]. It is evident that the incapacitation of NK cells during tumor progression is not only manifested in the relative resistance of AML blasts to NK cell targeting, but also in the regulation of NK cell function in the AML immune microenvironment.

For the past decades, the concomitant molecular mechanisms including soluble factors, cell-

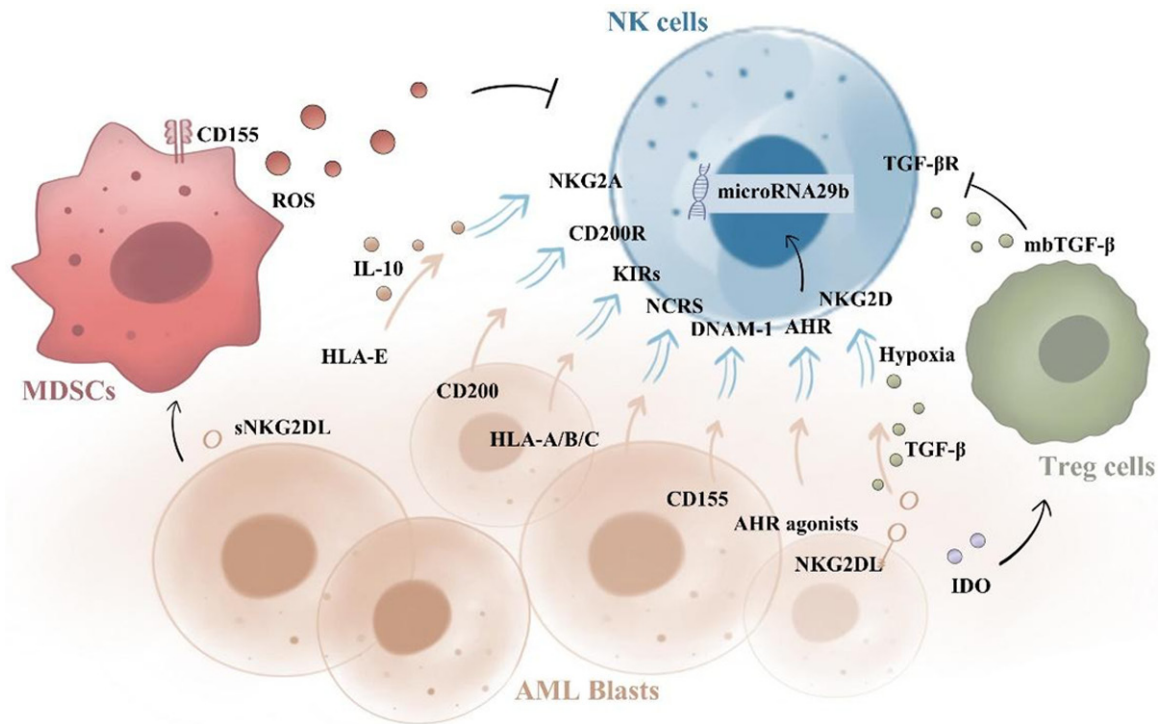


Figure 4. NK cells and the regulatory cell populations in the AML microenvironment. The interaction network of NK cells, the regulatory cell populations (MDSCs, Treg cells), and AML blasts in the AML microenvironment.

to-cell interactions, and other regulatory elements in the AML microenvironment have been extensively described (Figure 4). For instance, some investigators have reported the frequent downregulation of activated NK cell receptors in AML (e.g., NKG2D, DNAM-1, and the NCRs), which positively correlates to the leukemia burden in the patients [94-96]. Soluble NKG2D ligands (NKG2DL) shedded by the tumor cells and tumor surface NKG2D could trigger the NKG2D reduction in NK cells [97, 98]. The NKG2D receptor can also be downregulated by hypoxia and cytokines (e.g., TGF-β) [99]. The antigen CD155 expressed on AML blasts and MDSCs contributes to the downregulation of DNAM-1 on NK cell surface and renders tumor cells resistant to NK cell targeting [100]. Expression of NCRs is reduced in an effect reliant on NK and blast cell contact, which allows leukemic blasts to avoid NK cell recognition [94].

A series of hypotheses have been identified for the explanation of the escape of myeloid malignancies from NK cell recognition. For instance, NKG2A expression is commonly upregulated by increased levels of IFN-γ and IL-10 in AML blast,

which thus results in the upregulation of HLA-E (NKG2A ligand) on tumor cells [94, 101]. Besides, AML Blast cells release diverse soluble agonists for the upregulation of the aryl hydrocarbon receptor (AHR) in NK cells, which facilitate microRNA29b expression and leads to incomplete maturation and poor cytotoxicity [101]. Upregulation of the immunosuppressive cell surface glycoprotein CD200 expression on AML blasts or LSCs can help tumor cells escape from NK cell-mediated lysis by interaction with CD200R on NK cell surface [102]. A population of hypo-functional NK cells with KIR expression frequently arise in AML patients, which partially explains the absence of respective ligands in their complement of self HLA molecules [103].

