# Expansion Of Natural Killer Cells and Efficacy Against Hematologic Malignancies

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**Abstract.** The battle against cancer in human body relies heavily on natural killer (NK) cells. Therefore, NK cells have been showing great potential in cancer immunotherapy. Adoptive transfer of great amounts of NK cells and their cytotoxicity towards cancer cells are heavily researched because of the fact that the NK cells in patients are typically diminished in quantity and malfunctional because of pretreatment. In this review, we concentrate on the techniques for expanding the four main NK cell sources employed in adoptive cell transfer: peripheral blood, umbilical cord blood, NK cell lines and different types of stem cells. We also investigate the efficacy of NK cells derived from each cell source against hematologic malignancies by analyzing experimental data and propose possible reasons for the discrepancy. In the last part, we touch on the prospect of genetically modified NK cells. We concluded that each cell source has its own advantages and drawbacks in expansion and efficacy. In addition, by combining with cytokines, monoclonal antibodies, and other treatments, the cytotoxicity of NK cells in vivo against hematologic malignancies can be enhanced.

**Keywords:** NK cells; expansion; hematologic malignancies.

## 1. Introduction

In modern society, cancer is among the most prevalent causes of death. Among all types of cancer, hematologic malignances refer to a group of cancers that involves abnormal cloning of hematopoietic cells within the blood, bone marrow and lymphatic tissue. They can be classified into three categories: leukemia, lymphoma, and myeloma, depending on the type of cells affected. They are the most common cancer types, claiming more than 600,000 lives worldwide in 2020. In recent years, immunotherapy has become a promising approach in treating cancer, especially non-solid tumors, since it can provide targeted treatment through the circulating system with fewer side effects. One of the most investigated immunotherapies so far is T-cell therapy. However, the method has certain drawbacks including immune evasion, cytokine release syndrome and neurological issues.

Hence, natural killer (NK) cells are receiving more attention nowadays. They are a kind of lymphocytes that share the same progenitor with T cells and B cells. They respond rapidly to pathogens without the need for activation as part of the innate immune system. NK cells are distinctive in all kinds of lymphocytes due to the fact that their ability to recognize antigens is manipulated by the signals from the activating and inhibitory receptors rather than specific antigens. In the case of cancer, they directly attack cancer cells and don't rely on MHC-I molecules for recognition, in contrast with T cells. On the contrary, the inhibitory receptors of NK cells can identify MHC-I or class I like molecules. Therefore, they can target the cells that escape immune response of T cells and enhance T-cell performance. Studies have shown that they can provide efficient therapy both alone and combined with T cells. Utilizing basically four sources, numerous approaches have been devised to acquire significant quantities of NK cells. Different expansion methods are applied to each source and the cells obtained present varied cytotoxicity against hematologic cancers.

## 2. Peripheral Blood (PB) NK cells

Autologous NK cells are obtained from the body of the individual himself, primarily from the peripheral blood (PB). It is the first source to be investigated due to the conveniences of using the patients' own blood and smaller chance of inducing graft-versus-host disease, which is a post-

transplantation disorder where the graft cells recognize the host tissues as foreign and attack them (table 1) [1]. The NK cells are taken out of the patients' body, expanded ex vivo, reprogrammed to be activated to better target pathogens, and infused back to the patients' body.

**Table 1**. Advances and Limitations of NK cells from Different Sources [1]

| NK sources | Advantages | Limitations |
|---|---|---|
| PB-NK cells | Easily available<br>Reliable replication in vivo<br>Positive clinical feedbacks | Heterogeneous cell population<br>Difficult to implement genetical manipulations<br>Impotent responses |
| UCB-NK cells | NK progenitors exhibited<br>Greater proportion of NK cells<br>Capability of cryopreserving UCB | Heterogeneous cell population<br>Difficult to implement genetical manipulations<br>Incomplete maturation |
| NK cell lines | Defined, homogeneous property<br>Easy to grow<br>Genetically modified<br>Relatively high cytotoxicity<br>Applicable for "of-the-shelf" cell therapy | Requires irradiation<br>Lack certain receptors<br>Limited in vivo expansion and persistence |
| iPSC-NK cells | Defined, homogeneous property<br>Easy to implement genetical manipulations for enhancement<br>Good in vivo survival<br>Relatively high cytotoxicity<br>Applicable for "of-the-shelf" cell therapy | More complex developmental procedure |

### 2.1. Expansion of NK cells from PB

A mixture of cytokines, the activation effect of feeder cells or membrane molecules are applied for boosting NK cell expansion when growing PB-derived NK cells in vitro.

