CAR-NK Cell Therapy: A Promising Alternative to CAR-T Cell Therapy

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Abstract. Cancer is one of the deadliest diseases and has long been a leading cause of death. Although the newly introduced chimeric antigen receptors-engineered T cell (CAR-T cell) therapy has been an efficient method to kill cancer cells and inhibit metastasis, its high cytotoxicity, long time to develop, and high cost due to its patient-specific nature suggest that a new treatment is needed. In recent years, researchers have been paying increasing interest in implementing CAR on natural killer cells (NK cells), making them CAR-NK cells similarly to making CAR-T cells. This paper focuses on the process of CAR-NK cell therapy and the extent to which it excels over CAR-T therapy. From several published research and data, it can be concluded that CAR-NK therapy cell is a promising alternative to CAR-T cell therapy as it offers various key advantages including better safety, production convenience, and tumor targeting efficacy. However, challenges such as low cell persistence and transduction efficiency still need to be resolved by further studies and improvements.

Keywords: cancer · immunology · chimeric antigen receptor · NK cells

1 Introduction

CAR-T cell therapy is a new type of cancer treatment first approved by the FDA in 2017 that redirects T cells to target and kill cancer cells using Chimeric Antigen Receptors (CARs) [1]. CARs are synthetic receptors designed to help T cells recognize particular tumor antigens that would otherwise evade or sabotage the immune system [4, 5]. This treatment begins with the collection of the patient's blood and isolation of the patient's T lymphocytes, or T cells. Then, genes encoding CARs are delivered to the collected T cells through methods such as using viral vectors, before the cells are infused back into the patients, granting their immune systems the ability to identify and kill the tumor cells with cytokines [2, 3]. However, although CAR-T cell therapy is effective in eliminating various types of cancer cells, it faces several serious challenges. Notably, CAR-T cell therapy can induce severe side effects such as neurotoxicity and cytokine release syndrome (CRS), and its patient-specific nature would lead to an extremely high cost and long time to develop [6, 7]. With the limited success of CAR-T cell therapy, more researches have shifted to the development of CAR-NK cell therapy, identifying it as a potential alternative. This paper will review the process of CAR-NK cell therapy,

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analyze its unique characteristics compared to CAR-T cell therapy, as well as discuss the extent to which it excels over and can be the alternative to CAR-T cell therapy. As a result, this investigation will reveal the advantages of CAR-NK cell therapy and provide a reference for further research in cancer immunology.

2 Process of CAR-NK Cell Therapy

2.1 Collection of NK Cells

CAR-NK cell therapy begins with the collection of NK cells. NK cells can be derived from various sources, including cell lines, blood collection, and cell differentiation [8]. Each way has its advantages and drawbacks.

Cell lines, such as the NK-92 cell line, have high proliferation and transduction efficiency in vitro [8, 11], which means the cost and manufacturing time would be reduced, contributing to the off-the-shelf production advantage. However, the CAR-NK-92 cells derived from it must be lethally irradiated before infusion into the patient as they are malignant lymphoma cells [8], inhibiting them from replicating in the host's body and therefore unable to provide long-term protection for the patient [12].

Through blood collection, NK cells can be isolated from either peripheral blood (PB) or umbilical cord blood (UCB) of the patient/donor [8]. About 10% of the lymphocytes in peripheral blood are NK cells, and they can be easily isolated through the density gradient of peripheral blood mononuclear cells [13], making the procedure faster and cheaper. Also, unlike NK cells from cell lines, PB-derived CAR-NK cells do not require irradiation prior to administration, meaning they can expand in vivo and potentially provide longer protection [8]. However, the expansion still appears to be relatively poor as PB NK cells generally display a mature phenotype with a high proportion of CD56dim CD16+ NK cells, which increases cytotoxicity and decreases proliferation [9, 10]. Besides PB, NK cells can also be isolated from UCB through a similar process. In UCB, NK cells constitute around 30% of the lymphocytes compared to PB NK cells' 10% [13]. Also, UCB NK cells exhibit a higher proportion of CD56bright CD16- NK cells, a more immature phenotype [14, 15]. Although this would increase the proliferative capacity that their PB counterparts lack, UCB NK cells' reduced cytotoxicity would hinder their efficiency in killing cancer cells [13]. However, the cytotoxicity can reach the adult level with stimulation by different cytokines such as IL-15 [15].

