**The splendid future of CAR-NK cells in the treatment of gynecological cancers**

Yisen Cao1, Liying Wang2,3,4, Liang Wang3,4\*

1Fujian Medical University

2Laboratory of Gynecologic Oncology, Fujian Maternity and Child Health Hospital, College of Clinical Medicine for Obstetrics & Gynecology and Pediatrics, Fujian Medical University, Fuzhou 350001, Fujian, China

3Fujian Key Laboratory of Women and Children's Critical Diseases Research, Fujian Maternity and Child Health Hospital (Fujian Women and Children's Hospital), Fuzhou 350001, Fujian, China

4Fujian Clinical Research Center for Gynecological Oncology, Fujian Maternity and Child Health Hospital (Fujian Obstetrics and Gynecology Hospital), Fuzhou 350001, Fujian, China

\* Correspondence：[wangliang@fjmu.edu.cn](mailto:wangliang@fjmu.edu.cn)

\*Correspondence author: wang liang

**Abstract**

NK cells are an innate class of lymphocytes in the human body that can achieve non-specific killing of tumor cells without MHC restriction or prior sensitization. In recent years, targeted killing of tumor cells has become possible due to the development of diverse biological technology, particularly the chemical chimeric antigen receptors (CAR), and other technologies. CAR gives NK cells a new magic, and its extracellular domains of the recognition region are usually single-chain antibodies (scFv), which can be targeted to specific antigens. CAR-NK cells have shown excellent results in several preclinical studies and clinical trials for hematologic malignancies. However, their clinical application in the treatment of solid tumors is still insufficient. Currently, the treatment of gynecological tumors relies mainly on surgery, chemotherapy, and radiotherapy, which are often accompanied by significant side effects and limited efficacy. CAR-T cell therapy has shown efficacy in certain gynecological tumors. However, side effects that are still urgent problems in clinical applications such as Graft-Versus-Host Disease (GVHD) and Cytokine Release Syndrome (CRS) have been observed. In contrast, CAR-NK cell therapy shows potential advantages in this area. Based on the above, this review mainly focuses on the development of CAR-NK cell constructs and their promising applications for immunotherapy of gynecological malignancies, aiming to provide references for clinical trials and clinical studies.

**Keywords**

CAR-NK cell; Chimeric antigen receptor; Solid tumor; cell therapy; gynecological cancers

**Introduction**

Natural killer (NK) cells, which account for 10 - 15% of peripheral blood lymphocytes and are identified in humans by CD3−CD56+, mediate innate immunity as they can achieve effective function surveillance and elimination of cancer cells without MHC restriction or prior sensitization. Moreover, 90% of NK cells are mature NK cells with CD56dimCD16bright and play a role in mediating the immune function. But 10% of NK cells are CD56brightCD16dim as immature NK cells, are cytokine producers especially interferon gamma (IFN-γ), which plays an important role in immunomodulation (1). The state of NK cell activation is not a form of dualism by one switch or one signal pathway. Whether the tendency is offensive or not is a balance of activating receptors and inhibitory receptors of NK cells. The tumor microenvironment has figured out many barriers for NK cell attack both in cells like dendritic cells, suppressive macrophages, Treg cells, Myeloid-derived suppressor cells (MDSCs), and cancer-associated fibroblasts and cytokines like indoleamine 2,3-dioxygenase (IDO), transforming growth factor-beta (TGF-b) and prostaglandin E2 (PGE2) (2–4).

