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).
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 receptor 2 (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 (FcyRIIIa/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-IBBL 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 insertional 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 the 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.

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