NK cells can also be obtained from cellular differentiation [8]. CD34+ hematopoietic progenitor cells (HPCs) are one approach. CD34+ HPCs can be isolated from PB, UCB, bone marrow, and embryonic stem cells [8, 16] and developed ex vivo [17]. With cytokine treatment, CD34+ HPCs can be differentiated into CD3−CD56+ NK cells [17, 18]. Similar to PB NK cells, these differentiated NK cells exhibit strong cytotoxicity against targeted cancer cells but have relatively limited expansion capacity [19]. Compared to HPC, the newly introduced pluripotent stem cell (iPSC) method completely solves the problem. iPSCs can be efficiently genetically engineered with CARs and result in CAR-iPSCs, which are then differentiated into CAR-HPCs with various cytokines and then into NK cells [20]. Due to iPSCs homogeneity, one single CAR-iPSC can differentiate and expand into a substantial amount of homogeneous NK cells while maintaining, which may standardize the product [21].

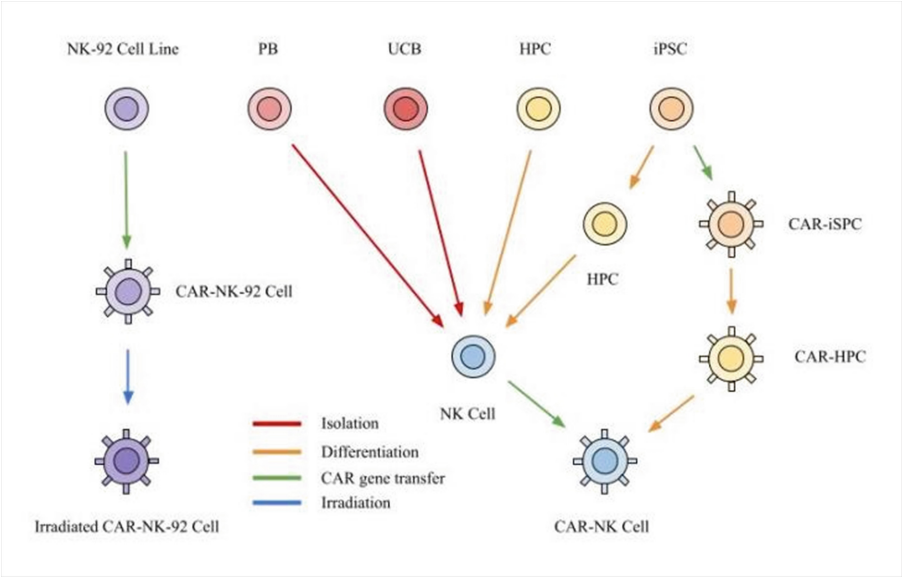

Fig. 1. CAR-NK Cell Sources

Figure 1 maps the sources of NK cells in CAR-NK cell engineering. NK-92 cell line with its high proliferation rate can be used to construct CAR-NK-92 cell. However, irradiation is required before infusion into the patient. Blood collection, which includes peripheral blood (PB) and umbilical cord blood (UCB), is a source of primary NK cells. Through means such as density gradient, NK cells can be isolated and used for engineering. NK cells can also be differentiated from CD34+ hematopoietic progenitor cells (HPC), which can be derived from a pluripotent stem cell (iPSC). Besides directly differentiating into HPC and subsequent NK cells, iPSCs can also be engineered with CAR before the differentiations.

2.2 CAR Structure

A typical CAR is composed of three domains: extracellular, transmembrane, and intracellular [8, 22]. The extracellular domain (also known as ectodomain), as its name suggests, is exposed outside of the cell [8]. This domain is generally formed by a single-chain antibody variable fragment (scFv), which consists of a light chain and a heavy chain connected to a linker [22]. This domain mainly serves to recognize targeted antigens by binding on it [8], and scFvs derived from different antibodies would target different expressions [23]. For example, scFv derived from the murine FMC63, a monoclonal antibody for CD19, would specifically target epitopes of CD19 [24]. The scFv is connected to the transmembrane domain by a flexible hinge (also known as a spacer) [8, 22]. The transmembrane is a hydrophobic alpha helix around the cell membrane that serves to anchor the CAR and therefore plays a crucial role in the stability of the receptors [8, 23]. Inside the cell is the intracellular domain (also known as endodomain), which consists of signaling modules to stimulate and activate the cell [22]. There is one

