Manufacturing of Natural Killer Cells for Treating Solid Malignancies

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Abstract

Objectives: Natural killer (NK) cells are an attractive and potent tool for cancer immunotherapy, however, *ex vivo* expansion of NK cells is required to achieve therapeutic cell dosages. As such, this review will discuss recent NK cell manufacturing methods applied in clinical trials for expanded NK cells for the treatment of solid tumors, as well as investigational NK cell manufacturing protocols. Given the unique challenges posed by the solid tumor microenvironment, the main objective of this review is to highlight key biological mechanisms associated with tumor homing and infiltration of NK cells and how manufacturing methods impact these functions.

Key Findings: For efficient adoptive NK cell therapy for the treatment of solid malignancies, NK cells need to extravasate from the blood stream, migrate through the tumor extracellular matrix, lyse cancer cells, activate other immune cells, and persist in the blood stream. The NK cell manufacturing process is complex, with each parameter influencing the expansion rate, and final NK cell number, purity, phonotype, and cytotoxicity. Many investigational and clinical NK cell manufacturing protocols generate high numbers of NK cells with greater cytotoxicity than freshly isolated NK cells. The expression of factors related to homing and migration in NK cells after *ex vivo* expansion is largely overlooked, but the few studies which have explored this indicate manufacturing processes can affect these critical mechanisms.

Conclusion: The current manufacturing protocols can generate high numbers of NK cells with increased cytotoxic functions, however understanding the effect of expansion on factors related to NK cell homing and migration is also important for treating solid malignancies. Furthermore, to progress the field of expanded NK cells for the treatment of solid tumors, improving "off-the-shelf" NK cell therapies and standardizing the manufacturing protocols and release criteria should be prioritized.

Keywords: Natural Killer Cell, Manufacturing NK cells, *Ex Vivo* Expansion, Adoptive NK Cell Therapy, Immunotherapy

1. Introduction

Cellular immunotherapies have become a powerful tool in treating cancer, especially for patients with limited treatment options. Adoptive transfer of engineered autologous T cells, which express chimeric antigen receptors (CARs) for a specific tumor antigen, has shown remarkable effects on inducing long term remission in patients with B lymphoblastic leukemia[1] and chronic lymphocytic leukemia.[2] As such, two anti-CD19 CAR T cell therapies have been approved by the US Food and Drug Administration (FDA) for patients with relapsed B cell acute lymphoblastic leukemia, refractory or relapsed large B cell non-Hodgkin lymphoma, and refractory lymphoma.[3–5] However, CAR T cells therapies suffer from various limitations, including severe side effects, variability in patient responses, and cell sourcing issues. The toxicities related to CAR T cells are most commonly cytokine release syndrome, neurotoxicities, and on-target, off-tumor effects.[6–8] While the majority of hematological cancer patients enter remission with CAR T cells, a portion of patients, anywhere from 15-50%, do not have a response to the therapy.[9,10] Autologous T cells must be used to avoid graft-versus-host disease (GvHD), however the manufacturing process is long and patient-specific, and it can be challenging to generate the consistent final cell products.[7,9–12] Furthermore, there is a high cost associated with CAR T cell therapy, further limiting the availably of this therapy. Utilizing allogeneic cells may overcome some of these limitations, and unlike T cells, natural killer (NK) cells provide a safe and effective source for allogeneic cell therapy. NK cells recognize and lyse virus-infected and transformed cells without the need for priming or knowledge of a specific antigen.[13–15] Instead, NK cell activation relies on the balance of signals from target cells, received through an array of activating and inhibitory receptors. Inhibition of NK cells relies on the expression of self-human leukocyte antigens (HLAs) on healthy cells; however, transformed cells downregulate these molecules leading to NK cell activation, in combination with other activating signals. Furthermore, NK cells have a relatively short lifespan of about 2 weeks *in vivo*,[13,16] reducing the risks of long-term adverse effects. As such, NK cells are a viable option for allogeneic cancer therapy and may overcome some of the limitations associated with T-cell therapies.

Both autologous[17–19] and allogeneic[20–28] NK cells have been used in numerous clinical trials to date, with minimal side effects and promising results in patients with hematological cancers. Importantly, allogeneic NK cells are less susceptible to GvHD while concurrently able to induce a potent graft-versus-tumor (GvT) response.[20–28] There has been significant clinical benefit in allogeneic NK cell therapy, specifically in patients with acute myelogenous leukemia (AML).[29–31] While, NK cell therapies have been effective in inducing remission in 50-80% of hematological cancer patients,[21,29–32] their use for solid tumors has been less effective, with approximately 20% of patients entering remission.[33,34] In certain

hematological cancers, NK cells may have a more readily available route of exposure to cancer cells, increasing the likelihood of NK cell-target cell contact and thus NK cell-mediated lysis. The solid tumor microenvironment (TME) poses additional challenges for NK cells, as it is highly suppressive to NK cell function and infiltration. The TME is composed of heterogeneous cancer cells with a high degree of plasticity, additional immune modulating stromal cells (i.e. cancer associated fibroblasts, tumor associated macrophages, and regulatory T cells), and typically a extracellular matrix of various proteins, all which combine to create challenging hurdles for NK cells. In addition, solid tumors can reside in less accessible areas of the patient. Due to the limited clinical efficacy and the unique challenges that solid tumors pose to NK cells, this review will focus on manufacturing *ex vivo* expanded NK cells and strategies to develop more effective NK cell therapies for the treatment of solid maliganaices.

Clinical trials of adoptive NK cell therapy for various malignancies have revealed that higher dosages[32] and multiple infusions lead to better clinical outcomes in patients.[35,36] For example, infused IL-2 activated allogeneic NK cells were inhibited by soluble stress ligands in the plasma of neuroblastoma patients, but this could be overcome in part by infusing a higher number of NK cells.[37] A typical dose of NK cells in clinical trials has been $1x10^6$ – $1x10^9$ cells/kg, with recent studies reaching up to 10^{10} cells/kg, and the maximum tolerated dose (MTD) has yet to be determined.[21,29–33,35,38–40] As NK cells comprise only 10% to 20% of the circulating lymphocyte population,[14,41] only a small amount can be isolated from a donor. While a standard unstimulated leukapheresis process can results in enough NK cells for a lower cell dosage, it is not enough for higher or multiple dosages. Therefore, there is a need to expand highly enriched NK cells *ex vivo* to reach a clinically relevant dose of cells for multiple infusions, to have a sufficient anti-tumor effect. Furthermore, during the expansion process, NK cells can be activated or engineered to be a highly functioning cell population, even greater than the starting cell source. Many different protocols have been used to generate a high number of potent NK cells, but they are not clinically translatable as they don't comply with set standards. Conversely, certain protocols to manufacture NK cells on a clinical-scale have been developed in compliance with current Good Manufacturing Practices (cGMP) standards. cGMP standards ensures the identity, potency, quality, purity, safety, and the intended effect of the final product is maintained, and is required for therapies in clinical trials. However, a well-defined clinical-grade standard manufacturing procedure has yet to be established, which could help progress NK cell therapies for solid tumors towards becoming a successful therapeutic option.

This review describes the various considerations for manufacturing NK cells for the treatment of solid tumors. Emphasis is be placed on the importance of tumor homing and migration of NK cells, by acknowledging the associated biological mechanisms and how manufacturing protocols consider and evaluate these critical NK cell functions. First, we highlight the different functions NK cells require to be

effective for the treatment of solid malignancies, followed by a summary of relevant clinical trials of *ex vivo* expanded NK cells for treating solid tumors. Next, the protocols for manufacturing *ex vivo* expanded NK cells will be compared. Lastly, methods to advance NK cell therapies for solid tumors and other future considerations for NK cell therapies will be discussed.

1.1 Natural Killer Cell Biology

NK cells are the major effector cell of the innate immune system and have both cytotoxic and cytokine-producing effector functions. NK cells are phenotypically defined by the expression of CD56 and the lack of CD3,[14,42] with the two main subsets of NK cells characterized based on the expression level of CD56. Approximately 90% of circulating NK cells are considered the cytotoxic population, defined by a low density of CD56 and high expression of FcgRIII (CD16), a receptor that binds the Fc portion of IgG ($CD56^{dim}CD16^{bright}$).[42,43] This population also has abundant perforin and granzyme granules, and the ability to rapidly release cytokines, such as tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), upon target cell recognition. The smaller population with a high density of CD56 and low expression of CD16 ($CD56^{bright}CD16^{dim/-}$) has largely an immunoregulatory role exerted through the secretion of many cytokines, like TNF-α and IFN-γ, and chemokines, such as RANTES and macrophage inflammatory protein 1α/β (MIP-1α/β).[42,43] Importantly, there is a subset of T cells which express CD56,[44–46] referred to as CD56+CD3+ cells, NK-like T cells (NKT cells), or cytokine induced killer cells, which need to be taken into account when relying on CD56 for NK cell identification and classification.

NK cells utilize a large array of surface receptors, which receive activating or inhibitory signals from target cells to monitor surrounding cells for phenotypic alterations and mount an appropriate response. Human NK cells can discriminate between normal and transformed cells on the basis of HLA expression; as healthy cells express HLAs that engage inhibitory receptors on NK cells, they are protected from NK cell mediated lysis. In a process called "licensing", naïve NK cells that engage self HLAs are educated to recognize self and remain quiescent, while also becoming activated to lyse target cells which lack or downregulate self-antigens.[47] According to the "missing self" hypothesis, activation of NK cell mediated cytotoxicity occurs by recognition of transformed cells that have lost or deficient HLA expression.[48,49] The "dynamic equilibrium concept" built upon the "missing self" hypothesis to explain the regulation of NK cell cytotoxicity,[13,50] as they rely on activating and inhibitory signals from target cells received through an array of surface receptors. Therefore, NK cell mediated cytotoxicity is activated by recognition of transformed cells that not only have lost or deficient HLA expression, but also have gained expression of stress-induced ligands, which interact with activating ligands on NK cells. This type of NK cell-mediated lysis relies on the release of lytic granules to induce target cell death, and corresponds with the release of pro-inflammatory cytokines and chemokines from the NK cell. After the initial target cell lysis, particularly active NK cells have the capacity to restart the activation cycle to

target and lyse three additional target cells, in a process called serial killing.[15,51–53] Interestingly, NK cells utilize granule-mediated cytotoxicity, as previously described, for initial killing events then switch to another method, death receptor-mediated cytotoxicity, for their final kill.[53] In this mechanism of killing, NK cells induce apoptosis of target cells through a caspase cascade, initiated by activation of caspase-8 and -10 at the death-inducing signaling complex formed in a target cell by interaction with death receptors on NK cells.[53]

Activating NK cell cytotoxicity through various activating receptors results in granule-mediated cytotoxicity. These include the different families of activating receptors - the natural cytotoxic receptors family (NCRs - NKp30, NKp44, and NKp46), the natural killer group 2 calcium-dependent lectin-like family (NKG2 C-lectin - CD94/NKG2C, NKG2D/E/H), nectin-binding adhesion molecules (DNAX accessory molecule 1, DNAM1), and killer cell immunoglobulin-like receptors (KIRs) with short cytoplasmic tails (KIR-S).[13,54] The NCRs recognize viral ligands, heat shock-associated proteins, or tumor antigens. The most characterized receptor, NKG2D, recognizes two groups of proteins – (i) major histocompatibility complex class I chain-related genes A and B (MICA and MICB) and (ii) UL16 binding protein family members 1-6 (ULBP1-6). The other NKG2 C-lectin receptors interact with HLA-E ligands on target cells, and KIR-S interact with HLA-C and other unknown ligands.[13,54] Death receptor-mediated cytotoxicity relies on activation of NK cells through the death receptors, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL).[53] Another mechanism of NK cell-mediated cytotoxicity is antibody-dependent cellular cytotoxicity (ADCC) through CD16 activation. CD16 can bind to the Fc region of antibody coated targets (opsonized), leading to activation signals that result in NK cell degranulation and perforin-dependent lysis of the target cell. This activation also leads to the production of cytokines and chemokines by the NK cell. Since the different subset of NK cells express different levels of CD16, the $CD56^{dim}CD16^{bright}$ population exhibits greater levels of ADCC compared to the $CD56^{bright}CD16^{dim/-}$ population.[55] Importantly, some families of NK cell receptors contain paired receptors that bind similar ligands but have distinctly different stimulatory functions.[54] For example, the main inhibitory receptors on NK cells are in the NKG2 C-lectin family (CD94/NKG2A/B), and the KIR family with long cytoplasmic domains (KIR-L).[13,54] The CD94/NKG2D receptor interacts with HLA-E molecules and the KIR–L ligands interact with “self-HLA ligands”, HLA-A, -B, and –C, molecules on target cells. It is important to note that the different populations of NK cells express different profiles of activating and inhibitory receptors. The immunoregulatory population, $CD56^{bright}CD16^{dim/-}$, expresses high levels of CD94,[56] NKG2A,[56] NKG2D and NKp46, and low levels of KIR-L and KIR-S.[57,58] On the other hand, the cytotoxic population, $CD56^{dim}CD16^{bright}$, expresses higher levels of KIR-L,[59] KIR-S,[59] NKp30, and DNAM1,[57,58] and lower levels of CD94[59] and NKG2A[57,58] than $CD56^{bright}CD16^{dim/-}$ cells.

Furthermore, the different populations exhibit different receptor repertoires depending on the tissue they are isolated from (i.e. peripheral blood vs lymph node).[14]

In addition to cytotoxic functions, NK cells also have a critical function of producing pro-inflammatory cytokines and chemokines, within minutes of stimulation, further distinguishing them from T cells which have a delayed response.[60,61] Stimulation by cytokines, such as interleukin 2 (IL-2), IL-12, IL-15, IL-18, and IL-21, alone, in combination, and/or engagement of activating receptors[14,62] leads to the production of cytokines, like TNF-α and IFN-γ, and chemokines, such as RANTES and MIP-1α/β.[42,43] Production of these molecules is critical to inducing an adaptive immune response, pro-inflammatory cytokine cascades in target cells, and death receptor and stress ligand expression on target cells.[13,14,62] Furthermore, stimulation by cytokines is required for NK cell differentiation, proliferation, and facilitating activation of cytotoxic functions. A combination of stimuli (multiple cytokines or cytokines and activating receptor engagement) results in greater NK cell proliferation and effector functions, relative to stimulation with a single cytokine.[63,64] In addition, there is evidence that the combination of IL-12, IL-15, and IL-18 stimulation can induce memory-like NK cells, which have enhanced NK cell function even after discontinuation or absence of the initial stimuli.[65] Upon re-stimulation with cytokines, cancer cell lines, or primary AML blasts, memory-like NK cells have superior IFN-γ production and potent cytotoxic functions.[65] Overall, the cytotoxic and cytokine/chemokine producing function of NK cells are critical for successful treatment of malignancies, and thus need to be enhanced during the manufacturing process to enable better functioning NK cell therapies.

1.2 Natural Killer Cell Functions in Solid Tumors

To manufacture NK cells for effective immunotherapy requires an understanding of their function and inhibition in solid tumors. The role of NK cells in cancer has been extensively studied and reviewed, including their role in immunosurveillance and the intense suppression of NK cells.[42,66–71] Briefly, NK cells are critical in the immunosurveillance solid tumors, as NK cell function has been negatively correlated with cancer occurrence and outcome.[72,73] These cells can control tumor growth by interacting directly with the cancer cells and by influencing the function of other innate and adaptive immune cells in the tumor microenvironment, based on mechanisms previously described. Notably, many studies reveal that the presence of tumor infiltrating NK cells is associated with a better prognosis in various solid tumors.[74–82] Indeed, the extent of tumor infiltrating NK cells was defined as an independent biomarker with great predictive value for complete response to an anti-HER2 antibody-based treatment in breast cancer.[83] A further role of NK cells in cancer is their ability to target and kill cancer stem-like cells, which are a chemo- and radio-resistant, quiescent cell type that is responsible for tumor relapse after therapy.[84–87] This is also true for undifferentiated tumors, which were highly resistant to chemotherapy,[35,87] and circulating tumor cells.[88] Hence, NK cells are attributed to controlling the formation of metastases.[88]

Though NK cells have the ability to control tumor progression and metastases, there are many mechanisms by which solid tumors suppress NK cell function.

