Mini-Review

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CAR-expressing NK cells for cancer therapy: a new hope

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SUMMARY Since the approval in 2017 and the amazing achievement of Kymriah and Yescarta, the number of basic researchers and clinical trials investigating the safety and efficacy of chimeric antigen receptor-expressing T cells (CAR-T cells) has been relentlessly increasing. Up to now, more than 200 clinical trials are listed on clinical trial database of NIH and the basic research is countless. However, the production of allogeneic CAR-T cells products is still expensive and has toxicity. Thus, more effort is needed to develop reliable off-the-shelf cellular therapeutic methods with safety and efficiency for the treatment of patients with cancer. As a kind of innate effector lymphocyte with potent antitumor activity, natural killer cells (NK cells) have attracted much attention. Until now, basic and clinical research has shown that chimeric antigen receptor-expressing NK cell (CAR-NK) therapy may play a significant anti-tumor role and its safety is higher than CAR-T cell therapy. In this review, we discuss advantages and shortages of employing CAR-NK cells as a novel cellular therapy against cancer.

Keywords CAR-NK, cancer, immunotherapy, clinical trial

1. Introduction

Natural killer (NK) cells, which were discovered over 45 years ago (*1*), are a type of cytotoxic lymphocyte critical to the innate immune system. NK cells launch rapid responses to virus-infected cells, acting at around 3 days after infection, and respond to tumor formation. Typically, immune cells detect the major histocompatibility complex (MHC) presented on infected cell surfaces, triggering cytokine release, causing the death of the infected cell by lysis or apoptosis. NK cells are unique, however, as they have the ability to recognize and kill stressed cells in the absence of antibodies and MHC, allowing for a much faster immune reaction. They were named "natural killers" because of the initial notion that they do not require activation to kill cells that are missing "self" markers of MHC class I. This role is especially important because harmful cells that are missing MHC I markers cannot be detected and destroyed by other immune cells, such as T lymphocyte cells (*2-4*). Previous research has suggested that lower activity of NK cells in peripheral blood is related to higher cancer risk, indicating that NK cells play a role in inhibiting cancer (*5,6*). NK cells in human peripheral blood are divided into two major subgroups: CD56bright and CD56dim NK cells. CD56bright NK cells are usually known as cytokine-producing cells with low cytotoxicity, while CD56dim NK cells are known for potential cytotoxicity (*7*). Since NK cells can identify and break up tumor cells, immunotherapy based on NK cells has been developed.

The chimeric antigen receptor (CAR) is a receptor protein that has been engineered to give immune cells the new ability to target a specific antigen protein. The receptors are chimeric because it is a fusion protein composed of an extracellular antigen binding domain, a transmembrane region, and intracellular activating signaling domains. The extracellular antigen binding domain, which is usually a single-chain variable fragment (scFv), can identify the specific antigen on the surface of tumor cells. Intracellular activating signaling domains, such as CD28, 4-1BB (CD137) and OX40, usually play a role of triggering the activation and killing effect of immune cells. CAR-expressing T cells can instantly identify the tumor surface antigen and then lyse the tumor cells. CAR-T cell immunotherapy has produced a great achievement in treating hematological tumors, such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and lymphoma. As we know, CD19 CAR-T therapy has shown complete remission rates as high as 90% in both children and adult patients with ALL (*8*). Although CAR-T cell immunotherapy has advanced rapidly, it still has several deficiencies in clinical application. CAR-T cell immunotherapy has shown a

low effect in the treatment of solid tumors (*9,10*). In addition, most CAR-T cell immunotherapies require autologous adoptive cell transfer because allogeneic T cells may cause graft-versus-host- disease (GVHD) unless addressing HLA barriers (*11,12*). Furthermore, CAR-T cell immunotherapy may lead to a few side effects, which may do harm to patients' lives, such as cytokine release syndrome. CAR-expressing NK cells have been reported to overcome the above deficiencies of CAR-T cells and showed a significant anti-tumor effect (*13,14*). In this review, we will discuss the opportunities provided by CAR-expressing NK cells and the challenges faced by CAR-NK cells.

