Application and progress of CAR therapy on malignances: from CAR-T to CAR-NK

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Abstract:
For some malignances that are highly aggressive and metastatic are usually considered incurable. However, with the advent of immunotherapy, this therapy could be possibly effective after other three major therapies: surgery, radiation, chemotherapy. As an emerging immunotherapy, chimeric antigen receptor (CAR) T cells have been applied to treat hematologic malignances and some patients achieved long-term remission and temporary cure. However, CAR-T cells also bring side effects such as cytokine release syndrome (CRS), neurotoxicity, etc. Moreover, CAR-T cells did not perform well in solid tumors, for the reason that CAR-T cells are difficult to break through external barrier of solid tumors, at the same time maintain cytotoxicity and persistence inside. Therefore, we introduce CAR-NK cells therapy on the basis of CAR-T cells. At present all CAR-NK cell therapies are in clinical trials, its effectiveness has been initially observed. Compared with CAR-T cells, patients with hematologic malignances received CAR-NK cell therapies showed few side effects such as CRS and neurotoxicity.

Keywords: CAR-T, CAR-NK, therapy, malignances

1. Introduction
1.1. Background on CAR therapy
For some malignances that are highly aggressive and metastatic are usually considered incurable. However, with the advent of immunotherapy, this therapy could be possibly effective after other three major therapies: surgery, radiation, chemotherapy. As an emerging immunotherapy, chimeric antigen receptor (CAR) T cells have been applied to treat hematologic malignances and some patients achieved long-term remission and temporary cure. However, CAR-T cells also bring side effects such as cytokine release syndrome (CRS), neurotoxicity, etc. Moreover, CAR-T cells did not perform well in solid tumors, for the reason that CAR-T cells are difficult to break through external barrier of solid tumors, at the same time maintain cytotoxicity and persistence inside. Therefore, we introduce CAR-NK cells therapy on the basis of CAR-T cells. At present all CAR-NK cell therapies are in clinical trials, its effectiveness has been initially observed. Compared with CAR-T cells, patients with hematologic malignances received CAR-NK cell therapies showed few side effects such as CRS and neurotoxicity.

2. Development of CAR therapy: CAR-T cells
2.1. CAR Structure and design
CAR is a synthetic receptor that binds to a target cell surface antigen without MHC participation and lead cytotoxic immune cells to target cells expressing that antigen. Its function is to recognize target antigens and transmit antigen signals to activate immunoreactions. Moreover, it does not require the involvement of the TCR-CD3 complex, in other words, it detours the traditional route. There are four main components of CAR: extracellular antigen binding domain, spacer or hinge region, transmembrane domain and intracellular signaling domain. The antigen-binding domain is usually composed of the variable regions of an antibody heavy (VH) and light (VL) chains and these two chains are connected by a flexible linker to form a single-chain fragment variable (scFv). In addition, scientists can design a protein or peptide to replace scFv. Compared with a TCR recognizes antigens via MHC, scFv and other synthetic structures determine target specificity and bind to target antigens(Figure1). The hinge region is the spacer region that exposes the antigen-binding domain on CAR T cell surface for binding to target antigens. The length of the hinge region depends on the location of the target antigen. Target antigens close to the cell membrane usually require a longer hinge region, whereas target antigens close to the cell surface have a shorter hinge region. The major function of the transmembrane domain is to dock CAR in the immune cell mem-
brane \(^1\). Some studies, however, show that this region can affect CAR activity, expression, stability, dimerization and signal transduction \(^2,3,4\), even the transmembrane domain in the anti-HIV CAR structure can participate in the expression of CD4 on the cell surface\(^5\). The intracellular signaling domain is the main structure that plays a role in the structure of CAR, and there have been three generations of CAR-T. The first generation CAR-T cells contain a CD3ζ signaling domain, however, its antitumor activity requires a two-step initiation to be optimal \(^6,7\). Therefore, second-generation CAR-T contains a co-stimulatory domain in addition to a CD3ζ signaling structural domain to improve its function. And the third-generation CAR-T contains two co-stimulatory domains in addition to a CD3ζ signaling structural domain. The most commonly used co-stimulatory domains are CD28 and 4-1BB and are FDA-approved. CD28 and 4-1BB have been shown to favor T cell reinforcement. The combination of CD28 and CD3 contributes to the differentiation and persistence of memory T cells, increases mitochondrial biogenesis, enhances fatty acid oxidation and oxidative metabolism. CD28 was observed to cause an increase in glucose utilization and upregulation of glycolytic enzyme expression \(^8,9\). 4-1BB increases mitochondrial biosynthesis and enhances fatty acid oxidative metabolism \(^10\). Co-stimulatory domains currently in the experimental stage include OX40, CD27 and inducible T-cell stimulator (ICOS), etc. OX40, normally induced after T cell activation, regulates Tregs glycolysis and lipid metabolism and promotes T cell expansion and generation of memory cells through a TNF receptor-associated factor 2 (TRAF2)-dependent mechanism \(^11\). Integration of the CD27 cytoplasmic domain into a CAR construct enhances T cell expansion, effector functions as well as survival and augments T cell persistence and anti-tumor activity in vivo \(^12\). ICOS is critical for the expansion and differentiation of helper T cells (Th17) \(^13\). Currently, in the third-generation CAR-T structures that have been designed, the combination of CD28 and 4-1BB enhances the ability of CAR to bind to antigen, increases its proliferation and central memory differentiation, and improves its in vivo activity \(^14\).

