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Synergizing CAR with memory-like NK cells

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Abstract. Natural killer (NK) cells are crucial for combating the tumor growth and spread of cancer. The receptors which inhibit and activate NK cells coordinate to recognize "self" or "missing-self" to determine whether NK cells should be activated to destroy the targets. NK cells exhibit intrinsic memory to haptens and viral infections despite lacking antigen-specific receptors. A combination of Interleukins may create cytokine-induced memory-like (ML) NK cells that respond more vigorously to tumors and lessen symptoms in cancer patients. Meanwhile, chimeric antigen receptors (CAR) have been utilized to modify NK cells. CAR-NK cells have been tested to be more powerful in treating solid tumors and hematological cancers in both pre-clinical and clinical trials. A few recent studies showed that when ML NK cells are armed with CAR, the resulting CAR-ML-NK cells demonstrated better survival in human xenograft models as well as enhanced responses against cancer cells *in vitro* and *in vivo*.

Keywords: NK cells, memory-like, CAR.

1. Introduction

NK cells were initially regarded as innate lymphocytes, which are generally considered not to possess memory of previous exposures. However, a subset of NK cells has surfaced over the last decade, demonstrating their capability of proliferation and long-lasting immunologic memory specific to an antigen. Another noteworthy phenomenon is that NK cells can differentiate into effectors with ML properties when exposed to a variety of cytokines, including IL-12, IL-15, and IL-18. When exposed again to antigens, these cytokine-induced ML NK cells secrete more interferons (IFNs) than the wildtype cells. But, unlike memory NK cells, these ML NK cells do not respond specifically to an antigen. Instead, they display more flexible and adaptable responses that allow them to identify and attack atypical tumor cells. Even after numerous rounds of cell division *in vivo*, the improved recall responses still persist. On the other hand, the generation of CAR-NK cells as a valid immunotherapy measure by applying CAR technology has become a research focus in the past few years. CAR-NK cells, after being engineered, may specifically and accurately target tumor cells. In consequence, Scientists postulated that the CAR-armed ML NK cells can be a more powerful tool in fighting cancers, and designed experiments to test their hypotheses.

2. Innate features of NK cells

Because their quick effector responses are non-specific and their lack of antigen-specific receptors on the surface, NK cells were first thought to be an innate defense of the immune system. Via two

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primary ways, NK cells defend the host against viral infection and the growth of tumors: (1) Their cytotoxic granules, which include perforin and granzymes, are released into the extracellular space to destroy the target cells. (2) They release proinflammatory cytokines that accelerate phagocytosis and lysis in macrophages, upregulate the expression of the major histocompatibility complex (MHC) class I on antigen-presenting cells, attract more immune cells, and encourage cytotoxicity.

Signals from the germline-encoded surface receptors are combined to control the activation and cytotoxic function of NK cells. Only an appropriate ratio of inhibitory to activating signals may launch the killing scheme of NK cells. NKG2A and multiple killer cell Ig-like receptor (KIR) family members are the primary NK cell inhibitory receptors in humans. Similar to KIRs, the Ly49s are found in mice. Both KIR and Ly49 receptors can recognize major histocompatibility complex Class I (MHC-I) molecules derived from the host, which aid with the development of NK cells in the "licensing" and "education" processes. This ensures their self-tolerance and capability to kill pathological cells that lack MHC-I molecules ("missing-self") [1]. If viral and tumor-derived ligands are emerging and signals for inhibitory receptors are missing, NK cells can effectively eliminate aberrant cells. NKG2C, NKG2D, and natural cytotoxicity receptors (NCRs), which identify ligands increased on stressed cells, as well as CD16, which mediates antibody-dependent cell-mediated cytotoxicity (ADCC), are the key NK cell activation receptors that have been found in humans. NCRs, such as NKp30, NKp44, and NKp46, are transmembrane proteins that resemble immunoglobulin (Ig). Patients with a variety of solid malignancies have been found to have significant amounts of soluble NCR ligands [2].

3. Adaptive features of NK cells-memory

Because their quick effector responses are non-specific and their lack of antigen-specific receptors on the surface, NK cells were first thought to be an innate defense of the immune system. Via two primary ways, NK cells defend the host against viral infection and the growth of tumors: (1) Their cytotoxic granules, which include perforin and granzymes, are released into the extracellular space to destroy the target cells. (2) They release proinflammatory cytokines that accelerate phagocytosis and lysis in macrophages, upregulate the expression of the major histocompatibility complex (MHC) class I on antigen-presenting cells, attract more immune cells, and encourage cytotoxicity.

