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NK Cell-based Immunotherapy for Acute Myeloid Leukemia: An Exciting Future

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Abstract:

Given that allogeneic hematopoietic transplantation has been achieved in the treatment of acute myeloid leukemia (AML) over the past decade by providing graft-versus leukemia effect. However, age at diagnosis, donor availability, the treatment-related toxicities of allogeneic hematopoietic cell transplantation, relapse/refractory disease and minimal residual disease (MRD) prior to transplantation remain major problems. Novel approaches in cellular immunotherapy have contributed to substantial improvement in the treatment of hematologic malignancies, while adoptive NK cells therapy has also emerged as a promising treatment option, to improve survival in AML patients in the context of transplantation and non-transplantation. In this review, we summarize biology of NK cells and current different strategies of adoptive NK therapy for treating AML in clinical studies, also discuss about future directions of NK cell-based immunotherapy for the treatment of AML.

Keywords: NK-cell, Immunotherapy, Acute myeloid leukemia

Introduction

Over the past decade, breakthroughs in genetic and molecular research, which provide new insights into how acute myeloid leukemia (AML) develops and is regulated by complex molecular networks, have resulted in more effective treatments. These include chemotherapy, targeted therapy, and allogeneic hematopoietic cell transplantation, which provide both chemo-ablative and immuno-ablative effects. While these therapeutic approaches have the potential to cure AML, the 5-year survival rate using these treatments is still around 50% for patients aged up to 60 years, and 5%-15% for patients aged > 60 years. Although allogeneic hematopoietic cell transplantation is a standard treatment for patients with relapse, previous studies have indicated that relapse rates and non-relapse mortality remain high, with overall survival (OS) rates of 20% to 30%.[1-7] Minimal residual disease status (MRD) before allogeneic hematopoietic cell transplantation has a convincing impact on predicting relapse and survival after transplantation in all patient risk groups, including patients in complete remission (CR1) and CR2.[7] Apart from being a prognostic factor beforetransplantation, MRD status has been shown to provide additional prognostic clinical outcomes in AML after induction and consolidation treatments, and can be an efficient tool to

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establish risk-adapted treatment, such as allogeneic hematopoietic transplantation in patients with intermediate risk.[7-11]

Several novel approaches in cellular immunotherapy have revolutionized the treatment of hematologic malignancies, while adoptive ex-vivo expanded NK cell-therapy has also emerged as a promising treatment option, to improve survival rates among patients with AML who are not eligible for allogeneic hematopoietic cell transplantation, and to improve survival rates among patients who are MRD-positive before transplantation.[12-30] Due to our improved understanding of NK-cell biology and cell manipulation techniques, the application of NK-cell immunotherapy for treating malignancies, of both of hematologic and solid cancers, has progressed rapidly in the past few years; for example, ex-vivo adoptive transfer of NK cells with or without in-vivo cytokines, combinations with antibodies or drugs that enhance NK-cell cytotoxicity, or drugs that sensitize tumor cells to NK-cell lysis and chimeric antigen receptor NK cells.[17,20,26,31-41]

This review summarizes the biology, sources, and functions of NK cells in the AML tumor microenvironment. The historical clinical study of NK cell-based immunotherapy, and recent novel strategies to improve the efficacy of NK cell-based immunotherapy to treat acute myeloid leukemia in transplantation and non-transplantation settings, are also discussed

NK-cell biology and functions

NK cells are a subtype of innate lymphoid cells recognized by CD56+ve and CD3-ve. They are morphologically characterized as large granular lymphocytes, the granules of which contain both perforin and granzyme B, responsible for NK cell-mediated killing.[39,42,43] Human NK cells derive from common lymphoid progenitor cells in the bone marrow, as B cells and T cells, and develop in the secondary lymphoid organs as well as the liver and spleen. NK cells do not express antigen-specific antigen receptors; however, with the integration of signals delivered by activating and inhibitory receptors show as Figure 1 that bind with ligands on target cells, NK cells can kill malignantly transformed cells and virus-infected cells without prior antigen recognition directly. They do this by: 1) using perforin and granzyme B; 2) inducing apoptosis by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) pathway; and 3) indirectly by antibody-mediated cellular cytotoxicity (ADCC), where antibodies bind target cells to low affinity IgG Fc receptor III on NK cells (CD16), known as the most potent activating receptor.[20,35,37,39] In addition to their cytotoxic capacity, NK cells can enhance the function and increase the number of other immune cells in the tumor microenvironment, including dendritic cells, T-helper cells, by secreting multiple cytokines and growth factors, such as interferon-γ, IL-13, TNF, XCL1, CCL4, and CCL5.[42,44-47]

Since the inhibitory signals from diverse inhibitory receptors provide immunological self-tolerance that has negative-feedback against the stimulating signal from activating receptors and prevent the killing of normal self-cells, the most important inhibitory receptors are killer-cell immunoglobulin-like receptors (KIRs); their ligands are generally major histocompatibility (MHC) class I. During development, an essential process for NK-cell tolerance, termed licensing, is the engagement between inhibitory KIR or NKG2A expressed on the surface of NK cells and several MHC class I (HLA-A, HLA-B, HLA-C, HLA-E) expressed on the surface of healthy cells, resulting in the maturation and function competency of NK cells[18,39,48-50,51,52-54] as show in Figure 2.

