Application of Natural Killer Cells in Cancer Treatment and Its Clinical Progress

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Abstract. One type of effector lymphocyte that is crucial to the innate immunity is the natural killer (NK) cell. With the increasing interests in immunotherapy, the value of NK cells utilization on treating cancers is gradually being recognized and thus is transforming to be proved by current clinical trials. NK-based cancer immunotherapy results in unique cytotoxic functions in a more direct and straightforward manner which targets both hematologic cancers as well as solid tumors. Concurrently, several clinical obstacles are encountered, including immunological escape, hardship of tumor access, and effector cell fratricide, which all await to be solved and require further studies on promoting the effectiveness while remaining immuno-safety. Pleasantly, multitudinous corresponding solutions are flourishing, and researchers are using their best endeavors to accelerate positive progress in NK-based immunotherapy. Believing in a promising future of NK-related cancer immunotherapy, this review will concentrate on NK cells' effector functions towards cancers, including the cellular mechanisms, present experimental progresses, and challenges which need to be addressed.

Keywords: Natural killer cells, Cancer immunotherapy, NK cell activation, NK cell cytotoxicity, Anti-tumor therapy.

1. Introduction

Being a complex network of various organs, cells, and substances, our immune system works together to protect our body from any potential threats caused by outer invaders or inner aggressor. Cancer is one example of homologous cells with altered characteristics such as uncontrolled growth and loss of cellular markers, which can be, theoretically, detected by the immune system as the "abnormal" self tissue followed by immune response and cell elimination. However, throughout each stage of the immune system's engagement with tumor cells, these altered host cells continue to evolve to obtain different ways in escaping the attack by our natural immune system. For decades, research on uncovering potential approaches to enhance cancer patients' immune system as well as the efficiency of immune attack have flourished, including Immune checkpoint inhibitors (ICIs), monoclonal antibodies (mAbs), T-cell transfer therapy, etc. Despite the appearances of various immunotherapy against cancer, the effectiveness of treatment is still not promising, and the demand in discovering another feasible treatment to boost patients' immune system is urged.

Natural killer (NK) cells-related immunotherapy is gradually gathering attention because of its specialty in killing cancer cells in either innate or adaptive ways. There is a growing body of information indicating that NK cells possess the capability to induce apoptosis in cancerous cells not just by direct cytotoxicity (release of granzyme and perforin), but also by mounting an antigen-specific response upon tumor reemergence [1]. Yet, the effectiveness of NK-based immunotherapy is still under evaluation since it has some exclusive shortages which annoys scientists just as its unique advantages which attract researchers. Accuracy of binding to tumor cells, ability of tumor infiltration, and time of persistence can all be problems waiting to be solved. Nonetheless, scientists still trust that NK-based immunotherapy can be a wonderful target for cancer treatment in the future, and indeed current NK-based clinical trials show increased and wide development prospects. The day that mature NK-based immunotherapy benefits cancer patients shouldn't be too far away.

This review aims to elucidate the fundamental roles of natural killer (NK) cells in the eradication of tumours, pathways underlying the anticancer actions of NK cells, progress made in NK-related clinical trials, and the barriers scientists encountered with various contemporary solutions.

2. The function of NK in tumor

NK cells are a subset of leukocytes that are classified as constituents of the ILC lineage, which is ILC1, due to its representative expression of certain transcription factors as well as chemical productions [2]. Besides ILC1, there are two other families of ILCs, which are ILC2s (targeting helminth infections) and ILC3s (guarding mucosal environments), that all can be derived from one common ILC precursor cell. NK cells have a crucial function in the recognition and eradication of cells that are classified as "non-self" or "alter-self". This capability is attributed to their distinctive capacity to generate interferon-gamma (IFN-γ) and cytotoxic effects on cells without the need for an antigen. The derivation and development of NK cells are affected by both the tissue microenvironments surrounding them and the chemicals they are exposed to as developmental cues [3]. The classification of NK cells to distinct categories is through the differential presence of cellular markers CD56 and CD16, either CD56dimCD16hi and CD56brightCD16lo [3]. The former ones are typically found in human blood and circulation, carrying out cytotoxic functions, whereas the later ones tend to reside in the secondary lymphoid organs and tissues, and function as the main cytokine producers. There are multiple other remarkable markers and molecules on the NK cell surface, which contribute to its functions, and particularly, a few of them are directly tied to how well it functions towards cancers.