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4. ML NK cells in cancer teratment

Anticancer responses were seen following cytokine-induced ML NK cell transplantation in animal models. The combined actions of the cytokines boosted the production of IFN and subsequent cytotoxicity against leukemia cells relative to untreated NK cells [3]. Similar preclinical results have been obtained in a wide range of cell lines and animal models of cancer, such as melanoma, ovarian cancer, and hepatocellular carcinoma, further verifying the potential of ML NK cells being used as an immunotherapeutic tool and their robust responses against tumor targets. Furthermore, Ni and colleagues found that *in vivo*, tumor growth was dramatically suppressed, and lifespan was improved

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by giving mice radiotherapy before giving them adoptive NK cells [4]. Overall, these *in vitro* and animal experiment findings support the hypothesis that ML NK cells are superior to their untreated counterparts when it comes to targeting tumor cells.

Additionally, employing ML NK cells to treat human patients with leukemia has improved outcomes. The induced ML NK cells can grow and replicate *in vivo*, leading to full remissions in patients who experience a relapse of acute myeloid leukemia (AML), as reported by Berrien-Elliott et al [5]. They also elucidated that ML NK cells are risk-free since they are well-tolerated and do not result in chronic relapsing syndrome, graft-versus-host disease, or neurotoxicity syndrome associated with immune cells. Similar findings were seen in a different study: the derived ML NK cells expanded rapidly *ex vivo* after infusion and sustained a ML behavior for at least three months[6]. Half of the patients evaluated on day 28 went into complete remission, and this lasted for as long as two years. These promising initial results raise the possibility of additional research into ML NK cell treatment and its potential application in the treatment of other cancers.

5. CAR technology applied to NK cells

One of the most common ways to create immune cells that are specific to tumor targets is through the utilization of chimeric antigen receptor (CAR). A CAR typically consists of three parts. The ectodomain outside of the cell has structures to recognize and bind to antigens; the middomain stabilizes the CAR on the membrane; and the endodomain relays activation signals to T cells or dendritic cells following antigen contact. The endodomain has many functional components, including three immunoreceptor tyrosine-based activation motifs, which are essential for signal transduction [7].

NK cells are usually armed with CARs used that have been applied for CAR-T. 4-1BB co-stimulatory domain has been shown particularly crucial to activate NK cells, and to generate tumor necrosis factor and IFN- γ. NK cells can eliminate CD19+ acute lymphoblastic leukemia (ALL) by producing anti-CD19 CAR with a 4-1BB co-stimulatory domain, despite the presence of inhibitory signals [8]. Recently, new types of CAR designs made just for NK cells have been developed. It has been demonstrated that the antitumor efficiency of NK cells is much enhanced as the cells express a CAR containing the NK-specific co-stimulatory domain 2B4 [9]. They rapidly proliferated, increased production of IFN-γ, and reduced apoptosis compared to traditional 4-1BB transduced NK cells. In addition, if CAR constructs are designed with different signaling domains (CD3ζ, DAP10, or DAP12), the resultant CAR-NK cells show different anticancer potency. A CAR based on DAP12 performed better than a CD3ζ-containing CAR, and a CAR having the CD3ζ signaling domain performed much better than a CAR containing the DAP10 [10].

6. CAR-NK in immunotherapy

Research has revealed that NK cells modified with CAR can accelerate infiltration into solid tumors and overcome a resistant tumor microenvironment, as well as increase their persistence and expansion *in vivo*. Leukemia, multiple myeloma (MM), lymphoma, and other solid cancers occurring in different organs have all responded well to treatment with CAR-NK.

Due to the abnormality of leukemia cells, malignant leukemic cells can elude the attack by NK cells. Patients with relapsed or refractory AML can safely receive NK92 cells modified with anti-CD33 CAR (CD33-CAR-NK92), which have been shown to be more lethal than parental NK92 cells against HL-60, a human cell line of AML. CAR-engineered NK92 cells with potent and focused anticancer activity were demonstrated in NOD-SCID IL-2R null mice by their ability to impede the progression of B-cell lymphoma [11].

CAR-NK cells are not only effective against leukemia, but they can also aid in the therapy of multiple myeloma (MM) and lymphomas. It was discovered that CAR-engineered cells could cause selective cytotoxicity against CD38+ MM cells obtained from the patients, suggesting that the CD38-specific nanobody-based CAR (Nb-CARs) may have therapeutic effects [12]. Non-Hodgkin's lymphomas, including T-cell lymphomas, are another potential target for CAR-NK cells. Different CD4+ human T-cell lymphoma and leukemia cell lines, as well as *ex vivo* patient samples, have been

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found to be selectively lysed by CD4-CAR-NK92 cells. Furthermore, *In vivo* studies indicated that CD4-CAR-NK92 cells may effectively eradicate lymphoma cells and increase the survival of xenograft mice [13].