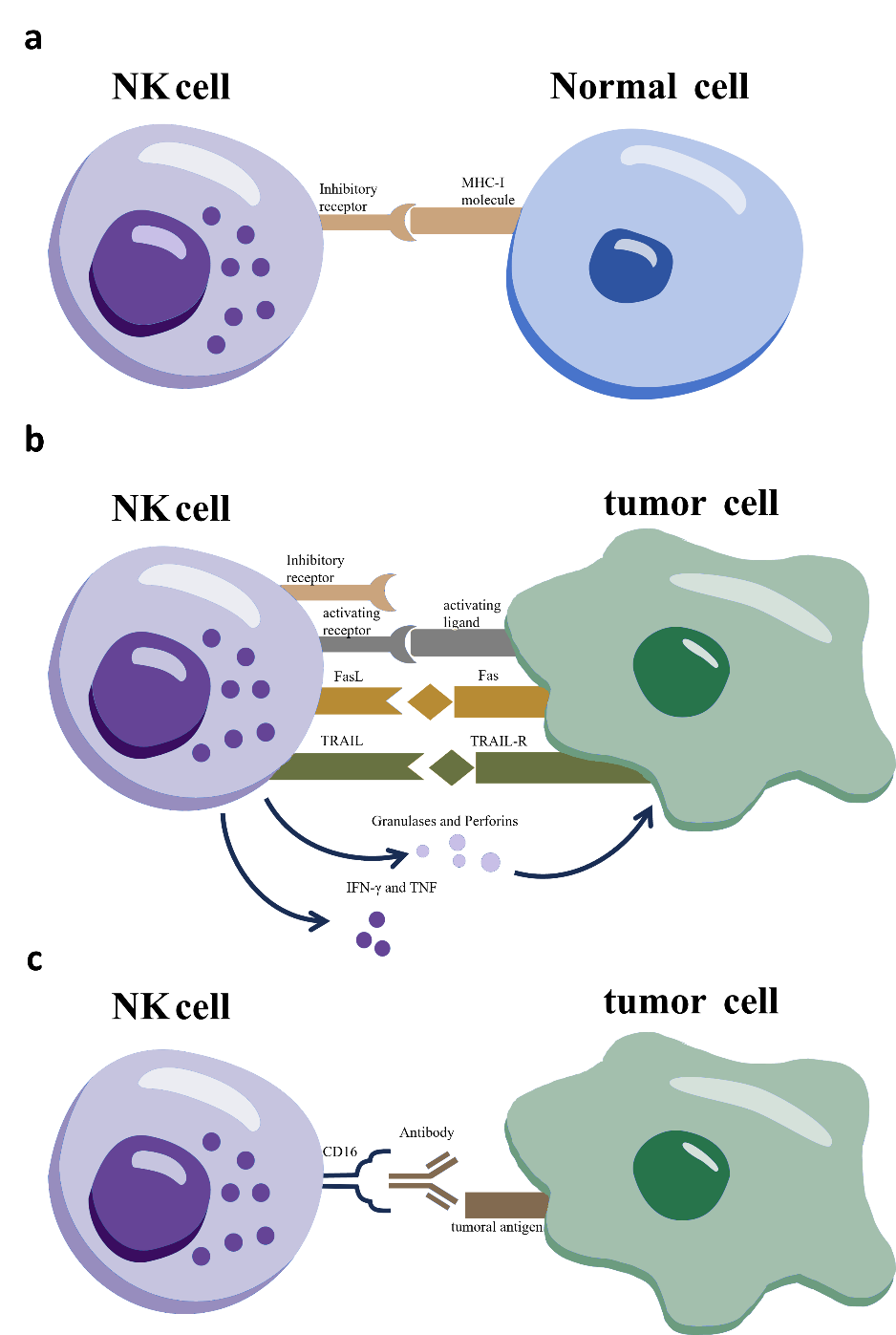
Gynecological cancer has become a major threat to women's health, and its current treatments (e.g., surgery, radiotherapy chemotherapy, etc.) have certain limitations, while the introduction of immunotherapy has made it possible for human beings to fight cancer effectively. CAR-NK cell therapy an emerging class of immunotherapy is still in the exploratory stage. The current preclinical studies and clinical trials applied to hematologic diseases have demonstrated the effective killing of tumor tissues. In recent years, there has been a growing interest in CAR-NK cell therapy for solid tumors. In solid tumor applications, CAR-NK cells are still slightly underutilized, but some preclinical studies and clinical trials have shown that they have a vast application prospect.