2.1. The receptor in NK

NK cells express two distinct types of receptors on their cell surface, namely activating receptors and inhibitory receptors, which also compose parts of the detecting system that NK cells utilize to carry out their effector functions. While NK cells do immune surveillance, the binding of ligands that can be found on healthy cells with the inhibitory receptors can protect healthy host cells from cellular lysis. This specific type of ligands is called MHC class 1, or HLA class 1 in people. It serves as an intuitive way for NK cells to differentiate between host healthy cells and non-self/altered cells since host-specific HLA class 1 is present on every nucleated cell. Two major families of HLA-detecting inhibitory receptors are KIR/CD158 and CD94/NKG2A. KIRs are specific for detection of HLA-A, B and C ligands, whereas NKG2A binds to HLA-E for effector function inhibition [4].

The activating receptors, on the other hand, recognize the presence of abnormal molecules and ligands appearing on cells that are "non-self" or "altered". Compared to the inhibitory ones, the category and family of activating receptors are more tangle some and complex since the novel ligands possessed by non-self/altered cells are truly random and unpredictable. NCR is one example. The extracellular regions of NCRs bind to various ligands. For example, it can bind to hemagglutinin on the surface of influenza virus. Particularly targeting cancer cells, NCRs bind to heparan sulfate, which carries out positive functions in accelerating tumor progression, therefore the tumor cells' metabolism will be interfered and eventually eliminated by NK cells [5]. Other activating receptors include NKG2D, CD94-NKG2C/E/H, CD226/224, etc. Similar to NCRs, these activating receptors aim to detect up-regulation of abnormal surface molecules and subsequently send downstream signals to trigger the cytotoxic functions.

Generally, tumor cells upregulate "non-self" but "beneficial" molecules such as molecules function to increase metabolic rate, and may downregulate "self" molecules. Through either way, tumor cells can be detected by NK cells, and the cytotoxic process should be triggered through distinguishable pathways.

2.2. Activation

When the receptors on the extracellular domain successfully bind to proper ligands, changes on the intracellular regain happens. ITIM is a protein sequence that can be found on the cytoplasmic domain of multiple inhibitory receptors, whereas ITAM is typically associated with previously-mentioned activating receptors. Both ITIM and ITAM are highly conserved and are extremely essential for signaling transduction. When the inhibitory receptor is triggered, the tyrosine residues contained by ITIM will be phosphorylated. The phosphorylation of ITIM tyrosine will subsequently recruit two types of protein tyrosine phosphatases, which are SHP-1/2 (contains Src Homogy domain 2) and SHIP [6]. Take SHP-1 as our example. The recruitment of SHP-1 can recognize and dephosphorylate LAT, another cellular marker present on the NK cell surface. LAT serves as an indispensable part of downstream signaling regarding cytotoxic functions, NK cells proliferation, and cytokine production, therefore, with dephosphorylation of LAT [6], the activation pathway is inhibited. One way that cancer cells can be discovered by NK cells during surveillance is by the downregulation of appropriate binding molecules for the inhibitory receptors. This leads to the reduction of NK cell effector inhibition, NK cell alertness, and cancerous cell elimination.

ITAM, on the other hand, functions in an opposite direction. It doesn't inhibit; instead, it strengthens the activation pathway, which leads to an eventual upregulation of the expression of some genes linked to NK cell killing processes. In this instance, LAT is not inhibited. It contacts with molecules called phospholipase C–γ (PLC-γ) in the intracellular domain of NK cells. PLC-γ1 is the predominant one, which is an enzyme with the function of converting PIP2 into inositol triphosphate (IP3) and DAG. Calcium ions can then be released when IP3 binds to IP3R on the ER. The abrupt rise in intracellular calcium concentration has the potential to trigger the production of specific genes and the activation of many transcription factors, hence initiating the effector actions of natural killer cells.