Cytokines are in charge of manipulating the growth and multiplication of NK cells. NK cell proliferation can be induced more efficiently by different cytokines combined than by only one type, but there's still room for improvement in the overall expansion fold. Hence, antibodies have been used simultaneously with cytokines for growing NK cells ex vivo. This method shows better expansion efficiency than the multi-cytokine method [2].

The feeder cell system involves lymphoblastoid cells, irradiated tumor cells, especially K562 cell line, and allogeneic peripheral blood mononuclear cells (PBMCs). These cells are genetically modified to express specific ligands and membrane particles to activate the proliferation pathways and enhance expansion of NK cells. The use of membrane particles goes a step further. It serves as an effective alternative to irradiated tumor cell lines and avoids the adverse effects when cancer cells are not completely separated from NK cells.

### 2.2. Efficacy of PB-NK cells against Multiple Myeloma

Studies have shown that NK cells originating from this source are able to proliferate in vivo and have cytotoxicity against multiple myeloma. But the effect is weaker compared with umbilical cord blood (UCB) NK cells [3]. Possible explanations may be that NK cells recognize the MHC-1 molecules from the same individual, and the immune responses of the NK cells infused are inhibited. Also, as autologous NK cells are often taken out of the patients who have been heavily pretreated, their efficacy and persistence in vivo remain limited. However, when combined with daratumumab, a type of monoclonal antibody, the efficacy significantly increases [3]. Cytokines and cells with ligands that activate immune-response pathways can also enhance cytotoxicity. Therefore, combination therapy is still prospective despite the unideal outcome using solely PB-NK cells.

## 3. Umbilical Cord Blood NK Cells

Since autologous NK-cell therapies are mainly ineffective, more attention nowadays is focused on allogeneic NK cells, which are divided into three main types. The first type comes from the UCB. In the PB, NK cells constitute about 10% of all lymphocytes, while in the UCB, they account for about 30%. Therefore, the UCB is a robust source for these cells.

### 3.1. Expansion of NK cells from the UCB

The use of feeder cells, including tumor cell lines, lymphoblastoid cell lines etc., is the first method for expanding NK cells from UCB. Different feeder cells result in various expansion fold and cell purity. Similar with PB-NK cells, feeder-cell free systems with cytokines and other stimulating factors are also implemented. These methods reduce the possibility of impurities where undesired cells are present in the outcome. In addition, with continuous improvements in these systems, they are becoming easier to implement. Under certain circumstances, highly pure NK cells with high efficacy can be yielded by a single type of cytokine.

### 3.2. Efficacy of UCB-NK cells against Leukemia

Studies have shown that these cells show weaker efficacy against K562, a type of myelogenous leukemia cell line. Compared with PB-NK cells, adhesion proteins and activating receptors, including CD2 and CD16, are expressed less frequently on UCB-NK cells, whereas inhibitory receptors, such NKG2A and CXCR4, are more prevalent [4]. These occur as a result of differentiated yet immature UCB-NK cells. Treatment with cytokines like IL-2 can promote UCB-NK cells maturation and therefore increase cytotoxicity. Moreover, monoclonal antibodies that block CD47 on acute lymphoblastic leukemia are able to reduce immunosuppression and enhance the efficacy of UCB-NK cells. Studies have shown that following NK cell line-based CD47 blocking, the proportion of apoptosis rose in Molt-4 cells from 13% to 52%, in Nalm-6 cells from 20% to 49%, and in Reh cells from 11% to 44% [5].

## 4. NK Cell Lines

The second type of allogeneic cell source is the NK cell line. They are clonal cells that come from one cell, which leads to their genetical homogeneity (table 1) [1]. Also, the expansion procedure is simple. NK cell lines readily double in number within 24 to 36 hours, and they can continue to proliferate ex vivo for more than 18 months [6].

### 4.1. Expansion of NK cells from cell lines

Unlike UCB-NK cells and PB-NK cells, the growth of NK cell lines doesn't rely on feeder cells and demands minimum intervention. Methods using cytokines are the most prevalent. NK cells are cultured with IL-2 as well as other stimulating factors and necessary components. Furthermore, for clinical-grade expansion, human plasma and serum-free expansion methods have both been reported. However, the use of human plasma increases the cost and potential health risks in clinical use [7]. Cells obtained through serum-free expansion methods show limited cytotoxicity and persistence in vivo. Hence, the choice of expansion method depends on clinical needs.