As previously stated, solid malignancies pose a greater hurdle to NK cells than hematological cancers because of the hostile and complex TME.[89] The solid TME is composed of various cell types and extracellular proteins, which create a physical barrier and a biochemical milieu that is highly immunosuppressive to NK cells. This results in insufficient NK cell infiltration,[90–93] with a majority of infiltrating NK cells remaining in the tumor stroma, thereby impeding the critical NK cell-cancer cell contact required for NK cell mediated cytotoxicity.[71,94–96] Furthermore, the NK cells within the tumor are functionally inhibited by many tumor derived immunosuppressive factors that either act directly on NK cells or indirectly by stimulating other immune cells, such as regulatory T cells (T_{regs}), to produce additional anti-inflammatory factors.[66,69,91,92] In addition to tumor infiltrating NK cells, peripheral blood NK cells also have decreased cytotoxic functions and activating receptor expression, relative to healthy patients.[77,92,97] To directly evade NK cells, cancer cells downregulate the expression and/or shed stress-induced ligands, such as MICA/B and ULBP1-6,[66,69] thus decreasing the extent of activating stimuli delivered to NK cells. To increase inhibitory signals relayed to NK cells, cancer cells upregulate HLA molecules and increase Fas and PD-LI expression.[66,69] Cancer cells and stromal cells in the TME also release a substantial amount of soluble suppressive molecules, such as transforming growth factor β (TGF-β), IL-6, IL-10, prostaglandin E_2 (PGE_2), and indoleamine 2,3-dioxygenase (IDO), which inhibit NK cell effector functions.[69,71,91] Cancer cells indirectly evade and inhibit NK cells, by recruiting anti-inflammatory immune cells, including T_{regs}, myeloid derived suppressor cells (MDSCs), and macrophages secreting reactive oxygen species (ROS), which all contribute to NK cell suppression through various mechanisms.[66,69] Additionally, the poor availability of nutrients, such as glucose and glutamine, in the TME inhibits NK cell metabolism, which leads to cellular dysfunction and decreased viability.[67] The hypoxic environment of the TME also causes dysfunction of tumor infiltrating NK cells, resulting in downregulation of NKp46, NKp30, NKp44, NKG2D, perforin, and granzyme.[98,99] Overall, the highly suppressive solid TME needs to be overcome, through many routes, to have effective adoptive NK cell therapies. As such, during the manufacturing process, the ability to develop potent NK cells to counteract some of these suppressive functions is of upmost importance.

1.3 Natural Killer Cell Infiltration into Solid Tumors

A critical hurdle for adoptive NK cell therapy for solid malignancies is the ability to home and migrate after infusion into the blood stream to the tumor location. In general, NK cells must extravasate from the blood stream in response to a chemokine gradient, and migrate through the tissue to a specific site of inflammation.[100] This process is dependent on the expression of several chemokine receptors by NK cells, such as CX3CR1, CXCR1-4, CCR1, CCR2, and CCR5.[75,100] To reach the site of inflammation,

NK cells need to extravasate from the blood stream which requires the adhesion molecule CD62L (L-selectin) to roll along the vascular endothelium.[101] Then the NK cell will sense the chemokine signal, and firmly adhere to the endothelium surface using β2 integrins, such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), and α4 integrins, such as VLA-4 (CD49d/CD29) and α4β7.[101] Then, in a process called locomotion, the cells use Mac-1 and LFA-1 to crawl to nearby endothelium borders for transendothelium migration (diapedesis).[101] The leukocyte squeezes in an amoeboid manner between the endothelial cells, requiring the use of PECAM-1 and CD99.[101] This is followed by migration through the extracellular matrix (ECM) of the tissue to the site of inflammation. Currently, little is known about NK cell migration in 3D matrices - NK cells may utilize mesenchymal migration, which is dependent on matrix metalloproteinases (MMPs) and integrin adhesion, or amoeboid migration, which is Rho-associated protein kinase (ROCK) dependent and MMP and integrin independent, or a combination of both mechanisms.[102–104] There is some evidence from 2D and 3D *in vitro* studies that NK cells utilize MMPs and integrins to migrate across and through protein-coated surfaces, suggesting mesenchymal migration in these studies.[105–108] Similarly, *in vitro* and animal studies of human and murine T cells have demonstrated the requirement of various proteases to degrade protein extracellular matrices.[109] Conversely, studies of T cell migration in *in vitro* 3D matrices demonstrated these cell primarily utilize amoeboid migration and contact guidance, through interaction with collagen fibrils independent of β1 and β2 integrins.[102,110,111] Furthermore, there is evidence from macrophages and dendritic cells that the architecture and stiffness of the 3D ECM dictates which mechanism of migration, mesenchymal or amoeboid, the cells primarily utilize.[103,104] Understanding factors important for migration and determining their expression in *ex vivo* expanded NK cells will be critical for the development of effective therapies for solid tumors. With this knowledge, protocols which conserve, or enhance, these factors can be prioritized for clinical trials or the NK cells could be engineered to express these factors during *ex vivo* expansion.

As for tumors, the exact mechanisms promoting or inhibiting NK cell infiltration are not completely known. A majority of solid tumors are poorly permeable to NK cells and infiltrating NK cells remain in the tumor stroma.[93,94,112] It is unclear if this is due to the chemokines secreted by tumor cells, suppressive factors secreted by tumor cells, physical properties of the tumor, or a combination of factors. Chemokine recognition has been implicated as an important factor for regulating NK cell infiltration. Tumor infiltrating NK cells are typically identified as $CD56^{bright}$,[113–115] except one study which identified $CD56^{dim}$ in the tumor bearing lymph node of melanoma patients.[116] This may be due to the specific chemokine profile produced by different tumors, as the chemokine profile depends on the cancer cells themselves, tissue origin, and the infiltrating immune cells.[117,118] This may also be due to the different chemokine receptor repertoire expressed by the different NK cell populations. Although there have been

many conflicting studies on the chemokine receptor expression in $CD56^{bright}$ and $CD56^{dim}$ NK cells, $CD56^{bright}$ NK cells seem to express chemokine receptors to a slightly greater extent than $CD56^{dim}$ NK cells,[119–121] which could explain the differences in tumor infiltration. The importance of chemokine recognition in NK cell migration is supported by genetically engineering cancer cells to express CXCL10[122] and engineering NK cells to express specific chemokine receptors[123–125] leads to greater tumor infiltrating NK cells. However, the chemokine receptor repertoire on NK cells can be modulated by many tumor-derived immunosuppressive factors, such as TGF-β. This cytokine can induce an upregulation of CXCR4 and CXCR3 expression on NK cells, but downregulates the expression of CX3CR1.[126,127] Still, the infiltration of NK cells cannot be attributed to chemokine recognition alone, since despite high levels of chemokines within the tumor, endogenous NK cell infiltration can still be limited[128] as is also observed with endogenous T cells.[111] The tumor ECM may also have a role in influencing NK cell infiltration. For example, colorectal cancer tumors with thick ECMs had low levels of endogenous leukocyte infiltration, while tumors with minimal ECMs had more infiltration.[129] Importantly, tumors with high intraepithelial endogenous leukocyte infiltration was significantly correlated with a lower level of tumor reoccurrence and longer disease-free survival, high levels of leukocyte infiltration in the ECM was not.[129] Similarly, areas of tumor ECMs with dense collagen I or fibronectin around a cancer cell cluster, determined by immunohistochemistry (IHC) and 2-photon imaging and second harmonic generation (SHG), had significantly less endogenous T cells than areas of tumors with loosely arranged collagen I networks.[110,111] Furthermore, T cells overlaid on tumor slices, imaged by real-time microscopy, migrated significantly slower in dense matrix regions relative to loose matrix regions, determined by IHC and SHG.[110] In *in vitro* studies, a similar finding was found that the migration distance and migration speed of[130] or number of invading T cells[111] were significantly reduced with increasing collagen I matrix density, and thus decreasing pore size. Furthermore, *in vitro* and animal models, have shown glycosaminoglycans inhibit lymphocyte tumor infiltration and contact with cancer cells.[131] Likely, NK cell tumor infiltration is controlled by the balance of chemoattractants, such as CXCL10, and chemorepellants, such as TGF-β, and the physical barrier of the ECM. Additionally, as NK cells utilize MMPs and integrins to migrate *in vitro*,[105–108] the expression of proteases and integrins by NK cells may be critical for their infiltration into solid tumors. For example, in hepatic tumors the presence of vitronectin corresponded to a greater presence and retention of NK cells,[132] demonstrating the benefit of interaction with this protein likely through integrins. Studies of endogenous and *in vitro* NK cell infiltration into solid tumor ECMs will be important to understanding important factors regulating this function which will be important for the development of adoptive NK cell therapies for treating solid tumors. For adoptive transfer of NK cells to be effective for solid tumors, *ex vivo* expanded NK cells need to retain, or increase, their expression of chemokine and adhesion receptors, and proteases.

1.4 Summary of NK Cell Functions

To advance NK cell manufacturing for clinical use, we need to understand the various barriers these cells encounter when infused into a patient. Adoptively transferred NK cells need to perform a wide array of functions to be able to infiltrate solid tumors and lyse cancer cells (**Figure 1**). The NK cells need to extravasate from the blood stream and migrate through the tumor ECM to establish the cell-cell contact with cancer cells that is needed for killing. The NK cells also need to remain functional upon entering the TME to lyse target cells and activate more NK cells and other immune cells, for an even larger anti-tumor response. Lastly, the NK cells need to persist, and ideally, expand in the patient after infusion for a durable and effective anti-tumor response. While increasing activating signals at the NK cell-cancer cell

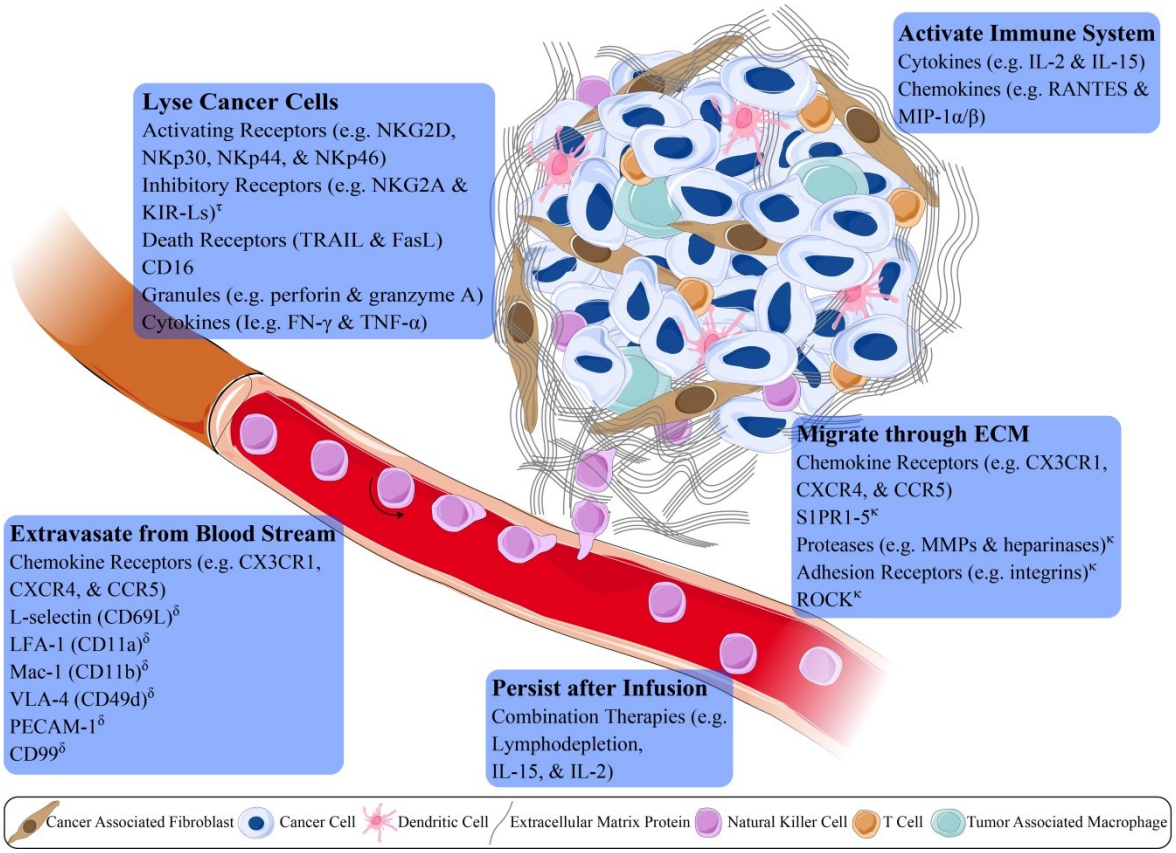

Figure 1. **Critical factors required for NK cells to be an effective adoptive therapy for solid tumors.** The infused NK cells need to be able to retain homing and migration function, to extravasate from the blood stream and migrate through the tumor ECM to reach the cancer cells. Furthermore, the cells need to remain functional to lyse cancer cells and activate other cells of the immune system. Importantly, after infusion, the NK cells need to persist, and ideally expand *in vivo* to have a lasting anti-tumor effect. δ signifies that this information is gathered from general leukocyte extravasation, not specific to NK cells. τ signifies there is conflicting reports on whether expression of inhibitory receptors makes NK cells better killers or not. κ signifies this information has been taken from *in vitro* studies of human NK cell migration or animal models of murine NK cell migration. S1PR1-5: sphingosine-1-phosphate receptor 1-5; ROCK: Rho-associated protein kinase. Figure was created using Servier Medical Art according to a Creative Commons Attribution 3.0 Unported License guidelines (https://creativecommons.org/licenses/by/3.0/). Simplification and color changes were made to the original cartoons.

synapse and other mechanisms to increase NK cell killing are the typical objectives for NK cell therapies, NK cell homing and migration are often underappreciated mechanisms. Additionally, when NK cells are expanded *ex vivo* prior to infusion, the expression of proteins related to NK cell killing are commonly determined, however proteins related to extravasation and migration are often overlooked. Yet, the expression of chemokine receptors, adhesion receptors, and proteases are likely critical for a successful NK cell therapy for solid tumors. Therefore, understanding the impact of different manufacturing methods for *ex vivo* expanded NK cells on their homing and migratory ability, and employing methods that increase, or maintain, these functions are critical for successful solid tumor therapies.

2. Clinical Trials of Expanded Natural Killer Cells for Solid Tumor Immunotherapy

There have been many reviews on the outcome of recently published and ongoing clinical trials of adoptive NK cell therapy.[133–136] As such, the following will focus on the NK cell sources, manufacturing methods, and clinical outcomes for recent clinical trials of *ex vivo* expanded NK cells for treating solid tumors (**Table 1**). Interestingly, while these therapies were for the treatment of solid tumors, only two studies analyzed the expression of chemokine receptors, important for NK cell homing and migration (**Table 1**). When acknowledging clinical outcomes, it should be noted that some of these clinical trials are Phase I and are not powered to show clinical efficacy. All the NK cell products used in clinical trials are cGMP-compliant to ensure the final product safe and potent. Prior to release of the final product for patient infusion, the product will undergo standard quality control testing including NK cell viability, NK cell purity, contaminating cell type and quantity, the endotoxin levels, and the presence of contamination (bacterial, fungal, viral, and mycoplasma).