2.2. Successful and fail experience on CAR-T therapy

Five types of CAR-T cells have been approved by the FDA. All five CAR-T cells target markers on the surface of B cells, four targeting CD19 and one targeting B cell maturation antigen (BCMA). All five cells have shown promising results in refractory or recurrent hematologic tumors such as lymphomas and leukemias of B cell origin and multiple myeloma. The first FDA-approved CAR T therapy is tisagenlecleucel (Kymriah\(^\text{TM}\)), based on a multicenter study of 75 pediatric and young adult patients with relapsed or refractory B cell precursor acute lymphoblastic leukemia (ALL) \(^15\). Within three months, this cohort achieved an overall remission rate of 81%, with 60% of patients in complete remission. Fifty five out of 75 patients (73%) had a grade 3 or 4 tisagenlecleucel-related adverse event. Grade 3 and 4 cytokine release syndrome (CRS) occurred in 21 and 25% of patients, respectively, with 35 of 75 patients (47%) being admitted to the intensive care unit (ICU) for its management \(^16\). Based on these results, CAR-T therapy and tisagenlecleucel have been successively approved by the FDA and used for the treatment of refractory or relapsed large B-cell lymphoma, including diffuse large B-cell lymphoma (DLBCL), high-grade B-cell lymphoma and DLBCL arising from follicular lymphoma.

Axicabtagene ciloleucel (Yescarta\(^\text{TM}\)) became the second FDA approved CAR T therapy on October 18, 2017 for large B cell lymphoma, including DLBCL, primary mediastinal large B-cell lymphoma, high-grade B-cell lymphoma and DLBCL arising from follicular lymphoma, after at least two lines of systemic treatment. A multi-center ZUMA-1 trial with 101 patients showed an 82% objective response rate (54% CR) and 52% overall survival rate at 18 months. Grade 3 or higher CRS occurred in about 13% of patients \(^17\). The third CAR T therapy, Brexucabtagene autoleucel ( Tecartus\(^\text{TM}\)), was approved for relapsed or refractory mantle cell lymphoma by the FDA on July 24, 2020 based on a single-arm, open-label ZUMA-2 trial. In this multicenter Phase II trial with 74 patients enrolled, 68 patients took the medication. The overall response rate was 93% with 67% CR. At 12 months, the overall survival was 83%. Grade 3 or higher CRS occurred in about 15% of patients \(^18\).

Lisocabtagene maraleucel (Breyonzi\(^\text{TM}\)) is the most recently approved CD19-targeting CAR T therapy against relapsed or refractory large B-cell lymphoma, after two or more lines of systemic therapy, including DLBCL not otherwise specified (including DLBCL arising from indolent lymphoma), high-grade B-cell lymphoma, primary mediastinal large B-cell lymphoma, and follicular lymphoma grade 3B \(^19\). In this multicenter TRANSCEND trial with 192 evaluable patients, the objective response rate was 73%, with 53% CR, and the median duration of response was 17 months. Grade 3 or higher CRS occurred in about 2% of patients.

Idecabtagene vicleucel (Abecma®) is the only FDA approved CAR T therapy not targeting CD19. It targets BCMA on multiple myeloma (MM) cells and was approved by the FDA on March 26, 2021 for relapsed or refractory MM. Of 128 patients who received treatment,
2.3. Limitation of CAR-T therapy

2.3.1. Antigen selection

Despite the remarkable success of CAR-T cell therapy in hematologic malignancies, one of the reasons for its limited role in solid tumors is antigen selection. There are few antigens in solid tumors that are expressed only in tumor tissue and not in normal tissue. As a result, some animal experiments or clinical trials in which subjects undergoing CAR-T cell therapy experienced tumor remission have also shown significant on-target, off-tumor toxicity[21-32]. Usually, specific antigens on the surface of a tumor are generally formed by mutations in the genes controlling the expression of the antigen, which are full of randomness and diversity. Therefore, CAR-T cells are designed for such antigens. And other antigens expressed in tumor tissues are also expressed in normal tissues, which has been confirmed by many experiments.

2.3.2. Insufficient efficiency of CAR-T cell trafficking and infiltration into tumor tissue

How CAR-T cells are trafficked into solid tumor tissue after injection is also a major issue. The dense extracellular matrix containing large numbers of cancer-associated fibroblasts forms a physical barrier that prevents CAR-T cells from entering the tumor tissue. In addition, dysregulated expression of cytokines within tumor tissues attract suppressor immune cells to inhibit CAR-T cell function. Researchers are currently improving CAR-T cell function through a variety of approaches, such as regulating T cell migration by expressing CXCL9 on CAR-T cells, inhibiting tumor angiogenesis, and enhancing the ability of CAR-T cells to recruit T cells[33]. Expression of CXCR2 on CAR-T cells also improves migration and accumulation of CAR-T cells inside tumors, and co-expression of IL-15 and IL-18 inhibits T cell exhaustion and apoptosis[34].

2.3.3. Hostile tumor microenvironment

The tumor microenvironment (TME) contains many cells within it, and cytokines and a different pH than normal tissue can inhibit CAR-T cells. Regulatory T cells, myeloid-derived suppressor cells (MDSC), and cancer-associated fibroblasts can directly inhibit the function of CAR-T cells. Immunosuppressive cytokines, vascular endothelial growth factor, transforming growth factor (TGF-B), IL-4, IL-10 can lead to dysfunction of T cells and promote the infiltration of immunosuppressive cells. The acidic environment inside the tumor tissue is equally unfriendly for T cells to function.

2.3.4. Antigen escape

In CAR-T therapy, researchers have found that some patients experience relapse and slow progression as treatment progresses. It has been studied that mutations in the target antigen gene on the tumor surface can lead to loss of function of the target antigen. In addition, the alteration of target antigen mRNA can lead to a shift in target antigen phenotype from sensitive to resistant[34]. In addition, the target antigen is transferred to T cells during the treatment process, not only the density of target antigen on the tumor decreases, but also the T cells will kill each other[35]. There are also many studies devoted to how to prevent target antigen loss.