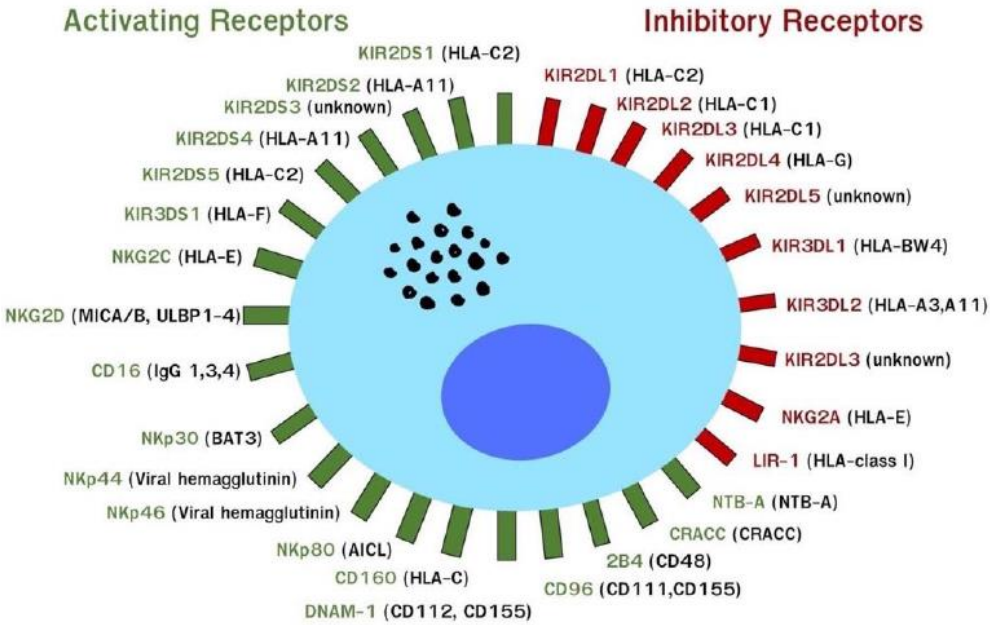

Figure 1 Summary of NK cell receptors and their ligands[14,16,20,42,49]