Table 1: Relevant published clinical trials of *ex vivo* expanded NK cells for the treatment of solid tumors

Phase	NK Cell Source	Tumor Type	Expansion Method	Quality Control:		Therapeutic Regime	Clinical Efficacy	Reference
				Expansion, Viability, and Purity	Phenotype and Function			
Phase I Phase II	Autologous PBMCs	Advanced lung tumor	13 day expansion of $4x10^7$ PBMCs in X-Vivo 15 medium and solutions from the human NK cell *in vitro* culture kit (HANK Bioengineering Co. Ltd) in T175 flasks (days 1-7) and in a cell culture bag (days 8-13).	• On day 12, QC was done for cell viability (≥80%) and CD56+CD3- NK cell purity (≥80%) • ~250-fold increase in CD56+CD3- NK cells • 92% viable cells • CD56+CD3- NK cell purity was 88%	• Increase in NKG2D, NKp30, NKp44, and NKp46 on CD56+ cells, relative to freshly isolated cells	All prior treatments were stopped. All patients received at least 1 (average 2-3) course of NK cell treatment (each course had 3 NK cell infusion, over 3 days). Each infusion was 3-$5x10^9$ NK cells. No more than 3 courses of treatment were received monthly.	3 months post infusion: 11/13 SD 2/13 PD	Xie et al.[38]

Phase II	Autologous PBMCs	Melanoma and renal cell cancer	21-23 day expansion of $5x10^8$ CD3+ depleted cells in AIM-V medium with 600 IU/mL IL-2, 30 ng/mL OKT3, and irradiated autologous PBMCs as feeder cells (1:10 ratio of NK to feeder cells) in T175 flasks and transferred to culture bags.	• On day 21-23, QC was done for cell number and CD56+CD3- NK cell purity • From pre-clinical study: ~160-fold expansion (minimum 80-fold) with 90% CD56+CD3- NK cells • From clinical study: ~8,000-fold expansion with 96% CD56+CD3- NK cells	• On day 21-23, QC was done for specific lysis to melanoma cells and CD16 and NKG2D expression • Average lysis of 88% to allogeneic melanoma cells (E:T cell ratio 10:1, 4hrs) • 48% CD16+ NK cells and 99% NKG2D+ NK cells	Patients received lymphodepleting, but nonmyeloablative, chemotherapy. Followed by $4.7x10^{10}$ cells consisting of 96% CD56+CD3- NK cells. Patients received at least 6 doses of a high dose of IL-2, and 6 out of 8 patients received a second cycle of IL-2, 20-27 days later.	No clinical response (8/8)	Parkhurst et al.[33]
Phase II Phase III	Autologous PBMCs	Advanced colon carcinoma	14-21 day expansion of PBMCs in AIM-V medium with 700 U/mL IL-2 and 1 ng/mL OK431 (bacterium, initial) in anti-CD16 coated flasks. Note: the starting number of cells was not specified.	• Prior to infusion, QC was done for cell number, CD56+CD3- NK viability, CD56+CD3- NK cell purity, and CD3+ T cell contamination • CD56+CD3- NK cell viability was 95% • CD56+CD3- NK purity was 95%	• Prior to infusion, QC was done for CD69 expression • Results not specified	Patients received lymphodepleting chemotherapy, and 9-10 days later received 2.4-$4.0x10^9$ CD56+CD3- NK cells. Patients received 6 infusions in 1 course within 3 weeks. Multiple courses were administered to patients in combination with the chemotherapy.	50.5 months median follow up time: • 5-year PFS rate, median PFS, and 5-year OS rate were significantly higher in NK cell group than control (chemotherapy alone) • 5-year PFS and median PFS were significantly higher in the group receiving >3 courses then the group receiving ≤3 courses	Li et al.[35]

Phase I	Autologous PBMCs	Advanced digestive cancer	21-22 day expansion of $5.6x10^6$ PBMCs in GT-T507α medium with IL-2, OK431 (bacterium, initial), and irradiated autologous T cells as feeder cells in flasks (days 1-6) and bags (days 7-21/22). Note: the ratio of PBMCs to feeder cells was not specified.	• Prior to infusion, QC was done on fresh cells for cell number and cell viability (>80%) • QC was done on frozen cells for CD56+CD3- NK cell purity (>50%) • ~4720-fold expansion of CD56+CD3- NK cells • CD56+CD3- NK cell purity was 90-96% • CD56+CD3+ NKT cell contamination was 9% • CD3+ T cell contamination was 4%	• QC was done on frozen cells for cytotoxicity and expression of cell surface markers • 98% NKG2D+ NK cells and 62% CD16+ NK cells • 45% CXCR3+ NK cells, 38% CXCR4+ NK cells, and 44% CX3CR1 NK cells • Average lysis of 90% to K562 cells (E:T cell ratio 6.25:1, 18hrs)	Only 3/14 patients had combined chemotherapy treatment. Each patient received 3 doses (day 0, 7, and 14) of $0.5x10^9$, $1.0x10^9$, or $2x10^9$ cells per dose.	1 month post last infusion: 5/10 SD 5/10 PD	Sakamoto et al.[137]
Phase I*	Autologous PBMCs	Malignant glioma	14 day expansion of $1x10^6$ PBMCs in RHAMα medium with 200 U/mL IL-2 and irradiated a kidney Wilms tumor cell line as feeder cells (10:1 ratio of PBMCs to feeder cells) in culture plate days (days 1-5/6) and flasks (days 6/7-14).	• Prior to administration, QC was done for cell number and CD56+CD3- NK cell purity • ~43-fold expansion • CD56+CD3- NK cell purity was 82%	• Prior to administration, QC was done for cytotoxicity • Average lysis of 95% and 63% to a kidney Wilms tumor cell line and a glioblastoma cell line, respectively (E:T cell ratio 1:1, unknown time)	All patients received 1 or more courses: 1 course was 3 injections/infusions once a week. Whole expanded lymphocytes, mainly NK cells, were injected into the tumor cavity ($0.4\text{-}2.3x10^9$ cells/course) and IV ($0.2\text{-}3.7x10^9$ cells/course), where possible. A minimal dose of IL-2 was also administered with the cells. Patients also received an IV injection of IFNβ every week.	4-5 weeks post last treatment: 2/9 SD 6/9 PD 1/9 MR	Ishikawa et al.[40]

Phase I	Autologous PBMCs	Gastric or colorectal cancer	18-24 day expansion of $5.6x10^6$ PBMCs in LymphoONE NK culture medium with IL-2, OK431 (initial), and irradiated autologous T cells as feeder cells in culture bags. After harvesting, cells were cryopreserved. Note: the ratio of PBMCs to feeder cells was not specified.	• Prior to infusion, QC was done on fresh cells for cell number and cell viability (>80%) • QC was done on frozen cells for CD56+CD3- NK cell purity (>50%) • 3,848-fold expansion of CD56+CD3- NK cells • CD56+CD3- NK cell viability was 99% • CD56+CD3- NK cell purity was 93% • CD56+CD3+ NKT cell contamination was 5% • CD3+ T cell contamination was 3%	• QC was done on frozen cells for cytotoxicity, and expression of cell surface markers • 98% NKG2D+ NK cells and 70% CD16+ NK cells • 73% CXCR3+ NK cells, 24.7% CXCR4+ NK cells, and 49% CX3CR1 NK cells • 3% PD-1+ NK cells, 35% LAG3+ NK cells, and 81% TIGIT+ NK cells • Average lysis of 99% to K562 cells (E:T cell ratio 6.25:1, 4hrs)	Patients received 3 combination courses, over 6 weeks (week 0, 3, and 6). Each combination course included chemotherapy and an IgG1antibody (Trastuzumab or Cetuzimab) 3 days prior to cryopreserved NK cell infusion. Each patient received 1of 3 doses: $0.5x10^9$, $1.0x10^9$, or $2x10^9$ cells per dose. Patient also received additional chemotherapy and IgG1 antibody 3 weeks prior to the first course (week -3) and 4 weeks after the last course (week 10).	4 weeks post last infusion: 4/6 SD (3 decreased in size of target lesion) 2/6 PD (1 w/o target lesions had decreased CEA levels and SD maintained for 4 months after final infusion)	Ishikawa et al.[39]
Phase I	Allogeneic PBMCs (unrelated healthy donors)	Various advanced solid tumors	14 day expansion of $2x10^5$ CD3+ T cell depleted cells/mL in SCGM medium with 500 IU/mL IL-2, 10 ng/mL OKT3 (initial), and irradiated autologous PBMCs as feeder cells (1:5 ratio of NK to feeder cells) in culture bags.	• Prior to infusion, QC was done for cell number, CD56+CD3- NK cell viability, CD56+CD3- NK cell purity, and CD3+ T cell, CD14+ monocyte, and CD19+ B cell contamination • 758-fold expansion of CD56+CD16+CD3-NK cells • CD56+CD16+CD3- NK cell purity was 98% • CD56+CD16+CD3- NK cell viability was 93% • CD3+ T cell, CD14+ monocyte, and CD19+ B cell contamination were <1%	• Prior to infusion, QC was done for cytotoxicity • Average lysis of ~83% to K562 cells (E:T cell ratio of 3:1, 4hrs)	Patients received either 1 or 3 (once weekly) NK cell infusions, at $1x10^6$, $3x10^6$, $10x10^6$, or $30x10^6$ NK cells/kg (body weight).	4 weeks post first infusion: 7/15 SD 8/15 PD	Yang et al.[138]

Phase I	Allogeneic PBMCs (related healthy donors)	Non-small cell lung cancer	21-23 day expansion of CD56+ selected cells in αMEM medium with L-glutamine, gentamicin, hydrocortisone, and 20 ng/mL IL-15. Note: the starting number of cells was not specified.	• On day 19-21, QC was done for CD56+CD3- NK cell purity • 23-fold expansion • CD56+CD3- NK cell purity was 98%	• On day 19-21, QC was done for phenotype and cytotoxicity • Average lysis of 23% to K562 cells (E:T cell ratio of 1:1, 4hrs). • >95% of NK cells were NKp30+, NKp44+, NKp46+, NKG2D+, and 2B4+	Patients received 2-4 IV infusions of NK cells 2 days after previous and 1 week prior to their next chemotherapy. Average NK cell dose was $4.15x10^6$ NK cells/kg ($0.2-29x10^6$ NK cells/kg). Median number of T cell infused was $0.12x10^6$cells/kg ($0.009-1.8x10^6$ cells/kg)	22 month median follow up: 2/15 PR 6/15 SD 7/15 PD (3 had local regression, 1 had stabilization of lung disease) • Median PFS and OS were 7 and 15.5 months, respectively • 1- and 2-year survival rates were 60% and 27% respectively	Iliopoulou et al.[139]
Phase I Phase II	Allogeneic PBMCs (healthy donors)	Non-small cell lung cancer	13-15 day expansion of PBMCs in solutions from the human NK cell *in vitro* culture kit (HANK Bioengineering Co. Ltd) and K562-mbIL15-41BBL cells as feeder cells (1:1 ratio of PBMCs to feeder cells) in culture bags. Note: the starting number of cells was not specified.	• On day 9, QC was done for cell viability (≥80%) and CD56+CD3- NK cell purity (≥85%) • 92% viable cells • CD56+CD3- NK cell purity was 86%	• On day 9, QC was done for cytotoxicity • Results not reported	Patients had cryoablation therapy of the lung, liver, and bone 5 days prior to NK cell infusion. 3 doses of NK cells were infused at 1 dose a day. Dosages were not clear, but the range likely is $2.6-3.3x10^9$ cells.	3 months post treatment: 7/30 CR 12/30 PR 6/30 SD 5/30 PD	Lin et al.[140]
Phase I Phase II	Allogeneic PBMCs (healthy donors)	Non-small cell lung cancer	13-15 day expansion of PBMCs in solutions from the human NK cell *in vitro* culture kit (HANK Bioengineering Co. Ltd) and K562-mbIL15-41BBL cells as feeder cells (1:1 ratio of NK cells to feeder cells) in culture bags. Note: the starting number of cells was not specified.	• On day 9, QC was done for cell viability (≥80%) and CD56+CD3- NK cell purity (≥85%) • Results not specified	• On day 9, QC was done for cytotoxicity • Results not specified	Patients received 3 doses of NK cells (1 dose a day, days 1-3), with a Cetuximab injection on the first day, and again on day 8 and 15, and 3 more doses of NK cells starting on day 16 (1 dose a day, day 16-18). Dosages were not clear, but the range likely is $2.6-3.3x10^9$ cells.	3 months post treatment: 4/27 PR 17/27 SD 6/27 PD • Median PFS and OS were 6 and 9.5 months, respectively, and were significantly longer than the control group (Cetuximab alone)	Liang et al.[141]
Phase I Phase II	Allogeneic PBMCs (healthy donors)	Hepatic Carcinoma	13-15 day expansion of $4-5x10^7$ PBMCs in solutions from the human NK cell *in vitro* culture kit (HANK Bioengineering Co. Ltd) and	• On day 9, QC was done for cell viability (≥80%) and CD56+CD3- NK cell purity (≥85%) • Results not specified	• On day 9, QC was done for cytotoxicity • Results not specified	Patients received 1-6 courses of 3 doses of NK cells (1 dose a day). Dosages range from $20-120x10^6$ cells/kg	3 months post treatment: 3/16 PR 8/16 SD 5/16 PD • Median PFS was 7.5 months	Qin et al.[36]

			K562-mbIL15-41BBL cells as feeder cells (1:1 ratio of NK cells to feeder cells) in culture flasks.				• Median PFS was significantly higher for patients who received >4 NK cell courses, than ≤4 courses	
Phase I Phase II	Allogeneic PBMCs (healthy donors)	Non-small cell lung cancer	12 day expansion of $4x10^7$ PBMCs in solutions from the human NK cell *in vitro* culture kit (HANK Bioengineering Co. Ltd) in culture flasks	• On day 12, QC was done for cell number, NK cell purity, NK cell viability (≥80%) • $10x10^9$ NK cells • CD56+CD3- NK cell purity was 90% • CD3- T cell contamination was 5%	• On day 12, QC was done for activating and inhibitory receptors and cytotoxicity • Average lysis of 85% and 38% to K562 cells (E:T cell ratio of 20:1, and 5:1, respectively, 4hrs) • 80% of NK cells were NKG2D+ (increased relative to freshly isolated NK cells) • 69%, 65%, and 64% of NK cells were NKp46+, NKp44+, and NKp30+, respectively (increased relative to freshly isolated NK cells) • 23% and 16% of NK cells were KIR2DL1+ and KIR2DL2+, respectively (decreased relative to freshly isolated NK cells)	Patients received two cycles of pembrolizumab (anti-PD-1) and NK cells. First course: patients receive pembrolizumab on day 1 and 1-3 infusions of NK cells ($3x10^9$ cells) on days 12-14. Second course: patients receive pembrolizumab on day 22 and 1-3 infusions of NK cells ($3x10^9$ cells) on days 26-28.	• The objective response rate was 36.4% for NK cells and pembrolizumab, and 18.5% for pembrolizumab alone • Median OS and PFS were 15.5 and 6.5 months, respectably, which were longer than pembrolizumab alone • Median OS was significantly higher for patients who received multiple courses than those who received a single course	Lin et al.[142]
Phase I	NK-92 cells	Various advanced solid tumors	7-9 day expansion of $2\text{-}5x10^4$ cells/mL in X-Vivo 10 medium with 1000 IU/mL IL-2 in culture bags.	• Before release, QC was done for cell number and viability (<80%) • ~26-34-fold expansion • Expanded until $0.5\text{-}1x10^9$ cells/L was reached	• Before release, QC was done for cytotoxicity (>50%) • Results not specified • $5x10^5$ cells/mL of NK-92 cells cultured for 24 hrs produced >5000 pg/mL IFN-γ and ~1900 pg/mL IL-10	Patients received 2 infusions (2 days apart) of irradiated NK-92 cells, at $1x10^9$, $3x10^9$, or $1x10^{10}$ cells/m^2 (body surface area).	28 days post last infusion: 1/15 SD 2/15 MR 12/15 PD Average OS was 280 days	Tonn et al.[143]

Phase I	NK-92 cells	Advanced renal cell cancer and melanoma	15-17 day expansion of $3.5x10^5$ cells/mL in 25 mL of X-Vivo 10 medium with 500 U/mL IL-2, asparagine, and L-glutamine in culture bags.	• Before release, QC was done for cell number and viability • Expanded until $1x10^9$ cells/culture bag was reached • NK-92 cell viability was ≥80%	• Before release, QC was done for cytotoxicity • Results not specified	Patients received 3 infusions (days 1, 3, and 5) of irradiated NK-92 cells, at $1x10^8$, $3x10^8$, $1x10^9$, or $3x10^9$ cells/m^2.	4 weeks post treatment: 4/12 SD 2/12 MR 6/12 PD	Arai et al.[144]

*The Phase of the study by Ishikawa et al.[40] could not be identified, and was assumed based on the nature of this study

CEA: carcinoembryonic antigen; CR: complete response; E:T cell ratio: effector to target cell ratio; hrs: hours; IFNβ: interferon β; IL-2: interleukin-2; IV: intravenous; Kg: kilogram (body weight); K562-mbIL15-41BBL: K562 cells expressing membrane bound IL15 and 4-1 BB ligand; MR: mixed response; NK cell: natural killer cell; OKT3: anti-CD3 monoclonal antibody; OK431: freeze dried bacterium; OS: overall survival; PBMCs: peripheral blood mononuclear cells; PD: progressed disease; PFS: progression free survival; PR: partial response; SD: stable disease; QC: quality control

2.1 Autologous Natural Killer Cells

Adoptive transfer of autologous NK cells is a safe and feasible approach to treating solid tumors, as it does not require a donor selection process and prevents any risk of GvHD from contaminating T cells in the final product. Since the patients' own cells will be infused, the risks associated with other peripheral blood mononuclear cells (PBMCs) in the final product are low, but still there are requirements for NK cell purity and amount of contaminating cells. In recent studies, the release criteria for the purity of autologous NK cell products ranges from greater than 50% to greater than 80%,[38,39,137] (**Table 1**) with a T cell contamination of approximately 5% or less.[145] *Ex vivo* expanded autologous NK cells, alone and in combination, have been used to treat patients with metastatic melanoma,[33] renal cell carcinoma,[33,146] gastric cancer,[35,39,137] colorectal cancer,[35,39,137] lung cancer,[38] and malignant glioma[40] (**Table 1**). In most clinical trials, non-depleted PBMCs were cultured in media with various stimuli in culture flaks or bags, to expand NK cells (**Table 1**).[35,38–40,137] Culturing non-depleted PBMCs resulted in a final NK cell purity of 82-96% (**Table 1**), with small percentages of contaminating NKT cells and T cells.[39,137] In an effort to improve purity, one study performed a CD3+ depletion of the PBMCs prior to expansion, resulting in a final NK cell purity of 90%.[33] This final NK cell purity is greater than some clinical trials (**Table 1**), but this additional step may not be necessary in an autologous setting. Though it is important to note, the specific stimulation used during the expansion has an effect on the final NK cell purity. In terms of the different stimuli, irradiated feeder cells are commonly known to increase NK cell expansion,[33,39,137] relative to cytokines alone,[38] due to the additional stimulation of NK cells through cell-cell contacts.[147,148] However, expanding NK cells with an irradiated kidney Wilms tumor cell line as feeder cells[40] resulted in a lower fold expansion than observed in the other clinical trials (**Table 1**). The stimulation used during NK cell expansion can significantly influence the expansion rate of the final NK cell product. The expansion time for all autologous NK cell therapies was 13-24 days of expansion after the cell isolation

(**Table 1**). This time has critical implications on the cost and the logistics of the therapy. In all studies, NK cell infusions were well-tolerated and no serious side effects were noted. In some studies, after infusion there was an increase in the number of circulating NK cells,[33,137] indicating at least a portion of the infused cells remained viable and contributed to the circulating NK cell population. The dosages ranged from $0.5x10^9$ to $4.7x10^{10}$ NK cells/kg (body weight), and in many studies 2 or 3 courses of 3 to 6 infusions were administered to the patients (**Table 1**).[33,35,38] Importantly, one study found the group of patients who received more than 3 courses of NK cell infusion had a better clinical response than the group of patients who received 3 or less courses.[35] The multiple high dosages in these clinical trials underscores the need for *ex vivo* NK cell expansion. A key advantage of autologous NK cell therapy is their strong safety profile, however they may be limited in their cytotoxic functions because of inhibition by self HLA molecules on the tumor cells and chronic suppression due to their exposure to the cancer cells prior to *ex vivo* manipulation.[27] However, given that they are safe, do not require a donor selection process, and expand to the same extent as NK cells from healthy patients,[149] autologous NK cells remain a viable source for NK cell therapies.