Of note, AML blasts are also adequate to hinder NK cell activity by recruiting diverse immunosuppressive cells. For example, soluble NKG2DL promotes the expansion of myeloid-derived suppressor cells (MDSCs) and skews macrophages to the more immune suppressive alternative phenotype via activation of STAT3 [104]. Tumor-induced MDSCs contribute to the inhibition of NK cells by producing a range of substances, including TGF-β, IL-10, reactive oxygen

species (ROS), and arginase [105]. Instead, regulatory T cell (Treg) expansion is supported by blast cell production of indoleamine 2,3-dioxygenase (IDO) [106]. These inhibitory cell subsets inhibit NK cell activity via a variety of mechanisms, including membrane-bound TGF β release and restriction of IL-2 bioavailability [107]. Meanwhile, hypoxia is associated with various inhibitory NK cellular effects, including the decreased activation receptor expression and impaired IFN- γ production [108].

Standardization and productization

Cell-based therapy, including stem cell therapy and cellular immunotherapy, is “live drug”-based medical therapeutics, which largely depends on cellular vitality and clinical therapeutic scheme. For instance, we and Zhang et al verified the variations in the outcomes of mice with acute graft-versus-host disease (aGvHD) and acute liver failure (ALF) after MSC transplantation due to the heterogeneity caused by donor sources and continuous passages, respectively [109, 110]. Similarly, as to CAR-NK cell preparation and the subsequent cellular immunotherapy, establishment of the guidelines and standardizations is the prerequisite for further clinical application and large-scale industrialization of new drug application (NDA).

To fulfill the objective of large-scale and clinical-grade CAR-NK cells for adoptive immunotherapy as well as novel therapeutic products of AML, there're several core issues should be adequately concerned. Firstly, a public cell bank of “seeds” is the cornerstone for the follow-up standardized NK cell preparation for oncological surveillance. Of the aforementioned cell sources, perinatal blood and stem cells display higher feasibility over those from the adult tissues (e.g., bone marrow, peripheral blood) as seed cells for heterogeneous and standard CAR-NK cell generation [16, 17]. As to seed cells from perinatal blood, the general requirements should encompass the aspects of quality control (QA) and quality assurance (QC), including informed consent and immune characteristics from healthy donors, blood collection under sterile conditions, dynamic monitoring during cold chain transport, sterility and virus testing (e.g., hepatitis B virus, cytomegalovirus, syphilis), living cell number in total mononuclear cells (TNCs) or resident NK (rNK) cells or hematopoietic stem cells (HSCs), cellu-

lar vitality (e.g., proliferation, apoptosis, senescence), labeling requirements, and cryopreservation. As to those from stem cells, a series of consensus and general requirements for stem cells have been released [16, 17, 111, 112]. For example, the Chinese Society for Stem Cell Research (CSSCR) published the first general guidelines for stem cell research and production in China entitled “General requirements for stem cells”, which detailed described the classification, ethical requirements, quality requirements, QC requirements, detection control requirements, and waste disposal requirements of stem cells [111]. Meanwhile, we and other investigators have also put forward various guidelines of diverse stem cell-based application and investigational new drug (IND) such as human pluripotent stem cells (hPSCs), HSCs and MSCs, which help assure the feasibility and consistency of the safety and quality of the stem cell seeds for CAR-NK cell induction [112-115].

Secondly, all intermediate manufacturing stages for large-scale *ex vivo* CAR-NK cell preparation should under GMP conditions and follows standard specifications, including seed cell thawing, GMP-grade cytokines (e.g., rhIL-2, rhIL-15), serum-free medium (e.g., X-VIVO medium, AIMV medium, SCGM medium), CAR-delivery reagents, CAR-NK cell enrichment, and the relevant pharmaceutical excipients [17, 116]. For example, Spanholtz and the colleagues verified the generation of GMP-grade NK cells with high purity and potency, and thus met the basic benchmarks for allogeneic CAR-NK cell-based cell products [117, 118]. Simultaneously, standard operating procedures (SOPs) and the molecular mechanisms for explaining CAR-NK cell function are also urgently needed for large-scale cell product development, such as the purity, specific subpopulations for specific indications, cellular vitality, cytokine release, manufacturing control, oncolytic activity towards AML cells, and transcriptomic characteristics. For example, we and other investigators indicated a variety of non-gene-edited NK cell subsets or gene-edited CAR-NK cells for AML administration, including the NKG2C⁺ memory-like NK cells and the CD94/NKG2C⁺Fc ϵ R1 γ long-lived subset [16, 17, 119, 120].