Cytokines are another example of another substance that can activate or suppress NK cells via various mechanisms. The balance between the opposite signals that an NK cell constantly receives determines whether it will become activated or remain quiescent.

3. Potential mechanism in anti-tumor therapy

3.1. Induce apoptosis

While T cells are asked to recognise foreign antigens before killing, NK cells can promptly trigger cellular death in modified cells, including tumor cells. NK cells contain granules that store a variety of chemicals and cytotoxic particles. Two of them which are associated with apoptosis inducing are perforin (Prf1) and granzyme (Gzm). The major function of Prf1 is to poke "holes" on the membrane of target cells to facilitate the delivery of Gzm into the target cells. GzmB is a protease which can induce the caspase activation consequently through the direct interaction with molecules under the BCL-2 family. Together with other pro-apoptotic mediators existing in the target cell, the classical caspase-mediated apoptosis, especially caspase-9 followed by caspase-3, can be triggered. Finally, caspase-3, as a death protease, would fragment the inhibitor for caspase-activated DNase (CAD), granting CAD to be internalized into the nucleus to cut the DNA of the target cell into pieces.

Besides GzmB, GzmM functions in a slightly different way through hindering the inhibitor of GzmB, allowing GzmB to function normally. GzmA also exists in the granules. In fact, GzmA is the most abundant form of granzyme contained by NK cells. Unlike GzmB and GzmM, GzmA targets mitochondria to cause the outflow of reactive oxygen species (ROS). Therefore, the mitochondrial metabolism is disrupted and eventually leads to cell death. GzmA can also work on cleaving proteins and molecules for DNA repair to facilitate GzmB in disturbing normal DNA sequences.

3.2. Ligand expression

NK cells express distinct ligands that attach to death receptors on tumor cells, serving as another method of inducing apoptosis in target cells. One generally expressed death receptor on tumor cells is the TNF receptor, which has various ligands such as TNF, FasL and TRAIL. When these ligands attach to the death receptors, there will be structural alterations of the receptors, and groups of adaptor protein, named FADD, are gathered. Followed by the recruitment of FADD, caspase 8 are enlisted and activated on their own to further activate caspase-3. As mentioned earlier, caspase-3 will ultimately lead to the cleavage of DNA materials and cell apoptosis [7].

3.3. Reduce immunosuppression

To allow the tumor cells to evade immune monitoring and endure as long as possible, they mediate the surrounding environment to become immunosuppressive. Such an immunosuppressive microenvironment can be created through multiple ways including soluble components, chemicals, and suppressive particles. To aid in tumor escape, these substances significantly reduce the NK cells' capacity for cytotoxicity. One way that NK cells do to relieve the suppressive impact is through cytokine production.

IFN-γ is a member of interferon family type II and has been long discovered to possess some antitumor effects. As a cytokine secreted by NK cells in quantity, IFN-γrescues immune systems against tumor cells in multiple ways. Firstly, as its primary responsibility, IFN-γcan be emitted and relocated to other parts of the body to alert and gather more immunological cells like neutrophils and macrophages. Specific to NK cells, IFN-γis able to aid perforin and granzymes [8], which are cytotoxic particles produced by NK cells, to start caspase-mediated tumor apoptosis. IFN-γalso interplays between other immune cells. For example, it induces macrophage polarization to result in more macrophages with M1 phenotype that is proinflammatory and more professional in chemokine secretion. IFN-γalso impacts the capability of antigen presentation by dendritic cells through upregulating MHC class II expression. For T cells, IFN-γcan manipulate their differentiation towards cytotoxic Th1 subset, and the differentiation of T regulatory cells, which are considered to be immunosuppressive, will be inhibited [8].