2.2 Allogeneic Natural Killer Cells

Allogeneic NK cells are also a viable option for immunotherapies. Indeed, allogeneic NK cells have gained significant interest for adoptive cell therapy for solid tumors due to the ability to have easy access to donors, to use a single donor for many patients and/or multiple doses in one patient, and to select donors based on HLA, KIR-HLA ligand mismatch, and CD16 genotype. There are many findings demonstrating KIR-HLA ligand mismatch between donors and recipients, in the GvT direction, leads to better outcomes in patients.[26] Furthermore, allogeneic NK cells do not directly cause GvHD, if the infused product is sufficiently depleted of T cells.[20–28] However, if there remains a high level of contaminating T cells, there are some reports of grade II or higher GvHD after allogeneic NK cell infusions.[150] Recent clinical trials have an allowable T cell contamination in the range of $0.25\text{-}5x10^5$ T cells/kg.[31,151–156] The percent of contaminating T cells in a final product can limit the total number of NK cells which can safely be infused into a patient, resulting in patients receiving less than the intended dose of NK cells in clinical trials. The most common source of allogeneic NK cells is from haploidentical or unrelated healthy donor PBMCs, however other sources include NK cells isolated from umbilical cord blood and the NK-92 cell line.

Ex vivo expanded allogeneic NK cells, alone and in combination, have been used to treat various advanced solid tumors,[138] lung cancer,[139–141] and hepatic carcinoma[36] (**Table 1**). Purifying NK cells with a depletion or selection step prior to initiating culture resulted in a higher final NK cell purity, of 93-98%,[138,139]than when all P BMCs were used to initiate culture (86%)[140] (**Table 1**). This exemplifies the need for purifying PBMCs prior to initiating culture in the allogeneic setting to ensure the final NK cell

purity is high and the presence of harmful contaminating cells is low. To stimulate the NK cells, 4 of the clinical trials utilized the human NK cell *in vitro* culture kit (HANK Bioengineering Co. Ltd) and some additionally used with a genetically engineered cancer cell line as feeder cells (K562 cells expressing membrane bound IL-15 and 4-1 BB ligand, K562-mbIL15-41BBL, **Table 1**).[36,140–142] This culture kit is proprietary and the exact stimulation is unknown, so comparisons about the different stimuli leading to NK cell expansion and purity cannot be drawn. Between the other two studies of allogeneic PBMC-derived NK cells, again the protocol with stimulated NK cells with feeder cells, had a higher NK cell expansion in fewer days[138] than the protocol which relied on IL-15 and hydrocortisone for stimulation[139] (**Table 1**). Importantly, the study with the higher NK cell expansion was able to infuse higher dosages of NK cells into patients (**Table 1**). As with autologous NK cell therapies, the expansion time of allogeneic therapies ranged from 13-23 days (**Table 1**). In these clinical trials, there were no adverse effects and all the NK cell dosages were well-tolerated. The dosages ranged from $0.009x10^6$ to $120x10^6$ NK cells/kg, with the number of total infusions ranging from 1-18 (**Table 1**). Again the need for multiple doses was exemplified, as one study found patients who received more than 4 courses of NK cell infusion (3 infusions per course) had a better clinical response than those patients who received 4 or less courses.[36] The amount of cells needed for multiple infusions could not be obtained without expanding the NK cells prior to infusion. While allogeneic NK cells may prove to be a more potent therapy than autologous cells, there are limitations with this cell source. The donor NK cell sources suffer from large donor-to-donor variability, and thus there is high variation in the expansion rate and potency of the final NK cell product from one batch to another. Also, allogeneic NK cells require an extensive donor selection process to identify an optimal donor, which can delay the initiation of therapy for the patient. Still, allogeneic NK cells are likely to be more effective than autologous cells and therefore continue to be used in many clinical trials.

2.3 Natural Killer Cell Lines

NK cell lines also offer a potential source for allogeneic cells for cancer therapy. There exists many NK cell lines, but the NK-92 and NK-92MI lines are the predominant ones utilized in clinical trials. The NK-92 cell line was established from a 50-year old male patient with rapidly progressive non-Hodgkin's lymphoma, whose bone marrow was diffusely infiltrated with large granular lymphocytes.[157] Since the cells are dependent on IL-2 for growth and survival in culture, IL-2 must be included with any additional stimulating factors.[157,158] The NK-92MI cell line was established to overcome the need for exogenous IL-2 by genetically engineering NK-92 cells to produce soluble IL-2. The NK-92 cell line is highly cytotoxic against many cancer cell lines and primary tumors, attributed to the low levels of inhibitory KIR receptors, and it also has excellent and defined expansion kinetics.[157,159] However, a limitation of this cell line is the lack of CD16 expression and therefore they cannot perform ADCC, which

decreases their overall cytotoxicity. Furthermore, NK-92 cells will proliferate continuously when sufficient stimuli and space is available. To prevent the aberrant proliferation of the cell line *in vivo,* NK-92 cells need to be irradiated prior to infusion into patients for safety reasons.[160–162] Therefore, for a sustained effect on tumor growth, repeated injections of irradiated cells are likely necessary. An important consideration for using NK-92 cells to treat solid tumors is their lack of CD62L expression, which may diminish their ability to extravasate from the blood stream to infiltrate the tumor.[158]

Despite these limitations, expanded NK-92 cells have been used for adoptive therapy in patients with renal cell cancer,[144] melanoma[144] and various solid tumors,[143] but have had little clinical efficacy with less than 17% of patient reaching stable disease after therapy (**Table 1**). In both clinical trials, the expansion methods were different in cell seeding densities and IL-2 concentrations, which influenced the NK cell dosages in patients (**Table 1**). In both studies, the NK-92 cells were well tolerated, with multiple doses ranging from $1x10^8$ to $1x10^{10}$ cells/m^2 (body surface area, **Table 1**). Importantly, this cell line could serve as an "off-the-shelf" therapy for cancer patients since it is a readily available, well-characterized, and uniform cell source.[157,159] NK-92 cells also have a well-defined and reproducible expansion rate in culture,[157,159] which makes the timeline for expansion, the final cell number, and function and phenotype of the final product highly consistent. An "off-the-shelf" NK cell therapy provides a universal source of cells, resulting in earlier initiation of therapy for each patient, by bypassing the donor selection process and having shorter expansion times (6-19 days) relative to PBMC-derived NK cells (**Table 1**). Furthermore, since this therapy strategy is not personalized and requires a less complex manufacturing protocol, the cost of the therapy would be significantly less than PBMC-derived NK cells. Given the relative logistical simplicity and therapeutic potential, clinical trials continue to evaluate adoptive NK-92 cell therapies (NCT02465957, NCT00900809, and NCT00990717).

2.4 Combination Therapies

Adoptive NK cell transfer has been combined with other therapies in clinical trials to aid in donor cell survival, activation, and expansion *in vivo,* ultimately to increase their clinical efficacy. In the 1990 to early 2000's many studies explored IL-2 infusion with or without adoptive NK cell therapy, which lead to three key findings - (i) low doses of IL-2 could be administered safely daily or 3 times weekly, (ii) IL-2 could increase the amount of circulating NK cells, and (iii) the recipients' lymphocytes compete with the infused NK cells for cytokines/chemokines and 'space'.[18,163–167] To address the latter, many clinical trials added a lymphodepleting protocol using high dose chemotherapy, prior to NK cell infusion,[33,163] as was done for T cell therapies. This process increases the efficacy of NK cell therapies by changing the balance between transferred cells and the endogenous lymphocytes, changing the cytokine milieu available to the transferred cells, and depleting the inhibitory T_{reg} cell population.[163,168] However, treating patients with a high dose of chemotherapy needs to be done cautiously as certain patients are high-risk for a variety of

toxicities, including prolonged neutropenia and the risk of infection, as well as other toxicities.[183] Many recent clinical trials combine a lymphodepleting and IL-2 therapy with adoptive transfer of NK cells, as the combination can lead to better clinical efficacy than either treatment alone.[133–136]

While a low dose of IL-2 is tolerated by patients, there are still concerns over expansion of immunosuppressive T_{reg} cells *in vivo* and inhibiting NK cell therapy.[163,169] Host T_{reg} cells can be resistant to the lymphodepleting chemotherapy and expand with low dose IL-2 therapy.[34,170] As such, clinical trials have explored the role of IL-15 to stimulate NK cells *in vivo,* which was proposed to expand NK cells (both endogenous and adoptively transferred) without activation and expansion of T_{regs}.[171–173] Clinical trials of adoptive NK cell therapy have shown IL-15 injections induced better rates of NK cell expansion *in vivo* than IL-2.[173] However, there have been dose-limiting adverse events of IL-15 injections including hypotension, thrombocytopenia, cytokine release syndrome, and neurotoxicity.[171–173] Furthermore, there has been some indications that IL-15 can stimulate T_{reg} cells *in vivo.*[171] As IL-15 infusions alone or in combination with adoptive NK cell therapy is relatively new, there are ongoing and recently completed clinical trials (NCT04290546, NCT01875601, and NCT01385423) of NK cell therapy with IL-15 therapy, commonly combined with lymphodepleting chemotherapy. These trials will help determine an optimal dose and dosing schedule to minimize adverse effects and T_{reg} expansion (if present), while activating NK cells *in vivo*.

Furthermore, *ex vivo* expanded NK cells for treating solid tumors have been combined with other therapies, including cryoablation[140] and FDA approved monoclonal antibodies (mAbs),[39,141,142] in clinical trials (**Table 1**). Combining NK cells with mABs aims to increase NK cell cytotoxicity through ADCC to have a greater clinical effect. Combination therapies with NK cells led to better outcomes for patients than either therapy alone (**Table 1**).[39,140–142] Additionally, a recent case study showed a combination of radiochemotherapy, autologous NK cells, and anti-PD-1 mAb induced long-term tumor control in a patient with stage IIIb non-small cell lung cancer.[174] As such, there are many ongoing clinical trials of adoptive NK cell and mAb therapy (NCT03554889, NCT01729091, NCT02650648, NCT03242603, NCT03209869). To further stimulate ADCC in NK cells, the development of heterodimeric bispecific mAbs have been developed which recognize CD16 on the NK cell and a target on the cancer cell, such as CD30, CD33, HER2, and many others.[38] There are a few ongoing clinical trials of NK cells and anti-CD16 bispecific antibodies in cancer patients (NCT04074746). Additionally, pre-clinical studies of trispecific antibodies, which engage CD16 and an activating receptor on the NK cells and a tumor specific antigen, have promoted greater NK cell activation and function *in vitro* than bispecific antibodies, and *in vivo* relative to an mAb.[175] These more specific antibodies could be combined with *ex vivo* expanded NK cells for a combination therapy in cancer patients, and may be more beneficial to either therapy alone.

2.5 Chimeric Antigen Receptor (CAR) Natural Killer Cells

Recent efforts have been directed towards engineered NK cells to overcome the suppressive effects of cancer cells for more efficient NK cell-mediated lysis. To develop more potent NK cell therapies, NK cells have been engineered to target specific surface antigens expressed by tumor cells using chimeric antigen receptors (CARs). CARs are composed of an extracellular signaling domain that recognizes a specific tumor antigen, a transmembrane region, and an intracellular domain.[176–178] CAR NK cells are a powerful immunotherapy and there have been many recent reviews about different pre-clinical and clinical trials of CAR NK cells.[176–178] As such, the following will only highlight the manufacturing methods of two recently published results from clinical trials of expanded CAR NK cells. Of note, there have been no published results of CAR NK cells for the treatment of solid tumors, but there are ongoing clinical trials (NCT03941457, NCT03940820, NCT03415100, and NCT03383978).

CAR NK cells may overcome the complex production process, high cost, and adverse effects associated with autologous CAR T cells, since allogeneic NK cells are well-tolerated and there is potential for "off-the-shelf" CAR NK cells therapies. Furthermore, CAR NK cells may offer greater potential for the treatment solid tumors than CAR T cells, as CAR NK cells have the ability to induce target cell death by both CAR-dependent and NK receptor-dependent mechanisms. The NK-92 and NK-92MI cell lines are an ideal cell source for an "off-the-shelf" CAR NK cell therapy.[162,179] These cell lines are a homogenous population prior to CAR modification, which will result in a more uniform final cell product, unlike peripheral blood NK cells sources. Additionally, transduction efficiency is more consistent in NK-92 cells compared with primary NK cells.[162,179] Another option for an "off-the-shelf" therapy is the use of cord blood NK cells to generate CAR NK cells, which can be supplied more readily from a blood bank, but there remains intra- and inter-donor variability in the cell source.[180] This variability can reduce the transfection efficiency and result in a heterogeneous final CAR NK cell product. CAR NK cells derived from both of these cell sources have been used in clinical trials for treatment of hematological cancers.[179,180] In a phase I clinical trial, in combination with lymphodepleting therapy, 3 doses of up to $5x10^9$ anti-CD33 CAR NK-92MI cells was deemed safe in patients with AML.[179] In a second phase I trial, patients with CD-19 positive lymphoid tumors received a single dose of up to $1x10^7$ CAR NK cells/kg, of cord blood derived anti-CD19 CAR NK cells with engineered expression of IL-15 and an inducible caspase-9 safety switch, in combination with lymphodepleting therapy.[180] The cord blood cells were purified by CD3+, CD19+, and CD14+ depletion and the remaining cells were cultured in the presence of irradiated K562 cells expressing membrane bound IL-21 and 4-1 BB ligand (K562-mbIL21-41BBL) as feeder cells and soluble IL-2.[180] The cord blood derived NK cells were expanded for 6 days, then transduced with the retroviral vector carrying the genes to be inserted, and expanded for an additional 9 days.[180] After therapy, the patients had no severe adverse events and

importantly, 64% of patients had a positive response to therapy.[180] Overall, "off-the-shelf" CAR NK cells appear to be a promising cancer therapy, since they have been well-tolerated by patients, have induced clinical responses in early studies, and have the potential to reduce the cost and complexity of the manufacturing process associated with current CAR T cell therapies.

2.6 Natural Killer Cell Persistence in Patients

NK cell persistence in the peripheral blood is currently used as a surrogate marker for the extent of tumor infiltrating NK cells and a predictor of therapy clinical efficacy. The persistence, and especially expansion, of donor NK cells, other than irradiated cell lines, *in vivo* has been suggested to be linked with a better clinical response.[30,33,181] There may be a correlation between the absolute number of NK cells in the peripheral blood and the percentage of NK cells in the tumor,[182] which may support this marker as a predictor for clinical efficacy. Currently, determining the pharmacokinetics of NK cells by detection in peripheral blood is the standard, however, there remains a need for a better understanding of donor NK cell bioavailability after infusion. This information would support the development of NK cells with increased homing and migratory abilities to tumors, through expansion and engineering methods. Increasing tumor homing and infiltration by NK cells could conceivably reduce the total number of NK cells required for a clinical effect, and possibly simplify the manufacturing process, as less NK cells would be required. Therefore, understanding NK cell biodistribution after infusion would help optimize NK cell therapies.