2. Advantages of CAR-NK cell immunotherapy

Although the early success of CAR-T cell immunotherapy, especially in treating hematological tumors, the extensive clinical application of CAR-T cell immunotherapy may be limited by autologous adoptive cell transfer and various side effects, such as GVHD, neurologic toxicities and off-target effects. Based on these problems, NK cell therapy has been suggested to be superior to CAR-T cell therapy (*15*). Particularly, NK cells have a few advantages in CAR-expressing immunotherapy.

First, CAR-expressing NK cells immunotherapy would be safer than CAR-T cells immunotherapy in clinical application, and the safety of NK cells has been validated in a few clinical fields. For example, a few phase I/II trials revealed that allogeneic NK cell infusions are tolerated well and did not cause GVHD and significant toxicities (*16-18*). Hence, the NK cell is an adaptable CAR driver that is not limited to autologous cells. One of the major side effects in CAR-T cell immunotherapy are off-target effects owing to the persistence of CAR-T cells. For example, CD19-targeting CAR-T cells can lead to significant and long-term B lymphocyte deficiency due to the cellular memory effect of T lymphocytes and the challenge of mature or progenitor B lymphocytes (*19*). Conversely, CAR-NK cells have a short life duration, which causes few off-target effects. Otherwise, the kinds of cytokines produced by NK cells are much different from those produced by T lymphocytes. Active NK cells normally produce IFN-γ and Granulocyte-macrophage colony-stimulating factor (GMCSF), but CAR-T cells usually induce a cytokine storm by secreting pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6.

Second, besides inhibiting cancer cells *via* a CAR-related mechanism by which NK cells recognize the tumor surface antigen *via* scFv, NK cells can suppress cancer cells by identifying various ligands through a variety of receptors (*20,21*), such as natural cytotoxicity receptors (NKp46, NKp44, and NKp30), NKG2D and DNAM-1 (CD226). These NK cell receptors normally recognize stress-induced ligands expressed on tumor cells under the pressure of immune cells or long-lasting therapy. Moreover, NK cells induce antibody-dependent cytotoxicity by FcγRIII (CD16). Thus, CAR-expressing NK cells can inhibit cancer cells through both CAR-dependent and NK cell receptor-dependent pathways to eliminate either tumor antigen positive cancer cells or cancer cells expressing ligands for NK cell receptors. The clinical trials have suggested that CAR-T cells can't eliminate cancer cells which are highly heterogeneous (*22*), but CAR-expressing NK cells could be able to effectively kill residual tumor cells that may change their phenotypes after long-term treatment.

Finally, NK cells are abundant in clinical samples and can be produced from peripheral blood (PB), umbilical cord blood (UCB), human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs), and even NK-92 cell lines. NK-92 cells provide a homogeneous cell population and can be easily expanded under proper culture conditions for extensive clinical applications (*23*). But, they must be irradiated before infusion owing to their tumor cell line origin. Conversely, active PB-NK cells express a broad range of receptors and could be utilized without irradiation, which enables them to be generated *in vivo*. NK cells derived from iPSCs or hESCs combine the merits of PB-NK and NK-92 cells for they show a phenotype similarity to PB-NK cells and are a homogeneous population. More importantly, CAR can be easily expressed in hESC- and/or iPSC-derived NK cells by employing non-viral transgenic methods (*24*).

3. Current status of CAR-NK cell immunotherapy

3.1. Hematologic cancer

Preclinical research has suggested that CD19-CAR-NK cells have high efficiency against hematological cancers and are easy to manufacture, which is a tremendous advance compared to current CAR-T cell immunotherapy (*25,26*). Clinical trials of CD19-CAR-T cell immunotherapy have revealed high complete responses in patients with hematological cancers (*27,28*). CD19-CAR modified NK cells are expected to show a better anti-tumor effect owing to the merits of CAR-NK cell immunotherapy in hematological cancers. Clinical trials have suggested that CD19-CAR-expressing NK cells could be a good therapeutic method for patients suffering from lymphoid malignancies (*29*). Besides CD19, CAR-NK cell clinical studies for lymphoma and leukemia also target CD7 (NCT02742727) and CD33 (NCT02944162). Although CAR-T cell immunotherapy has undergone a large number of clinical trials for hematological cancers, only several clinical CAR-NK cell therapies against hematological malignancies are under way (Table 1).