CAR-T cell therapy has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of hematologic malignancies and has also shown excellent results (5), but it still has unacceptable side effects; such as graft-versus-host-disease (GVHD) and cytokine release syndrome (CRS) (6–8). As an innovation of CAR-T cell therapy, CAR-NK cell therapy has been shown to have excellent safety in previous studies (9), and its efficacy for malignant tumors is not weaker than that of CAR-T cell therapy in experiments. Therefore, we hope to find perhaps feasible paths for the treatment of gynecologic malignancies by reviewing previous and undergoing studies of CAR-NK cells for malignant tumors in this review, to provide some reference for clinical work.

**1. The basement role of NK cells against cancer in the normal immune mechanism**

Whether NK cells are active or not is the result of the coordination of inhibitory receptors and activating receptors. In normal situations, the inhibitory killer immunoglobulin (Ig)-like receptors (KIRs) on NK cells can recognize major histocompatibility complex class I (MHC I) in normal cells and KIRs can contain the activating receptors on NK cells, and the killer NK cells cannot start (1). However, the virus-infected cells and malignant cells always lose the MHC molecule, the KIRs will disengage and the activating receptors will start a natural killer effect on “non-self” cells. The reorganization mechanism of NK cells is “non-self”, which is different from T cells against target cells. Activating NK cells can secret perforin and granzyme. Perforin can permeabilize the target cell membrane to help granzyme penetrate the target cell to elicit cell apoptosis (10). Another way to induce cell apoptosis is that the “death receptors” on target cells are bound and activated by “death ligands” secreted by NK cells such as FasL and tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) (10). The apoptotic pathway directly results in cell lysis for target cells. The killer role played by NK cells is not antigen-specific, however, NK cells can depend on the specificity of antibodies to target antigen on abnormal cells, which is antibody-dependent cell-mediated cytotoxicity (ADCC). First, antigens of target cells are coated with antibodies. Second, the IgG antibodies have their Fc region exposed and interact with Fc receptors in NK cells. The Fc receptors on NK cells are CD16 and CD32. At last, once those receptors are activated, degranulation happens and cytotoxic granules are released for the target cells to induce cell apoptosis (10). TNF not only directly induces apoptosis in tumor cells, but also promotes the secretion of IFNγ from NK cells, which in turn enhances the direct cytotoxicity of NK cells by promoting the release of granzyme and perforin. Besides the direct kill cytotoxicity, NK cells can also recruit T cells and macrophages to the infected site in the body by production of IFN and TNF (10). NK cells are important cytolytic and cytokine-producing effector cells of the innate immune system and possess the ability to lyse tumor cells without the need for the presentation of tumor-specific antigens.

NK cells are innate lymphocytes that recognize and lyse transformed cells and virally infected cells without prior activation. Therapy tactics based on the body's immune system and cancer immune microenvironment are gradually practical and are mainly divided into two types cell-based immunotherapy and non-cell-based immunotherapy (antibodies, cytokines, and extracellular vesicle.



**Figure 1 Functions of Natural Killer (NK) Cells** | NK cells express a variety of receptors with activating or inhibitory functions, and the balance of activating and inhibitory signaling controls the activation or non-activation of the cytotoxic effects of NK cells. Normal cells express MHC-I molecules on their surface, and when inhibitory receptors on NK cells bind to these molecules, the function of NK cells is inhibited and their cytotoxicity is suppressed (Figure a). In abnormal cells (tumor cells, for example), their MHC-I molecules are usually down-regulated, while the ligands of NK cell activating receptors (e.g., NKG2DL, etc.) are typically up-regulated. The mutual binding of activating ligands and activating receptors (e.g., NKG2D) leads to the absence of inhibitory signals, and the enhancement of activating signals induces the activation of NK cells and cytotoxicity against target cells. The release of cytokines to the recruitment of other immune cells (e.g., macrophages, etc.) enhances the immune response (Figure b). In addition, NK cells are activated through CD16 recognizable antibodies bound on target cells, allowing them to exert cytotoxicity on target cells through ADCC action (Figure c).