Adoptive transfer of NK cells in combination with lymphodepleting and cytokine therapy helps the persistence of NK cells *in vivo.* Without lymphodepleting therapy, donor NK cells were non-functional and not present in the patients' blood,[30,33] but with a low dose lymphodepleting therapy and IL-2 injections, patients had transient donor NK cell persistence.[30,33] On the other hand, patients who received a high dose of lymphodepleting therapy, an NK cell infusion, and IL-2 injections had a marked rise of endogenous IL-15 and expansion of donor NK cells, which peaked at day 7 post infusion.[30] While combination therapies can lead to the expansion of donor NK cells and thus a better clinical outcome, the donor NK cells typically disappear within 2 weeks.[30,33,181] As NK cells have a 2 week lifespan,[13,16] this finding demonstrates the cell population is not significantly expanding *in vivo*, likely due to the lack of cytokines for stimulation. This short persistence is unlikely to be sufficient for a lasting effect on tumor growth, and thus multiple infusions will be required for a sustained clinical effect. This is evident by multiple NK cell courses leading to a better clinical effect in patients than fewer courses (**Table 1**). As many NK cell therapies have limited *in vivo* persistence, the manufacturing protocol requires multiple cell isolations from the donor and high expansion rates of NK cells, or, alternatively, an "off-the-shelf" NK cell therapy may overcome some of these requirements.

Increasing donor NK cell persistence, and expansion, *in vivo* is a key challenge for expanded and genetically engineered NK cells are aiming to address. Doing so would simplify NK cell dosing regimens, in terms of number and timing of infusions, reduce costs, minimize adverse effects, and improve convenience. *Ex vivo* stimulated NK cells infused in combination with IL-15 have demonstrated increased persistence *in vivo.* For example, after IL-12, IL-15, and IL-18 activated memory-like NK cell infusion and IL-15 superagonist therapy in AML patients, NK cells were detected in the peripheral blood up to 2 months after infusion.[184] Moreover, cord blood derived anti-CD19 CAR and IL-15 expressing NK cells, previously discussed, were detected at low levels in patients for at least 12 months.[180] The patients which responded to the therapy had a significantly higher CAR NK cell expansion 3-14 days after infusion, than those patients who did not respond,[180] comparable to findings from CAR T cell studies.[2] The persistence, expansion, and anti-tumor activity of the CAR NK cells may be due to the engineered ability to produce IL-15 and the lymphodepleting regime prior to cell infusion. These studies suggest a role of IL-15 in NK cell persistence and expansion *in vivo*, and provide rationale for continued work into increasing NK cells to persistence and expansion in patients after infusion.

2.7 *In Situ* Injections of Natural Killer Cells

In the majority of clinical trials, adoptively transferred NK cells are infused intravenously, however some studies have pursued more localized delivery of NK cells as a way to improve tumor infiltration, distribution, and effector-to-to-target (E:T) cell ratios. This can be achieved through intra-tumoral injections or intra-arterially injections upstream of the tumor site. In an attempt to reduce the need for systemic tumor homing, e*x vivo* IL-2 stimulated allogeneic NK cells were infused directly into the hepatic artery in patients with liver metastases of gastrointestinal carcinoma, in combination with Cetuximab and IL-2 therapy, resulting in a clinical response in 3 out of 9 patients.[181] With this mode of infusion, the NK cells may more readily sense the chemokine gradient from the metastases and extravasate from the blood stream. Another method to bypass the need for tumor homing and extravasation from the blood stream is to inject the NK cells intra-tumorally. In a recent case study, a patient with relapsed and refractory Ewing sarcoma received 3 intra-tumoral injections of IL-2 expanded NK-92 cells, which was safe to the patient and the tumor regressed in size, but metastases in other locations were detected and did not regress in size.[185] Intra-tumoral injections are likely to have a local effect on tumor regression; however, to control the development and growth of metastases, intravenous infusions of NK cells may also be necessary. In one trial which explored this, patients received multiple injections of autologous expanded NK cells in the tumor cavity and intravenously, followed by IL-2 injections, which resulted in a clinical response in 2 out of 9 patients.[40] Another opportunity to deliver NK cells intra-tumorally is during tumor resection surgeries. An ongoing clinical trial is seeking to determine the MTD or maximum feasible dose of *ex vivo* expanded ErbB2-specific NK-92/5.28z CAR NK cells[162]

injected into the wall of the resection cavity during relapse surgery for patients with recurrent or refractory ErbB2-positive glioblastoma.[186] If patients in this clinical trial respond to the treatment, it may provide rationale for intra-tumoral injections of donor NK cells during other tumor resection surgeries. More localized administrations of NK cells may enable increased NK cell-cancer cell interactions and greater NK cell-mediated lysis, but this may not bypass the need for multiple injections to achieve a lasting clinical effect.[40,186] Still, intra-tumoral injections require certain technical considerations in terms of administrations of the cells to ensure good distribution of the cells, and these should not be underestimated. *In situ* delivery of NK cells may be a promising option for solid tumors, but the manufacturing process will still require *ex vivo* expansion of the cells to reach the required cell numbers.

2.8 Summary of Findings from Clinical Trials

High dosages of *ex vivo* expanded NK cells, from various cell sources and expansion methods, have been safe for patients, but few NK cell therapies have resulted in remission of solid malignancies in patients. However, the clinical trials of NK cells have revealed insight into the therapeutic potential of these cells, the dosing regimen, and methods to expand NK cells to clinically relevant cell numbers (**Table 1**). The clinical trials have illustrated the need for multiple NK cell infusions for better clinical responses. From these clinical trials there is also insight into differences between autologous and allogeneic NK cell sources. Compared to high dosages (10^{10} cells/kg) of autologous NK cells, lower dosages (10^{6} cells/kg) of allogeneic NK cells resulted in higher clinical efficacy (0% versus 28% complete or partial response, respectively) (**Table 1**). Further clinical trials of higher cell dosages of allogeneic NK cells in solid tumor patients will provide insight into the clinical benefit of increasing NK cell dosages. With these clinical trials comes the need to *ex vivo* expand NK cells to greater cell numbers, which will require manufacturing methods that can generate sufficient cell numbers of NK cells with increased cytotoxicity, relative to freshly isolated NK cells. An alternative approach to NK cell therapy is utilizing engineered NK cells, with increased cytotoxicity and persistence, relative to naïve NK cells, which may reduce the number of cells required for a clinical effect. Understanding the optimal therapeutic approach may be further addressed as more clinical data is accrued on CAR NK cells. Additionally, with a better understanding of NK cell bioavailability after infusion, NK cell therapies with increased homing and migratory functions can be developed. The clinical data from ongoing studies will reveal critical information into NK cell dosing regimens, which will greatly influence manufacturing NK cell protocols.

3. Manufacturing Natural Killer Cells for Solid Tumor Immunotherapy

The outcome of clinical-grade and large-scale NK cell expansion is dictated by a number of related and interacting parameters. The final number of cells, and the function and phenotype of the final product can therefore be altered by modifying one or more of the factors. The manufacturing parameters (**Figure 2**), including cell source, number of cells to initiate the culture, cell density during expansion, and culture vessel are all considered to optimize the process for generation of a large number of potent NK cells. The clinical trials of *ex vivo* expanded NK cells all relied on open culture systems, which can increase the risk contamination and the operator-to-operatory variability may have an effect on the final cell product. Many recent investigational manufacturing protocols (**Table 2**) utilize GMP-compliant materials, closed culture systems, which reduce operator involvement and the risk of contamination. Pre-clinical investigations aim to simplify the manufacturing protocol with closed systems and reduce the amount of operator involvement, while also increasing the consistency in the final cell product, the proliferation of the NK cells over time, and the function of the final NK cell product. Therefore, these investigations provide insight into where the field of NK cell expansion may be headed and into the effect

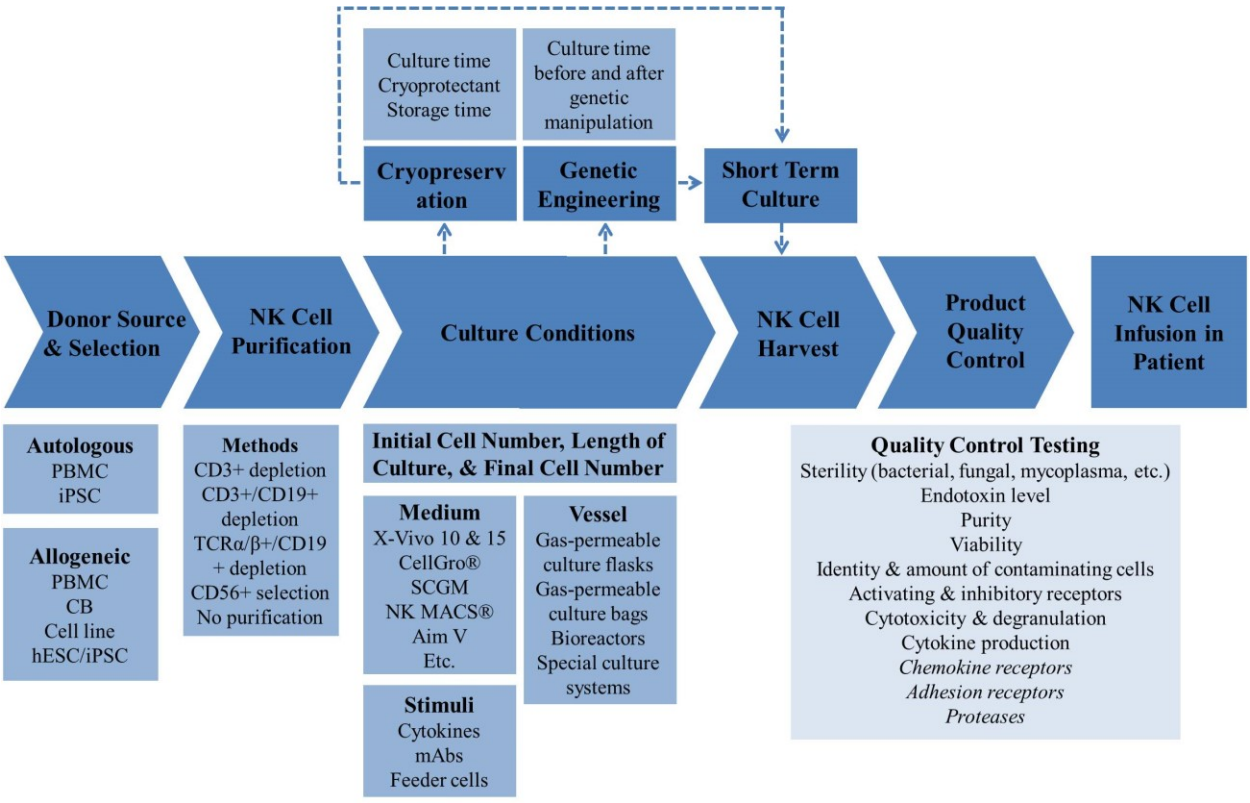

Figure 2. **Parameters of the manufacturing process which influence the final NK cell product.** Each step of the manufacturing NK cell process and the factors in those steps influence the outcome of the final cell product, determined by quality control testing. The common quality control testing methods are listed, and those in italicized text are important considerations for solid tumors but are not often studied. The dashed lined arrows represent optional steps in the manufacturing process. NK: natural killer, PBMC: peripheral blood monocytes, CB: cord blood, hESC: human embryonic stem cells, iPSC: induced pluripotent stem cells, mAbs: monoclonal antibodies.

of expansion method on the phenotype and function of the final cell product (**Table 2**). In investigational studies, the end products are evaluated for phenotype and cytotoxic functions; however the impact of expansion protocols on NK cell functions related to homing and migration is limited and may be important in advancing the development of NK cell therapies for solid malignancies

Table 2: Recent investigational NK cell manufacturing protocols

NK Cell Source	Purification Method	Initial Cell Seeding	Base Medium	Additives	Culture Vessel	Culture Length	Cryopreservation	Final Product				Reference
								Expansion, Viability, and Purity	Phenotype	Cytotoxicity and Others	Homing and Migration	
CB CD34+ cells	N/A	Not specified	GBGM® SCGM	Human serum, GM-CSF, G-CSF, IL6, IL-7, SCF, IL-15, and IL-2	Wave Bioreactor ™ or BIOSTAT® CultiBag RM System	6 weeks	N/A	• ~2,100-fold expansion in Wave (~1,300-fold in bags) • Total CD56+CD3- NK cells ~$2.65x10^9$ • CD56+CD3- NK cell purity was 92% in Wave (71% in bags) • CD56+CD3- NK cell viability was >90% • CD14+ monocyte and CD15+ monocyte cells contamination was <5% • No contamination of CD34+ cells, CD3+ T cells, or CD19+ B cells	• Wave expanded NK cells had increased expression of NKG2D, NKp30, NKp44, and NKp46 relative to bag expanded NK cells	• Increased degranulation (CD107a expression) in response to K562 (18hrs) cells by wave expanded NK cells relative to bag expanded NK cells • Cytotoxicity to K562 cells (E:T cell ratios of 1:1 and 10:1, 18 hrs, ~31% and ~58% cytotoxicity, respectively)	Not Determined	Spanholtz et al.[187]
CB-derived NK cells	Human NK cell isolation kit (negative selection)	$2x10^6$ purified NK cells	CellGro® SCGM	200 U/mL IL-2 (Proleukin) and irradiated K562 clone 9.mbIL21 as feeder cells (2:1 ratio of NK cell to feeder cells)	Not specified	14 days	N/A	• 2221-fold expansion • $4443.6x10^6$ NK cells • Viability after transduction was ≥95%.	Not Determined	• Transduction of the viral vector to express iC9/CAR.CD19/IL-15 was 66.6% on day 14 • CB-derived CAR NK cells were more efficient at killing target cells than wild type CB-derived NK cells and patient PBMC-derived CAR NK cells	Not Determined	Liu et al.[188]
NK-92/5.28.z (anti-ErbB2 (HER2)	N/A	$5x10^4$ cells/mL	X-Vivo 10 rTF (conta	HI human plasma and	VueLife® 750-C1 cultur	5 days	N/A	• QC standards were viability >80%, CAR expression >95% • ~12-fold expansion	Not Determined	• Specific cytotoxicity to ErbB2+ target cells (E:T cell ratio of 10:1, 2 hrs, quality control standard: >50%), immediately	Not Determined	Nowakowska et al.[162]

CAR NK-92 cell line)			ins recom binant transf errin)	500 U/mL IL-2 (Prole ukin)	e bags			• Immediately after irradiation, viability was 92% and CAR expression was 97% • Average doubling time of 32.76 hrs during the 5-day culture • Negative for culture microbes (BacT/Alert) (Sterility), mycoplasma, and endotoxin		after irradiation was 83% • NK-92/5.28.z cells released more granzyme B, IFN-γ, sFasL, IL-8, IL-10 in response to K562 cells (E:T cell ratio of 10:1, 2hrs) and unstimulated relative to NK-92 cells • The irradiation amount (10 Gy) they chose resulted in complete inhibition of cell proliferation, and after 5 weeks no viable cells were detectable		
PBMC (healthy and MM patient)	N/A	$2x10^6$ total cells /mL	CellGro® SCGM	Human serum, 500 U/mL, IL-2 (Prole ukin), and 10 ng/mL anti-CD3 mAb (OKT 3) (initial)	Wave Bioreactor™ (cultured in flasks for first 5 days)	20 days	N/A	• 77-fold expansion (770-fold expansion in flasks for the entirety of culture) • CD56+CD3- NK cell purity was 38% (44% in flasks for the entirety of culture) • CD56+CD3- NK cell viability was >93% at all times and 98% at day 20 • CD56+CD3+ NKT cell contamination was 14% (16% in flasks for the entirety of culture) • CD14+ monocyte contamination was <3% • CD19+ B cell contamination was <1%	• NK cells expanded in the Wave, bags, and flasks had comparable expression of activation markers and inhibitory and activating receptors • NK cells expanded in the Wave had increased expression of CD27, CD158b, and NKG2D relative to freshly isolated NK cells • NK cells expanded in the Wave had decreased expression of CD7 and NKp46 relative to freshly isolated NK cells • Wave and flask expanded NK cells had comparable expression of surface markers, but Wave NK cells had more NK2GD and NKp44 expression	• NK cells expanded in the Wave, bags, and flasks has comparable cytotoxicity to K562 cells (E:T cell ratios of 3:1 and 10:1, 4 hrs, ~55% and ~70%, respectively)	• NK cells expanded in the Wave had decreased expression of CD62L relative to freshly isolated NK cells • Wave and flask expanded NK cells had comparable expression of surface markers, but Wave NK cells had more CD11b expression	Sutlu et al.[189]
PBMC (healthy and MM patient)	N/A	$5x10^5$ total cells /mL	CellGro® SCGM	Human serum, 500 U/mL, IL-2	VueLife® culture bags (72-mL)	20 days	N/A	• 530-fold expansion (1,100-fold expansion in flasks for the entirety of culture) • CD56+CD3- NK cell purity was 31% (53%	• NK cells expanded in the Wave, bags, and flasks had comparable expression of activation markers and inhibitory and	• NK cells expanded in the Wave, bags, and flasks has comparable cytotoxicity to K562 cells (E:T cell ratios of 3:1 and 10:1, 4 hrs,	• NK cells expanded in the bags had decreased expression of CD62L relative to freshly isolated NK cells	Sutlu et al.[189]