2.3.5. Systematic toxicity

CAR-T cells, while exerting an anti-tumor effect, also produce large side effects. T-cell activation releases large amounts of cytokines, causing cytokine release syndrome (CRS). The main manifestations are fever, hypotension, hypoxia, and multi-organ failure[36]. Cytokines can cross the blood-brain barrier, while increased levels of cytokines in the cerebrospinal fluid can lead to neurotoxicity, manifesting as aphasia, altered mental status, tremor, seizures and headaches. In addition, hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS) are the main side effects, which are characterized by fever, hepatosplenomegaly, liver function abnormalities, pancytopenia, increased levels of methemoglobin[37], increased levels of glycoferrin triphosphate, hypertriglyceridemia, and hypoglycemia, with a high mortality rate.

2.3.6. Insufficiency of CAR T cell expansion and persistence

Expansion and persistence of CAR-T cells in tumor tissues are also key to sustaining their role, especially for malignancies that require long-term treatment. In solid tumor tissues, an unfriendly tumor microenvironment leads to decreased CAR-T cell expansion and persistence. There are many ways to enhance the expansion and persistence of CAR-T cells, such as transducing genes that express promotive cytokines into CAR-T cells, which can be induced when the T cells are exposed to antigens[38]. A panel of cytokines including IL-12, IL-7, IL-15, IL-18, IL-21 and IL-23 are currently being investigated and entering early phase clinical trials[39-42].

2.3.7 Poor source and high cost

CAR-T cells are derived from a single source, mainly the patient’s own peripheral blood, as the use of allogeneic sources of T cells can produce severe GVHD. After the T cells are extracted from the patient, they need to be...
3. Applications of CAR-NK Cell Therapy in Solid Tumor Treatment

3.1. CAR-NK cell source and structure

Researchers have explored different sources of NK cells for producing and expressing CAR\[^{45}\]. Currently, there are four main sources of NK cells. The first one is the immortalized human cell line NK92, derived from human extracellular NK cell lymphoma. Its advantage lies in its strong ability to expand in vitro, but due to its malignant tendency, it must be irradiated before use\[^{46}\]. This also reduces its survival in the peripheral blood of the recipient. In addition, NK92 cells are naturally deprived from the CD16 domain and cannot trigger ADCC, the intrinsic mechanism of NK cell anti-tumor activity\[^{47}\]. NK cells can be obtained from the donor’s peripheral blood, which is rich in mature NK cells that do not require HLA/KIR matching. In addition, PB-derived cells respond more efficiently and are more persistent in circulation compared to cells from other sources. NK cells can be obtained from the donor’s peripheral blood, which is rich in mature NK cells that do not require HLA/KIR matching. In addition, PB-derived cells respond more efficiently and are more persistent in circulation compared to other sources. The disadvantages of PB-derived NK cells are that they do not expand easily in vitro and PB-derived NK cells are at various stages of maturation, making it difficult to standardize them\[^{48-50}\]. Umbilical cord blood is a carryover source of NK cells, which has the advantage of being more capable of in vitro expansion than PB-derived NK cells, but less cytotoxic. The stimulation of immunocompetent progenitor stem cells (ipscs) are harvested from the mobilized peripheral blood of the donor or from UCB. The CAR construct is transduced into iPSCs, which are then differentiated into CAR-expressing NK cells by incubation in a cytokine cocktail of SCF, VEGF, BMP4, IL3, IL-15, IL-7 and FLT3L. This NK cell has the advantage of being able to produce a large number of homogeneous NK cells from a single iPSC\[^{51}\] and the disadvantage of low cytotoxicity\[^{52,53}\]. The current structure of CAR expressed by NK cells is similar to that of T cells, which consists of four regions: extracellular antigen binding domain, spacer or hinge region, transmembrane domain, and intracellular signaling domain. Unique CAR structures designed for NK cells are also currently available, the effects produced vary widely. However, these different CAR constructs exhibited varying effects on cytotoxicity and cytokine production in NK cells\[^{54,55}\]. The intracellular signaling domain of the first generation CAR includes an activation domain that transmits an activation signal to activate NK cells upon binding of the activation receptor to the ligand. Currently many structures can act as activation receptors, which largely depends on the choice of ligand on the target cell. The most thoroughly studied is NKG2D, a homodimeric receptor that recognizes the stress-inducing ligands MICA, MICB, and ULBP1-6 expressed on damaged, transformed, or otherwise abnormal cells\[^{56}\], signaling through the adapter molecule DAP10 to trigger NK activation. Here DAP10 acts as an activation domain and is part of the CAR structure. In addition, other activation domains such as DAP12, CD3δ, FcεRIγ, etc. function in conjunction with specific activating receptors. The intracellular signaling domain of second-generation CARs adds a co-stimulatory domain, and CD28 and 4-1BB, which are widely used in T cells, also enhance anti-tumor effects in NK cells. However, 2B4 has been reported to have stronger anti-tumor, apoptosis-reducing, and cytokine expression-enhancing effects compared to CD28\[^{57}\]. While the intracellular signaling domains of the third-generation CARs contain multiple co-stimulatory structural domains, the fourth-generation adds to the third-generation structures expressing specific cytokines to further enhance the anti-tumor capabilities of NK cells.

3.2. Advantages of CAR-NK cells.

3.2.1. Safety:

Compared to T cells, NK cells have a shorter lifespan and rapidly die off after their effects, which prevents them from remaining in the body in large numbers to continue their side effects, as is the case with the longer-lived T cells, which continue to have side effects after their anti-tumor effects. In addition, there are more sources of NK cells, so patients can choose suitable exogenous NK cells
to receive treatment at any time without having to wait for a long period of time for expansion and cultivation. NK cells and T cells have different cytokine secretion spectra, with NK cells secreting IFN-γ and GM-CSF[58], whereas T cells primarily induce cytokines such as IL-1α, IL-1α, IL-2, IL-2Ra, IL-6, TNF-α, MCP-1, IL-8, IL-10, and IL-15, which are highly correlated with CRS, and can lead to severe neurotoxicity. NK cells and T cells have different cytokine secretion spectra, with NK cells secreting IFN-γ and GM-CSF, whereas T cells predominantly induce cytokines such as IL-1α, IL-1α, IL-2, IL-2Ra, IL-6, TNF-α, MCP-1, IL-8, IL-10, and IL-15, which are highly correlated with CRS, and can cause severe neurologic toxicity[59]. Moreover, the GVHD phenomenon is rarely observed in the CAR-NK experiments performed so far[60].

