Research Progress of NK Cell-Based Immunotherapy for Tumors

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***Abstract*—Nowadays, immunotherapy plays an important role in the comprehensive treatment of tumors. As an immune cell, Natural Killer (NK) cells have many advantages in tumor immunotherapy and will become a potential tumor immunotherapy. This dissertation will investigate three types of NK cell-based immunotherapies, namely NK cell infusion therapy, immune checkpoint blockade therapy, and CAR-NK therapy. The potential of immunotherapy based on NK cells has been demonstrated in various clinical trial cases. At the same time, it shows that there are still some key questions about NK cells, such as how to prepare NK cells, the selection of NK cell donors, and how to improve the function of NK cells. This article reviews the research progress of NK cell-based immunotherapy, which is expected to provide new ideas for the clinical treatment of tumors.**

***Keywords*—CAR-NK, immune checkpoint blockade, NK cell, tumor immunotherapy**

I. Introduction

Tumor refers to a new organism formed by the proliferation of local tissue cells in the body under the action of various carcinogenic factors [1]. Malignant tumors pose a significant threat to the body. It can cause infinite proliferation, causing damage to the structure and function of normal tissues and organs, and serious tumors can endanger human life. At present, due to the research, the powerful killing mechanism enables NK cells to demonstrate enormous clinical potential in the treatment of both hematomas and solid tumors [2]. The tumor microenvironment is the environment in which cancer cells live, including blood vessels, fibroblasts, immune cells, bone marrow-derived inflammatory cells, and extracellular matrix [3]. There is growing evidence that innate immune cells (macrophages, neutrophils, dendritic cells, and natural killer cells) as well as adaptive immune cells (T cells and B cells) are involved in tumor progression in the tumor microenvironment [4].

Tumor immunotherapy is the application of immunological principles and methods to specifically remove small residual tumor lesions, inhibit tumor growth, and break immune tolerance by activating immune cells in the body and enhancing the body's anti-tumor immune response [5]. Tumor immunotherapy is to overcome the mechanism of tumor immune escape, so as to reawaken immune cells to remove tumor cells. Due to its small side effects and obvious treatment effects, it is gradually becoming the development direction of tumor treatment in the future and is known as the fourth major tumor treatment technology after surgery, radiotherapy, and chemotherapy [6]. With the deepening of the research on the mechanism of action of NK cells, immunotherapy based on NK cells has been applied to the clinical treatment of tumors [7]. Compared with traditional treatments, NK cell therapy has a better effect and fewer negative effects, so it has attracted much attention [8]. This article mainly describes the application and research progress of NK cell-based immunotherapy in tumor treatment, which aims to provide new ideas for tumor immunotherapy.

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II. Existing NK Cell Immunotherapy Ideas

A. Definition of NK Cell

NK cells are one of the important components of the body's innate immune system, which can kill target cells without specific antigen stimulation, and have the functions of immune clearance and immune surveillance [9]. NK cell is also the third type of lymphocyte found after B cells and T cells, which are an important component of the body's innate immune response and is often considered to be the first line of defense against viral/bacterial infection, stimulating dendritic cells and B cell maturation by producing cytokines that support helper T cell polarization and T cell activation, thereby bridging and coordinating innate and adaptive immune responses [10]. NK cells account for 10%~15% of peripheral blood lymphocytes, mainly derived from hematopoietic stem cells in bone marrow, developed and differentiated from NK precursor cells, and are important effector cells in the tumor immune microenvironment [11]. As the first line of defense of the human immune defense system against the invasion of foreign pathogenic microorganisms, NK cells have a strong ability to lyse cells. They can rapidly and non-specifically identify pathogenic microorganisms or infected target cells, playing an important role in the early antitumor and antiviral phases of the body.