Thirdly, for further IND development, CAR-NK cells should fulfill the principles, including the guidance, quality management, regulations,

processes, pre-IND meeting, and IND application for obtaining the permission for launching clinical trials [21, 113]. Therefore, all procedures in the intermediate stages during industrialization and IND application must follow the successive guidelines for drug development, such as the basic research stage, pharmacy stage, pharmacology stage, toxicology stage, IND application stage, and clinical trials stage [16, 113, 121]. For instance, we and the collaborator took advantage of diverse biological tests and single-cell RNA sequencing technology for the decoding of the multifaceted biological and transcriptomic profiling of adoptive NK cells and CAR-NK cells, which would collectively facilitate the further development of the standardization and productization for the safety and efficacy assessment of CAR-NK cell-based regimens and cell products.

Fourthly, in the context of the intractable clonal heterogeneity and tumor microenvironment (TME) complexity, the next-generation of comprehensive regimens for ultimately once and for all conquering the inflexible AML should orchestrate the traditional remedies and the novel cancer immunotherapies, including surgery, chemoradiotherapy, immune checkpoint inhibitors, transplantation of non-gene-edited immune cells or gene-edited CAR-T cell and CAR-NK cells [16, 122-124]. Meanwhile, the potential complications during CAR-T cell-based immunotherapy should also be systematically tested and eluded during CAR-NK cell-based treatment algorithms for AML, such as aGvHD, CRS, immune-related adverse events (irAEs), and immune effector cell-associated neurotoxicity syndrome (ICANS) [40]. Of note, Bachanova *et al* found that the efficacy of the infused haploidentical NK cells against AML was weakened by recipient Treg (regulatory T cells), which highlighted the feasibility of complete clearance rate of refractory AML by depleting the Treg with IL-2-diphtheria fusion protein (IL2DT) in host [17, 125].

Prospective and challenges

For decades, immunotherapy has highlighted the feasibility of efficient administration of diverse hematologic malignancies, and in particular, the great success of CAR-T cell-based regimens have strikingly upgraded the level of cancer immunotherapy [84]. However, the

intractable untoward effects of CAR-T cells (e.g., GvHD, CRS, neurotoxicity) continually frustrate cancer patients, which commonly result in high recurrence and mortality rates. Therewith, more and more researchers turn to genetically modified NK cells as alternative and “off-the-shelf” CAR immune effector cells, which are adequate to across HLA barriers without causing the aforementioned untoward effects [84]. In details, CAR-NK cells are adequate to recognize and eliminate AML cells with high specificity and cytotoxicity on the basis of the surface antigens exclusively expressed on cancer cells. CAR-NK cells are less toxic than CAR-T cells and minimally cause CRS or ICANS, which are the most common and serious side effects associated with CAR-T cell therapy [126]. CAR-NK cells reveal a lower risk of off-target effects as CAR-T cell therapy because CAR-NK cells exempt from major histocompatibility complex (MHC) matching [2]. AML cells can suppress the immune system, making them resistant to conventional therapies [16]. CAR-NK cells have efficiently ameliorated this immunosuppressive effect and thus enhance the killing effect upon AML cells. Differ from CAR-T cells, CAR-NK cells have gotten rid of the matching restrictions, which thus eliminate the need for individualized preparation for AML patients [90, 126]. Overall, CAR-NK cells have possessed significant advantages (e.g., high specificity, cytotoxicity, and resistance to immunosuppression), which collectively make them as a promising option for AML treatment, in particular, in patients who endure the toxicity of CAR-T cell therapy (Table 3).

Differ from the advantages of genetically modified T cells (e.g., TCR-T cells, CAR-T cells), the high-efficiency and cost-effective procedures as well as the standardization for suitable CAR-NK cell products preparation is still challenging [84]. Of note, Soldierer *et al* recently reported the optimal lentiviral delivery into primary human NK cells by comparing viral entry enhancers and lentiviral pseudotypes, and modifying the internal promoters for lentiviral CAR construct [84]. With the aid of CD34 microbead-assisted enrichment, CAR-NK cell products with over 95% purity could be prepared for potential clinical usage or preclinical testing the cytotoxicity of CAR-NK cells upon primary AML blasts and AML cell lines [84]. Nevertheless, the more efficient alternative

CAR-NKs for AML immunotherapy

Table 3. Comparison of CAR-T cells and CAR-NK cells in cancer immunotherapy

Parameter	CAR-T cells	CAR-NK cells
Cell sources	Mostly autologous T cells for avoiding GVHD	PBMCs, UC-MNCs, p-MNCs, NK cell lines, iPSCs, hESCs, HSCs
Transduction efficiency	Higher	Lower
<i>In vitro</i> expansion	Better	Worse
<i>In vivo</i> persistence	Better	Worse, limited <i>in-vivo</i> survival without cytokines [127]
Safety	GVHD, CRS and neurotoxicity [127]	No
Killing capacity	Kill tumor cells carrying TAA in a MHC-independent manner, slow killing response	Kill tumor cells regardless of their MHC status, rapid killing response

technologies and platforms for overcoming the shortcomings in delivering CAR construct including the liposome nanoparticles (LNP) and CRISPR/Cas9-based genetic engineering approaches are urgently needed to eradicate r/AML [126].