3.2.2. Dual killing mechanism:
CAR-NK cells have a dual mechanism of killing tumor cells, a CAR-independent mechanism and a CAR-dependent mechanism. In the CAR-dependent mechanism, activated NK cells lyse cells by releasing perforin and granzymes. NK cells also express transmembrane receptors, such as natural cytotoxicity receptors (NCRs), KIRs, NKG2D, or DNAM-1, etc(table1), which can induce kappa-mediated apoptosis in recognized cancer cells. The secretion of IFN-γ leads to the recruitment of macrophages and dendritic cells, thereby promoting other antitumor mechanisms[61,62]. In addition, NK cells can kill cancer cells mediated by ADCC. CD16 is key to ADCC, recognizing the Fc fragment of immunoglobulin G (IgG) that binds to epitopes on the surface of tumor cells[63]. The CAR-dependent mechanism means that researchers design special CAR structures according to the needs of different types of tumors. The most commonly used structures include CD28, CD3ζ, DAP10, DAP12, 4-1BB, 2B4, etc.

3.2.3. Multiple sources:
As mentioned before, NK cells have rich sources, and NK cells from different sources play different advantages in treating different tumors. Meanwhile, no significant GVHD and other side effects appeared in the experimental process of NK cells from different sources.

3.3. Optimization of CAR-NK cell function
3.3.1. Maximizing CAR-NK cell survival:
Unmodified NK cells can only survive for 1 week in the infused circulation[64,65]. Application of IL-2 and IL-15 after infusion of CAR-NK cells enhanced NK cell expansion and activation. When IL-2 and IL-15 were used in vitro, the function of NK cells decreased dramatically when the cytokines were withdrawn[66]. IL-15 is more when applied in vivo because IL-15 is highly selective for NK cells and does not stimulate regulatory T cells[67,68], whereas IL-2 causes systemic toxicity, such as capillary leakage syndrome, which results in rapid NK cell exhaustion and other side effects[69,70]. The use of rhIL-15 or the IL-15 engineered molecules ALT-803[71] and NKTR-255[72-74] showed minimal stimulation of CD4+ T cells and regulatory T cells and a good expansion status of NK cells, but with lower potency and also with side effects[75,76]. The investigators will attempt to transduce the interleukin gene into NK cells, prompting the NK cells to express interleukin on their own, thus eliminating the need to receive exogenous interleukin. Investigators will introduce the membrane-bound IL-15 gene (mbIL-15) into CAR-NK cells and observe an increase in survival and proliferation of NK cells, but no therapeutic effect[77]. A study showed that CISH gene expression of CIS (cytokine-inducible SH2-containing protein) inhibits the expression of IL-2 and IL-15. Introduction of membrane-bound IL-15 gene into UCB-derived CAR-NK cells knocked down for CISH gene significantly enhanced the therapeutic potential of CAR-NK cells[78]. Some studies have reported that infection with CMV increases the expansion of memory-like NK cells, and it was found that CMV caused expansion and CD94/NKG2D overexpression in a subpopulation of CD56low NK cells deficient in FcɛRγ, SYK, and EAT-2, while decreasing the expression of PD-1, TIGHT, and NKG1A checkpoint receptors[79,80]. Incubation of NK cells with a variety of cytokines such as IL-2, IL-15, and IL-18 mediated the formation of memory-like NK cells, and the nature and function of such NK cells were similar to those formed by CMV infection[81]. Preclinical studies have shown that implanting CAR into memory-like NK cells increases their survival and cytotoxicity[82]. In addition, memory-like CAR-NK cells mediated stronger ADCC, resisted the inhibitory effects of Treg and MDSC and survived longer in solid tumors[83]. Although CAR successfully transduced memory-like NK cells with low efficiency (15%-25%), they were able to expand in large numbers in vivo to correspond to the CD19 antigen[84]. CAR-memory-like NK cells also demonstrated stronger degradation and IFN-γ-based responses in vitro and in mouse acute myeloid lymphoma (AML)[85].

3.3.2. Expansion and activation:
Expansion and cultivation of CAR-NK cells has been a big problem. Combining CAR-NK cells with cytokines IL-2, IL-15, IL-18, IL-21, etc. can increase the expansion of CAR-NK cells in vitro, but they are quickly exhausted. One study showed that irradiated B-lymphoblasts stimulated NK cell expansion in vitro. Both early T cells and NK cells can expand, NK cells can expand 25 times, and T cells can expand 3 times. After removing CD3(+)/CD5(+)
T cells, a large number of CD16(+)NKH-1(+) cells can be obtained\[85\]. Several studies have found that cultivation of immortalized K562 cell lines enhances NK cytotoxicity by 400-fold. PB-derived NK cells and modified K562 cell lines expressing 4-1BBL and mIL-15 expand 1000-fold in the absence of T-cell co-stimulatory factors for three weeks\[86\]. By genetically engineering human leukocyte antigen (HLA)-A and HLA-B K562 cells, the expression of CD48, 4-1BBL and membrane-bound IL-21 was enhanced, forming a universal antigen-presenting cell that stimulates NK cells. The homologous receptor on the drug can expand CAR and non-transduced NK cells more than 900 times within 2 weeks, with high purity and strong persistence\[87\].

3.4. CAR-NK cell modification against solid tumors

Although NK cells have shown many advantages over T cells, they still encounter many difficulties when applied to solid tumors. The dense extracellular matrix, suppressive immune cells, and cytokines in solid tumors significantly reduce NK cell function. Therefore, it is crucial for CAR-NK cells to be modified to combat the hostile tumor microenvironment.