### 4.2. Efficacy of NK-92 against Hematologic Malignancies

The most common type of NK cell line used in NK cell therapy is NK-92. High efficacy against multiple types of leukemia, lymphoma, and myeloma is demonstrated by the NK-92 cell line. Few inhibitory receptors may account for this potency. For instance, in a 4-hours chromium-51 release experiment, NK-92 cells efficiently eliminated both K562 and Daudi cells, with data showing that 84% and 86% of cancer cells were eradicated respectively in the two cases [6]. It is also important to note that normal PBMCs and hematopoietic cells did not experience any cytotoxicity in the

experiment. The cytotoxicity of NK-92 was verified in immunodeficient animal models xenografted with human leukemia and malignant melanoma in vivo [8]. In these models, the animals tested demonstrated extended survival periods.

All the above shows that NK-92 has great potential in cancer immunotherapy against hematologic malignancies. However, despite all the merits, they are aneuploids, which leads to their genetical instability. Therefore, they require irradiation prior to infusion. Even with multiple infusions or accompanied by IL-2 cytokines, the non-irradiated cells fail to engraft. On the other hand, it has been shown that cells treated with radiation can eliminate tumor cells. but the irradiation process reduces in vivo persistence, presenting an obstacle in durable clinical efficacy. Four clinical studies are now testing the efficacy of one type of genetically edited NK-92-derived product haNK, applied with avelumab, an anti-PD-L1 antibody, and the cancer vaccination NANT [9].

## 5. Stem-Cell Derived NK cells

NK cell lines, UCB-NK cells, or PB-NK cells have been used in a majority of NK cell treatments so far. However, as previously mentioned, there are significant disadvantages in each of them. These drawbacks can be overcome, but stem-cell derived NK cells have emerged as another type of more promising source, since they can offer standardized, 'off-the-shelf' therapies for every patient. Stem cells which have unlimited proliferation potential, enables generation of nearly an infinite number of NK cells. This measure improves the homogeneity and reproducibility of the source and standardizes the starting material in NK cell therapies. Also, these cells exhibit higher potential for genetic engineering for enhanced efficacy (table 1) [1].

### 5.1. Types of Stem-Cell derived NK cells

Two major stem cell types, embryonic stem cells (ESCS) and induced pluripotent stem cells (iPSCs), are employed in NK cell therapy. Between day 3 and day 5 after fertilization, the embryo, also known as a blastocyst at this stage, includes an inner layer of cells that has the potential of forming any tissue, organ and cell type in the human body. The embryos used in clinical research are fertilized in vitro and employed after informed permission has been received. Even so, the use of ESCs evokes ethical concerns, as genetic manipulations on human embryos are strictly limited.

Therefore, more attention nowadays is focused on iPSCs. Researchers discovered that under certain conditions, some mature, fully differentiated adult cells can be reprogrammed back to pluripotency. Transcription factors, known as Yamanaka factors, have been applied to realize this process, stimulating the expression of major genes that regulate pluripotency. After expansion, iPSCs are differentiated into NK cells. This type of stem cells is free from ethical concerns and the material is easily accessible.

In addition to ESCS and iPSCs, hematopoietic stem cells can be collected from the cord blood as well. It is by far the only stem-cell related product approved by the FDA. However, the amount of stem cells in cord blood is small. Hence, the method of using cord blood to obtain stem cells remains to be improved.

### 5.2. Expansion of NK cells from iPSCs

To obtain sufficient amount of NK cells, feeder cell system is used for expansion of iPSCs. The feeder cells used are mainly irradiated K562 cells and irradiated PBMCs, yielding an expansion fold from about 102 to 106 in three weeks. Furthermore, iPSCs have to be differentiated into NK cells to be applied into adoptive cell transfer. After years of research, some basic protocols have been developed concerning the differentiation process. There are two phases in the differentiation process. In the first phase, iPSCs differentiate into a type of immature stem cells that are capable of developing into mature blood cells, called the hematopoietic progenitor cells. In the second phase, these cells differentiate into NK cells [10].