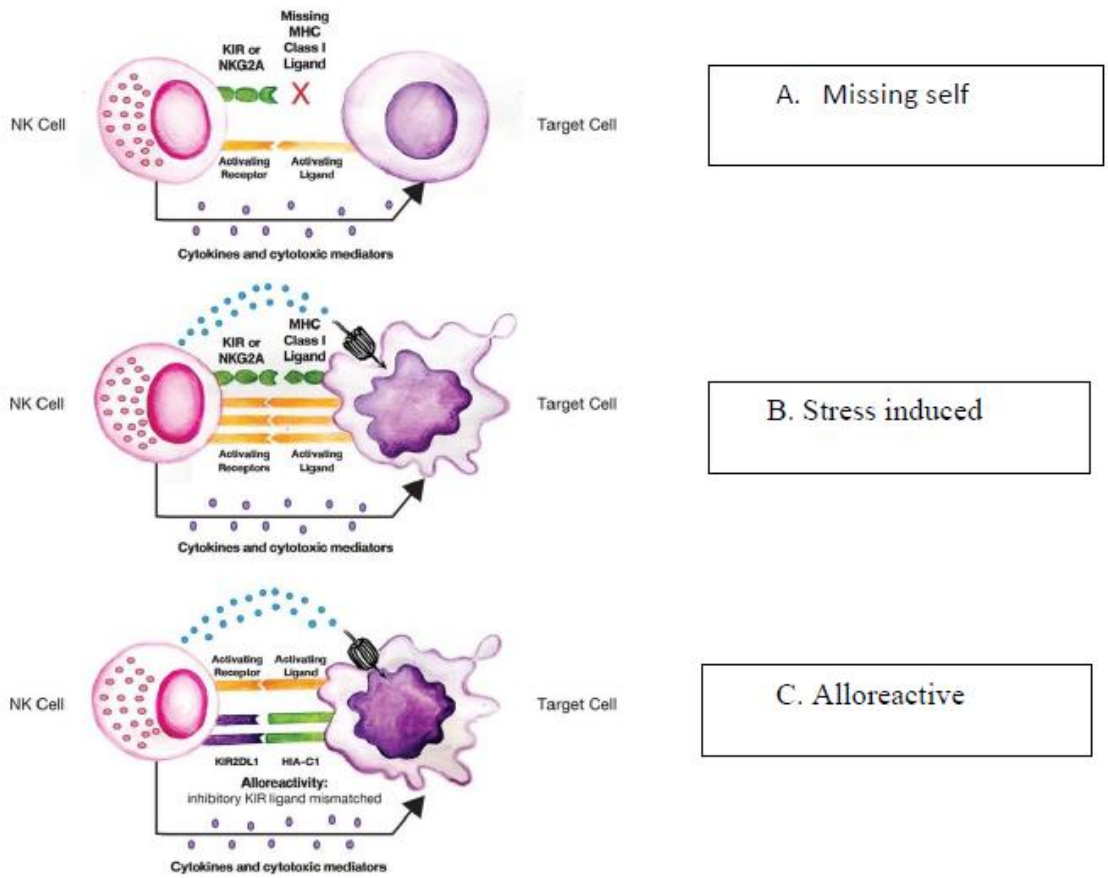

Figure 2 NK cells respond to transformed cell via (A) Transformed cells enhance target cells killing by decreased expression of MHC class I resulting in NK cells activation (B) Transformed cells upregulate ligand for NK cells activating receptor follow by enhancing the activating signal which promote cytokines release and cytotoxicity against the target cell (C) In the transplantation, NK cells alloreactivity can be promoted by selection of donor who will have inhibitory KIR ligand mismatched.[18,20,49,56,92]

Unlike T cells, NK cells in the allogeneic infusion do not result in graft-versus-host disease (GVHD). Importantly, several preclinical studies have noted that they may have the potential to protect GVHD by attacking host-antigen presenting cells.[51-54] Preclinical and clinical studies showed that NK cells play a key role in eliminating residual leukemia cells after haploidentical stem cell transplantation by KIR ligand mismatch, thus leading to further studies investigating the competence of adoptive immunotherapy with NK cells to eradicate leukemia.[48,55] While such a strategy can help improve survival and in-vivo proliferation of allogenic NK cells by using lymphodepleting treatment, many patients still experience serious side effects, such as pancytopenia and serious infection.[21,24] Moreover, the injection of IL-2 potentially causes a systemic side-effect from cytokine syndrome and the stimulation of regulatory T cells. [20,24,30]

Reduced NK-cell function in the AML tumor microenvironment

While preclinical studies showed that ex-vivo expanded NK cells can kill various types of cancer cells, only a small number of patients in clinical trials showed a response to adoptive NK-cell therapy because cancer cells can develop various mechanisms to escape NK-cell immune surveillance.[20,43,56]

In patients with AML, the function of NK cells in controlling AML-cell growth is impaired due to multiple immunosuppressive mechanisms in the AML tumor micro environment. Three major components are involved in functional defect of NK cells in the AML tumor microenvironment, resulting in the immune escape of AML cells.

First, aberrant epigenetic mutations of AML cells, resulting in the low expression or shedding of the natural killer group 2D (NKG2D) ligands that bind to NKG2D and natural cytotoxicity receptors (NCRs), which provide potent activating signals for NK cells. Moreover, the increased expression of inhibitory molecules, such as PD-L1and CD200, is also observed in AML cells. Coles et al. reported that increasing the expression of CD200 on AML cells may reduce NK-cell cytotoxic activity and promote AML-cell escape from NK-cell killing.[57-60]

Second, because the cytotoxicity function of NK cells is determined by the integration signals from activating and inhibitory receptors, the defective maturation of those receptors in AML patients results in NK-cell dysfunction. The increased expression of inhibitory receptors, such as KIR2DL2/DL3 and natural killer group2A (NKG2A), and the decreased expression of activating receptors, such as NKG2D, DNAX accessory molecule-1 (DNAM-1), natural cytotoxicity triggering 30 (NKp30), NKp46, natural cytotoxicity receptors (NCRs) can be found during AML development, leading to NK-cell dysfunction.61-64 Interestingly, the functional defect of NK cells persists, even in patients who achieve complete remission after chemotherapy. Moreover, upregulation of programmed cell death ligand-1 (PD-L1) and PD-L2 also observed in AML cells.[61-65]

In the AML tumor microenvironment, the complex interaction between the extracellular matrix, soluble factors, such as transforming growth factor (TGF)-B, IL-6, IL-10, prostaglandin E2, indolamine 2, 3-dioxygenase (IDO), and immunosuppressive cells, such as myeloid derived suppressor cells (MDSCs), regulatory T cells (Treg), and tumor-associated macrophages (TAMs) can suppress the function of NK cells, as well.[38,66-69]

Role of NK cells in allogeneic hematopoietic cell transplantation

NK cells are among the first immune cells to recover after the graft of stem cells into bone marrow, and play an important

role in preventing relapse by graft-versus-leukemia effect, to protect graft-versus-host disease by attacking recipient antigen-presenting cells and some infections, even where their functions are still impaired compared with the NK cells of healthy donors.[51,52,54,70-74] The beneficial effect of NK-cell alloreactivity in the context of allogeneic hematopoietic cell transplantation was first described in 2002. Ruggeri et al. found alloreactive NK-cell cytotoxicity against AML cells among AML patients who received donor inhibitory KIRs and host HLA ligand mismatches in bone-marrow graft haploidentical transplantation. This study demonstrated superior disease-free survival and a better 5-year survival rate (65%) for the inhibitory KIRs ligand mismatch group, compared with only 5% for the control group.[19] Since the family of 14 polymorphic KIR genes can be classified into 2 haplotypes, KIR A haplotypes comprises only one encoding activating KIR receptor gene (KIR2DS4) while KIR B haplotype comprises of 5 encoding activating KIR receptor genes (KIR2DS1, 2DS2, 2DS3, 2DS5, 3DS1) the effectiveness of alloreactive NK cells was also demonstrated in unrelated donor transplantation from donors with the KIR B haplotypes, which contain more activating KIRs gene.[55] Rapid NK-cell recovery after transplantation is also associated with better transplantation outcome.[18] Taken together, many investigators have explored the possibility of introducing the adoptive transfer of NK cells to treat acute myeloid leukemia patients in the clinical setting.