Tabel 1. Clinical trials of CAR-NK cell immunotherapy against cancers

Cancers	Targets	Origin	Phase	*Ref.*
Leukemia and lymphoma	CD19	Umbilical cord blood	I/II	NCT03579927
Leukemia and lymphoma	CD19	Umbilical cord blood	I/II	NCT03056339
Leukemia and lymphoma	CD19	NK-92	I/II	NCT02892695
Leukemia and lymphoma	CD7	NK-92	I/II	NCT02742727
Acute lymphocytic leukemia	CD19	Haploidentical donor NK cells	II	NCT01974479
Acute lymphocytic leukemia	CD19	Expanded donor NK cells	I	NCT00995137
Acute myeloid leukemia	CD33	NK-92	I/II	NCT02944162
Solid tumors	MUC1	Unknown	I/II	NCT02839954
Solid tumors	NKG2D ligands	Autologous or allogeneic NK cells	I	NCT03415100
Glioblastoma	HER2	NK-92	I	NCT03383978
Non-small cell lung cancer	Unknown	CCCR-NK-92	I	NCT03656705

Ref. resource: clinicaltrials.gov.

3.2. Solid tumors

In previous studies, it has been suggested that the NK-92 cell line can be effectively transduced with various CARs against different cancers for experiments in preclinical research and currently in clinical trials. CAR-NK-92 cells were extremely successful in targeting tumor cells and exerting anti-tumor cytotoxicity against several resistant solid tumors, such as epithelial cancers, by targeting human epidermal growth factor receptors (HER1, HER2), neuroectodermal tumors by GD2, brain tumors by HER1 and HER2, and ovarian cancers also by HER2 (*13,30-32*). But there are several limitations for using this cell line. For transformed NK-92 cell lines from undifferentiated NK-cell precursors (*33-35*), they are short of antibody-dependent cell-mediated cytotoxicity (ADCC)-inducing CD16 receptors, which share a similar situation with other NK cell lines (*36*). As a result, these NK cells fail to recognize tumor-targeted antigens by ADCC mechanisms. To supply these gaps, NK-92 cells were genetically modified to express the high-affinity V158 variant of the Fc-gamma receptor (FcγRIIIa/CD16a, termed haNKTM) and to produce endogenous, intracellularly retained IL-2 (*37,38*). In a phase I clinical trial underway it will be evaluated for safety and efficacy of haNKTM cells in treatment of patients with unresectable and locally advanced or metastatic solid tumors (NCT03027128; Table 1).

Another deficiency is the absence of some killer-cell immunoglobulin-like receptors (KIRs), with the absence of KIR2DL4 (CD158d) on the surface of NK-92 cells, which may lead to potential stimulation of GVHD (*39-41*). Therefore, attention should be paid that activated CAR-expressing NK-92 cells must be irradiated with at least 10 Gy before infusion into patients with cancers, resulting in a lower cell persistence and a loss of effector-mediated anti-tumor functions (*41*). Despite these deficiencies, preclinical research has suggested that CAR-expressing NK-92 cells could target a broad range of cancer antigens (*42,43*). Up to now, only a few clinical trials using CAR-expressing NK cells against Hematologic cancer and particularly against solid tumors have been launched (Table 1). Lately, a phase I/II trial has aimed to validate the safety and efficacy of CAR-NK cells in patients with overexpressed MUC1-positive solid tumors, particularly carcinomas (hepatocellular, pancreatic, breast, colorectal, gastric), non-small cell lung cancer (*31*), and glioblastoma (NCT02839954; Table 1).

4. Barriers to clinical application of CAR-NK cell immunotherapy

4.1. Mass production of NK cells

The first barrier to CAR-NK cell immunotherapy is the expansion of NK cells *in vitro*. The number of NK cells from a single-donor is insufficient for therapy, which makes the expansion and activation of NK cells very critical (*44*). This production process normally takes two to three weeks to culture NK cells with certain cytokines (IL-2 or in combination with IL-15 or anti-CD3 mAb) (*45*). The combination of IL-2 and IL-21 were also utilized to improve NK cells proliferation (*46,47*). The studies suggested that the combination of IL-2 and IL-21 showed a higher inhibitive effect on proliferation of cancer cells than employing IL-2 alone (*46,47*). In spite of irradiated K562-mb15-4-1BBL cells used as feeders could improve growth of cells in the production process of NK cells proliferation, the availability of donor cell number remains a barrier (*48*). In addition, T cells must be entirely eliminated to protect against GVHD. Achieving sufficient NK cells is critical for treatment of patients with cancers. However, owing to the production process limitations of expanding to a great number of cells, it is difficult to broadly perform in clinical applications.