**2. The construct of CAR-NK cells and the differences between CAR-NK and CAR-T cells**

In terms of the characteristics of NK cells and T cells, the activation of NK cells does not depend on the MHC class I molecular. Transplantation of allogeneic NK cells does not induce graft-versus-host disease (GVHD) or other alloimmune or autoimmune toxicities due to the mismatch between the KIRs on their surface and the HLA molecules of the recipient's normal tissue cells. If autologous NK cells are used for adoptive therapy, they bind to MHC molecules on the surface of the autologous body and produce inhibitory signals, avoiding damage to normal tissue cells associated with NK cell adoptive therapy (6). This characteristic permits the existence of commercial “off-the-shelf” allogeneic NK cells from healthy donors (7). The emergence of CAR-T cells has taken the immunotherapy of tumors to a new level, while at the same time, its other major side effect is still not to be ignored, which is cytokine release storm (CRS), in which T-cells release severe pro-inflammatory factors leading to non-tumor killing of tissues; whereas NK-cells have a lesser side effect of not leading to cytokine release storm. NK cells have a relatively limited lifespan which can avoid the “on target, off tumor” effect to a certain extent even without suicide genes (11). Different from CAR-T cells, the intracellular costimulatory domain is not only dependent on CD3z pathway which contains three immunoreceptor tyrosine-based activation motifs (ITAMs) for CAR-NK cells. DAP12, an exclusive adaptor molecule associated with activating NK cell receptors, has a single ITAM that can bind Syk and ZAP70 kinase to active NK cells with enhanced stimulate effector and cytokine release compared with CD3z (12). Especially, fusing NKG2D as the extracellular recognition region of a CAR and DAP12 as the intracellular domain of a CAR has a more active effector because DAP12 is the endogenous signal to active NKG2D receptor (7).

**Table 1 The differences between CAR-NK and CAR-T cells**

|  |  |  |  |
| --- | --- | --- | --- |
|  | CAR-T cells | CAR-NK cells |  |
| The basic characteristics of immune cells | Restricted by MHC I  Cause GVHD  Cause CRS  Comparative long lifespan | MHC I independency  Do not induce GVHD  Do not cause CRS  Comparative short lifespan | (11)  (6,7)  (8)  (13) |
| The design of the intracellular domain | CD3z with or without other costimulatory molecular | CD3z with or without other costimulatory molecular  DAP12 is more cytotoxic than CD3z | (10) |

**3. The current application of CAR-NK cells in cancer**

In recent years there has been a growing interest in CAR-NK cell therapy for the treatment of cancer, and the enrollment of clinical trials on CAR-NK therapy is increasing (9), which facilitates a more in-depth evaluation of the efficacy and safety of CAR-NK cell therapy. The following is a partial summary of the application of CAR-NK cell therapy for the treatment of cancer.

Several reviews have summarized the CAR-NK used for cancer (3,14–16). In solid cancer, the antigens are variant, and cancer types gradually increase these days. The anti-HER2-primary NK cells had been engineered to eradicate breast cancer (17). For glioblastoma, anti-HER2-NK92 cells have been studied under development or in the clinic and there is a clinical trial (NCT03383978) going on now(18,19). In gastric cancer, anti-HER2-NK92 cells have synergized with apatinib in a preclinical study(20). In addition, CAR-NK cells targeting Claudin 18.2 (CLDN18.2) in immunotherapy for gastric and pancreatic cancers are also being recruited (NCT06464965). Anti-Robo1-CAR-NK-92 cells combined with brachytherapy inhibit pancreatic carcinoma in mouse models (21). Also under the synergistic effect of radiotherapy, anti-CPG3-NK92 cells assembled with CXCR2 showed strong cytotoxicity against Hepatocellular carcinoma (HCC), however, the anti-tumor effect varied under different radiation doses (22). Anti-GPC3-NK cells are about to be utilized in volunteers with primary HCC in a clinical trial (NCT06652243). In colorectal cancer, three advanced patients treated with NKG2D-CAR-NK cells by local infusion had tumor regression targeting NKG2D ligands (7). Anti-HER1-CAR-NK cells demonstrated potent tumor-killing activity against Head and Neck Squamous Cell Carcinoma but showed up-regulated expression of CD44v6, which may require a multi-target combination therapy strategy (23).