Role of adoptive NK-cell therapy in hematopoietic cell transplantation

Infusions of donor purified NK cells after haploidentical are feasible and well-tolerated. This treatment facilitates engraftment and provides graft-versus-leukemia alloreactivity without acute graft-versus-host disease.[74] Ciurea et al. conducted a phase-1 dose escalation study (1 x 10^5/kg to 1 x 10^8/kg) to determine the safety, feasibility, and maximum tolerated dose of membrane-bound interleukin 21 (mbIL21) expanded donor NK cells infused before and after haploidentical HSCT for high-risk myeloid malignancies. Thirteen patients aged 18-60 years were enrolled into the study. Ex-vivo expanded NK cells were infused on days -2, +7, and +28. No infusion-related reactions or dose-limiting toxicities were observed. All of the patients were engrafted with donor cells. Seven patients (54%) developed grade 1-2 acute GVHD, but no grade 3-4 acute GVHD was observed. Eleven of the 13 patients (85%) were still alive and in remission at last follow-up (median, 14.7 months). This trial demonstrated the efficacy and feasibility of infusing high doses of ex-vivo expanded NK cells by using feeder cells after haploidentical HSCT without serious adverse effects or increased grade 3-4 acute GVHD.[27] Another phase-I study aimed to investigate the effect of haploidentical donor NK-cell infusion early at day 6 and day 9 versus day 13 and day 20 after haploidentical transplantation in refractory acute myeloid leukemia. While the early infusion of adoptive NK cells was not associated with a reduction in the progression of leukemia, it was associated with increased toxicity from cytokine-release syndrome. In addition, a higher expression of NKp30 (> 90%) on donor NK cells was associated with higher complete remission.[75]

Interestingly, the adoptive transfer of haploidentical NK cells can be infused safely before hematopoietic-cell infusion as part of the conditioning regimen for transplantation. A phase I clinical study conducted by Lee et al. demonstrated that the infusion of IL-2 activated haploidentical donor NK cells at day 8 after busulfan, fludarabine and ATG, as part of the conditioning regimen for treating high-risk myeloid malignancies, was safe. Relapse-free survival was

associated with the number of infused NK cells.[76]

The adoptive transfer of haploidentical NK cells can also be used to clear residual leukemic cells in relapse/refractory AML patients who are eligible for transplantation. Bjorklund et al. recently published a phase I/II study aiming to evaluate the safety and efficacy of overnight IL-2 activated haploidentical NK-cell infusion in 16 patients with relapse refractory high-risk AML, MDS. The patients received a less-toxic lymphodepleting regimen with fludarabine at a dose of 25 mg/m^2 per day at days -7 to -4, cyclophosphamide at a dose of 25 mg/kg per day at days -3 and -2, and TLI (total lymphoid irradiation) at day -1 before infusion of haploidentical NK cells at a median infused dose of 6.7×10^6 cells/kg (range, 1.3–17.6×10^6 cells/kg). The NK-cell infusions were well-tolerated, and common adverse events were chills and nausea. Six of 16 patients achieved complete remission, and 5 proceeded to allogeneic hematopoietic cell transplantation. Five of 5 patients who achieved complete remission had detectable donor NK cells at day 7 and day 14 post-infusion.[26] In another clinical study, Vela et al. published a post-hoc analysis assessing the safety and efficacy of infusing expanded NK cells from haploidentical donors by using K562-mb15-41BBL as feeder cells in patients from two clinical trials. A total of 18 patients with relapsed or refractory ALL, AML, or bi-phenotypic acute leukemia were enrolled. Their mean age was 12 years (range 12.3 years). All patients received salvage chemotherapy prior to infusion of NK cells at a maximum dose of 1×10^8 /kg in a total of 2 cycles and injection of low-dose IL-2. All infusions were well-tolerated with no graft-versus-host disease or serious infusion-associated adverse events. Thirteen patients achieved complete remission, and 10 patients proceeded to allogeneic hematopoietic cell transplantation. Four patients were alive and leukemia-free > 750 days post-transplantation. These studies suggest that relapse/refractory AML and MDS cells were susceptible to adoptive NK-cell therapy and that the infusion of haploidentical NK cells may convert patients to being candidates for transplantation, so providing the opportunity for cure.[29] In addition, these findings may apply to developing a strategy to clear MRD before proceeding to transplantation, due to the negative impact of being MRD-positive on transplantation outcome.[11,77]