4.2. The methods to transduce CAR into NK cells

For development of CAR-NK cell immunotherapy, it will be critical to choose the proper method to transduce CAR into NK cells. So far, viral vectors and non-viral

vectors have both been employed to transfer CAR.

Transfection vectors, including viral vectors and non-viral vectors, are broadly utilized in the production of CAR-NK cells because they can stably integrate into the human genome. Although the transfection efficiency of retroviral vectors is high, it may give rise to insertional mutagenesis, carcinogenesis and other adverse effects (*30*). Although lentiviral vectors show a lower incidence rate of insertion mutagenesis, their transfection efficiency is as low as 20% for NK cells from peripheral blood (*45*). The transfection efficiency of lentivirus vectors is high enough for NK cells from cord blood (*49*). However, the transfection efficiency of lentivirus for NK cells from peripheral blood has room for improvement. Previous research has suggested that suppressing the intracellular antiviral system may increase lentivirus transfection level of NK cells, providing an affordable and safe method for CAR transduction into NK cells (*50*).

Transfection with mRNA for CAR-NK cells has also been considered to be a practical and safe transduction method. Research has revealed that receptor expression level 24 hours after electroporation with the mRNA method was more than 80% and NK cells transfected with mRNA showed obvious cytotoxicity in a xenograft cancer model (*48*). Lately, a research result suggested that "on-target off-tumor" toxicity, which is an important limiting factor for the clinical application of CAR-modified immunotherapies, may be effectively avoided by transfection with mRNA (*51*). However, the antitumor effect of CAR-NK cells transfected with mRNA by electroporation method will be transitory because the expression level of CARs will last no more than three days (*52*).

5. Conclusion and outlook

Both cord blood and peripheral blood-derived CAR-NK cells and CAR-NK-92 cell line are comprehensive medicinal products combining critical characteristics: they are genetically modified and employed as cellular immunotherapy. The complete production process following Good Manufacturing Practice (GMP) requires ten days to several weeks using Teflon bags, flasks, continuous-flow devices, stirred-tank bioreactors and the Miltenyi's Prodigy system (*44*). Compared to CAR-T cells, CAR-NK cells have the merit of "off-the-shelf" production, but still are confronted with several challenges. These challenges include improvement in cell expansion, making the activation of cytotoxicity more efficient, and finally finding the best reconstruction methods for NK cells (*53*).

Although CAR-NK cell immunotherapy has been proved to be effective for inhibiting cancers, the long-term anti-cancer effect is still ambiguous. The combination therapy provides a novel prospect for CAR-based cell immunotherapy. In a few previous studies, researchers suggested chemotherapy may also improve the efficiency of CAR-NK cell immunotherapy. Chemotherapeutic drugs could not only eliminate the existing cell populations to establish new niches for the proliferation of NK cells, but also can lead to a genotoxic stress response to increase tumor cell sensitivity to NK cells (*54*). Clinical trials have revealed that the chemotherapeutic drugs could remarkably enhance the tumor inhibitive effect of CAR-NK cells (*55*). In a preclinical study, it has been reported that the combination of CAR-T cell immunotherapy and radiotherapy shows a synergistic effect against glioblastoma (*56*). However, the synergistic effects of CAR-NK cell immunotherapy and radiotherapy remain unclear (*57*). Thus, further research is needed to better understand the relationship between the two therapeutic methods. Moreover, the CRISPR/Cas9 technique has become an increasingly popular gene editing tool due to its advantages in editing the genomes of multiple organisms precisely (*58*). A few studies have suggested that editing a CAR by using the CRISPR/Cas9 technique could cause homogeneous CAR expression and improve cytotoxicity efficiency (*59*). If so, then the CRISPR/CAS9 technique may have the capacity to improve the efficiency and safety of CAR-NK cells by editing genes of primary NK cells and manufacturing stably transduced NK cells.