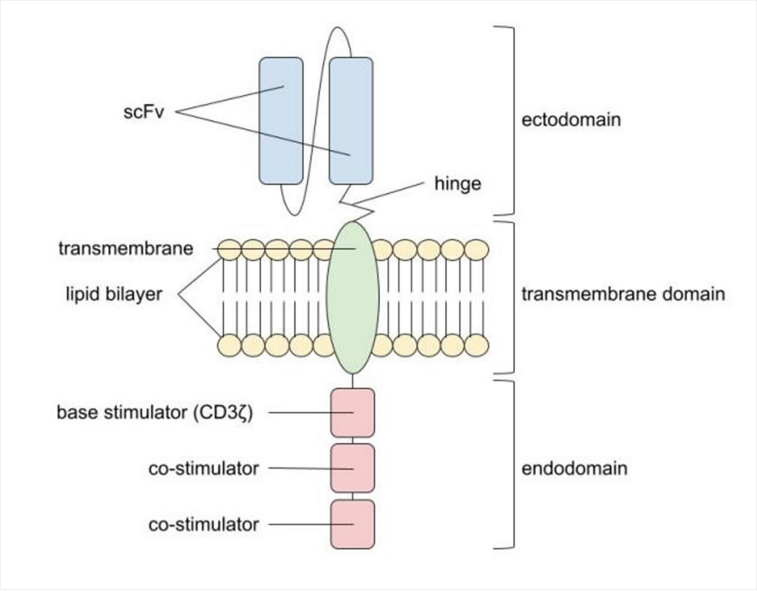

Fig. 2. Structure of Chimeric Antigen Receptor

basic stimulator, which only contains the CD3ζ chain [22, 25]. However, CD3ζ alone is usually not enough to activate the full potential of the cell and therefore is ineffective against tumors [22, 25]. To solve this problem, additional co-stimulator such as 4-1BB and 2B4 are added to the domain to enhance the signaling [25, 26]. As a result, CARs are divided into three classes: first-generation CARs (CD3ζ alone), second-generation CARs (CD3ζ and one co-stimulator), and third-generation CARs (CD3ζ and two or more co-stimulators) [23].

Figure 2 shows the structure of a typical Chimeric Antigen Receptor (CAR). CAR is divided into three domains, ectodomain outside of the cell, a transmembrane domain within the lipid bilayer of the cell, and endodomain inside the cell. Ectodomain consists of a single-chain antibody variable fragment (scFv), which is used to recognize antigen expressions, and a hinge, which connects the scFvs with the transmembrane. The transmembrane domain has a hydrophobic alpha helix that anchors the CAR to the cell membrane. Endodomain includes several stimulators, the base stimulator, which is usually CD3ζ, and a few co-stimulators to help fully activate the CAR.

2.3 CAR Gene Transfer

After the collection of NK cells is the transfer of genetic material into the cells for CAR expression. There are two major approaches to this step, viral transduction, including using retroviral and lentiviral vectors, and non-viral transfection, usually done by electroporation [27].

Retroviral transduction is the first viral method used to genetically engineer NK cells [28]. Although it is a reliable method used for and improved over decades, one major

Table 1. Characteristics of CAR Gene Transfer Methods

Retroviral transduction	Lentiviral transduction	DNA or mRNA transfection
– depends on cell mitosis – cause insertional mutagenesis and genotoxicity	– depends on envelope protein – potential for high transduction efficiency	– high transduction efficiency – low cell persistence

limitation of using retroviral vectors to integrate the genome is that it requires the cell to be in mitosis when their nuclear membranes dissolve [27, 28], which would hinder the production of NK cells. Studies have shown that the CAR transduction efficiency of primary NK cells ranges from 27.2 to 51.9% after one round of transduction [29]. This limitation can be mitigated to an extent while transduction is performed on NK-92 cell lines due to their highly active expansion compared to primary NK cells, showing results of higher efficiency [30]. However, its insertional mutagenesis and genotoxicity still persist as its innate limitation [28].

Lentiviral transduction is a more recent method that excels for its ability to transfer genes without the cell being in mitosis and its reduced insertional mutagenesis and genotoxicity [28]. Unlike retroviral vectors, whose transduction efficiency requires cells' active division, Lentiviral vectors' transduction efficiency depends more on their envelope protein [27, 28]. Although lentiviral vectors' transduction is generally lower than their retroviral counterparts, it has the potential to be significantly improved when incorporated with certain envelope proteins [28]. In a recent study, a baboon envelope glycoprotein pseudotyped lentiviral vector was found to have 70% transduction efficiency in incorporating CD19 CARs into primary NK cells [32]. However, further studies may be required to validate its practicality on other types of CARs.

The most common non-viral transfection method is through mRNA or DNA electroporation, a cost-effective process in which short electric pulses are used to induce small pores on the cell membrane, allowing molecules such as mRNA or DNA to pass and transfect the cell [28]. This offers a number of advantages. Electroporation can be done regardless of the cell's mitosis activity, and its transfection efficiency is generally higher than that of viral methods at around 40–60% in NK cells, sometimes reaching 80–90% with minimal defects when using high purity CAR mRNA [28, 34]. Similar to mRNA transfection, DNA transfection also exhibits high transduction efficiency but with a lower cell viability because DNA normally experiences a harsher condition reaching the nucleus [27]. Furthermore, CAR-NK cells created through non-viral transfection display a lower variance between individual cells, which may contribute to standardizing of the product [28]. However, one major setback is that the genetic material is usually not integrated into the cell's genome. This would mean that the CAR expression would only last and decline over a short period of time (3–5 days for mRNA and up to 15 days for DNA electroporation) and is impossible for long-term storage [27, 33] (Table 1).