State-of-the-art literatures have indicated the promising effect of CAR-NK cells in pioneering clinical trials. Interestingly, Valeri *et al* found that the impact of downregulation or loss of CAR upon CAR-NK cell-based cytotherapy was minimal, which was distinguish from the CAR-T cell therapy for oncotherapy [126]. Of note, considering the lessons from CAR-T cell treatment, the potential emergence of CAR-NK cell-related therapeutic resistance should also be envisioned [126]. Additionally, the influence of tumor microenvironment (TME) upon CAR-NK cell-based cancer immunotherapy and the resultant “tumor escape” during AML should also be paid attention to [16]. Meanwhile, there are still several challenges need to be overcome for CAR-NK therapy to become a standard treatment for AML [126]. One of the main challenges is identifying the optimal target antigen for CAR-NK cells in AML [4, 81]. Unlike solid tumors, AML cells express a wide range of antigens, and not all of them are suitable for targeting with CAR-NK cells [32]. Furthermore, the heterogeneity of AML cells can also pose a challenge, as not all AML cells express the same antigens [2].

Cellular viability is the prerequisite for CAR-NK cell-based cancer immunotherapy and stem cell-based regenerative medicine [16, 110]. Despite the exploratory research of diverse sources for CAR-NK cell preparation, yet the inherent defect in *in vivo* persistence largely hinders the large-scale application of CAR-NK cells in clinical practice [17]. For the purpose,

Du *et al* recently took advantage of the piggy-back system for the co-expression CAR-NKG2D and IL-15, which could further augment the anti-AML activity and *in vivo* persistence of PB-NK cells [17]. Simultaneously, we recently reported the application of cytokine cocktail-based cell programming strategy for high-efficiency generation of NK cells from peripheral blood and perinatal blood (umbilical cord blood, placental blood) within 14 days [56, 59, 60]. Of note, human pluripotent stem cells (e.g., hiPSCs, hESCs) with self-renewal and multi-lineage differentiation capacity have been recognized as advantageous sources for hematopoietic stem cell (HSC) preparation and the subsequent CAR-NK cell generation [43, 68].

Overall, adoptive CAR-NK cell-based cancer immunotherapy has become a revolutionary new pillar and extensively expanded the therapeutic landscape in metastatic solid tumors and hematological malignancies including refractory and recurrent AML (r/AML). To fulfill the clinical demands and new drug application, it's of great importance to systematic and detailed dissection of the biofunction and underlying molecular mechanisms from the aspects of cell sources, target selection, CAR-modification and delivery, therapeutic application in clinical trials, together with the standardization and productization of CAR-NK products. In the near future, the comprehensive treatment options for efficiently conquering AML are hopeful by combining traditional remedies with the novel CAR-NK cell-based immunotherapy.

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Disclosure of conflict of interest

None.

Abbreviations

AML, Acute myeloid leukemia; CAR-T, chimeric antigen receptor-transduced T; hPSCs, human pluripotent stem cells; hiPSCs, human induced pluripotent stem cells; hESCs, embryonic stem cells; MSCs, mesenchymal stem/stromal cells; allo-HSCT, allogeneic hematopoietic stem cell transplantation; CAR-NK, CAR-transduced NK; GvHD, graft-versus-host disease; CRS, cytokine release syndrome; AML-MRC, AML with myelodysplasia-related changes; BM-MSCs, bone

marrow-derived mesenchymal stem/stromal cells; scFv, single-chain variable fragment; VSTs, virus-specific T cells; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; irAEs, immune-related adverse events; ADCC, antibody-dependent cell mediated cytotoxicity; GVL, graft-versus-leukemia; MACS, magnetic activated cell sorting; CIML-NKs, cytokine-induced memory-like NK cells; MNCs, mononuclear cells; PSCs, pluripotent stem cells; TAAs, tumor-associated antigens; NPM1, nucleophosmin-1; MDSCs, myeloid-derived suppressor cells; IDO, indoleamine 2,3-dioxygenase; ALF, acute liver failure; SOP, standard operating procedures; TME, tumor microenvironment; ICANS, immune effector cell-associated neurotoxicity syndrome; MHC, major histocompatibility complex.

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