TNF, or TNF-α, is another significant cytokine regarding tumor elimination and regression which could induce direct cell necrosis and cell death through the binding with its membrane-bound receptors, including TNF receptor 1 (TNFR-1) and TNFR-2. Further downstream activation of adaptor molecules and proteins such as receptor interacting protein (RIP) will be triggered to ultimately unleash the transcription of certain genes related to cell death. Similar to granzyme and ligand-induced apoptosis, caspases, especially caspase-3, 7, and 8, are signaled to trigger classical apoptosis. Necrotic cell death is another outcome achieved by the secretion of ROS mediated by TNF secretion [9]. However, TNF should be considered as a double-edged sword, and its capability regarding tumor suppression should be evaluated by the balance between its pro- and anti-tumor functions. For example, the binding of TNF and its receptors can also activate the gene transcription for the nuclear factor-κB (NF-κB). Research shows that continually excessive expression of NF-κB results in various tumor-facilitating consequences, including promoting angiogenesis, preventing cellular apoptosis, encouraging tumor cell proliferation, etc [10].

4. Current application of NK-therapy

Immunotherapy regarding NK cells has become a hotspot for decades. In comparison to T cell immunotherapy, the utilization of NK cells mitigates the likelihood of GVHD development [11] so that the cell sources will not be limited to the patients only but broaden to any possible healthy donors. Even before the allogeneic infusion, the ex vivo manipulation of the obtained NK cells is more applicable, and the in vivo impacts led by the NK cells effector functions are more profound and far-reaching as well. In consideration of its unique advantages over therapies related to other immune cells, NK cell immunotherapy continues to be flourishing currently.

4.1. Non-engineered NK immunotherapy

Due to NK cells' ability to eliminate tumor cells in a direct, HLA-independent manner, NK cells can be adoptively transferred to the patients without the limitation of one patient-one donor principle. The infused NK cells carry inhibitory receptors, for example KIRs, that are targeting donor-specific MHC class 1 molecules [12]. On the other hand, the tumor cells would exhibit patient-specific MHC class 1 molecules, or even downregulate the MHC class 1 expression to escape immune surveillance. A "mismatch" between MHC class 1 proteins and inhibiting receptors on transplanted NK cells will result from either circumstance, leading to loss of inhibiting signals to NK cells cytotoxic effector functions. Researchers' responsibility, in this case, is to obtain NK cells and expand to a relatively large quantity ex vivo to prepare for cell infusion, and hematological malignancy is still the major obstacle for those non-engineered NK cells to tackle.

4.2. Engineered NK immunotherapy

The main research emphasis regarding NK cellular modification centers on the expression of the CAR structures on NK cells, referred to as CAR-NK therapy. CAR is an "artificial" receptor which is designed specifically for an antigen aiming to target, which, in this case, is the antigen uniquely on the cancer cells. Three domains are present in a CAR structure: (1) an ectodomain, which is also the antigen recognition and binding domain, (2) a transmembrane domain, which joins the NK cell membrane with the CAR structure, and (3) an endodomain which contains ITAMs so that the further downstream activating signals can be propagated. Following the development and proliferation, mature NK cells will be transfected with vectors containing the intended CAR structures ex vivo. This will result in a population of qualified NK cells that consistently display CAR structures on their cell surfaces. After the infusion, these CAR-NK cells would hopefully function just as NK cells that can "naturally", and only, recognize tumor cells and eventually eliminate them.

Until August 2023, there were 74 CAR-NK based clinical trials, mostly in early study phases. Besides the limited numbers of CAR-NK related clinical trials, the progress in study results is not optimistical as well. No trials exhibit clear and specific results. This is fair since the first CAR-NK related clinical trials occurred in 2009, whereas the clinical trials related to CAR-T, which is the previous, and even current, research center of CAR-related immunotherapy, started around 20 years earlier. However, given the growing number of active trials, experts continue to see promise for CAR-NK immunotherapy.

5. Current shortage and strategy

Despite accelerating advancement in NK immunotherapy, among all the NK-related clinical trials, only around 11% of them progress with results. This situation may be a result of combinations of various ex vivo and in vivo challenges regarding NK cells manipulation, expansion, infusion, cytotoxicity, and persistence.