3 Advantages of CAR-NK Cell Therapy

Though similar to the older CAR-T cell therapy, the new CAR-NK cell therapy offers several key advantages due to its biological feature, including better safety, more convenient manufacturing, and greater efficacy in recognizing tumor cells.

First, CAR-NK cell therapy is superior in safety compared to CAR-T cell therapy [33, 35]. One major drawback of CAR-T cells is the highly proinflammatory cytokines, including TNF-a, IL-1, and IL-6, which T cells secrete [33]. Such cytokines are usually associated with serious adverse effects such as neurotoxicity and CRS and have the potential to further endanger the patients' health condition [6, 33]. On the other hand, activated NK cells typically produce a different range of safer cytokines consisting of IFN-y and granulocyte-macrophage colony-stimulating factor (GM-CSF), which eliminates the potential of inducing those negative effects as several clinical trials have proven its safety [35].

Second, due to the high risk of graft vs host disease (GVHD) in T cell-based therapies, the construction of CAR-T cells has to be patient-specific, resulting in the unfeasibility of mass production of CAR-T cells and therefore an extremely high cost of the therapy of over $700,000 in average per patient [7, 36]. On the other hand, NK cells exhibit a significantly reduced level of alloreactivity, which means NK cells can be isolated from donors regardless of their HLA matching result and mass engineered into products that can be used on any patient without risk of GVHD [33] Therefore, CAR-NK cell therapy can achieve high feasibility of off-the-shelf manufacturing with minimal cost and maximum availability.

Third, although CAR-T cells have already proven to be highly competent in targeting and killing specific tumor cells, they are not as effective at eliminating cells with diverse phenotypes due to their limited manner of detection [33, 39]. On the other hand, CAR-NK cells have even better efficacy with their additional intrinsic capacity [33, 37]. Unlike CAR-T cells, which detect tumor antigens through solely scFv, a CAR-dependent mechanism, CAR-NK cells preserve their intrinsic capacity to identify various ligands on tumors using their native receptors. These include the cytotoxicity receptors, NKG2D and DNAM-1 [35, 38]. As a result, CAR-NK cells are capable of eliminating tumor cells with vastly heterogeneous antigen expressions.

4 Challenges of CAR-NK Cell Therapy

Although CAR-NK cell therapy offers several key advantages as suggested in the section above, it still faces some obstacles including low transduction efficiency and in vivo persistence.

One major setback is CAR-NK cells' significantly shorter persistence in vivo compared to CAR-T cells. Although CAR-NK cells have been reported to persist for more than 12 months, most clinical trials suggest the general durability of days to weeks at most depending on factors such as cell type and transduction method [12, 27, 33, 40]. On the other hand, current CAR-T cell products can easily retain their functionality and expand in vivo for years [41]. As a result, current CAR-NK cell therapy cannot provide long-term protection against cancer with one single infusion, and subsequent infusions may be required to fully eliminate tumors for some patients.

Table 2. Comparing CAR-NK Cell Therapy and CAR-T Cell Therapy

	CAR-NK Cell	CAR-T Cell
Safety	No risk of neurotoxicity and CRS, reduced risk of GVHD	Risk of neurotoxicity, CRS, and GVHD
Efficacy	Higher with CAR and native receptors	High with CAR
Convenience	Higher with off-the-shelf manufacturing	Lower due to the need for HLA matching and high price
Transduction efficiency	Lower due to lack of efficient transduction method	Higher
Persistence	Lower	Higher

Despite the various methods used to improve the transduction efficiency of CAR-NK cells, CAR-NK cells currently still have notably lower transduction efficiency compared to T cells. This is due to the lack of an effective gene transfer method. Viral transduction is an effective approach for T cells. However, for NK cells, it cannot ensure a high level of transgene expression without serious adverse effects such as mutagenesis [33, 35]. On the other hand, although non-viral methods can achieve higher transduction efficiency without risk, their expression persistence is limited as mentioned [27, 33] (Table 2).

5 Conclusion

Overall, CAR-NK cell therapy shows great promise as a potential alternative to CAR-T cell therapy. By implementing CAR on NK cells through a similar process to CAR-T cells, CAR-NK cells exhibit high efficacy in targeting and eliminating tumors like CAR-T cells. Furthermore, studies and experiments indicate that the CAR-NK cells excel in safety and tumor treatment efficacy over CAR-T cells and can be made into an "off-the-shelf" product due to its non-patient specific nature, eliminating the latter's flaws of inducing serious side effects and the long and expensive production. However, with limited data, CAR-NK cells' notably low transduction efficiency and cell persistence and expansion raise concerns as only a few studies suggest the opposite. As a result, although it is expected for CAR-NK therapy to be the next alternative to CAR-T therapy in cancer immunology, further investigations and improvements are needed.

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