B. The Functions of NK Cell

1) Cytotoxicity

Most of the NK cells in the peripheral blood circulation express CD56dim, which can continuously kill infected cells and malignant transformed cells, and release cell-lytic granules containing granzyme and perforin through immune protrusion, inducing apoptosis of target cells [12]. NK cells directly release cytotoxic particles, such as perforin and granzyme, through exocytosis, and activate cysteine aspartate protease-related signaling pathways to induce apoptosis of tumor target cells. Perforated protein is a glycoprotein produced by lymphocytes with a killing function, which can form a transmembrane channel on the target cell membrane so that the target cell can undergo osmotic lysis, and at the same time promote granzyme to enter the cell and activate the autolytic endonuclease, then eventually lead to cell death. Perforated proteins play a key role in NK cell-mediated antiviral, antitumor effects [13].

Cytotoxicity plays an important role in the body's immune defense. Cell-mediated killing provides an effective defense barrier against other factors that affect the body, such as tumor proliferation. In recent years, in the process of studying NK cell killing, it has been found that there is a Pore-Forming Protein (PEP) related to cytotoxicity in the cytoplasm of these cells [14]. PEP is also known as perforin1. The mechanism by which PEP causes death and injury is very similar to complement-mediated cytotoxicity. They all have varying degrees of similarity in structural, functional, or immunological properties. Therefore, it has been suggested that all cytotoxic effects may have a common killing mechanism. In 1983, under electron microscopy, the cytoplasm of NK cells contained a certain number of particles that could normally be observed in the Golgi apparatus close to the perforated side of the bar. After the effector cell surface receptor sends a signal, it can be seen that these secreted particles are diffusely dispersed on the target cell membrane and locally release some cytotoxic substances, resulting in damage to the target cell membrane. In addition, in vitro, PEP can polarize cultured cells and move cytoplasmic granules towards target cells, and can lyse many tumor cells.

2) Expression of multiple tumor necrosis factors

Tumor necrosis factor, a cytokine produced primarily by activated macrophages, NK cells, and T cells, is a common tumor marker that can participate in the body's immune response [15]. NK cells can express a variety of tumor necrosis factors, such as Fas Ligand (FasL) and tumor necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) [16] They can bind to the corresponding receptor and thus induce target cell death. The CD16 receptor on the surface of NK cells can recognize the Fc segment of antibodies bound to the surface of target cells, and exert the function of lysing target cells through Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) in a granzyme B-dependent mechanism. FasL and TRAIL can bind to Fas and TRAIL receptors on the surface of tumor cells, respectively, to induce the activation of apoptosis-associated proteases in target cells and trigger receptor-dependent apoptosis stimulation in target cells [17]. Activated NK cells can secrete a variety of chemokines to recruit T cells, monocytes, and neutrophils, and secrete cytokines such as IFN-γ, TNF-α, GM-CSF, and IL-10 to regulate dendritic cells, macrophages, and T cells, thereby mobilizing more immune cells to participate in the anti-tumor immune response. IL-2 is a cytokine widely used in clinical cancer treatment. In multiple myeloma, IL-2 may lyse cell tumors by promoting the perforin effector mechanism of NK cells activated by the NKG2D pathway, enhancing the killing activity of CD16NK cells. Different from the antitumor effect of IL-2 alone, the combination of IL-2 and IL-8 can significantly enhance NKG2D expression. After overnight treatment with IL-15 after tumor exposure, NKG2D expression was enhanced. IL-15 is a cytokine structurally similar to IL-2 and may also have the potential to treat solid tumors.

3) Immune response

In the human immune system, T and B cells can recognize ligands of different origins by rearranging surface receptors. NK cells do not have this property, but NK cells can directly recognize target cells through activated and inhibitory receptors. NK cells can effectively recognize and remove cancer cells. Its receptors are mainly divided into two categories, which are consistency receptors and activated receptors [18]. These two classes of receptors transmit inhibitory signals and activation signals to the cell, respectively. When NK cells receive a greater inhibitory signal than the activation signal, NK cells are in a suppressed state and cannot produce an immune response. When NK cells are activated, they can generate an immune response, and recognize and eliminate virus-infected cells and cancer cells. NK cells can effectively kill infected cells and tumor cells and save normal tissue cells from damage. This tolerance is primarily mediated by MHC class I molecules that are widely distributed on the surface of healthy tissue cells. When NK cells interact with normal tissue cells, NK cells are not activated because the expression of MHC class I molecules on the surface of normal cells far exceeds the expression of activated ligands, binding to killer inhibitory receptors, so that the inhibitory signal is dominant. Since MHC molecules have an inhibitory effect on NK cells, NK cells should have strong killing ability against cells with low MHC expression. This feature helps NK cells kill tumor cells with low MHC expression and virus-infected cells.