5.1. Tumor ignorance

During tumorigenesis, especially in later stages with the presence of concrete tumor, the expression of tumor-specific antigens is consciously being down-regulated in order to escape immune surveillance, resulting in immunological ignorance. Immunosurveillance carried by NK cells becomes less effective as well, which contributes to less desirable effector functions as well as tumor elimination. Multiple therapeutic methods are under investigation, aiming to restore NK cells' capability regarding detecting altered tumor cells. Previously mentioned CAR-NK therapy is one of the approaches researchers are studying aiming to increase the effectiveness of tumor targeting by introducing a fully manmade novel receptor to NK cells. Besides figuring out a new receptor, armouring naturally existing receptors on NK cells is also feasible. When NK cells are activated and their cytotoxic activity begins, the cellular marker CD16 is crucial, and its detection towards cancer antigens can be improved upon the addition of monoclonal antibodies (mAbs). CD16 can bind the Fc

portion of certain monoclonal antibodies, which is artificially designed for the antigens. With more specific targeting and tighter binding to the tumor antigens, ADCC is a mechanism through which NK cells can induce cell death [11]. Even though both approaches require the production of new protein structures, modification to pre-existing receptors seems to be less labor-intensive since it is missing the transfection-expression step compared to CAR-NK therapy.

5.2. NK cell fratricide

Another situation which benefits tumor cells and weakens NK cell effector function is NK cell fratricide. In other words, the engineered NK cells target not only the tumor tissue but also NK cells themselves concurrently. For example, numerous myeloma over-express a cellular marker named CD38, which serves as a wonderful target for researchers to design corresponding receptors/mAbs. However, NK cells express a considerable amount of CD38 as well [11]. Therefore, the engineered NK cells with increased targeting towards CD38, after infusion, may fratricide, which bring about declined NK cell effector function as well as early NK cell depletion. One possible strategy to overcome this drawback is through genetic modification to knock down the NK cells' expression of CD38. Researchers found that the essential period for CD38 expression is during the ex vivo expanding time slot. Therefore, applying the CRISPR/Cas9 technology to the expanding NK cell population in the laboratory to disrupt CD38 expression before infusing into the patients can be achieved to enhance the efficiency for NK-mediated cancer removal and the longevity of NK cells in humans.

5.3. Tumor infiltration

Compared to T cells, which also carry out cytotoxic function, NK cells exhibit low levels of tumor infiltration. Natural Cytotoxicity Triggering Receptor 1 (NCR1) encodes a protein named NKp46, which is the "symbol" of NK cell presence [13]. The weakened expression of NCR1 in NK cells indicated that less NKp46 was being produced, and, in turn, less NK cells were infiltrated into solid tumor tissues. One current response is through gene alteration to over-express certain chemokine receptors. Chemokine belongs to the family of cytokines, but it functions especially in attracting and guiding immune cells to the site of need by creating a concentration gradient. One pro-inflammatory chemokine, IL-8, is generally excessively secreted by both solid tumors and hematological malignancies, and its receptor, CXCR1, can be found on NK cells. Related mice xenograft samples showed that NK cells with overexpression of CXCR1 display an boosted level of infiltration for multiple IL-8 secreting tumors in vivo [14]. This highlights the possibility of altering the level of presence of specific receptors for certain chemokines to enhance tumor targeting.

6. Summary

NK-based cancer immunotherapy is progressively growing into a valuable treatment for both solid tumor and hematologic malignancies. The growing quantity of clinical trials based on NK and the strategies that were constantly being discovered in coping with corresponding challenges further prove scientists' confidence in accelerating NK immunotherapy development and to generally increase the success rate of cancer treatment. Further studies may be needed to reduce the side effects brought by NK therapy such as CRS. To be more precise, the detrimental effects that come along with TNF-α secretion—one of the main cytokines generated by NK cells—should be thoroughly studied in order to eliminate any chance of fostering an environment that supports cancer.

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