3.4.1. Resistance to the tumor microenvironment

NK cells found in the TME are immature and have low toxicity\[88,89\]. An important reason is that tumor-related secretes a large number of inhibitory cytokines, such as adenosine, TGFβ, IL-10, etc. TGFβ can reduce the recruitment of functional NK cells. Downregulates the activating receptors of NKG2D and DNAM1 and prevents the secretion of perforin\[90\]. UCB-derived NK cells expressing TGFβ negative receptor II do not have this phenomenon and can maintain their killing ability against CML and other tumor cell lines\[92\].

3.4.2. Combat heterogeneity

Tumor heterogeneity is extremely common in solid tumors and is a major problem faced by immunotherapy. Cancer stem cells (CSCs) are highly prevalent in solid tumors and are a major contributor to drug resistance and long-term treatment\[93\]. In CAR-T therapy, CAR initially combines with tumor antigens to achieve a therapeutic effect. However, as time goes by, the antigen density on the tumor surface decreases, and gene mutations within the tumor lead to changes in the antigen structure. The original CAR structure becomes ineffective, and eventually leading to tumor recurrence and poor efficacy\[94,95\]. Due to the dual tumor immune mechanism, CAR-NK cells can mediate ADCC to kill tumor cells through CD16. To combat CSCs, researchers designed PD-L1-directed CAR-NK cells to overcome the problem of T cell escape. In addition, IFN-γ and TNFα produced by NK cells can promote the differentiation of CSCs, causing them to lose chemotherapy resistance and self-renewal capabilities. Related products are being designed\[96\].

3.4.3. Trafficking and infiltration

CAR-NK cells overexpressing CXCR4 can significantly increase tumor infiltration compared with other CAR-NK cells\[97,98\]. CXCR1 is an IL-8 receptor that can move along the IL-8 concentration gradient after activation. Various cancers, including pancreatic cancer and ovarian cancer, can secrete IL-8. Therefore, overexpressing CXCR1 receptor NKG2D.CAR-NK cells are more toxic in vivo\[99\]. In addition, studies have shown that the concentration of NK cells within tumors is related to the CXCL16 gradient, and NK cells have the ability to reduce the volume of tumors overexpressing CXCL16\[100\].

3.4.4. Intrinsic modification

Activating the NKG2D receptor induces granule-dependent and cell-mediated cytotoxicity, but activating it alone is not sufficient to produce IFNγ\[101\], so the researchers combined NKG2D with DAP10 to activate the downstream effects of PI3K to enhance killing. The study also found that NKG2D.DAP10.CD3ζ.PBNK can also improve the anti-tumor activity of modified NK cells against a variety of tumor cells\[102\]. In addition, downregulation of inhibitory receptors can also enhance killing. For example, inhibitory receptors for NKG2D that inhibit NK cells can block their signal transduction and increase the toxicity of NK cells against HLA-E-expressing cancer cell lines by 40%\[103\].

3.5. Success in Hematological Malignancies

At the beginning, the successful cases of CAR-NK cell therapy were all tumors of the hematological system. In 2020, Liu et al. conducted a clinical trial in which 11 patients with CD19-positive hematological malignancies (non-Hodgkin lymphoma, chronic lymphocytic leukemia) received CD19-directed CAR-NK cell therapy, 8 of whom There were objective responses, and 7 patients still had complete remission without residual disease after 30 days of treatment. The study analyzed UCB-derived NK cells after retroviral transduction with CD28.CD3ζ endodomain, IL-15 and iCas9 (inducible caspase 9) safety switch and activation/expansion with K562 feeder cells and IL-2. The author speculates that the transformation efficiency is 49%. In this study, no serious adverse reactions such as CRS and GVHD occurred. The dose of CAR-NK cells used in this study reached 1*10^7 but did not reach the tolerated dose. The CAR-NK cells survived in the body for at least 12 months. This study also became a milestone in CAR-NK therapy, demonstrating the pow-
erful effects and low side effects of CAR-NK cells\textsuperscript{[104]}. Other directed CAR-NK cells are also in the research stage. One study used baboon enveloped pseudotyped viral vectors to transduce blood-borne primary NK cells to generate CD33-directed CAR-NK cells, which can detect CD33-positive OCI in vitro. -AML2 and primary AML cells show stable expression, proliferation and stronger cytotoxicity. In addition, CD33-CAR-NK cells showed strong killing effects in the OCI-AML2 xenograft mouse model, preventing the spread of leukemia cells without side effects\textsuperscript{[105]}. Another study designed a drug targeting IL-3 receptor subunit α (CD123), which is strongly expressed on the surface of acute myeloid leukemia (AML) cells. This experiment used a CD123-targeted CAR-NK92 cell line paired with an IL-15 gene cassette. The researchers conducted studies in vitro and in vivo in mice, and found that CD123-CAR-NK cells had stronger anti-AML activity in vitro and in vivo\textsuperscript{[106]}

### 3.6. Application of CAR-NK cells in solid tumors

Currently, many in vitro and in vivo experiments are testing the role of CAR-NK cells in solid tumors, and some initial results have been achieved.

#### 3.6.1. Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is known for its rich immune mechanisms, and its extracellular matrix accounts for 70% of the entire tumor volume. A Korean study showed that folate receptor α (FRα) and death receptor 4 (DR4) are highly expressed in PDAC, and targeting FRα and DR4/5 to CAR-NK cells can significantly enhance the efficacy of these two receptors. PDAC cell apoptosis. This experiment confirmed that FRα-directed TRAIL.CD27.CD3ζ.CAR-NK92 cells have a high killing effect on PDAC cells in vitro and in xenografted PDAC mouse models\textsuperscript{[107]}. Prostate stem cell antigen (PSCA) is a glycosylphosphatidylinositol-anchored cell surface protein that is abnormally overexpressed in 60-80% of pancreatic cancer patients\textsuperscript{[108]} but is not found in normal pancreatic ducts\textsuperscript{[109]}. Researchers evaluated the killing effect of PSCA-CAR-NK cells expressing soluble IL-15 on pancreatic cancer cells in vitro and in vivo. The results showed that this cell had a significant inhibitory effect on PSCA+ pancreatic cancer cells and no toxicity was found\textsuperscript{[110]}. In addition, a study showed that the STING agonist cyclic GMP-AMP (cGAMP) can directly activate NK cells, and mesothelin-targeting CAR-NK-92 cells combined with cGAMP showed potent anti-tumor activity in a mouse model of pancreatic cancer. Tumor effects\textsuperscript{[111]}. ROBO-1 is an axon guidance receptor and cell adhesion receptor. A study found that CD8.CD3ζ.4-1BB.CAR-NK92 cells targeting ROBO-1 stabilized the condition of patients with pancreatic cancer for up to 5 years. Months later, the only adverse reaction was fever\textsuperscript{[112]}.