### 5.3. Efficacy of iPSC-NK cells against Hematologic Malignancies

In comparison with PB-NK cells, it has been revealed that iPSC-NK cells exhibit more durable anti-tumor functions on K562 myeloid leukemia cells. Although cells from these two sources exhibit similar cytotoxicity in early stages, iPSC-NK cells maintained better antitumor function later on [11]. This can also be supported by another experiment testing the killing effects of a type of gene-edited iPSC-NK cells against MM.1R human multiple myeloma cells [12]. There were three rounds of experiments. During the first round, cytotoxicity of the two types of cells is similar. However, in the second round and third round, iPSC-NK cells enhanced their cytotoxicity while PB-NK cells lost its ability to kill cancer cells. The data indicates that iPSC-NK cells can produce efficient and durable innate cytotoxicity. Furthermore, the monoclonal antibody daratumumab can enhance the cytotoxicity against multiple myeloma and possibly other types of hematologic malignancies [11]. Even without the aid of cytokines, they are capable of producing potent, long-lasting in vivo anticancer activity. Therefore, this type of NK cells has the potential of offering successful therapy, either applied alone or in conjunction with daratumumab. Even though the future seems promising, there are still technological obstacles that remain to be overcome. Only one type of stem-cell related product has been approved by the FDA so far, which is hematopoietic progenitor cells derived from cord blood. As iPSC emerged as a promising field in fairly recent years, more research is required to avoid immune rejection and ensure successful transplantation and engraftment.

## 6. Genetically modified NK cells

Genetically modified cells are a unique source in that they utilize all the sources mentioned above and add genetic manipulations to the cells. Following the recognition process between inhibitory receptors and the MHC-1 molecules, NK-cell activation is inhibited. However, to avoid the detection of CD8+ T cells, many cancer cells suppress MHC-1 expression on the cell membrane. Hence, genetically modified NK cells can provide an excellent supplement for CAR-T therapy, as they robustly target the cells that manage to escape the immune detection of T cells. They can also recruit T cells to the cancer site and activate their immune response.

Genetically modified NK cells include CAR-NK cells, and other genetic modifications which increase cytotoxicity or in vivo persistence. Major inhibitory receptors can be removed, as well as adding activating receptors and adhesion molecules to restore antibody-dependent cellular cytotoxicity. As mentioned before, the in vivo persistence of NK cells can be enhanced with the use of recombinant cytokine IL-2. However, side effects include immune inhibition of T cells at low doses and significant cytotoxicity at high doses. Therefore, to address this issue, IL-2 related genes are introduced to the NK cells. Consequently, they are able to synthesis IL-2 and the growth is not dependent on external cytokines.

The first clinical trials are being conducted following significant advancements recently. However, several drawbacks remain to be solved. Viral-based methods are currently the most common way to realize expression of exogenous genes. Nevertheless, expression levels and efficiency of gene transmission are varied. Further research is required to initiate the best protocol. Non-viral methods, on the other hand, have lower efficiency, and the expression of the target genes can be short-lived, so they are far from large-scale implementation. Besides, continuous and detailed safety surveillance is needed after genetical manipulations.

## 7. Conclusion

Clinical research in the domain of NK-cell immunotherapy for liquid cancers has yielded promising results. Various NK cells sources undergo different expansion procedures and have shown varied efficacy against hematologic malignancies in adoptive cell transfer. Their cytotoxicity can be enhanced with cytokines or combination treatment with cancer vaccines or other methods. Despite positive results, there are still certain drawbacks. NK cells exhibit relatively limited lifespan in vivo,

which limits their clinical efficacy. Moreover, the efficacy against solid tumors remains to be investigated. Two factors contribute to the low efficiency. Firstly, the tumor microenvironment inhibits the immune response of NK cells. Secondly, it is more challenging to target solid tumors with the immune cells in the circulatory system. To address these issues, cytokines and other combination treatments can be infused with NK cells to regulate their lifespan and proliferation. In addition, genetical modifications of different kinds can be implemented to increase pertinence and cytotoxicity, including altering membrane receptors and transduction of cytokine-related genes. On a broader scale, the advances in laboratory techniques should be better translated into clinical applications as more data on clinical trials is needed to verify the performance and safety of NK cell therapy. Also, standardized protocols are necessary before large-scale treatment can be put into practice. Besides, as NK cells have different subtypes, more research remains to be done on the growth of a particular type of NK cell and its efficacy on a specific type of tumor. Although challenges exist, we believe that with new technological advancements in genetic manipulation, iPSCs etc., the great potential of NK cells can be realized in the future.

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