References

1. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. cytotoxic cells with specificity for mouse moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol. 1975; 5:112-117.
2. Farag SS, Caligiuri MA. Human natural killer cell development and biology. Blood Rev. 2006; 20:123-137.
3. Konjević G, Vuletić A, Mirjačić Martinović K. Natural killer cell receptors: alterations and therapeutic targeting in malignancies. Immunol Res. 2016; 64: 25-35.
4. Benson DM Jr, Caligiuri MA. Killer immunoglobulin-like receptors and tumor immunity. Cancer Immunol Res. 2014; 2:99-104.
5. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. Lancet. 2000; 356:1795-1799.
6. Orange JS, Ballas ZK. Natural killer cells in human health and disease. Clin Immunol. 2006; 118:1-10.
7. Björkström NK, Ljunggren HG, Michaëlsson J. Emerging insights into natural killer cells in human peripheral tissues. Nat Rev Immunol. 2016; 16:310-320.
8. Maude SL, Frey N, Shaw PA, *et al.* Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med. 2014; 371:1507-1517.
9. Feng KC, Guo YL, Dai HR, Wang Y, Li X, Jia HJ, Han WD. Chimeric antigen receptor-modified T cells for the immunotherapy of patients with EGFR-expressing advanced relapsed/refractory non-small cell lung cancer. Sci China Life Sci. 2016; 59:468-479.
10. Gilham DE, Debets R, Pule M, Hawkins RE, Abken H. CAR-T cells and solid tumors: tuning T cells to challenge