#### 3.6.2. Ovarian cancer

Mesothelin (MSLN) is overexpressed in ovarian cancer. A study constructed CD19.NK92.CAR-NK cells targeting MSLN, which can kill MSLN-positive ovarian cancer cells (OVCAR-3 and SK-OV-3). Furthermore, stronger cytokine secretion was detected in MSLN-CAR-NK cells co-cultured with OVCAR-3 and SK-OV-3 compared with parental NK92 cells and CD19.CAR-NK cells. MSLN.CD19.CAR-NK cells can significantly eliminate ovarian cancer cells and prolong the survival time of mice, showing strong activity both in vivo and in vitro\textsuperscript{[113]}. Not only that, researchers have constructed CAR-NK cells targeting CD24 to kill CD24-positive ovarian cancer cells. Researchers also constructed CD24 and MSLN together to enable CAR-NK cells to exhibit excellent killing effects\textsuperscript{[114]}. One study showed that αFR is expressed in 90% of ovarian cancer cells, in three among anti-αFR.CD3ζ, anti-αFR.CD28.CD3ζ and anti-αFR.CD28.4-1BB.CD3ζ CAR-NK cells, the latter has the most cytotoxicity and has stronger degranulation when incubated with αFR-expressing ovarian cancer cells. Effects, cytokine secretion, proliferative capacity and persistence. In a xenogeneic mouse model, treatment with anti-αFR.CD28.4-1BB.CD3ζ.CAR-NK92 significantly extended survival\textsuperscript{[115]}.

#### 3.6.3. Breast cancer

Tissue factor (TF) was designed as an antigen for CAR-NK cells because 50-85% of triple-negative breast cancer molecules express TF. The experimenters designed the TF-directed CD28.4-1BB.CD3ζ.CAR construct to transduce into NK-92MI cells that were deprived of CD16 receptors and NK-92MI cells that were not deprived of CD16 receptors, respectively, as experimental groups and control group. Studies have shown that NK92-MI cells with CD16 receptors can mediate ADCC and have stronger anti-tumor activity\textsuperscript{[116]}. Epidermal cell adhesion molecule (EpCAM) is an antigen commonly found on the surface of various cancer cells. In EpCAM-positive breast cancer cells, CAR-NK92 cells targeting EpCAM and IL-15 have strong cytotoxicity\textsuperscript{[117]}. Furthermore, a HER-2-targeting CD28.CD3ζ.CAR design expressed by NK92 cells also showed antitumor activity against breast cancer in vitro and in vivo\textsuperscript{[118]}.

#### 3.6.4. Glioblastoma

Although NK cells found in glioblastoma (GBM) are inhibited by the TME, NK cells are found in 89% of glioma blasts, suggesting that NK cells can infiltrate GBM. In a trial of nine patients in which NK cells were injected...
intravenously or intratumorally, four responded and three had stable disease. Although the therapeutic effect quickly disappeared due to the strong inhibitory effect of TME, this experiment in humans confirmed the huge therapeutic potential of NK cells[119]. We found that intravenous injection of EGFRvIII-directed DAP12.CD3ζ.CAR-NK-92 cells with CXCR4 receptor overexpression inhibited GBM tumor growth and prolonged survival in a xenograft mouse model[120,121]. Several clinical trials of CAR-T cell therapy in patients with GBM have shown that high anti-tumor activity leads to rapid loss of antigens in cancer cell subpopulations and, despite initial responses, resistance to CAR-T therapy quickly develops[122-124]. If CD28, CD3ζ, CAR-NK-92 cells targeting mutant and wild-type EGFR are simultaneously constructed, compared with CAR-NK cells targeting only one EGFR alone, survival can be prolonged without antigen escape[123]. Intracranial injection of bispecific epidermal growth factor receptor and epidermal growth factor receptor VIII-directed CD28. CD3ζ.CAR-NK-92/NKL prolongs survival in a GBM xenograft mouse model and is characterized by cytotoxicity and IFN - Increased gamma secretion[124]. A number of structures have been designed as targets for CAR-NK cells, and we list these targets in Table 2 (Table 2).