C. NK Cell-Based Immunotherapy

As natural immune cells, NK cells play an important role in immune surveillance and tumor clearance. Therefore, it is often used in clinical medicine.

1) Infusion therapy

a) Theory

NK cells can be isolated from peripheral blood for in vitro expansion using cytokines in autologous and allogeneic environments as adoptive immunotherapy for hematologic malignancies and solid tumors [19]. NK cell reinfusion therapy refers to the transfusion of NK cells derived from peripheral blood, liver cells, and induced pluripotent stem cells into the patient, which can induce

the activation and proliferation of damaged NK cells in tumor patients and increase the number of NK cells [11]. NK cell infusion therapy is mainly divided into font NK cell infusion therapy and allogeneic NK cell reinfusion therapy. As with other adoptive cell therapies, autologous NK cell reinfusion therapy collects NK cells from the patient's own body and expands processes, and activates them in the exosome. The NK cells are eventually infused back into the patient. Allogeneic NK cell infusion therapy involves extracting NK from healthy donors. NK cells are important natural immune cells in the body and can recognize tumor-killer cells and virus-infected cells without prior sensitization. HLA hemizygous healthy donors partially attenuated the effect of inhibitory receptors on NK cell function due to the lack of some NK cell immunoglobulin receptors. Therefore, the function of NK cells is enhanced, so that they can better exert tumor-killing ability. Adoptive reinfusion NK cell therapy has gradually become an important concept of anti-leukemia treatment in hematopoietic stem cell transplantation.

b) Clinical examples

Gliomas account for about 50% of intracranial tumors which is the most difficult tumor to treat in the brain [20]. Surgery plus radiotherapy and chemotherapy are still difficult to achieve satisfactory results. In recent years, the application of immune cells to treat glioma as an ideal new way is gradually valued by the majority of medical workers. As early as 2006, a patient with recurrent glial tumors underwent immune cell therapy with satisfactory results [21]. The improvement in the quality of life of patients may be attributed to the immunomodulatory effect of β-endorphin. β-endorphin is a macromolecule composed of 31 amino acids with powerful analgesic and sedative effects [22]. The serum of β-endorphin in healthy people is 5.8 pg/ml, while 109 CD56+ positive tables can produce 20 ng of β-endorphin. According to the number of NK cells infusion each time in this case, the β-endorphin produced by NK cells in the body may be greater than 30 ng. Therefore, it may have a beneficial effect on the recovery of immunity and the improvement of quality of life. After receiving 5 of this auto-NK cell immunotherapy, MRI radiographic reality and intracranial recurrent glial tumor disappeared. NK cells successfully cured one case of recurrent glioma, which provided a feasible method for continuing immune cell therapy for glioma in the future. Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) is an effective treatment for hematologic malignancies, but recurrence is still a major complication after transplantation, affecting the efficacy [23]. At present, the formal treatment methods for post-transplantation recurrence include chemotherapy and combined Donor Lymphocyte Infusion (DLI), but although DLI significantly alleviates post-transplantation recurrence, it increases the risk of post-infusion Graft-Versus-Host Disease (GVHD). Cao *et al.* [24] conducted preliminary studies on the immunological changes and clinical efficacy of NK cell reinfusion into relapsed patients after transplantation, showing that adoptive NK cell reinfusion was used in patients with relapse after transplantation without adverse reactions, and the symptoms of GVHD were relieved in two of the patients. At the same time, the expression of NK cell surface-activated receptors NKp30, NKp46, and NKG2D was significantly higher than that in the non-response group, indicating that the secretion level of CD107a in the NK killing ability index also tended to be higher in the response group, which further indicated that NK cells with more activated receptors after reinfusion had a better prognosis in tumor cell patients.