				(Proleukin), and 10 ng/mL anti-CD3 mAb (OKT3) (initial)				in flasks for the entirety of culture)	activating receptors • NK cells expanded in the bags had increased expression of CD27, CD158b, and NKG2D relative to freshly isolated NK cells • NK cells expanded in the bags had decreased expression of CD7 and NKp46 relative to freshly isolated NK cells	~55% and ~70%, respectively)		
PBMC (healthy)	RosetteSep™ Human CD3 Depletion Cocktail	~70 x10^6 purified NK cells	Alpha Medium	Human serum, L-glutamine, gentamycin, 1000 U/mL IL-2 (Proleukin), and propriety activation cocktail	Z®RP Bioreactor 50M coated with a propriietary activating cocktail	12-22 days to reach ~1 x 10^9 cells	Cryopreserved on day 12-22 (final day of culture) (all testing was performed on immediately thawed cells and cells after 48 hrs in culture with IL-2)	• CD56+CD3- NK cell purity >85% • CD14+ monocyte cell contamination ~6% • CD3+ T cell, CD19+ B cells, and CD56+CD3+ NKT cells contamination were all <2%	• Expressed NKp30, NKp44, NKG2D, DNAM1, CD16, and TRIAL immediately after thawing, which increased after 48 hrs in IL-2 • Did not express or expressed low levels of NKp46 and FasL immediately after thawing, but increased expression after 24 hrs in IL-2	• Cytotoxic to K562 cells (E:T cell ratio of 5:1, 4 hrs, 40-80% lysis, after 48 hrs in IL-2) • Also cytotoxic to PaCa5061 and SKOV3 cells • Degranulated (CD107a expression) in response to K562 cells (E:T cell ratio of 1:2, 6 hrs, 25% CD107a+ cells, after 48 hrs in IL-2) • Produced IFN-γ in response to K562 cells (E:T cell ratio of 1:2, 6 hrs, 67% IFN-γ+ cells) (after 48 hrs in IL-2)	• Expressed CXCR3, LFA-1, and VLA-4 immediately after thawing, which increased after 48 hrs in IL-2 • Expressed CXCR4 immediately after thawing, but did not change in expression after 24 hrs in IL-2 • Did not express or expressed low levels of CCR7 and CD62L immediately after thawing, but increased expression after 24 hrs in IL-2	Bröker et al[190].
PBMC (healthy)	CD3+ depletion and CD56+ selection, done within the Prodigy® system	2-4 x 10^6 purified NK cells/mL	NK MACS® expansion media	Human type AB serum, 1 U/mL IL-21 (initial), 500 U/mL IL-2 (Proleukin), 140 U/mL	ClinMACS Prodigy®: static until day 6, gentle shaking following that	14 days	Cryopreserved samples on day 0, 3, 6, 8, 10 and 14 in 20% DMSO. Stored for 3 months, then cultured in IL-21, IL-2,	• 6-fold expansion • CD56+CD3- NK cell purity 99% • CD56+CD3- NK cell viability 86% • 140x10^3 CD3+ T cells • 156.3x10^3 CD56+CD3+ NKT cells	• Fresh expanded (not cryopreserved) NK cells had increased expression of NKG2D, NKp30, NKp44, NKp46, FasL, CD253, CD69, and CD137 relative to freshly isolated cells	• Fresh expanded NK cells had increased killing against K562 cells (E:T cell ratios of 1:1 and 5:1, 4 hrs) relative to freshly isolated cells (E:T cell ratio of 1:1 cytotoxicity was ~83% relative to ~60%; E:T cell ratio of 5:1 cytotoxicity was ~98% relative to ~84%) • Fresh expanded NK cells had increased degranulation (CD107a expression) in response	Not Determined	Oberschmidt et al.[191]

				IL-15, and NK MACS® supplement			and IL-15 for 24, 48, and 72 hrs. (Expansion, viability, degranulation, cytotoxicity, and CAR transduction were determined after thawing)			to K562 cells (E:T cell ratios of 1:1 and 5:1, 4 hrs) relative to freshly isolated cells (E:T cell ratio of 1:1 , CD107a expression was 37% relative to 10%; E:T cell ratio of 5:1 , CD107a expression was 13% relative to 2%) • Fresh expanded NK cells had increased secretion of TNF-α, IFN-γ, granzyme A and B in response to K562 cells (4 hrs) relative to freshly isolated cells • Cells frozen on day 10 of expansion had best ability to expand after thawing, were the most cytotoxic, and had the best percent degranulation. • Cells transduced with a CAR retroviral vector on day 14 had the highest transfection efficiency (~7%), but cells transduced on day 8 and 14 were the most cytotoxic		
PBMC (healthy)	TCR-α/β+/CD19+ depletion and CD56+ selection, done within the Prodigy® system	5.25 x10^5 purified NK cells /mL	TexMACS GMP Medium	Human AB serum, 500 U/mL IL-2 (Proleukin), and irradiated EBV-LCL cells as feeder cells (1:20	Clini MACS Prodigy®: static until day 7, gentle shaking following that	14 days	N/A	• 787-fold expansion • 1.3x10^9 CD56+CD3- NK cells • CD56+CD3- NK cell purity was 81% • CD14+ monocyte contamination was 12% • TCR-γδ+CD56+CD3+ NKT cell contamination was 5%	• Automated and manual (same protocol but in T75/culture bags with medium additions) expanded NK cells had downregulated expression of CD16 relative to fresh NK cells • Automated expanded NK cells had decreased expression of CD57 and NKp80, and increased expression of TRAIL, FasL, NKG2D, DNAM1, NKp30, NKp44, NKG2C, CD94, and	• Automated expanded NK cells had comparable cytotoxicity to K562 cells (E:T cell ratio of 10:1, 4 hrs, ~80%) as manually expanded NK cells • Automated expanded NK cells has comparable production of proinflammatory cytokines and degranulation (CD107a expression) to K562 cells (E:T cell ratio of 2:1, 4 hrs) as manually expanded NK cells • No shortening of	• Automated cells had decreased expression of CX3CR1 and CD62L relative to fresh NK cells (by flow cytometry) • Automated expanded cells had increased expression of CCR5, CXCR6, CCR2, and CX3CR1, and decreased expression of CCR7 relative to fresh NK cells (by gene expression) • Automated expanded cells had increased expression of CX3CR1	Granzin et al.[192]

				ratio of NK cells to feeder cells)					NKG2A relative to fresh NK cells (by flow cytometry) • Automated expanded cells had increased expression of TRAIL, FasL, NKG2A, and TIGIT, relative to fresh NK cells (by gene expression) • Automated expanded cells had increased expression of NKG2C and KIR2DL2/L3, and decreased expression of NKG2A and NKp44 relative to manually expanded cells	telomere length in expanded NK cells	relative to manually expanded cells	
PBMC (healthy)	CD3+ depletion and CD56+ selection, done within the Prodigy® system	$1x10^6$ purified NK cells /mL	NK MACS® expansion media	Human AB serum, NK MACS® supplement, 1000 U/mL IL-2 (Proleukin), 1 U/mL IL-21 (initial), and irradiated autologous PBMCs as feeder cells (1:20 ratio of NK cells to feeder	T-flasks	14 days	N/A	• 84-fold expansion • $81.4x10^6$ CD56+CD3- NK cells • CD56+CD3- NK cell purity was 98% • CD56+CD3- NK cell viability was ~90% • CD3+ T cell contamination was <1% • CD14+ monocyte, CD19+ B cells, and CD56+CD3+ NKT cell contamination were all <2%	• Expanded NK cells had increased expression of NKG2D, NKp30, NKp44, CD69, CD137, FasL, and CD253 relative to freshly isolated NK cells	• Expanded NK cells had increased cytotoxicity to K562 cells (E:T cell ratios of 1:1 and 5:1, 4 hrs) relative to freshly isolated cells (E:T cell ratio of 1:1 cytotoxicity was 95% relative to 37%; E:T cell ratio of 5:1 cytotoxicity was 97% relative to 76%) • Expanded NK cells had increased degranulation (CD107a expression) and secretion of TNF-α, IFN-γ, granxyme A/B, and granulysin in response to K562 cells (E:T cell ratios of 1:1 and 5:1, 4hrs) relative to freshly isolated NK cells and NK cells expanded similarly but without IL-21 • Expanded NK cells were transduced with alpharetroviral vectors encoding anti-CD123 CAR, 3 days later transfection frequency was 22%, then 6 days later frequency	Not Determined	Klöß et al.[193]

				cells)						decreased to 11%		
PBMC (healthy)	CD3+ depletion and CD56+ selection	$2x10^5$ purified NK cells/mL	CellGro® SCGM	Autologous plasma, 500 U/mL IL-2, 50 ng/mL IL-15, and irradiated autologous PBMCs as feeder cells(1:2 ratio of NK cells to feeder cells)	T-flasks	14 days	Cryopreserved cells after expansion in 2/10 volumes of DMSO (only viability, degranulation, and cytotoxicity were determined after thawing)	• 15.7-fold expansion • CD56+CD3- NK cell purity was 97% • $CD56^{bright}$ population was 94% • CD56+CD3- NK cell viability was 96% • CD3+CD56- T cell contamination was <1% • Culture remained sterile, free of mycoplasma, and endotoxin levels were <1 EU/mL. • Average viability after freezing and thawing way 90%	• Fresh expanded NK cells had increased expression of NKG2D, DNAM1, NKp30, NKp44, CD158a, and CD158e relative to freshly isolated NK cells	• Cryopreserved, expanded NK cells had comparable degranulation (CD107a expression) in response PMA and ionomycin to fresh expanded cells (~88% relative to ~84% CD107a expression) • Cryopreserved expanded NK cells had increased degranulation (CD107a expression) to K562 cells (E:T cell ratio of 1:1, 2:1, 5:1, and 10:1, 3hrs) relative to freshly isolated NK cells • Fresh expanded NK cells had slightly higher cytotoxicity to K562 cells (E:T cell ratios of 6.25:1 and 12.5:1, 4 hrs) relative to cryopreserved expanded NK cells (E:T cell ratio of 6.25:1 cytotoxicity was ~70% relative to ~59%; E:T cell ratio of 12.5:1 cytotoxicity was ~76% relative to ~59%)	Not Determined	Torelli et al.[194]
PBMC (healthy)	CD3+ depletion	$2x10^5$ purified NK cells/mL	CellGro® SCGM	Autologous plasma, 10 ng/mL anti-CD3 mAb (OKT3) (initial), 500 IU/mL IL-2 (Proleukin),	A-350N culture bag	14 days	N/A	• 691-fold expansion • CD56+CD3- NK cell purity was 98% • CD56+CD16+ NK cell purity was 97% • CD56+CD3- NK cell viability was 95% • CD3+ T cell , CD14+ monocyte, and CD19+ B cell contamination were all <1%	• Expanded NK cells had increased expression of NKG2C, NKp30, NKp44, CD25, and CD69 relative to freshly isolated NK cells • The proportion of CD158a+b+e+, CD158a+e+, and CD158b+e+ expanded NK cells was decreased relative to freshly isolated NK cells	• Expanded NK cells had increased cytotoxicity to K562 cells (E:T cell ratio of 01:1, 4 hrs) relative to freshly isolated cells (~78% relative to ~40%, respectively) • Expanded NK cells had increased degranulation (CD107a expression) and intracellular TNF-α and IFN-γ expression in response to K562 cells (E:T cell ratio of 1:1, 4hrs) relative to freshly isolated NK cells	• Expanded NK cells had increased expression of CD62L and CXCR4 relative to freshly isolated NK cells	Lim et al.[195]

				and irradiated autologous PBMCs as feeder cells (1:5 ratio of NK cells to feeder cells)								
PBMC (healthy)	(1) 2 CD3+ depletion, (2) 1 CD3+ depletion, or (3) 1 CD3+/CD19+ depletion, all followed by CD56+ selection	1x10^6 purified NK cells/mL	X-Vivo 10	Human fresh frozen plasma and 1000 U/mL IL-2 (proleukin)	VueLife® culture bags	12 days	Cryopreserved cells after expansion in 20% DMSO (only viability and recovery were tested for after thawing)	• Large donor-to-donor variation: (n=2) 30-fold expansion, (n=12) 4-fold expansion, (n=3) no expansion • The use of 100 U/mL Il-2 and 10 ng/mL IL-15, did not enhance proliferation, relative to only 1000 U/mL IL-2 • CD56+CD3- NK cell purity was >98% • Of all CD3+ cells - 66% were CD56+CD3+ NKT cells and 33% were CD3+ T cells • The viability of cryopreserved NK cells immediately after thawing was 60% and had an NK cell recovery of 84%	• Fresh expanded (not frozen) NK cells had decreased expression of CD16 relative to freshly isolated NK cells • Fresh expanded NK cells had increased expression of CD69, NKG2C, NKp30, NKp44, and NKp46 relative to freshly isolated NK cells	• Fresh expanded (not frozen) NK cells were cytotoxic against K562 cells (E:T cell ratios of 1:1 and 10:1, 4 hrs, 64% and 92%, respectively) • If freshly isolated NK cells had >10% monocyte contamination, the NK cells were more cytotoxic to K562 cells (E:T cell ratios of 1:1 and 10:1, 4 hrs) than fresh NK cells with <10% monocytes	Not Determined	Koehl et al.[153]
PBMC (healthy)	CD3+ depletion	Not specified	X-Vivo 10	Human AB serum, Penicillin-Streptomycin, 500 U/mL	T175 flasks	21 days	N/A	• 459-fold expansion • CD56+CD3- NK cell purity was ~60% • CD3+ T cell contamination was ~13% (highly variable and could not be controlled) • No CD19+ B cell or CD14+ monocyte	• Expanded NK cells had increased expression of NKG2D and NKp30 relative to freshly isolated NK cells	• Expanded NK cells had increased cytotoxicity against K562 cells (E:T cell ratio of 10:1, 5 hrs) relative to freshly isolated NK cells (HLA-C-mismatched: ~60% relative to ~12%; matched: ~45% relative to ~11%)	Not Determined	Besser et al.[196]

				IL-2 (Proleukin), and irradiated autologous PBMCs, pretreated with anti-CD3 mAb (OKT3), as feeder cells (1:10 ratio of NK cells to feeder cells)				contamination		• Used blocking antibodies to determine NKG2D and NKp30 were directly involved in killing melanoma cells		
PBMC (healthy)	CD3+ depletion and CD56+ selection. Then single KIRa+, KIRb+, and KIRe+ NK cells were sorted	$6x10^8$ single KIR+ NK cells	CellGro® SCGM	Human AB serum, penicilin, streptomycin, 10 ng/mL anti-CD3 mAb (OKT3), 200 U/mL IL-2 (Proleukin), 10 ng/m	Baxter LifeCell culture bags	19 days	N/A	• 160-390-fold expansion for the different single KIR+ populations • 0.18-0.72x10^8 total single KIR+ CD56+CD3- NK cells • single KIR+ CD56+CD- NK cell purity was 98%; • single KIR+ CD56+CD3- NK cell viability was 84% • CD3+ T cell contamination was <1%;	• Determined the KIR profiles, and selected for different single positive groups	• The different single KIR+ cells were cytotoxic against K562 cells (E:T cell ratios of 5:1 and 10:1, 4 hrs, ~75% and ~78%, respectively) • The single KIRa+ and KIRb+ cells were more cytotoxic to AML cells (E:T cell ratio of 10:1, 4 hrs) than the bulk expanded NK cells (KIRa+ ~29% relative to ~16%; KIRb+ ~19% relative to ~13%)	Not Determined	Siegler et al.[154]