Clinical trials using adoptive NK-cell infusions

In light of the limitations: age at diagnosis, donor availability, and the treatment-related toxicities of allogeneic hematopoietic cell transplantation, the use of autologous NK-cell infusions was the first focus of adoptive NK-cell therapy to treat AML patients who are not candidates for allogeneic hematopoietic cell transplantation, due to the convenience of using the patient's peripheral blood and there being no requirement for lymphodepleting chemotherapy.[10] Wang et al. conducted a study to evaluate whether cytokine-induced killer (CIK) cells from patients with relapse or refractory AML can be expanded efficiently for clinical use, and to evaluate the efficacy of CIK in eradicating leukemic cells. Eleven patients with relapse/refractory AML were enrolled. The CD3+CD56+ cells in AML-derived CIK cells were expanded approximately 1,020-fold. The proportions of CD3+and CD3+CD56+ CIK cells from patients with AML were similar to those from healthy donors, and showed similarly high cytotoxicity against leukemic cell lines. Two patients had dramatically decreased blast cells in the peripheral blood by 2 weeks' post-infusion, then gradually increasing. No infusion-related adverse events were observed in all patients. This study demonstrated that the autologous CIK from the patients can be

expanded efficiently and comparable to the phenotypes of healthy donors.[28] However, subsequent clinical trials using this strategy in hematologic malignancy and solid cancers failed to achieve responses, even though infused autologous NK cells can expand in vivo.[78] The inhibitory effect of MHC class I expressed on cancer cells and the immunosuppressive status of patients due to their being heavily pretreated prior to cell collection resulted in poor NK-cell expansion and dysfunction. Thus, the investigators turned to developing clinical trials using allogeneic NK-cell therapy to treat hematologic malignancies.[56,57,79]

Given that KIR-HLA ligand mismatches had improved 5-year survival rates in haploidentical hematopoietic cell transplantation, NK cells form a haploidentical donor that may potentially have graft-versus-leukemia effect in non-hematopoietic cell transplantation, as well. Miller et al. tested haploidentical, related-donor NK-cell infusions in a non-transplantation setting to determine safety and in-vivo NK-cell expansion. Nineteen patients with poor AML prognoses were enrolled. All patients received lympho-depleting treatment with fludarabine and cyclophosphamide, and infusions of adoptive NK cells from haploidentical donors. All patients received subcutaneous IL-2 injections after the infusion of NK cells. Five patients achieved a hematological complete remission. Importantly, four patients were KIR ligand mismatched in the graft-versus-host direction and 3 of 4 (75%) achieved complete remission. This study demonstrated that the infusion of short-term IL-2-activated allogeneic haploidentical NK cells in patients with refractory leukemia can induce remission, while lympho-depleting treatment can cause serious adverse events, such as infections, but play an important role in the in-vivo expansion of infused adoptive NK cells.[24] The finding from this study highlighted lympho-depleting chemotherapy is necessary for adoptive allogeneic cellular therapy, including allogeneic NK cell-, CAR-T cell- and CAR-NK-cell therapy. Alloreactive NK-cell activity and clinical benefits have also been also observed in childhood AML. Rubnitz et al. conducted a pilot study to determine the safety, feasibility, and engraftment of haploidentical NK-cell infusions in 10 children with acute myeloid leukemia in first complete remission. The patients received fludarabine and cyclophosphamide as lymphodeplete treatment, followed by an infusion of KIR ligand mismatched NK cells and 6 doses of IL-2. All patients had engraftment for a median of 10 days. No nonhematologic toxicity and no graft-versus-host disease were observed. The 2-year event-free survival estimate was 100%, and median follow-up was 964 days.[22] The findings from this study indicated that the infusion of haploidentical NK cells is a promising consolidation therapy for pediatric patients with AML who are not eligible for transplantation. NK cell mediated cytotoxicity was also studied in elderly AML. Kottaridis et al. reported a phase-I clinical study illustrating the feasibility and toxicity of haploidentical NK-cell infusion in 7 patients with high-risk, elderly AML patients not eligible for allogeneic stem-cell transplantation. The median age for diagnosis was 65 years. The patients included in this clinical study received lympho-depleting treatment with fludarabine at a dose of 25mg/m^2 for 3 days and TBI as a single dose of 2 prior to infusion of NK cells, at a dose of 1 x 10^6/kg. At six months post-treatment, 3 patients treated in CR remained in remission (37.5%), while one patient who was infused at partial remission (PR)1 had achieved CR1 50 days post-infusion. All patients developed prolonged pancytopenia after lympho-depleting treatment. The infusion of NK cells was well-tolerated,

and no patient had infusion-related toxicity. The median time to relapse was 253.5 days post NK infusion (range, 58 to 845 days) and the median overall survival was 468.5 days (range, 148 to 1180 days). This study showed that the infusion of allogeneic NK cells could induce prolonged remission among high-risk elderly AML patients, but immunosuppressive treatment with fludarabine and TBI could include serious hematologic toxicity and serious infection, especially pneumonia and fungal infections due to prolonged neutropenia.[21]