2) Immune checkpoint blockade

a) Theory

Immune checkpoint therapy is used to block signaling pathways by using antagonists of co-inhibitory molecules or ligands, as well as other drugs [25]. It is exposed to immunosuppression in tumor patients, which in turn stimulates the activation of related immune-killer cells and enhances their ability to kill tumor packs. Due to the occurrence of resistance to ICs blockade therapy, this has become a major obstacle to the clinical application of other immune cell-related ICs blockade therapy [26]. Therefore, the natural killer properties of NK cells are crucial. NK cells are not affected by the expression of MHC class 1 molecules, so they can directly identify and kill tumor cells. In recent years, many new NK cell immune checkpoint molecules, such as non-human leukocyte antigen-1 specific inhibitory receptors (such as PD-1, TIGIT, CD96, TIM-3) and agonist receptors (such as NKG2D), have been discovered to control the dysfunction of NK cells in the tumor microenvironment [27]. In addition, NK cells express HLA-1 specific inhibitory receptors and agonist receptors. Such NK cell receptors mediate multiple signaling processes, and the balance of signals determines whether NK cells kill target cells or remain inactive. Abnormal regulation of signaling leads to NK cell dysfunction, resulting in down- and up-regulation of NK agonist receptor expression in some cancers. Abnormal signaling of immune checkpoint receptors in NK cells causes NK cells to malfunction. Therefore, blocking immune checkpoint molecules is an important strategy to improve NK cell function [28]. Many clinical studies have confirmed that these immune checkpoint inhibitors have clinical efficacy in different types of cancer.

b) PD-1

Programmed cell death protein 1 (PD-1), also known as CD279, belongs to the immunoglobulin super family, and present in T cells and NK cells, and is an important immunosuppressive molecule [29]. PD-1 is expressed on NK cells in malignant pleural fluid in both primary mesothelioma and metastatic tumors, including lung, intestinal, uterine, breast, and bladder cancers. PD-1 binds to its ligands PD-L1 and PD-L2, mediating NK cell inactivation. For example, in vitro experiments for radiotherapy-tolerant non-small cell lung cancer escape immune surveillance of NK cells by self-high expression of PD-L1 and induction of NK cells to increase PD-1 expression. And in the lymphoma tumor model. PD-1 promotes the escape of tumor cells from NK cell responses by inhibiting immune surveillance of NK cells. PD-1 has made remarkable achievements in preclinical

research and clinical trials in the blockade treatment of tumors. Pidilizumab is an anti-PD-1 monoclonal antibody that has been put into clinical trials in recent years, which can block the killing activity of NK cells in patients with multiple myeloma [30]. High-density activated human raw NK cells can kill colorectal cancer cells in 3D culture that do not rely on PD-L1 expression, suggesting that allogeneic activated NK cells may be an effective treatment for such cancers.

c) TIGIT

T cell immunoreceptor with lg and Immunoreceptor Tyrosine-Based Inhibition Motif (ITIM) domains, TIGHT, which is an immune checkpoint molecule that inhibits NK cell activation and consists of the LGV domain, the type 1 transmembrane domain, ITIM, and Immunoglobulin Tail Tyrosine (ITT). In tumor immune surveillance, TIGIT acts similarly to PD-1 in tumor immunosuppression. In addition, it plays an important role in the activation and maturation of NK cells. Blocking TIGIT allows NK cells to resist inhibition from myeloid-derived suppressor cells. Down-regulation of TIGIT expression inhibits the proliferation of colorectal cancer cells. In some specific preclinical tumor models. TIGIT's blockade regulates the effector function of cells and may have therapeutic effects in cancer patients. In addition, the expression of TIGIT was significantly associated with the progression of colorectal cancer. Therefore, TIGIT can be used as a biological marker or prognostic marker for evaluating cancer.