In hematologic malignancies, CAR-NK cell immunotherapy has shown exciting results. In several recent studies on multiple myeloma (MM) (24,25), CS1-CAR-NK cells were able to effectively inhibit the proliferation of tumor cells and effectively increase the survival of tumor-bearing mice in an allogeneic implant mouse model (24). The three types of CAR-NK cells equipped with targeting CD19 also achieved selective cytotoxicity against tumor cells (25). In addition, GPRC5D and BCMA (NCT06045091) are also alternative targets (26,27). In a new study, Yang et al. (28) demonstrated that in the absence of target antigens, CAR-NK cells targeting CD19/CD20 were constructed and more significantly exhibited cytotoxicity against acute lymphoblastic leukemia (ALL).

However, data from preclinical and clinical trials have shown that CAR-NK cells demonstrate good efficacy and safety in the treatment of solid tumors (9). There are still other issues that need to be addressed, some of which we will mention below.

**Table 2 Incomplete Summary of CAR-NK Cell Clinical Trials**

|  |  |  |  |
| --- | --- | --- | --- |
| Row | Gov Identifier | Conditions | Status |
| 1 | NCT03383978 | HER2-positive glioblastoma or its variant gliosarcoma | Active, not recruiting |
| 2 | NCT06464965 | the positive expression of CLDN18.2 ≥ 10%；pancreatic cancer and gastric cancer | Recruiting |
| 3 | NCT06652243 | Glypican-3 (GPC3)-Positive Advanced Hepatocellular Carcinoma | Not yet recruiting |
| 4 | NCT05922930 | at least 1+ TROP2 expression; Ovarian Cancer | Recruiting |
| 5 | NCT05703854 | CD70-positive osteosarcoma or mesothelioma | Recruiting |
| 6 | NCT05574608 | CD123-positive acute myeloid leukemia (AML) | Recruiting |
| 7 | NCT06045091 | relapsed/refractory multiple myeloma or plasma cell leukemia | Recruiting |
| 8 | NCT05673447 | CD19-positive diffuse large B cell lymphoma | Recruiting |
| 9 | NCT06696846 | relapsed/refractory T-lymphoma and acute myeloid leukemia; CD70 positive | Recruiting |
| 10 | NCT06307054 | acute myeloid leukemia; CLL1-positive | Recruiting |
| 11 | NCT03692767 | Relapsed and Refractory B Cell Lymphoma; CD22-positive | Recruiting |

**4. The novel technology to develop CAR-NK cells**

On-target and off-tumor toxicity is a risk for CAR therapy because rare expression of tumor-associated antigens in normal tissues is subversive for normal tissues (29).

**4.1 Possible modes of immune escape of tumor cells**

In the absence of obvious pathological conditions, tumor cells may shed their specific targeting molecules through the action of metal shearing enzymes on the surface. This, on the one hand, reduces the specific targeting proteins on the surface of tumor cells. On the other hand, the targeting proteins under the shear may specifically mask the activated receptors on the surface of CAR-NK cells. Consequently, this causes the inefficiency of CAR-NK cells in killing tumor cells (30,31).

**4.2 Measures for CAR-NK cells to respond to low-expressed antigens**

On the one hand, tumor cells can be localized by multi-targeting to achieve the killing effect on tumor cells. In a recent study, Zhang and his colleagues designed CAR-NK cells targeting both NKG2DL and ErbB2 to address the interference of soluble NKG2DLs (32). The bispecific CAR structure can solve the problem of down-regulation of cancer antigens with insufficient precision (33). On the other hand, the advantages of RNA electroporation transfection become apparent in the context of possible immune escape pathways from tumor cells—the RNA electroporation transfection results in transient expression of CAR. CAR-killing activity has been enhanced transiently. An RNA CAR has been adopted to target NKG2D ligands by NKG2D-CAR-NK in colorectal cancer patients (7). In this study, the electroporation transfection technique increased the expression of NKG2D, thereby improving the killing ability of CAR-NK cells. Recently, it has also been reported that LNP transfection of umbilical cord blood-derived NK cells is more advantageous than the electroporation method. However, the receptor function of mRNA-LNP-treated NK cells needs to be further evaluated (34).