an inveterate foe. Trends Mol Med. 2012; 18:377-384.
11. Kalaitsidou M, Kueberuwa G, Schütt A, Gilham DE. CAR T-cell therapy: Toxicity and the relevance of preclinical models. Immunotherapy. 2015; 7:487-497.
12. Qasim W. Allogeneic CAR T cell therapies for leukemia. Am J Hematol. 2019; 94:S50-S54.
13. Zhang C, Oberoi P, Oelsner S, Waldmann A, Lindner A, Tonn T, Wels WS. Chimeric antigen receptor-engineered NK-92 cells: an off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity. Front Immunol. 2017; 8:533.
14. Boissel L, Betancur-Boissel M, Lu W, Krause DS, Van Etten RA, Wels WS, Klingemann H. Retargeting NK-92 cells by means of CD19- and CD20-specific chimeric antigen receptors compares favorably with antibody-dependent cellular cytotoxicity. Oncoimmunology. 2013; 2:e26527.
15. Klingemann H. Are natural killer cells superior CAR drivers? Oncoimmunology. 2014; 3:e28147.
16. Miller JS, Soignier Y, Panoskaltsis-Mortari A, *et al.* Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. Blood. 2005; 105:3051-3057.
17. Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, Pui CH, Leung W. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. J Clin Oncol. 2010; 28:955-999.
18. Shaffer B, Le Luduec JB, Forlenza C, Jakubowski AA, Perales MA, Young JW, Hsu KC. Phase II study of haploidentical natural killer cell infusion for treatment of relapsed or persistent myeloid malignancies following allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2016; 22:705-709.
19. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med. 2011; 3:95ra73.
20. Sun C, Sun HY, Zhang C, Tian ZG. NK cell receptor imbalance and NK cell dysfunction in HBV infection and hepatocellular carcinoma. Cell Mol Immunol. 2015; 12:292-302.
21. Sun C, Sun HY, Xiao WH, Zhang C, Tian ZG. Natural killer cell dysfunction in hepatocellular carcinoma and NK cell-based immunotherapy. Acta Pharmacol Sin. 2015; 36:1191-1199.
22. Maude SL, Frey N, Shaw PA, *et al.* Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med. 2014; 371:1507-1517.
23. Tam YK, Martinson JA, Doligosa K, Klingemann K. *Ex vivo* expansion of the highly cytotoxic human natural killer-92 cell-line under current good manufacturing practice conditions for clinical adoptive cellular immunotherapy. Cytotherapy. 2003; 5:259-272.
24. Ni ZY, Knorr DA, Bendzick L, Allred J, Kaufman DS. Expression of chimeric receptor CD4ζ by natural killer cells derived from human pluripotent stem cells improves *in vitro* activity but does not enhance suppression of HIV infection *in vivo*. Stem Cells. 2014; 32:1021-1031.
25. Liu E, Tong Y, Dotti G, *et al.* Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. Leukemia. 2018; 32:520-531.
26. Oelsner S, Friede ME, Zhang CC, Wagner J, Badura S, Bader P, Ullrich E, Ottmann OG, Klingemann H, Tonn T, Wels WS. Continuously expanding CAR NK-92 cells display selective cytotoxicity against B-cell leukemia and lymphoma. Cytotherapy. 2017; 19:235-249.
27. Levine BL. Performance-enhancing drugs: design and production of redirected chimeric antigen receptor (CAR) T cells. Cancer Gene Ther. 2015; 22:79-84.
28. Weiland J, Elder A, Forster V, Heidenreich O, Koschmieder S, Vormoor J. CD19: a multifunctional immunological target molecule and its implications for blineage acute lymphoblastic leukemia. Pediatr Blood Cancer. 2015; 62:1144-1148.
29. Romanski A, Uherek C, Bug G, Seifried E, Klingemann H, Wels WS, Ottmann OG, Tonn T. CD19-CAR engineered NK-92 cells are sufficient to overcome NK cell resistance in B-cell malignancies. J Cell Mol Med. 2016; 20:1287-1294.
30. Hu Y, Tian ZG, Zhang C. Chimeric antigen receptor (CAR)-transduced natural killer cells in tumor immunotherapy. Acta Pharmacol Sin. 2018; 39:167-176.
31. Zotto GD, Marcenaro E, Vacca P, Sivori S, Pende D, Chiesa MD, Moretta F, Ingegnere T, Mingari MC, Moretta A, Moretta L. Markers and function of human NK cells in normal and pathological conditions. Cytometry B Clin Cytom. 2017; 92:100-114.
32. Schönfeld K, Sahm C, Zhang CC, Naundorf S, Brendel C, Odendahl M, Nowakowska P, Bönig H, Köhl U, Kloess S, Köhler S, Holtgreve-Grez H, Jauch A, Schmidt M, Schubert R. Selective inhibition of tumor growth by clonal NK cells expressing an ErbB2/HER2-specific chimeric antigen receptor. Mol Ther. 2015; 23:330-338.
33. Tonn T, Becker S, Esser R, Schwabe D, Seifried E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. J Hematother Stem Cell Res. 2001; 10:535-544.
34. Maki G, Klingemann HG, Martinson JA, Tam YK. Factors regulating the cytotoxic activity of the human natural killer cell line, NK-92. J Hematother Stem Cell Res. 2001; 10:369-383.
35. Gong JH, Maki G, Klingemann HG. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia. 1994; 8:652-658.
36. Matsuo Y, Drexler HG. Immunoprofiling of cell lines derived from natural killer-cell and natural killer-like T-cell leukemia-lymphoma. Leuk Res. 2003; 27:935-945.
37. Jochems C, Hodge JW, Fantini M, Fujii R, Morillon YM, Greiner JW, Padget MR, Tritsch ST, Tsang KY, Campbell KS, Klingemann H, Boissel L, Rabizadeh S, Soon-Shiong P, Schlom J. An NK cell line (haNK) expressing high levels of granzyme and engineered to express the high affinity CD16 allele. Oncotarget. 2016; 7:86359-86373.
38. Klingemann H, Boissel L, Toneguzzo F. Natural killer cells for immunotherapy - advantages of the NK-92 cell line over blood NK cells. Front Immunol. 2016; 7:91.
39. Cooley S, Xiao F, Pitt M, Gleason M, McCullar V, Bergemann TL, McQueen KL, Guethlein LA, Parham P, Miller JS. A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self-MHC is developmentally immature. Blood. 2007; 110:578-586.
40. Faure M, Long EO. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. J Immunol. 2002; 168:6208-6214.
41. Suck G, Odendahl M, Nowakowska P, Seidl C, Wels