d) CTLA-4

CTLA-4 is mainly expressed in CD4+ and CD8+ cells, and is not expressed on the surface of naive T cells and quiescent effector T cells, but can be expressed on the surface of Treg cells [31]. CYTLA-4 exerts immunosuppressive effects primarily by competing with CD28 for binding ligands. Previously, the researchers only detected CTLA-4 expression on IL-2-activated NK cells and found that CTLA-4 inhibits the production of NK cell Interferon-γ (IFN-γ) by binding to B7-1 [32]. Recent studies have shown that healthy human NK cells activated by IL-2, IL-12, and IL-18 can also express CTLA-4. Another study has shown that the combined application of IL-2 and CTLA-4 inhibitors can significantly increase the infiltration of immune cells in melanoma, and reduce the content of NK cells depleted in tumors, thereby improving the killing of immune cells and inhibiting tumor growth [33].

3) CAR-NK

a) Theory

Regarding CAR, the earliest studies focused on T cells. The CAR structure is designed based on signals that NK cell activation relies on, including activation receptor signaling, co-stimulation signaling, and cytokine signaling [34]. The CAR structure of CAR-NK cells includes extracellular, transmembrane, and intracellular segments, and the early CAR-NK design continues the CAR structure in CAR-T cells [35]. The extracellular segment of CAR-NK is mainly a single-stranded variable region of antibodies. The transmembrane segments commonly used in CAR-NK is CD3ζ and CD8. The intracellular segment is responsible for cell activation after CAR-NK receives antigen signals from target cells, and is a linear structure of co-stimulatory molecules and signal domains recruited downstream of signaling [36].

b) The process of CAR-NK

Through a cell separator, NK cells are extracted from the patient's blood. Tumor-associated target proteins were then screened. Different tumors cause different genetic mutations and express different proteins. After leukocyte removal, NK cells are isolated and sent to the laboratory and altered by adding a specific Chimeric Antigen Receptor (CAR) gene. This allowed them to be engineered into CAR-NK cells. In a laboratory setting, genes encoding specific antigen receptors are integrated into NK cells, resulting in CAR receptors on the cell surface. NK cells can be obtained from a variety of sources for CAR-NK production, such as immortalized human NK cell lines, donor peripheral blood, archived samples of umbilical cord blood, or supervised differentiation of pluripotent cells. Depending on the source, harvested NK cells vary with their maturation stage and viability, which is reflected in the different anti-tumor efficacy of the resulting CAR-NK cells. However, continuous optimization of protocols for obtaining NK cells from a variety of sources and scaling up production will inevitably provide ready-made cancer treatments that comply with Good Manufacturing Practices (GMP).

c) Target CD-19

CD19 is one of the most widely expressed surface antigens in B-cell malignancies including acute lymphoblastic leukemia, chronic lymphocytic leukemia, and non-Hodgkin lymphoma. It is therefore an ideal target antigen for immunotherapy. Patients with recurrent B-cell NHL currently lack effective treatment and have a poor prognosis. CAR-modified NK-92 cells have been shown to be effective in killing CD19-positive cell lines as well as primary cells resistant to modified NK-92 [37]. For the treatment of NHL, direct injections of CAR-expressing NK-92 cells into tumor lesions have been shown to be very effective [38].

d) Target HER2

HER2 is commonly expressed in many epithelial tumors and overexpressed in many cancers, including breast, ovarian, and lung cancers [39]. Overexpression of HEER2 is associated with aggressiveness and poor prognosis of malignant tumors, and breast cancers with HER2 amplification are more aggressive and have a higher risk of central nervous system metastases. Moreover, the residual tumor of malignant brain tumor after surgery hinders many chemotherapy drugs due to the blood-brain barrier, resulting in unsatisfactory treatment effect. CAR-NK-92 cells targeting HER2 can be delivered to tumor sites within the brain via MRI-guided focused ultrasound and accurate microvascular transport [40].