**4.3 Risks of non-tumor-targeted toxicity of CAR-NK cells and their management**

The improved strategy of enabling CAR-NK cells to overexpress the appropriate ligands in response to low antigen expression in tumor cells undoubtedly poses another risk which is an off-target effect. More specifically, many target proteins that are abundantly expressed on the surface of tumor cells are also expressed in normal tissue cells. For example, macrophages and dendritic cells also express NKG2DL (35), and thus there is a potential risk of off-target effects of CAR-NK cell therapy. Although CAR-NK cells have a relatively short lifespan, usually around two weeks (13), and therefore have relatively limited potential for damage, non-tumor toxicity in this case cannot be ignored. In recent studies, it has been shown that it is possible to introduce a suicide gene within the CAR molecule of the CAR-NK cells, the icasp9 suicide switch (36). For patients who require a strategy of repeated infusions of CAR-NK cells, the risk of non-tumor-targeted toxicity can be managed by the addition of certain chemicals that rapidly terminate the continued activity of the CAR-NK cells after the CAR-NK cells in the body have performed their task well, to minimize or prevent their impact on normal tissues (37).

**5. The elusory state of CAR-NK cells against gynecological cancers**

Cervical, endometrial, and ovarian cancers constitute the most gynecologic malignancy with both high morbidity and mortality. Cervical cancer is the most common gynecologic cancer and the second leading cause of cancer death in women behind breast cancer and the mortality of cervical cancer patients in poor counties is twice that of women in affluent counties (38). Endometrial cancer is the fourth most common cancer and the fifth most common cause of cancer death in women (39). Ovarian cancer accounts for 3.6% of all cancer cases but 4.3% of all cancer deaths, which is the second most common cause of gynecologic cancer death next to cervical cancer, the seventh most common and the eighth most common cancer death in women (40). Since the immunotherapy strategy represented by CAR has shown significant progress in hematologic malignancies and other solid tumors, it is more urgent to conceive the application of immunotherapy in gynecological cancers for women's health.

It is very worth considering where are we and where are we going in the realm of CAR therapy strategies against gynecological cancers. Totally, CAR-T is more widely studied for gynecological cancers than CAR-NK cells.

The majority of endometrial cancers (Type I) are estrogen-related (41). Chemotherapy, radiotherapy, and hormonal therapy are recommended as adjuvant treatments for endometrial cancer, meanwhile, the status of biomarker-driven targeted therapy for endometrial carcinomas has lagged (42). Recently, it has been shown that MISIIR is overexpressed in ovarian and endometrial cancers but not in normal tissue cells, and that CAR-T cells targeting MISIIR achieved excellent results in lysing MISIIR-overexpressing cervical cancer model mice and patient-derived tumor cells (43). Nowadays, the distinctive molecular and genomic profiles have been understood especially in Type II endometrial cancer and the understanding of the immune microenvironment in endometrial cancer tumors has gradually evolved, so molecular-targeted therapy or immunotherapy is urgently needed (44).

The antigens and CAR design methods to engineer T cells or NK cells for ovarian cancer, especially epithelial ovarian cancer are versatile (45–50). Antigen-targeted therapy for ovarian cancer is at an accelerated speed owing to the marvelous exploration of specific or associated antigens in ovarian cancer. One research constructed anti-CD133-CD28-41BB-CD3ζ-CAR-NK92 cells against CD133-positive ovarian cancer cells and another research designed anti-CD24-CD28-41BB-CD3ζ-CAR-NK92 cells to specifically kill CD24-positive ovarian cancer cells, and both CD133 and CD24 are cancer stem cell markers which can be used for other cancers (46,49). FRα, which is overexpressed in 90% of ovarian cancer, had been targeted by the second-generation CAR-NK92 cells and displayed potent cytotoxicity against FRα-positive ovarian cancers (45). In an ovarian cancer xenograft model, anti-mesothelin CAR-NK cells derived from iPSCs can inhibit cancer growth *in vivo* (50).