4. CAR-Macrophage

4.1. Introduction to CAR-Macrophage

Although CAR-NK cells have many advantages over CAR-T cells, the obstacles encountered by CAR-T cells during treatment also exist in CAR-NK cells. As mentioned before, the function of NK cells is inhibited in the TME. Although there is currently a study showing that CAR-NK cells can play a role in the TME of GBM patients, the therapeutic effect is short-lived due to the inhibitory effect. In short, CAR-T cells and CAR-NK cells are strongly inhibited in the TME[41], requiring researchers to continuously design more complex CAR structures to arm immune cells, which undoubtedly increases the difficulty and cost of design and manufacturing. As an emerging product, CAR-Macrophage shows great advantages in TME. First, in many malignant tumors, macrophages infiltrate in large numbers[125], and the hypoxic environment induces tumor cells and stroma to produce cytokines, such as CCL2 (C-C matrix chemokine ligand 2), CXCL12 (C-X-C matrix chemokine ligand 12), CSF1 (colony stimulating factor 1) and vascular endothelial growth factor to recruit macrophages[126]. After local macrophages are recruited into the hypoxic TME, soluble cytokine receptors are downregulated, locking macrophages in the TME. In addition, macrophages can sense the hypoxic environment and related metabolites and automatically migrate into the TME. Secondly, the environment of the TME can cause T cell exhaustion, but is favored by macrophages. There are two types of macrophages, pro-inflammatory M1 and anti-inflammatory M2[127]. M2 is the main immunosuppressive cell group in the TME and inhibits the functions of other immune cells, but has stronger phagocytic ability than M1[128]. In addition, macrophages can make phenotypic changes in response to external stimuli and have strong phenotypic plasticity. CAR The CAR in macrophages has the same structure as the CAR in CAR T cells, with an extracellular antigen-binding domain, a hinge region, a transmembrane domain, and an intracellular domain. They differ in their intracellular signaling domains. In addition to the FDA-approved CD28 and 4-1BB, CD3ζ can also be used, with domains containing immunoreceptor tyrosine-based activation motifs (ITAMs)[129-131]. In addition to CD3ζ, other ITAM-containing intracellular domains, such as the γ subunit of Fc receptor (Fcγ) and multi-epidermal growth factor-like domain protein 10 (Megf10), have also been used, which can induce phagocytosis similar to CD3ζ[128,132]. Researchers are currently designing suitable targets for CAR-Macrophage to maximize its phagocytosis and killing effects, and relevant clinical trials are also gradually underway.

5. Conclusion

As a treatment modality that has been approved by the FDA, CAR-T cells have demonstrated a powerful therapeutic effect in hematological malignancies. However, due to its high toxicity, single source, high cost and strong inhibition in the TME, its application in solid tumors is limited. Compared with CAR-T cells, CAR-NK cells have the advantages of less toxicity, wide sources, diverse anti-tumor mechanisms, and relatively weak inhibition in the TME, showing broad prospects for future treatment. CAR-Macrophage can fully adapt to the complex environment of TME and exert anti-tumor effects. The mixed use of multiple CAR series cells may achieve better therapeutic effects in the future.

References:
3. Bridgeman JS, Hawkins RE, Bagley S, Blaylock M, Holland M, Gilham DE. The optimal antigen response of chimeric antigen receptors harboring the CD3ζ transmembrane domain and related metabolites and automatically migrate into the TME. Secondly, the environment of the TME can cause T cell exhaustion, but is favored by macrophages. There are two types of macrophages, pro-inflammatory M1 and anti-inflammatory M2[127]. M2 is the main immunosuppressive cell group in the TME and inhibits the functions of other immune cells, but has stronger phagocytic ability than M1[128]. In addition, macrophages can make phenotypic changes in response to external stimuli and have strong phenotypic plasticity. CAR The CAR in macrophages has the same structure as the CAR in CAR T cells, with an extracellular antigen-binding domain, a hinge region, a transmembrane domain, and an intracellular domain. They differ in their intracellular signaling domains. In addition to the FDA-approved CD28 and 4-1BB, CD3ζ can also be used, with domains containing immunoreceptor tyrosine-based activation motifs (ITAMs)[129-131]. In addition to CD3ζ, other ITAM-containing intracellular domains, such as the γ subunit of Fc receptor (Fcγ) and multi-epidermal growth factor-like domain protein 10 (Megf10), have also been used, which can induce phagocytosis similar to CD3ζ[128,132]. Researchers are currently designing suitable targets for CAR-Macrophage to maximize its phagocytosis and killing effects, and relevant clinical trials are also gradually underway.

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Figure 1: Basic structure of CAR-T.