WS, Klingemann HG, Tonn T. NK-92: an 'off-the-shelf therapeutic' for adoptive natural killer cell-based cancer immunotherapy. Cancer Immunol Immunother. 2016; 65:485-492.
42. Boissel L, Betancur M, Wels WS, Tuncer H, Klingemann H. Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leuk Res. 2009; 33:1255-1259.
43. Uherek C, Tonn T, Uherek B, Becker B, Schnierle B, Klingemann HG, Wels W. Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. Blood. 2002; 100:1265-1273.
44. Klingemann H. Challenges of cancer therapy with natural killer cells. Cytotherapy. 2015; 17:245-249.
45. Shimasaki N, Coustan-Smith E, Kamiya T, Campana D. Expanded and armed natural killer cells for cancer treatment. Cytotherapy. 2016; 18:1422-1434.
46. Oberschmidt O, Morgan M, Huppert V, Kessler J, Gardlowski T, Matthies N, Aleksandrova K, Arseniev L, Schambach A, Koehl U, Kloess S. Development of automated separation, expansion, and quality control protocols for clinical-scale manufacturing of primary human NK cells and alpharetroviral chimeric antigen receptor engineering. Hum Gene Ther Methods. 2019; 30:102-120.
47. Granzin M, Stojanovic A, Miller M, Childs R, Huppert V, Cerwenka A. Highly efficient IL-21 and feeder cell-driven *ex vivo* expansion of human NK cells with therapeutic activity in a Xenograft mouse model of melanoma. Oncoimmunology. 2016; 5:e1219007.
48. Shimasaki N, Fujisaki H, Cho D, Masselli M, Lockey T, Eldridge P, Leung W, Campana D. A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. Cytotherapy. 2012; 14:830-840.
49. Boissel L, Betancur M, Lu WQ, Wels WS, Marino T, Van Etten RA, KlingemannH. Comparison of mRNA and lentiviral based transfection of natural killer cells with chimeric antigen receptors recognizing lymphoid antigens. Leuk Lymphoma. 2012; 53: 958-965.
50. Sutlu T, Nyström S, Gilljam M, Stellan B, Applequist SE, Alici E. Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. Hum Gene Ther. 2012; 23:1090-1100.
51. Hung CF, Xu XQ, Li LH, Ma Y, Jin Q, Viley A, Allen C, Natarajan P, Shivakumar R, Peshwa MV, Emens LA. Development of anti-human mesothelin-targeted chimeric antigen receptor messenger RNA-transfected peripheral blood lymphocytes for ovarian cancer therapy. Hum Gene Ther. 2018; 29:614-625.
52. Li L, Liu LN, Feller S, Allen C, Shivakumar R, Fratantoni J, Wolfraim LA, Fujisaki H, Campana D, Chopas N, Dzekunov S, Peshwa M. Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. Cancer Gene Ther. 2010; 17:147-154.
53. Oberschmidt O, Kloess S, Koehl U. Redirected primary human chimeric antigen receptor natural killer cells as an "off-the-shelf immunotherapy" for improvement in cancer treatment. Front Immunol. 2017; 8:654.
54. Fine JH, Chen P, Mesci A, Allan DSJ, Gasser S, Raulet DH, Carlyle JR. Chemotherapy-induced genotoxic stress promotes sensitivity to natural killer cell cytotoxicity by enabling missing-self recognition. Cancer Res. 2010;70: 7102-7113.
55. Klapdor R, Wang S, Hacker U, Büning H, Morgan M, Dörk T, Hillemanns P, Schambach A. Improved killing of ovarian cancer stem cells by combining a novel chimeric antigen receptor-based immunotherapy and chemotherapy. Hum Gene Ther. 2017; 28:886-896.
56. Weiss W, Weller M, Guckenberger M, Sentman CL, Roth P. NKG2D-based CAR T cells and radiotherapy exert synergistic efficacy in glioblastoma. Cancer Res. 2018; 78:1031-1043.
57. Seyedin SN, Schoenhals JE, Lee DA, *et al.* Strategies for combining immunotherapy with radiation for anticancer therapy. Immunotherapy. 2015; 7:967-980.
58. Zhang F, Wen Y, Guo X. CRISPR/Cas9 for genome editing: progress, implications and challenges. Hum Mol Genet. 2014; 23:R40-46.
59. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJC, Hamieh M, Cunanan KM, Odak A, Gönen M, Sadelain M. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017; 543:113-117.

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