Because most cervical cancer has a defined pathogenesis that is high-risk HPV-associated, targeted therapy strategies have paid more attention to antigens affected by HPV. Agents to stimulate the immune system against HPV-transformed cells have been tested in cervical cancer. For instance, ADXS11-001 is a live attenuated Listeria monocytogenes (Lm) bioengineered molecule, which can promote CTLs to infiltrate cervical cancer by mimicking antigen presenting process (51). Monoclonal Antibodies C1P5 (anti-HPV E6) and TVG701Y (anti-HPV E7) were investigated in mice models with cervical cancer and exerted inhibition of tumor growth (52). Recently, single immune checkpoint blockade has emerged in improved efficacy for cervical cancer and immune checkpoint inhibitors combined with HPV therapeutic vaccine, chemotherapy or radiotherapy would be considered in the clinic (53–55). Pembrolizumab, a humanized monoclonal antibody against the programmed cell death protein-1 (PD-1) receptor has been approved in PD-L1-positive cervical cancer (56). Adoptive immunotherapy for cervical cancer is in urgent need but without adequate research so far. For example, the tumor-infiltrating T lymphocytes (TILs), which are derived from fragments of metastatic tumor with reactivity against the HPV16 or HPV18 E6 and E7 antigens resulted in clinical regression for patients with metastatic cervical cancer (57). With regard to T cell receptor (TCR), there is a Phase 1-2 clinical trial of TCR therapy targeting HPV16 E7 antigen in recruiting for patients with HPV-associated cervical cancer (ClinicalTrials.gov identifier: NCT02858310). CAR-T cells that target HER2 have been explored in cervical cancer in clinical trials and CD47 in preclinical studies (58,59). The antigens of CAR-T cell therapy involved in cervical cancer are GD2, PSMA, Muc1, and mesothelin (ClinicalTrials.gov identifier: NCT03356795 and NCT01583686), yet there is no CAR-NK cell study for cervical cancer.

Epithelial cervical cancer (CALO and INBL) cell lines produce MICA and MICB which can facilitate tumor proliferation, however, NKG2D receptor is also detected in those cervical cancer cells that were thought exclusive in NK cells or some CTLs. The anomaly may be the mechanism for cancer cells to escape immune cytotoxicity. As the NKG2D receptors combine MICA and MICB molecules in situ in cancer cells, there is no binding region or signal communication for NKG2D receptors in immunocompetent cells (60).

Why seldom CAR is utilized in cervical cancer compared with other solid cancers is the scarcity of specific and exclusive cancer-associated antigens in cervical cancer when designing a T-cell targeted therapy. Candidate antigen for CAR should be expressed both widely even totally on cancer cell surface and rarely even none on normal tissues. The rate of HER2 overexpression in cervical cancer is 38%-94% and the rate of mesothelin overexpression is about 25%, which are the current antigens targeted by CAR-T cells in cervical cancer (58).

**Conclusion**

Traditional therapies exhibit limited therapeutic efficacy on gynecological malignant tumors, and CAR-NK cells, as a novel immunotherapy strategy, show great potential in gynecological oncology.

By genetically engineering NK cells to precisely target tumor cells and efficiently kill cancerous tissues, CAR-NK cell therapy has demonstrated superior efficacy, enhanced safety, and more convenient treatment options than traditional treatments and CAR-T cell therapy in pre-clinical studies and clinical trials. It has shown inhibition of tumor tissues in gynecological malignancies such as ovarian, cervical, and endometrial cancers, and it can effectively reduce patient suffering and prolong patient survival. In recent studies and trials, CAR-NK cells have been shown to have synergistic effects with traditional treatment modalities (such as radiotherapy), bringing light to the majority of patients suffering from gynecological malignancies.

Currently, CAR-NK cells are still in the exploratory stage. We are confronted with numerous issues, such as selecting tumor antigen targets, optimizing CAR structure, immunosuppression of tumor microenvironment, and self-interacting CAR-NK cells. However, with the continuous optimization of biotechnology, the above problems will be gradually overcome and CAR-NK cell therapy will make greater breakthroughs in precision medicine and personalized treatment of gynecological malignant tumors, and is expected to become a core component of gynecological malignant tumors treatment system, and bring the dawn of a cure to the majority of patients.

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**Competing interests**

The authors declare that they have no competing interests.

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