e) Clinical examples

B-line lymphoma and leukemia: Based on the successful application of CAR-T in B-line tumors, CAR-

NK has also achieved remarkable results in B-line lymphoma and leukemia. The results of clinical studies of CAR-NK targeting CD19 in the treatment of B-line tumors showed that CAR-NK cell therapy achieved a response rate of 73% and a complete response rate of 64% in patients [41]. The feasibility and efficacy of the clinical application of CAR-NK was determined for the first time. The results of a Phase 1 and Phase 2 trial of anti-CD19 CAR-NK cell therapy in 11 patients with relapsed or refractory CD19-positive cancer showed that most patients responded to CAR-NK cell therapy without serious side effects [42]. CAR-T cell infusion targeting CD19 for the treatment of recurrent B-ALL and some recurrent Non-Hodgkin Lymphoma (NHL), with a complete clinical response rate of 70%~90%. Among all solid tumors, Glioblastoma (GBM) obtained the broadest preclinical data on CAR-NK cell therapy. Although NK cells found in GBM tissue have been found to be inhibited by TME, the fact that approximately 89% of GBM is naturally infiltrated by NK cells suggests that the transport of CAR-NK cells into tumor tissue is justified. In a study of 9 GBM patients, intravenous or intertumoral infusion of unmodified, activated autologous NK cells has been evaluated, with 4 patients developing partial/mixed responses to the test therapy and 3 achieving disease stabilization [43]. The outcomes obtained were transient due to the strong immunosuppressive effect of TME. However, the findings suggest that NK cells have considerable potential in GBM treatment.

III. The Valuable Issues of NK Cells Therapy in Clinical Treatment

A. CAR-T

In recent years, tumor immunotherapy has attracted much attention. Among them, the most commonly used in clinical treatment are CAR-T cells, which have a great effect on tumor treatment.

1) Advantages of CAR-T

CAR-T cell therapy belongs to adoptive cell therapy, which is based on CAR structure, combining the high affinity of antigens and antibodies with T cell killing, and enabling T cells to specifically recognize tumor antigens and kill tumor cells through genetic modification [44]. CAR-T cell therapy has many advantages in immune cells. For example, it uses CAR specifically bound to cell surface antigens to precisely identify target cells and kill them to treat tumors. It also avoids tumor cells from escaping through MHC downregulation, because the CAR structure allows T cells to directly recognize tumor cells. At the same time, CAR recognizes not only peptide antigens but also carbohydrates and glycolipid antigens. In turn, the range of tumor antigen targets is expanded, so that one CAR may be effective for multiple tumors containing the same target antigen.

2) Limitations of CAR-T

a) Off-target effects

Because CD19 is expressed only in B-cells, CD19-targeting CAR-T can be effectively used in the treatment of B-cell leukemia. However, solid tumors are heterogeneous and lack the expression of specific tumor-associated antioxidants such as CD19. On the one hand, the expression of tumor-associated antigens Mesothelin (MSLN), HER2, MUC1, etc. all showed heterogeneity, resulting in CAR-T can only preferentially clear tumor cells with high antigen expression and not completely clearing the tumor, eventually leading to tumor recurrence. On the other hand, most of the target antigens of tumor treatment will also have trace amounts of expression in normal tissues of the human body, resulting in CAR-T cells killing tumor cells while also causing damage to normal tissue cells, resulting in off-target effects [45]. Therefore, improving the specific targeting ability of CAR-T in tumors is a key way to solve the challenge of tumor-specific antigenic deficiency.

b) Tumor microenvironment

Unlike hematological tumors, there are a large number of fibrous matrix and immunosuppressive cells in the solid tumor microenvironment, which can protect tumor tissue against attack by immune cells through both physical and immune barriers [46]. In terms of physical barriers, fibrous connective tissue and other stromal components in the solid tumor microenvironment physically prevent immune cells from contacting tumor cells and preventing immune cell infiltration. The stroma consists mainly of an extracellular matrix, of which Heparin Sulfate Proteoglycans (HSPG) are the main components of CAR-T cells that must enter the tumor to degrade. In addition, the low internal pH and low oxygen content of highly dense solid tumor tissue prevent CAR-T cells from sufficient chemotaxis, infiltrate the tumor, and replicate in large numbers.