While subcutaneous injection of IL-2 to stimulate in-vivo NK-cell proliferation and activation is associated with better outcomes in adoptive NK-cell therapy, the injection of IL-2 also stimulates regulatory T cells. Regulatory T cells (Treg), CD4+ CD25+ Foxp3+, expand rapidly after IL-2 is injected, and inhibit NK-cell proliferation and cytotoxicity. IL-2 diphtheria toxin (IL-2DT) is a recombinant cytotoxic fusion protein composed of the amino acid sequences for diphtheria toxin, followed by truncated amino-acid sequences for IL-2. IL2DT selectively deplete IL-2 receptor (CD25)-expressing cells, including regulatory T cells.[67] Bachanova et al[30] conducted a clinical study to determine the effect of regulatory T cells by using IL-2 diphtheria toxin (IL-2DT) for host Treg depletion. Fifty-seven refractory AML patients were enrolled. Patients were divided into 2 arms. All patients received cyclophosphamide and fludarabine, followed by NK-cell infusion, while one arm received IL-2DT injection. In the 15 patients who received IL-2DT, donor NK-cell expansion was detected in 27%, while in 42 patients who did not receive IL-2DT, the rate was only 10%. Regulatory T-cell depletion by IL2DT was associated with improved complete remission rates at day 28 (53% vs 21%; P = 0.02) and improved disease-free survival at 6 months (33% vs 5%; P < 0.01). Moreover, in the IL2DT cohort, NK-cell expansion correlated with peripheral blood regulatory T-cell depletion (< 5%) at day 7 (P < 0.01). This study demonstrated the efficacy of adoptive transferred NK cells to treat AML and highlighted the negative impact of host Treg.

Role of memory-like NK cells

NK cells function is involved in the innate immune response and can develop immunological memory, similar to the function of B cells and T cells in adaptive immunity. These adaptive NK cells are CD56 dim, CD57+, and CD94/NKG2C+ which is the activating HLA-E receptor that plays an important role in graft-versus-leukemia effects, and decreased expression of FeRγ, SYK, EAT-2, and PLZF. Moreover, this particular NK-cell subset has a DNA methylation pattern similar to cytotoxic T cells, and produces more IFN-γ and tumor necrosis factor-∝ that distinguish it from conventional NK cells.[80-83] Several clinical studies have reported an association between the activation and proliferation of these adaptive NK cells and lower relapse rates among HSCT patients experiencing human CMV reactivation after allogeneic hematopoietic cell transplantation. Cichocki et al. reported the outcome in 674 allogeneic HSCT recipients whose CMV reactivated with lower leukemia relapse (26%, P = 0.05) and superior disease-free survival (DFS) (55% P = 0.04) at 1 year compared with CMV-seronegative recipients who experienced higher relapse rates (35%) and lower DFS (46%).[84] The study of the reconstituting NK cells found that CMV reactivation is associated with higher frequencies and absolute numbers of CD56dimCD57+ NKG2C+ NK cells, particularly after RIC HCT. The expansion of these cells at 6 months' post-transplant was also associated with a lower risk of 2-year relapse.

Interestingly, this memory-like NK cell (CIML) can be generated by brief activation

with cytokines IL-12, IL-15, and IL-18, resulting in increased STAT5 signaling and CD 25 expression, and exhibiting enhanced cell proliferation, interferon gamma production, and cytotoxic functions for several weeks.[85] Romee et al[23] conducted a first-in-human phase I, dose-escalation clinical study to identify the maximum tolerated dose of memory-like NK cells administered to 9 patients with relapse refractory AML who were not candidates for hematopoietic cell transplantation (age 60-73 years). Patients were treated with fludarabine and cyclophosphamide, followed by allogeneic donor IL-12, IL-15, and IL-18 pre-activated NK cells in escalating doses: 0.5×10^6/kg (dose level 1), 1.0×10^6/kg (dose level 2). After the infusion of NK cells, the patients received 6 doses of low-dose rhIL-2 injections. Memory-like NK cells in the blood peaked at 7-14 days' post-infusion, and decreased in number after of 9 patients achieved CR. This study demonstrated that allogeneic human memory-like NK cells can potentially exhibit anti-leukemia functions after being transferred into relapse/refractory AML patients with active disease. In an ongoing study, NCT04354025, which is currently recruiting patients, the phase 2 clinical trial aims to investigate the effectiveness of cytokine-induced memory-like natural killer (CIML NK) cells in combination with chemotherapy as a treatment for refractory or relapsed pediatric AML. Fludarabine, cytarabine and filgrastim (FLAG) chemotherapy is used to lower leukemic burden and suppress the recipient's immune system to provide an environment for in-vivo CIML NK cell expansion. can potentially exhibit anti-leukemia functions after being transferred into relapse/refractory AML patients with active disease. In an ongoing study, NCT04354025, which is currently recruiting patients, the phase 2 clinical trial aims to investigate the effectiveness of cytokine-induced memory-like natural killer (CIML NK) cells in combination with chemotherapy as a treatment for refractory or relapsed pediatric AML. Fludarabine, cytarabine and filgrastim (FLAG) chemotherapy is used to lower leukemic burden and suppress the recipient's immune system to provide an environment for in-vivo CIML NK cell expansion. Overview of selected published clinical trials of NK cell infusion in AML are shown in Table 1.