				L IL-15, and irradiated autologous PBMCs as feeder cells (1:10 ratio of NK cells to feeder cells)								
PBMC (healthy)	CD3+ depletion	$4x10^5$ purified NK cells /mL	LGM-3	Human Serum, 500 IU/mL IL-2 (Proleukin), and irradiated autologous PBMCs as feeder cells (1:10 ratio of NK cells to feeder cells)	Culture flasks; The initial flasks had a 1 µg/mL anti-CD16 mAb coating	21 days	N/A	• 5421.6-fold expansion • $2.16x10^9$ NK cells • CD56+CD3- NK cell purity was 99% • ~96% CD16+ NK cells • CD3+ T cell contamination was <1%	• Expanded NK cells had increased expression of NKG2D, DNAM1, 2B4, NKp30, NKp44, and NKp46 relative to freshly isolated NK cells	• Expanded NK cells had increased degranulation (CD107a expression, E:T cell ratio of 1:1, 4hrs) and IFN-γ secretion (E:T cell ratio of 10:1, 4hrs) toward K562 cells relative to freshly isolated NK cells • Expanded NK cells had increased cytotoxicity to K562 cells (E:T cell ratios of 5:1 and 10:1, 4hrs) relative to freshly isolated NK cells and NK-92 cells • Cytotoxicity at E:T cell ratios of 5:1 and 10:1 to K562 cells was ~81% and ~89% respectively	Not Determined	Lee et al.[197]
PBMC (healthy)	CD3+ depletion	$3.33x10^5$ purified NK cells	CellGro® SCGM	Autologous plasma, 10 ng/mL	A-350N and A-1000 NL	21 days	Cryopreserved cells after expansion in 5%	• 15,000-fold expansion • CD56+CD3- NK cell viability was 94% • CD56+CD3- NK cell purity was 98% • CD3+ T cell , CD14+	• Fresh expanded (not frozen) NK cells had increased percentages of NK cells expressing NKG2D, NKp30, NKp44, and NKG2A	• Fresh expanded NK cells were cytotoxic against K562 cells (E:T cell ratios of 1:1 and 10:1, 4 hrs, 59% and 86%, respectively)	• Fresh expanded NK cells had increased percentages of NK cells expressing CXCR3 relative to freshly isolated NK	Min et al.[198]

		/mL		anti-CD3 mAb (OKT 3) (initial), 500 IU/mL IL-2 (Proleukin), and irradiated autologous PBMCs as feeder cells (1:5 ratio of NK cells to feeder cells)	culture bag		DMSO and 25% dextran. Frozen for 3-6 months. (only viability and recovery were tested for after thawing)	monocyte, and CD19+ B cell contamination were all <1% • No difference in NK cell recovery between fresh and cryopreserved expanded NK cells, immediately after thawing • NK cell viability was reduced in cryopreserved NK cells (92%) relative to fresh expanded NK cells (95%), immediately after thawing • Cell number and viability were not influenced by short term (24 or 48 hrs) in culture with IL-2 after thawing	relative to freshly isolated NK cells • Fresh expanded NK cells had decreased percentages of NK cells expressing NKRP-1 and CD158-NK cells relative to freshly isolated NK cells • Cryopreserved NK cells had decreased percentages of NK cells expressing NKp46, immediately after thawing, relative to fresh expanded NK cells	• Fresh expanded NK cells degranulated (CD107a expression) and produced IFN-γ and TNF-α in response to K562 cells (E:T cell ratio of 1:1, 4hrs) • No difference in cytotoxicity, degranulation, or produced IFN-γ and TNF-α between fresh and cryopreserved expanded NK cells, immediately after thawing • Cytotoxicity was not influenced by short term (24 or 48 hrs) in culture with IL-2 after thawing • Cryopreserved NK cells had lower tumor inhibition toward SNU354 xenograft mice than fresh expanded NK cells • Increasing the dose of cryopreserved NK cells 2-fold, lead to a significant increase in tumor inhibition *in vivo* • Fresh or cryopreserved NK cells had greater killing against Raji cells *in vitro* and *in vivo* in combination with an anti-CD20 mAb, relative to NK cells alone	cells • No change in the percentages of CXCR4 or CXCR5 NK cells	
PBMC (healthy)	CD3+ depletion and CD56+ selection.	Not specified	RPMI 1640	Fetal Bovine Serum, Penicillin, streptomycin, 200 U/mL IL-2,	Not specified in some studies, G-Rex in others	14 days	N/A	• ~200-fold expansion of CD56+CD3- NK cells in 14 days • CD56+CD3- NK cell purity was 94% • CD3+ T cell and CD19+ B cell contamination were <1% • ~700-fold expansion of CD56+CD3- NK cells in 21days	• 87% of NK cells were CD56+CD16+ • Expanded NK cells had increased expression of NKG2D, NKp30, NKp44, and NKG2A relative to overnight IL-2 activated NK cells • Expanded NK cells had decreased expression of CD57 and CD158 relative to overnight	• Expanded NK cells were more cytotoxic to hematological and solid tumor cancers than overnight IL-2 activated NK cells (E:T cell ratio of 1:1, 4hrs) • Expanded NK cells had comparable cytotoxicity as NK cells expanded with K562-mbIL21-41BBL feeder cells (E:T	• Expanded NK cells had increased expression of CD54 (ICAM-1), LFA-1, and CXCR6 relative to overnight IL-2 activated NK cells • Expanded NK cells had decreased expression of CD62L and CXCR4 relative to overnight IL-2 activated NK cells	Ojo et al.[199]

				and irradiated OCI-AML 3 cells expressing membrane bound IL-21 as feeder cells (1:5 ratio of NK cells to feeder cells)				• ~10,973-fold expansion of CD56+CD3- NK cells in 5 weeks • 89-fold expansion of CD56+CD3- NK cells in 14 days in G-Rex	IL-2 activated NK cells	cell ratio of 1:1, 4hrs) • Expanded NK cells controlled the growth of primary sarcoma tumors and tumor metastases in the lungs *in vivo* • Mice with lymphoid leukemia survived longer with expanded NK cells relative to control mice		
PBMC (healthy)	N/A	0.1x 10^6 NK cells /mL	CellGro® SCGM	Fetal bovine serum, Glutamax, 50 IU/mL IL-2, and 200 µg/mL PM15 particles	Not Specified	13-21 days	N/A	• ~2,200-fold expansion of CD56+CD3- NK cells on day 21 • CD56+CD3- NK cell purity was 95% • ~572-fold expansion of CD56+CD3- NK cells on day 16 • Only a ~396-fold expansion of NK cells in 16 days stimulated with K562-mbIL15-41BBL feeder cells instead • From n=10 donors, CD56+CD3- NK cells expanded 250-fold in 13 days, and 1,265-fold in 17 days • From n=10 donors, CD56+CD3- NK cell purity was ≥86% at day 21 • CD3+ T cell contamination was 9% • CD56+CD3+ NKT cell and CD19+ B cell contamination were	• Expanded NK cells had a larger $CD56^{bright}$ population • Expanded NK cells has increased expression of NKG2D, NKG2C, NKp80, NKp30, NK-44, NKp46, CD244, TRAIL, FasL, KIR2D, KIR2DL5, and NKG2A relative to freshly isolated NK cells • Expanded NK cells had increased expression of NKp44 and KIR2DL5 relative to NK cells expanded with feeder cells • Expanded NK cells had decreased expression of CD16 relative to freshly isolated NK cells	• Expanded NK cells were cytotoxic against multiple cancer cell lines (E:T cell ratio of 1:1 and 5:1, 4 hrs) • Increased E:T cell ratio had greater cytotoxicity than lower ratios (91% and 72% lysis of K562 cells a 5:1 and 1:1 ratio, respectively; E:T cell ratio of 1:1 and 5:1, 4 hrs) • Expanded NK cells were more cytotoxic to multiple cancer cell lines and patient-derived samples than NK cells expanded with feeder cells (E:T cell ratio of 2:1 and 1:1, 1.5 hrs)	• Expanded NK cells had decreased expression of CD11b relative to freshly isolated NK cells • Expanded NK cells had increased expression of CD11a relative to freshly isolated NK cells • Expanded NK cells had decreased expression of CD62L relative to NK cells expanded with feeder cells	Oyer et al.[200]

								<5%				
PBMC (healthy)	N/A	0.1x 10^6 NK cells /mL	CellGro® SCGM	Fetal bovine serum, Glutamax, 50 IU/mL IL-2, and 200 µg/mL PM21 particles	Not Specified	14-28 days	N/A	• ~835-fold expansion of CD56+CD3- NK cells on day 14 • CD56+CD3- NK cell purity was >90% on day 14 • Only a ~424-fold expansion of NK cells expanded with PM15 particles on day 14 • >100,000-fold expansion of CD56+CD3- NK cells on day 28 (NK cell expansion with PM15 particles stalled at day 22) • On day 14, CD56+CD3- NK cell purity was ~80% • On day 14, CD56+CD3+ NKT cell contamination was ~6% • On day 14, CD3+ T cell contamination was ~18%	• 97% of CD56+CD3- NK cells expressed CD16 • ~50% of CD56+CD3- NK cells expressed CD94 • A low percent of CD56+CD3- NK cells expressed NKG2A • 74% of CD56+CD3- NK cells expressed NKG2A • 75% of CD56+CD3- NK cells expressed NKG2D • 26% of CD56+CD3- NK cells expressed TRAIL • ~33% of CD56+CD3- NK cells expressed KIR2D	• Expanded NK cells were cytotoxic against multiple leukemia cell lines (E:T cell ratio of 10:1 and 1:1, 2 hrs) • Expanded NK cells were cytotoxic against autologous cancer cells (E:T cell ratio of 10:1 and 1:1, 2 hrs)	Not Determined	Oyer et al.[201]
PBMC (healthy)	N/A	Not specified	RPMI 1640	Fetal Bovine Serum, 500 U/mL IL-2, and irradiated Jurkat T cells (KL-1 cells) as feeder cells (1:1 ratio of NK	Culture flasks	14 days	N/A	• 130-fold expansion of CD56+CD3- NK cells • ~$10x10^7$ CD56+CD3- NK cells • CD56+CD3- NK cell purity was ~90% • CD3+ T cell contamination was ~10% (on day 10)	• Expanded NK cells had upregulated expression of NKp30, NKp44, 2B4, DNAM-1, NKG2D, CD25, and CD69 relative to freshly isolated NK cells • Expanded NK cells had downregulated expression of CD16 relative to freshly isolated NK cells	• Expanded NK cells were more cytotoxic to K562 and KL-1 cells than NK cells expanded with only IL-2 (E:T ratio of 20:1 and 2:1, 4 hrs) • At an E:T ratio of 10:1 NK cells to K562 cells , expanded NK cells lyses 65% of cancer cells while IL-2 expanded NK cells lysed 11% of cancer cells. • When expanded NK cells and KL-1 cells were injected into mice together, near complete disappearance of cancer cells was observed	• Expanded NK cells had upregulated expression of ICAM-1, CD11a, CD48, CD2, CD49d, and CD58 relative to freshly isolated NK cells	Lim et al.[202]

				cells to feeder cells)								

AML: acute myeloid lymphoma: ErbB2: erythroblastic oncogene B2/HER2; E:T cell ratio: effector to target cell ratio; CAR: chimeric antigen receptor; CB: cord blood; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; HER2: human epidermal growth factor receptor 2; HI: heat inactivated; hrs: hours; iC9: inducible caspase-9; IFN-γ: interferon γ; IL-2: interleukin 2; IL-6: interleukin 6; IL-7: interleukin 7; IL-8: interleukin 8; IL-10: interleukin 10; IL-15: interleukin 15; IL-21: interleukin 21; K562-mbIL15-41BBL: K562 cells expressing membrane bound IL15 and 4-1 BB ligand; K562-mbIL21-41BBL: K562 cells expressing membrane bound IL21 and 4-1 BB ligand; mAb: monoclonal antibody; MM: multiple myeloma; NK: natural killer cell; N/A: not applicable; OKT3: anti-CD3 monoclonal antibody; PBMCs: peripheral blood mononuclear cells; PMA: phorbol-12-myristate-13-acetate; PM15: plasma membrane vesicles from K562-mbIL15-41BBL feeder cells; PM21: plasma membrane vesicles from K562-mbIL21-41BBL feeder cells; SCF: stem cell factor; sFasL: soluble Fas ligand

3.1 Source of Natural Killer Cells

Sources of NK cells for expansion protocols include NK cells isolated from peripheral blood (autologous or allogeneic donor)[153,154,189,193,196,197,203] and cord blood,[180,187] NK cells derived from human embryonic stem cells (hESC)[204] and induced pluripotent stem cells (iPSCs),[204–207] and the NK cell line, NK-92.[161,179] Peripheral blood mononuclear cells (PBMCs) can be used in an autologous and allogeneic setting, but with allogeneic NK cells a donor selection process must occur, typically based on KLA, KIR, and CD16 genotype. It is highly advantageous to select a NK cell donor with a KIR-HLA mismatch in the anti-tumor direction,[30,32,36,156,181,208] which is done through donor KIR and recipient HLA genotyping.[209,210] To further select an optimal donor, although not as common in clinical trials, is to select donor NK cells that are homozygous in the high affinity CD16 receptor, 158VV, rather than donors that express the low affinity receptor, 158FF.[211–213] PBMCs can be isolated by leukapheresis or whole blood/buffy coat. Due to the advantage of aseptic collection in a closed system, PBMC collection by leukapheresis is likely to be preferred for GMP-compliant expansion of NK cells. However, there is donor variability in the number of PBMCs that can be isolated and the proportion of NK cells in the PBMCs, all affecting the amount of purified NK cells available to initiate culture. Furthermore, there is variability in the phenotype and function of NK cells isolated from different donors, based on age, sex, weight, as well as others, which reduces consistency in the expansion rate and in the final NK cell product. Using PBMCs as a cell source resulted in variable expansions (11-fold to 5421-fold) and purities (31% to 99%), due to the different culture medium and vessels used for NK cell expansion (**Table 2**). As PBMC-derived NK cells are largely used as a patient-specific therapy there are associated limitations including the lengthy donor selection process for allogeneic NK cells, the large donor-to-donor NK cell variability, and the high cost of the therapy. The development of a readily available "off-the-shelf" NK cell therapy to treat many patients could overcome these limitations. While some clinical trials have used "off-the-shelf" NK cell therapies, further work into cryopreservation methods and increasing their cytotoxicity needs to be done to develop effective therapies for solid tumors. As such, the majority of clinical trials have been performed with PBMC-derived NK cells, but there is growing interest in alternative sources, some of which have been used in clinical trials[180,214] (**Table 1**).

Cord blood-derived NK cells have been *ex vivo* expanded and used in a clinical trial, with no adverse events.[180] Cord blood obtained from a cord blood bank serves as a more readily available source of NK cells, as there are 730,000 cord blood units worldwide.[215] Screening can be done on the different units of cord blood to select optimal units, based on KIR genotype and high affinity CD16 receptor, to be donor cells to multiple patients. NK cells from the cord blood have the potential to function, in terms of cytokine production and cytotoxicity, similarly to adult peripheral blood NK cells, as there is a mixture of

immature and mature cells.[216,217] Since, NK cells constitute a slightly larger portion of cells in cord blood than in adult peripheral blood,[218] potentially more NK cells could be isolated from cord blood than adult peripheral blood.[219] In one study which compared the two cell sources, cord blood-derived NK cells proliferated slightly better during a 2 week culture, than PBMC-derived NK cells.[219] However there are conflicting reports on the cytotoxicity of expanded NK cells from the two cell sources, with studies observing cord blood-derived NK cells have increased,[220] decreased,[219] and comparable[216] cytotoxicity to target cells relative to PBMC-derived NK cells. The discrepancies between the different studies may be due to the different manufacturing methods used to expand the NK cells and the donor-to-donor variability in the starting NK cell source. To progress cord blood-derived NK cells forward, expansion methods which result in NK cells with at least comparable cytotoxicity to peripheral blood NK cells should be prioritized. Two different methods for expanding cord blood-derived NK cells have been used - isolating and expanding NK cells from the blood[180,188,217] and expanding NK cells derived from isolated CD34+ progenitor cells (**Table 2**).[187] Expanding NK cells isolated from the cord blood, resulted in a 2221-fold expansion in 14 days. [180,188] Alternatively, isolating CD34+ cells from cord blood, then differentiating them to NK cells and expanding, resulted in a 2100-fold expansion in 6 weeks.[187] However, it should be noted that these two protocols used different expansion methods, which may account for differences in expansion rates, in addition to the starting cell source. Although differentiating CD34+ cells into NK cells also led to a highly pure final product (approximately 92%),[187] it took significantly longer to reach clinically relevant numbers compared to expanding the NK cell population from the cord blood. Furthermore, the differentiation process is very labor intensive, requiring frequent media changes. These factors must be considered when developing clinically translatable NK cell expansion protocols, as shorter expansion times and less operator involvement are likely preferred. While isolation of NK cells or NK cell differentiation of CD34+ cells from cord blood are both viable options for expanding and activating NK cells, isolating NK cells may be more appealing for clinical translation.

Pluripotent stem cells have the potential for generating high numbers of NK cells and can be a potential source for an "off-the-shelf" therapy to treat multiple patients. Different pluripotent stem cell lines could be screened, during the process of establishing the cell line, for favorable KIR and CD16 expression prior to their clinical use.[221] As with other cell sources, the expansion method influences the final NK cell product, but the choice of starting cell source affects the efficiency and kinetics of reprogramming and the practicality of deriving clinical-grade NK cell. Human ESC cell lines,[204] iPSC cell lines,[204,206,207] and iPSCs generated from T cell-depleted PBMCs[205] have been used as starting material for differentiation into NK cells. Differentiation of these cell sources into NK cells requires the use of xenogeneic stromal feeder cell lines[205] or human spin embryonic bodies (EBs),[204,206,207] which eliminates xenogeneic cells to provide more defined conditions for NK cell development and may be more amenable

to clinical scale-up. Expanding NK cells from either method with irradiated K562-mbIL21-41BBL (Clone 9.mbIL21) feeder cells and IL-2 resulted in roughly an 80-fold expansion in 21 days and a final purity of 99% NK cells.[204] The expanded NK cell population from both methods had comparable cytotoxicity to K562 cells and phenotypes, which were largely retained from pre-expansion, except for increased expression of CD16 and inhibitory KIRs.[204] From this study, using the same hESC cell line to generate NK cell by different methods (feeder cells or EBs) resulted in largely different NK cell purities but comparable phenotypes; further expanding these different NK cells with the same method results in highly comparable final products. As such, using the spin EB method, with less operator involvement, to differentiate hESCs into NK cells may be more amendable to clinical-grade manufacturing.