B. Advantages of CAR-NK Compared to CAR-T

The transduction of patient-derived T cells by CARs has become a milestone in the treatment of patients with hematological tumors in recent years. CAR-T cell therapy has become one of the most sophisticated examples of personalized medicine. However, although CAR-T cell therapy has been shown to have antitumor activity, it has some drawbacks: production is time-consuming and requires advanced technical support, and the therapy is very expensive and has significant toxicity characteristics [47]. Given these drawbacks, replacing T cells with NK cells, known as CAR-NK therapy, maybe a better option, one that maintains the effectiveness of CAR-T but without drawbacks [48]. The fact that CAR-NK cell therapy does not require HLA or KIR (killer Ig-like receptor) compatibility, does not involve severe toxicity and is available on a large scale from multiple sources makes it a truly off-the-shelf product and a very promising natural successor to CAR-T cell therapy.

In contrast, CAR-NK cell therapy has a better safety profile than CAR-T. It reduces the likelihood of CRS, Immune effector Cell-Associated Neurotoxic Syndrome (ICANS), and Graft-Versus-Host Disease (GVHD). At the same time, the tumor-killing mechanism of CAR-NK cells is more diverse. In addition, CAR-NK has a versatile existing product preparation and is more feasible.

Because NK cells come from a wider range of sources, allogeneic NK cells are safer. Therefore, it can solve the current problems of long production cycles and individual demand for CAR-T products.

C. Defects in NK Cell Therapy

1) Transport disorders of NK cells

Enhancing the migration and infiltration of CAR-NK cells to tumors is of great significance for improving the anti-tumor effect of immune effector cells in vivo [49]. Since chemokines are essential in leukocyte transport to tumors and involve interactions between activated endothelial cells and circulating lymphocytes, the addition of chemokine receptors to CAR may enhance NK cell infiltration of solid tumors. CXCR4 has chemotaxis against CXCR12/SDF-1α, which is the same as the control group and YTSMR1. 1-DAP12 compared to YTSMR1. 1-DAP12/CXCR4 had greater migration ability against U87-MG EG**-** FRvIII/SDF1-α target cells, reducing average tumor growth in xenograft mice. In addition, transfection of CXCR1 enhances the migration of NKG2D CAR-NK cells to IL-8-secreting tumor cells, effectively reducing tumor burden.

2) Targeted antigen suppression

Tumor heterogeneity exists in almost all solid tumors, which poses a significant barrier to CAR-engineered cell therapy [50]. However, in addition to MHC I's recognition of tumor surface-specific markers, NK cells themselves can also rapidly kill targeted cells through multiple mechanisms without any antigen triggering. In addition, the preparation of dual and/or multiplex-specific targeted CAR may lead to solutions. GEN-BLER S *et al.* countered GBM by generating CAR NK-92 that double-targeted EGFR and EGFRvIII, reducing the risk of immune escape and prolonging the survival time of tumor-bearing mice.

D. Key Issues in NK Cell Therapy

1) Preparation of NK cells

Products enriched with NK cells obtained by leukocyte isolation should be infused strictly according to operating specifications. Allogeneic NK cell transfusions containing T cells need to be inactivated below a critical threshold to avoid inducing a graft-versus-host response. The amplification experimental design provides a large number of activated NK cells for adoptive therapy, however, phenotypic alterations, lineage deviations, and selective expansion of specific subsets of NK cells are possible. It is also necessary to investigate the extent to which in vitro treatment alters NK cells' ability to interact, transport, and homing between cells. In studies of adoptive therapy of T cells, it has been found that more potent in vivo antitumor effects can be obtained by utilizing a population of primitive immune cells with strong proliferative capacity. If these are applied to NK cells, it suggests that activated NK cells that were first used in experiments or short-term in vitro culture during adoptive immunotherapy have better anti-tumor potential than long-term activated NK cells.