Source and method of NK-cell isolation and activation

NK cells used for adoptive transfer in clinical trials can be collected from leukapheresis products. Peripheral blood mononuclear cells (PBMC) are enriched by centrifugation on a density gradient. T cells are removed using magnetic beads to remove CD3+ mononuclear cells with or without CD56+ selection, then highly purified NK cells can be isolated from PBMC. Some investigators also remove B cells using anti-CD19 beads.[24,43,86-88]

NK cells in peripheral blood are in a resting state. Grimm et al[89] generated lymphokine activated killer cells containing cytotoxic T cells and NK cells by incubating fresh lymphocytes with interleukin-2, resulting in the development of cytotoxic activity against autologous and solid tumor cells. Since then, activation with IL-2 has become a common method to stimulate NK cells; however, IL-2 also stimulate Treg cells, which can suppress in-vivo NK-cell expansion. For an alternative activation method, to avoid in-vivo NK-cell suppression, Miller and his group[30] demonstrated Treg depletion by introducing the use of IL-2DT instead of IL-12. Apart from IL-2, IL-15 is also used to activate NK cells. Preclinical studies in-vitro demonstrated that, compared to IL-2, IL-15 might enhance the expression of activating receptor and maintain cytotoxicity for longer. Pre-activation with IL-2, IL-15 and IL-18

Table 1 Overview of selected published clinical trials of NK cell infusion in AML

Ref	Indication	Source	Lympho-depletion	In vivo IL-2	N	Transplantation	Outcome
(74)	AML, CML	Haploidentical	HSCT conditioning	No	5	Post Haplo HSCT	CR in 4 patients, increased donor chimerism 2/5, median F/U 12 months
NCT00799799 (24)	AML	Haploidentical	Flu/Cy	Yes	19	No	CR in 5 patients,3/4 of patients with KIRL mismatch
(22)	AML	Haploidentical	Flu/Cy	Yes	10	No	2 years event-free survival of 100%
NCT00799799 (48)	AML	Haploidentical	Flu/CY	Yes	13	No	Three of 6 patients in CR, and disease-free after 34, 32, and 18 months
(23)	AML	Haploidentical	Flu/Cy	Yes	9	No	OR 55%, CR in 4 patients
(96)	AML	Partially HLA-matched cord	Flu/Cy	No	10	No	2/4 of MRD-positive patients became MRD-negative, which was sustained for 6 months
(26)	AML, MDS	Haploidentical	Flu/Cy/TLI	No	16	Preceded HSCT	CR in 5 patients, 1 in PR, 5 patients proceeded to HSCT
(27)	AML, CML	HSCT donor	HSCT conditioning	No	13	days 22, 17, and 128 post-transplant	CR in 7 of 8 AML patients. All were alive at median F/U 14.7 months
NCT01385423 and NCT02395822(108)	AML	Haploidentical	Flu/Cy	rhIL-15 IV or SQ	40	No	CR in 7 patients, CRS was observed in 56% of patients given SC rhIL-15
NCT00703820(109)	AML	Haploidentical	Flu/Cy	yes	21	No	Neither decreased relapse nor improved overall survival compared with chemotherapy alone
NCT00274846 and NCT01106950(30)	AML	Haploidentical	Flu/Cy	IL-2, IL-2DT	57	No	IL2DT associate with improved complete remission rates at day 28 (53% vs 21%; P 5 .02) and disease-free survival at 6 months (33% vs 5%; P < .01)
(75)	AML	Haploidentical	HSCT conditioning	No	91	First group day 6 and 9, second group day14 and 21 post-transplants	CR rate 57% in first month. Higher expression of NKp30 (>90%) on donor NK cells was an independent predictor of higher CR and reduced leukemia progression
NCT01520558 (25)	AML in CR1	Haploidentical	Flu/Cy CNDO-109-NK cells	No	12	No	RFS by dose level was 105 (3×10^5), 156 (1×10^6), and 337 (3×10^6) days. Exceeding 42.5 months in 2 patients

for short periods of time has also been used to generate cytokine-induced memory-like NK cells in a clinical study.[23,85] Other reagents have been used to activate NK cells include glycogen synthase kinase 3 inhibitor[90], and OKT3, which is an anti-CD3 antibody used to suppress T-cell expansion.[91]

Given that the clinical response of NK-cell therapy directly correlated with the number of infused cells, ex-vivo manipulation to increase the number of NK cells is required. Since NK cells can only be found in 10-15% of peripheral blood lymphocytes, it may be difficult to prepare enough NK cells for infusion at 1:1 effector- to targeted-cell ratio, or for multiple infusions by single apheresis.[18,20,43,86,87,92] Ex-vivo NK-cell expansion and cytotoxicity can be improved by adding feeder cells, such as autologous PBMC, Epstein-Barr virus-transformed lymphoblastoid cells (EBV-LCL), and K562-mbIL-15-4-1BBL cells, in the culture process. Subsequent culture protocols incorporating EBV-LCL as feeder cells with cytokine-containing media could augment NK cell expansion in the range 80–10,000-fold within 14–21 days.[86,93,94] Another protocol uses K562-mbIL-15-4-1BBL and stem cell growth tissue culture medium in GMP culture condition for 10 days can produce median 376-fold NK-cell expansion; as a result, enough expanded NK cells for 4 infusions can be obtained from one leukapheresis.[43,56,87]