In addition to its success in treating blood cancers, it has been shown that CAR-NK works in treating solid tumors. The epidermal growth factor receptor (EGFR) is a prototypical tyrosine kinase receptor that has been extensively studied for its role in tumorigenesis. When applied *in vitro* to a panel of breast cancer cell lines, EGFR-specific CAR-NK92 cells demonstrated stronger cytotoxicity and more IFN- γ generation than native NK cells [14]. In xenograft mice models, infusion of EGFR-CAR-NK92 cells into the brain could significantly reduce glioblastoma development and improved overall survival [15]. Recent studies have revealed that tumor infiltration increased by CAR-NK cells can improve the effectiveness of NK therapy in human ovarian cancers, and this has been achieved by expressing CAR and the chemokine receptor CXCR1 simultaneously [16]. When tested against GD2+ neuroblastoma cells, ganglioside GD2-specific CAR-NK cells demonstrated considerably higher cytotoxicity than parental NK cells. In addition to blocking tumor growth, GD2-CAR-NK cells showed significant trafficking into solid tumors [17].

7. CAR boosts the potency of ML-NK cells

Scientists predicted that adding CAR into cytokine-induced ML NK cells could be a more successful technique for immunotherapy because these ML NK cells showed improved growth and antitumor responses following adoptive transfer.

The first demonstrated efficacy in mice was achieved by Gang et al [18]. By using retroviral transduction to introduce an anti-CD19 antibody linked to the endodomain CD3ζ chain as a co-stimulatory factor, ML NK cells derived from the peripheral blood were transformed to express a CD19-CAR. The resultant CAR-ML-NK cells were more efficacious in producing interferon, degranulating, and killing NK-resistant lymphoma cells than ML NK cells and conventional CAR-NK cells. Human xenograft lymphoma models also showed that CD19-CAR and ML responses of NK cells working together might effectively control lymphoma *in vivo* and increase survival rates.

More recent research has examined the possibility that ML NK equipped with CAR has a more potent response. In one study, researchers found that targeting nucleophosmin-1 (NPM1)-mutated AML with a neoepitope-specific CAR greatly improved anticancer reactions without causing off-target damage in ML NK cells. Increased efficacy against NPM1-mutated AML cell lines and leukemic blasts derived from patients was observed [19]. In xenograft models of AML, CAR-ML NK cells were long-lived and dramatically improved patient outcomes. The most lethal form of gynecological cancer, epithelial ovarian carcinoma (OVC), was hypothesized to be treatable by equipping ML NK cells with a CAR-targeting mesothelin. Around 80% of OVCs express mesothelin, which leads to increased metastasis, resistance to chemotherapy, and poor overall survival. When compared to unmodified ML NK cells, ML NK cells equipped with anti-mesothelin CAR displayed markedly higher cytotoxicity and induced much more apoptosis [20].

8. Conclusions

Over the past decade, the non-specific cytotoxicity and the intrinsic memory responses of a subset of NK cells have established adoptive transfer of NK cells as a successful immunotherapy approach. Artificially manufactured ML NK cells may recognize and destroy cancer cells with greater flexibility and less reliance on specific antigens. This is made possible by the use of a mixture of cytokines. There are mounting indications that ML NK cells can provide a stronger antitumor response than their untreated counterparts. Meanwhile, CAR technology can promote the selectivity and precision of NK cells to target the stressed cells. Patients with cancer who have undergone an autologous or allogeneic NK cell transplant may benefit from the CARs because they may improve NK cell trafficking to tumor sites. Consistently encouraging pre-clinical results of CAR-NK cells have emerged from different independent research. Several *in vitro* animal studies have revealed that combining the ML response of

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NK cells with the effectiveness of CAR is promising. Different kinds of cancer that are resistant to NK cells can be treated with CAR-ML-NK cells.

Moreover, a few studies suggested that when NK cells were used together with chemotherapies, radiotherapies, or monoclonal antibodies, the antitumor effects could be boosted than using the traditional treatment methods alone. On the other hand, NK cells can be engineered to express other molecules, such as cytokines and antibodies, that can promote NK cell expansion or trafficking into solid tumors. Or, there are some molecules can that can potentially switch TME to facilitate the antitumor responses. Presuming synergizing certain treatments may enhance the antitumor response further and promote patient survival, more future studies may be conducted in combination with therapies in the hope that some combinations may yield more favorable outcomes.

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