2) How to improve the function of NK cells

Developing new pathways to activate endogenous NK cells or using drugs to modulate host tumor cells will overcome tumor tolerance by increasing the expression of activated NK cell receptor ligands or making them more sensitive to NK cell killing. Therefore, future clinical research will focus on the ability to develop hematopoietic growth factors, such as ligands for receptor tyrosine Kinase (KIT) and FMS-Like Tyrosine kinase 3 (FLT3), which are used to activate and expand NK cells in vivo. Other strategies have been used in clinical trials, such as the use of monoclonal antibodies to block inhibitory KIR, thereby enhancing NK cell recognition of tumor cells. NK cells can also be genetically engineered before adoptive transfer to patients. As an alternative to the use of KIR-blocking antibodies, small interfering RNAs (siRNAs) may be considered to silence inhibitory receptors. Alternatively, by overexpressing activated receptors on NK cells or by introducing novel chimeric receptors that recognize ligands expressed by tumor-binding signaling components, thereby triggering immune cell function.

3) Criteria for NK cell donor selection

A necessary condition for NK cell allogeneic reactions is the lack of one or more KIR ligands presented to the donor. Although individual donors and recipients do not match at the genetic levels regarding KIR and HLA, when KIR's own HLA-I molecule and CD94-NKG2A are both lacking, the size of the alloreactive cell population can vary from 1% to 50%. Thus, prediction of treatment effect is based on assessment of NK cell populations and selection of donors with a large number of alloreactive NK cell populations, which is feasible for donors of KIR haplotype A, where genetic analysis can be used to determine the size of the alloreactive cell population in combination with phenotyping by multiparametric flow cytometry analysis when these donors lack most of the activating KIR gene. However, this is more complicated for donors of KIR haploid B with a large number of activated KIRs, and most of the antibodies used to measure KIR expression do not distinguish the inhibitory type from the activated KIRs.

IV. Conclusion

Some surgeries and chemotherapy can treat and remove tumors, but they still have defects. Among them, the biggest problem is that it cannot be completely eradicated, resulting in secondary relapses, which necessitate continuous treatment. Therefore, immune cells such as NK cells can provide new treatment options from the perspective of tumor immunotherapy. NK cell immune checkpoint blockade therapy or CAR-NK therapy are gradually undergoing clinical trials. At the same time, there are already many clinical examples to prove its potential.

NK cells also have certain advantages over other immune cells, such as can accurately identify target cells and kill them through CAR specifically bound by cell surface antigens to treat tumors. It also avoids tumor cells from escaping through MHC downregulation, because the CAR structure allows T cells to directly recognize tumor

cells. At the same time, it can recognize carbohydrates and glycolipid antigens, thereby expanding the range of tumor antigen targets. Compared to T cells, NK cells are safer and have a wider range of sources.

Even so, NK cells still have limitations. The heterogeneity of solid tumors poses a huge obstacle to CAR-NK cell therapy. The preparation of double-targeted CAR-NK may lead to new solutions. In addition, NK cells still need to solve some key problems. The prediction of therapeutic effect is based on the evaluation of the NK cell population and the selection of donors with a large allogeneic active NK cell population. At the same time, overcoming tumor tolerance by increasing the expression of activated NK cell receptor ligands or making them more sensitive to NK cell killing, will focus future clinical research on the ability to develop hematopoietic growth factors. In addition, if a population of primitive immune cells with strong proliferative ability is applied to NK cells, it has better anti-tumor potential.

If the existing key problems of NK cells can be solved and multiple clinical trials can be carried out, NK cell immunotherapy will become an indispensable method in tumor treatment.

CONFLICT OF INTEREST

The author has claimed that no conflict of interest exists.

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