Interestingly, NK cells can be generated from hematopoietic stem cells. CD34+ hematopoietic stem cells (HSPC) are isolated from umbilical cord blood and expanded for 14 days, then CD34+cells are exposed to stem cell factor, IL-2, IL-15, IL-17 and other growth factors for 21-28 days. NK cells can also be generated from induced pluripotent stem cells (iPSC) by promote differentiation into hematopoietic stem cell first, then differentiating CD34+ to be CD3-CD56+ cells.[95] Dolstra et al[96] reported the outcome of the first-in-human clinical study using NK cells generated from CD34 + isolated from partially HLA- matched umbilical cord to treat 10 elderly AML patients in morphological complete remission. The patients received HSPC-NK cells after lympho-depleting chemotherapy without in-vivo cytokine boosting. Neither GVHD nor infusion related toxicity was observed, and 2 of 4 MRD-positive patients became MRD-negative. Remarkably, NK cells derived from master clonal iPSC banking could provide greater benefit than primary NK cells from autologous or allogeneic donors in terms of being immediately available, with high numbers of infused cells, and donor selection for KIR B haplotype.

Conclusion and future directions

The results from preclinical studies and clinical trials of adoptive transfer NK cells in patients with AML suggest a promising treatment strategy. The treatment options for adoptive NK cells include induction remission in relapsed/refractory AML, consolidation treatment in patients who are not eligible for transplantation, eradication of MRD before proceeding to transplantation, and potential use as a strategy for early relapse treatment in patients who have mixed chimerism after transplantation. Compared with chimeric antigen receptor T-cells, adoptive NK-cell therapy provides better safety profiles, including a lower incidence of severe GVHD and cytokine-release syndrome. Moreover, adoptive NK cells are easy to prepare under good manufacturing practice standards, which may have an "off the shelf" benefit for treating patients in a short period of time.[97,98] However, a number of issues need be resolved for NK-cell immunotherapy. First, how to promote in-vivo expansion and proliferation because most patients have a short duration of response due to a short lifespan and the poor in-vivo expansion of infused NK cells.

Second, strategies to overcome various immune escape mechanisms in the AML tumor microenvironment. Current ongoing clinical trials will provide knowledge for developing and guiding future treatment strategies in NK cell-based immunotherapy.

Study design will be a key factor to determine efficacy and bring the best of NK cell-based immunotherapy to the treatment of AML. Importantly, the optimal number of infused cells must be evaluated; theoretically, the number of infused cells should reach an effector-target ratio that can control AML cells in different disease statuses, because even in patients who achieved morphological complete remission after induction chemotherapy may still have 10^9-10^{10} residual leukemic cells.[99] Since the incidence of severe GVHD or severe cytokine-release syndrome were very low compared to CAR-T cells, an effector-to-target cell ratio > 1:1 should be applied to study design. In order to improve in-vivo persistence and proliferation, genetic modification, such as HLA-knockdown or augmentation of in-vivo proliferation by transduction of membrane-bound receptor for cytokines, or hematopoietic growth factor, such as erythropoietin or thrombopoietin, should be evaluated.[13,20,31,56,93,94,100]

Given our knowledge of NK-cell biology and NK-cell dysfunction in the AML microenvironment, a combination of multiple treatment modalities may optimize outcomes for adoptive NK-cell therapy. By selecting donors with KIR ligand mismatches to improve NK-cell alloreactivity.[48,49,101,102] Using ex-vivo activation and genetic modification of NK cells to improve toxicity and in-vivo proliferation. Since NK-cell functions and activation depend on integration signals from activating and inhibitory receptors, monoclonal antibodies can be used to promote NK-cell toxicity and improve clinical outcomes. Preclinical findings targeting tumor-associated antigen, such as CD33, CD37, CLL-1, and FLT3[103–105], have shown promising outcomes, while monoclonal antibodies that bind to inhibitory receptors, such as anti-KIR antibodies and anti-PD1 antibody[106], are under investigation for the clinical treatment of AML. In addition, immunomodulatory drugs can be used to improve the outcomes of NK-cell therapy by using drugs that augment NK-cell function, such as lenalidomide, which indirectly augments NK-cell cytotoxicity and proliferation through the release of IL-2 and IFN-γ from surrounding T cells and dendritic cells[107], or using drugs that make AML cells more sensitive to NK cell-mediated lysis, such as bortezomib,[20,40,41] which could enhance the expression of HLA-E on the surfaces of cancer cells. In summary, NK cell-based immunotherapy is a new hope for treating acute myeloid leukemia, but key to the success of future clinical studies is the incorporation of modalities that enhance NK-cell cytotoxicity, promote in-vivo proliferation and survival, home in to the tumor site, and the development of novel drugs and expansion methods.

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