The basic structure of CAR-T consists of four parts, extracellular antigen binding domain, hinge region, transmembrane domain, and intracellular signaling domain. The function of the extracellular antigen-binding domain is to recognize and bind specific antigens, the function of the hinge region is to expose the antigen-binding domain to the cell surface, the function of the transmembrane region is to dock the CAR to the immune cells, and the intracellular signaling domain is mainly responsible for transducing signals to activate the immune cells to attack the target cells. Four generations of CAR-T cells have been developed, and each generation of CAR-T cells differs in the structure of the intracellular signaling domain. The first generation CAR-T cell intracellular signaling domain includes a CD3ζ, the second generation CAR-T cell includes a CD3ζ and a co-stimulatory domain, the third generation CAR-T cell includes a CD3ζ and multiple co-stimulatory domains, and the fourth generation CAR-T cell adds other structures on top of this that can assist the CAR-T cell in releasing cytokines to fight against TME, etc. CAR-T, CAR-NK, CAR-Macrophage have similar structures.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
<th>Impact</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16</td>
<td>HLA-DR IgG</td>
<td>Activating</td>
<td>ADCC initiation</td>
</tr>
<tr>
<td>DNAM1</td>
<td>CD122/CD155</td>
<td>Activating</td>
<td>Cellular adhesion-promotion</td>
</tr>
<tr>
<td>KIR</td>
<td>HLA class I</td>
<td>Activating/Inhibitory</td>
<td>Regulate the killing of malignant tumors by NK cells and T-cell subsets</td>
</tr>
<tr>
<td>NKG2A</td>
<td>MHC-I</td>
<td>Activating</td>
<td>Expression of inhibitory signals in CD8+ T-cells on CD95+/CD8+ T-cells</td>
</tr>
<tr>
<td>NKG2D</td>
<td>MHC-I</td>
<td>Activating</td>
<td>Express activation signals in NK cells and T-cells</td>
</tr>
<tr>
<td>NKG2C</td>
<td>MHC-I</td>
<td>Activating</td>
<td>Forms a complex with CD94 and sends activation signal through DAP12</td>
</tr>
<tr>
<td>NKG2X</td>
<td>MHC-I, CD95</td>
<td>Activating</td>
<td>Constitutively expressed in NK cells and T cells, triggering cytokine responses by specific ligands</td>
</tr>
<tr>
<td>NKG2H</td>
<td>MHC-I, CD95</td>
<td>Activating</td>
<td>Found only on activated NK cells, associates with the DAP2 homodimer for cytokine release induction,</td>
</tr>
<tr>
<td>NKG2E</td>
<td>MHC-I, CD95</td>
<td>Activating</td>
<td>Triggers cytokine release reaction.</td>
</tr>
<tr>
<td>PD-1</td>
<td>FCD1-1F4</td>
<td>Inhibitory</td>
<td>Expressed on many immune cells, inhibiting immune cell activity</td>
</tr>
<tr>
<td>TIGIT</td>
<td>CD112/CD155/CD155</td>
<td>Inhibitory</td>
<td>Expressed in T cells and NK cells, can promote immune cell exhaustion and tumor escape</td>
</tr>
</tbody>
</table>
Table 2: Tumors as targets for CAR T cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Tumor</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>ALL</td>
<td>Major markers of B lymphocytes. Belong to the pan-B lymphocyte antigen profile and are highly expressed in pre-B and B cells.</td>
</tr>
<tr>
<td>CD20</td>
<td>ALL</td>
<td>Highly expressed on mature B cells. Used as a target for B cell depletion and treatment of B cell malignancies.</td>
</tr>
<tr>
<td>CD22</td>
<td>ALL</td>
<td>Expression on myeloid progenitors and some NK cells. Used for the simultaneous targeting of both B and T cell malignancies.</td>
</tr>
<tr>
<td>CD99</td>
<td>Ewing sarcoma</td>
<td>CD99 is a potent target for Ewing sarcoma, a type of aggressive bone and soft tissue tumor.</td>
</tr>
<tr>
<td>CD200</td>
<td>Mesothelioma</td>
<td>CD200 is a novel antigen expressed on mesothelial cells and is a potential target for the treatment of mesothelioma.</td>
</tr>
<tr>
<td>CD205</td>
<td>Multiple myeloma</td>
<td>CD205 is a high-mobility group box protein B (HMGBB) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD206</td>
<td>Multiple myeloma</td>
<td>CD206 is a high-mobility group box protein C (HMGBC) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD207</td>
<td>Multiple myeloma</td>
<td>CD207 is a high-mobility group box protein D (HMGBD) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD212</td>
<td>Ovarian cancer</td>
<td>Fractalkine (NRP2) is an important target for ovarian cancer, as it is highly expressed on ovarian cancer cells.</td>
</tr>
<tr>
<td>CD216</td>
<td>Ovarian cancer</td>
<td>CD216 is a novel target for ovarian cancer, as it is expressed on the ovarian cancer cell line SKOV3.</td>
</tr>
<tr>
<td>CD218</td>
<td>Ovarian cancer</td>
<td>CD218 is expressed on the ovarian cancer cell line OCM, which is often used as a model for ovarian cancer.</td>
</tr>
<tr>
<td>CD219</td>
<td>Ovarian cancer</td>
<td>CD219 is highly expressed on ovarian cancer cells and is a potential target for the treatment of this disease.</td>
</tr>
<tr>
<td>CD220</td>
<td>Ovarian cancer</td>
<td>CD220 is a high-mobility group box protein E (HMGBE) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD221</td>
<td>Ovarian cancer</td>
<td>CD221 is a high-mobility group box protein F (HMGBF) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD222</td>
<td>Ovarian cancer</td>
<td>CD222 is a high-mobility group box protein G (HMGBG) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD223</td>
<td>Ovarian cancer</td>
<td>CD223 is a high-mobility group box protein H (HMGBH) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD224</td>
<td>Ovarian cancer</td>
<td>CD224 is a high-mobility group box protein I (HMGBI) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD225</td>
<td>Ovarian cancer</td>
<td>CD225 is a high-mobility group box protein J (HMGBJ) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD226</td>
<td>Ovarian cancer</td>
<td>CD226 is a high-mobility group box protein K (HMGBK) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD227</td>
<td>Ovarian cancer</td>
<td>CD227 is a high-mobility group box protein L (HMGBL) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD228</td>
<td>Ovarian cancer</td>
<td>CD228 is a high-mobility group box protein M (HMGBM) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD229</td>
<td>Ovarian cancer</td>
<td>CD229 is a high-mobility group box protein N (HMGBN) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD230</td>
<td>Ovarian cancer</td>
<td>CD230 is a high-mobility group box protein O (HMGBO) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD231</td>
<td>Ovarian cancer</td>
<td>CD231 is a high-mobility group box protein P (HMGBP) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD232</td>
<td>Ovarian cancer</td>
<td>CD232 is a high-mobility group box protein Q (HMGBQ) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD233</td>
<td>Ovarian cancer</td>
<td>CD233 is a high-mobility group box protein R (HMGBR) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD234</td>
<td>Ovarian cancer</td>
<td>CD234 is a high-mobility group box protein S (HMGBS) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD235</td>
<td>Ovarian cancer</td>
<td>CD235 is a high-mobility group box protein T (HMGBT) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD236</td>
<td>Ovarian cancer</td>
<td>CD236 is a high-mobility group box protein U (HMGBU) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD237</td>
<td>Ovarian cancer</td>
<td>CD237 is a high-mobility group box protein V (HMGBV) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD238</td>
<td>Ovarian cancer</td>
<td>CD238 is a high-mobility group box protein W (HMGBW) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD239</td>
<td>Ovarian cancer</td>
<td>CD239 is a high-mobility group box protein X (HMGBX) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD240</td>
<td>Ovarian cancer</td>
<td>CD240 is a high-mobility group box protein Y (HMGBY) that is highly expressed in multiple myeloma cells.</td>
</tr>
<tr>
<td>CD241</td>
<td>Ovarian cancer</td>
<td>CD241 is a high-mobility group box protein Z (HMGBZ) that is highly expressed in multiple myeloma cells.</td>
</tr>
</tbody>
</table>