There are controversial ethical issues associated with the use of human ESCs; therefore a use of pluripotent stem cells from other source may be more appealing for an "off-the-shelf" NK cell therapy. An established iPSC cell line has been used to generate NK cells (spin EB method), which were then expanded with irradiated Clone 9.mbIL21 feeder cells and IL-2.[206] The NK cells expanded several log-fold in 21 days and were highly pure NK cells (greater than 97%), with high expression of NKp44, NKp46, and NKG2D.[206] Importantly, expanded iPSC-derived NK cells and PBMC-derived NK cells had comparable anti-tumor function in ovarian tumor bearing mice.[206] In an effort to decrease the expansion time, T cell-depleted PBMCs were used to generate iPSCs for differentiation into NK cells using a feeder cell approach.[205] After 9 days of expanding those NK cells with K562-mbIL15-41BBL cells (Clone 4.mbIL15) feeder cells and IL-2, there was a 74-fold expansion of highly pure NK cells (99%), and the cells had low expression of CD16 and inhibitory KIRs.[205] Focusing on inhibitory receptor expression, the hESC- and iPSC-derived and expanded NK cells had high expression of inhibitory KIRs and CD16,[204,206] but lower than PBMC-derived NK cells.[206] This may suggest that NK cells from these cell sources are more cytotoxic than cells from iPSCs derived from T cell-depleted PBMCs, due to some indications that KIR-positive NK cell subsets are more functional than KIR-negative subsets.[222] However, at an E:T cell ratio of 2.5:1 NK cells to K562 cells, hESC-derived and expanded NK cells' cytotoxicity was approximately 15%,[204] iPSC-derived and expanded NK cells' cytotoxicity was 40%,[206] and non-T cell PBMC-iPSC9-derived and expanded NK cells' cytotoxicity was approximately 81% (at a 2:1 ratio).[205] Relative to PBMC-derived NK cells, NK cells derived from hESC or iPSC cells were less cytotoxic to multiple solid tumor cell lines[204,206] while NK cells derived from iPSCs from T cell-depleted PBMC were more cytotoxic to multiple solid tumor cell lines and colorectal cancer primary tumor cells.[205] In addition to differences in starting cell source, the differences in cytotoxicity may be due to the expansion protocol and the variability in the donor PBMC-derived NK cells. Pluripotent stem cells have the ability to generate highly pure NK cells, but the differentiation process is labor intensive and time consuming (greater than 4 months) and longer expansion times may be required to generate high numbers of NK

cells from the hESC and iPSC cell lines. Overall, consideration must be taken into the starting cell source for pluripotent stem cells to be used to generate and expand NK cells for clinical use.

Another option for an "off-the-shelf" NK cell therapy, which has been used in clinical trials, is the NK-92 cell line. Since the starting material is a homogenous, established cell line, after expansion the final product is highly pure and uniform. The main considerations which can influence the expansion rate are the concentration of IL-2, inclusion of other stimuli, and concentration of cells in culture. NK-92 cells have been expanded in culture 14-fold in 5 days[162] and 211- to 250-fold in 2-2.5 weeks,[161] with greater than 85% viability (**Table 2**).[161,162] The NK-92 cell line is highly cytotoxic and has been found to be more cytotoxic toward K652 and Raji cells than expanded peripheral blood NK cells.[147] This cell line provides a good source of highly cytotoxic NK cells, but there are some limitations with this cell line as previously discussed, such as the need to genetically engineer the expression of CD16 and/or CD62L expression to unleash their full cytotoxic potential.

3.2 Purification of Natural Killer Cells

Isolating NK cells from peripheral and cord blood requires purification of the cells from other contaminating cell types which can cause harm if infused into the recipient. In autologous settings, T cells may be beneficial to the overall anti-tumor effect and are tolerated by the patient, however, in allogeneic settings, T cells can cause severe GvHD.[20–28] Another source of contamination is B cells, which can lead to autoimmune disorders and passenger lymphocyte syndrome in patients.[223] An allowable B cell contamination in allogeneic NK cell adoptive therapy is commonly 5% or less CD19+ B cells.[145,151] As such, the final product must be highly pure in NK cells, with little contaminating cell populations.

There are different methods to purify NK cells, and a trade-off between NK cell purity and recovery that has to be considered when selecting the purification method (**Figure 2**). The methods which have been used to are CD3+ depletion, CD3+/CD19+ depletion, CD56+ selection, or CD3+ depletion followed by CD56+ selection (**Table 2**). Purifying NK cells is commonly done prior to expansion, to minimize the space occupied and reagents consumed by the contaminating cells, which may result in less operator input during the expansion and/or greater proliferation of the NK cells. Clinical-grade purification of NK cells by CD3+ depletion followed by CD56+ selection leads to highly purified NK cell products with a median purity ranging from 90-98.6%.[151,153,154,194,224,225] However, this high purity comes at the expense of considerable loss of NK cells during the purification process, with the recovery of NK cells ranging from 19-77%, as there is large donor-to-donor and protocol variability.[151,153,154,194,224,225] Alternatively, purifying NK cells with only a CD3+ depletion step leads to a significantly lower purity of NK cells ranging from 10-50%, with high CD19+ B cell and CD14+ monocyte contamination (both greater than 20%),[151,196,225] but a greater recovery of NK cells of 79%.[151,225] Lastly, purifying NK cells with a CD56+ selection step resulted in a NK cell purity of 66%, with 30% NKT cells.[147] In clinical trials

of expanded NK cells for the treatment of solid tumors, only CD3+ depletion[33,138] or CD56+ selection[139] have been used, however CD3+ depletion followed by CD55+ selection has been used to purify NK cells for patients with hematological cancers.[146,208] To demonstrate the differences in purification method, three different methods used in clinical trials of overnight activated NK cells[28,226] were compared based on NK cell purity and recovery, and time required for the process: (i) 2 CD3+ T cell depletion steps (program 2.1, $D2.1_{2depl}$), (ii) 1 CD3+ T cell depletion step (program 2.1, $D2.1_{1depl}$), and (iii) 1 CD3+ T cell depletion step (program 3.1, $D3.1_{1depl}$), all followed by a CD56+ selection step.[153] While D2.1 (1 or 2 depletions) resulted in significantly better T cell depletion than $D3.1_{1depl}$, the program was extremely time consuming and the recoveries of NK cells were lower than $D3.1_{1depl}$.[153] Importantly, the NK cell purity (70-98%) and viability (79-98%) did not differ between the different methods.[153] Therefore there is a trade-off between level of T cell depletion and the time is takes to conduct the depletion steps, which must be considered when manufacturing NK cells. Additionally, a CD3+/CD19+ depletion step has been used to simultaneously remove T and B cells from the PBMCs. Compared to a CD3+ depletion step, a CD3+/CD19+ depletion resulted in comparable NK cell purities (38% and 31%, respectively) and recoveries (74% and 76%, respectively), greater T cell and B cell depletion, and increased monocyte presence (31% and 49%, respectively).[151] Each method is associated with a different time and cost to purify NK cells, and results in different NK cell purities and recoveries, and also the type of contaminating cells (**Figure 2**).

All of the previously mentioned purification methods require one system to purify the cells which will then be moved to another vessel for culture, which involves operator input and has a risk of contamination. Recently the CliniMACS Prodigy® was released, which combines the purification process in the same system as the culturing and washing processes, allowing all the work for a batch of cells to be completed in a single fully closed, automated instrument. Isolating NK cells using CD3+ depletion followed by CD56+ selection in this system, led to highly purified NK cell products of 93-95%, while maintaining a higher and less variable NK cell recovery of 60-66%,[191,193] than this purification method in other systems. Additionally, this system has been used to deplete T cells by the T cell receptor (TCR)-α/β, rather than the more common target, CD3.[192] This process was performed based on the finding that TCR-α/β+/CD-19+ depletion resulted in a more efficient T cell reduction than CD3+/CD19+ depletion.[227] After TCR-α/β+/CD-19+ depletion then CD56+ selection in the CliniMACS Prodigy®, there were no detectable T or B cells, and the NK cell purity ranged from 71-92%, with the remaining contaminating cells being monocytes and NKT cells.[192] The high purification and consistent NK cell recovery gives the Prodigy® system an advantage over other methods for purifying NK cells. Isolating NK cells based on CD3+ or TCR-α/β+ T cell depletion should be further explored to determine which method produces more consistent and reliable results.

Since any contaminating T cells will proliferate under expansion conditions,[34,170,171] some protocols choose to simplify the manufacturing process by culturing all PBMCs, especially for autologous NK cell therapy.[38,189,228,229] In these studies, the final purity of NK cells and presence of NKT cells, T cells, B cells, and monocytes highly depends on the expansion medium, stimuli, and culture vessel.[38,189,228,229] As such, the final NK cell purity can range from 31%[189] to 96%.[228] In clinical trials of expanded NK cells, this method has been most commonly used (**Table 1**), since it removes the purification step, thus reducing the associated time, money, and the risk of contamination during this step. In the allogeneic setting, CD3+ depletion or CD56+ selection may be used at the end of the expansion protocol to remove contaminating cells, if necessary. During culture, the presence of other PBMCs may help to activate and expand the NK cell population. PBMCs may become activated themselves by cytokines, like IL-2 and IL-15 commonly used in expansion protocols, which would increase their production of additional cytokines supportive to NK cell activation.[147] Also, dendritic cells and monocytes can help activate and expand NK cells through cell contact.[148] In one study found unstimulated NK cell products with a higher percent of monocytes (greater than 10%) were more cytotoxic to target cells than unstimulated NK cells products with less than 10% monocytes (**Table 2**),[153] highlighting the role of monocytes in aiding in NK cell activation. However a disadvantage of culturing all PBMCS is the contaminating cell types consume space and media, thus the process may require more operator involvement for medium changes and splitting of cells into multiple containers, if necessary. Culturing all PBMCs to expand the NK cell population has been used in clinical trials, but there are advantages of initiating culture with purified NK cell populations.

3.3 Medium and Stimuli for Expanding Natural Killer Cells

The complete culture medium for expanding NK cells incorporates a combination of the following: base medium, serum supplements, clinical-grade cytokines, monoclonal antibodies (mAbs) or other small molecules, and feeder cells. The components of the complete culture medium have a large influence on expansion time, final NK cell number, and the phenotype and function of the final cell product (**Figure 2**). Each expansion method has a different final NK cell number, purity, viability, phenotype, and cytotoxic potential (**Table 2**), due to the specifics of the manufacturing process (**Figure 2**). In pre-clinical studies the most common base media used to support NK cell survival and expansion have been X-Vivo™ 10[147,196] and 15,[161] GellGro® SCGM,[152,194,228,229] and NK MACS®[193] (**Table 2**). In clinical trials the most commonly used base media have been X-Vivo™ 10[143,144] and 15,[38] AIM-V,[33,35] GellGro® SCGM,[138] while other studies have used different mediums[39,40] (**Table 1**). It is important to note that the different base media will have an effect on the NK cell expansion. For example, NK MACS® was found to outperform TexMACS, X-Vivo 10, and GellGro® SCGM in terms of NK cell expansion by over 20-fold, NK cell viability, and amount of contaminating T cells.[193]

NK cells require stimulation to expand and activate, and in clinical-grade expansion protocols this has been done with the addition of cytokines, small molecules, mAbs, and feeder cells (**Table 2**). Many protocols use a combination of stimulatory signals to maximize NK cell proliferation and activation, such as IL-2, IL-15, and IL-21. These cytokines function as growth and differentiation factors for NK cells, both *in vitro* and *in vivo*, and regulate the expression of different surface receptors.[14,62–64,148,230] Feeder cells stimulate NK cells by the production of soluble factors and direct cell-cell contact. Feeder cells used to expand NK cells include autologous PBMCs,[154,195,200] umbilical cord stem cells,[231] Epstein-Barr virus (EBV)-immortalized lymphoblastoid cell lines,[146,192,203,232] cancer cell lines,[202,233] or genetically modified cancer cell lines.[152,228,234,235] While cytokine stimulation has a lower risk of contaminating feeder cells in the final product, expanding to a large numbers of NK cells required prolonged culture periods (3-6 weeks, **Table 2**). Alternately, using feeder cells as stimuli for expanding NK cells is a faster process, resulting in the same cell number, if not more, in 8-14 days (**Table 2**). A risk of using feeder cells is they could remain in the final cell product and cause adverse effects in patients. To minimize this risk, feeder cells are irradiated prior to initiation of culture to keep the cells alive but halt their proliferation capacity.[152,193–195,198,203,228,235] Using feeder cells in the expansion of NK cells has many advantages but, it is important to identify and completely remove the feeder cells from the NK cells prior to release of the final cell product.

Using IL-2, IL-15, IL-21 (initial), or any combination results in a maximum expansion of approximately 10-fold after 2 weeks in culture (**Table 2**).[147,153,190,191,200,236] An initial stimulation to the culture with IL-21 can help increase NK cell proliferation, but continuous stimulation will ultimately decrease NK cell numbers.[232] NK cells stimulated with only cytokines commonly have increased cytotoxicity to target cells and increased expression of NKG2D, NKp30, NKp44, and FasL, relative to freshly isolated NK cells (**Table 2**).[153,190,191,236] Very few expansion protocols are able to increase the expression of the NCR NKp46, but in two separate studies of IL-2 stimulation alone[153] and a combination of IL-15, IL-2, and IL-21 (initial bolus),[191] NKp46 expression was increased relative to freshly isolated NK cells. The expression of inhibitory KIRs and NKG2A in expanded NK cell is often overlooked, but one study found IL-15 expanded NK cells had increased expression of some inhibitory KIRs and NKG2A, in addition to more commonly evaluated activating receptors.[236] To further expand NK cells, protocols have included the anti-CD3 mAb, OKT3, at the initial stages of culture, to stimulate remaining NKT or T cells in culture to produce additional cytokines for NK cell activation.[189] Stimulating NK cells with IL-2 and OKT3 resulted in a 77- to 1100-fold expansion of cells, depending on the culture vessel,[189] which is significantly greater than expansion rates with IL-2 alone. However, the purity of NK cells was very low (less than 54%) and the T cell contamination is relatively high (approximately 41%).[189] While

the expansion rate is higher than cytokines alone, in an allogeneic setting, the final cell product would need to be purified to remove the T cell contamination prior to infusion.

Irradiated autologous PBMCs can support the stimulation and expansion of NK cells,[147,148] as previously mentioned, but they are as feeder cells, they are irradiated to prevent their growth while still providing support to the NK cells. Expanding PBMC-derived NK cells with irradiated feeder cells and various cytokines can result in 15- to 84-fold expansion in 14 days, depending on the cytokine cocktail used, with highly pure final products (**Table 2**).[193,194] Studies have also explored the use of OKT3, IL-2, and irradiated PBMC feeder cells, which resulted in increased expansion of PBMC-derived NK cells, relative to studies without OKT3, with a 691-fold expansion in 14 days[195] and 15,000-fold expansion in 21 days,[198] both with 98% NK cells. As with expanding NK cells with cytokines alone, stimulating NK cells with autologous PBMC feeder cells, cytokines, and OKT3 most commonly increased the expression of NKG2D, NKp30, and NKp44, relative to freshly isolated NK cells (**Table 2**)[193–195,198] Importantly, the expression of inhibitory KIRs was often determined in these studies, which helps to determine the whole receptor profile on the cells. Overall, expanding NK cells with IL-2, IL-15, and autologous PBMC feeder cells results in upregulated expression of specific inhibitory KIRs, relative to freshly isolated NK cells.[194] While one study of expanded NK cells with IL-2, OKT3, and autologous PBMC feeder cells (21 days) resulted in increased the amount of NKG2A+ cells and decreased the amount of CD158a-b-e- cells,[198] another study using the same expansion method (14 days) resulted in decreased the amount of CD158a+b+e+, CD159a+e+, and CD158b+e+ cells,[195] all relative to freshly isolated NK cells. Autologous feeder cells can be highly effective in expanding NK cells to high cell numbers in a shorter amount of time than cytokines alone, and generating cell products more cytotoxic than freshly isolated NK cells (**Table 2**).

Another common feeder cell line used to expand and activate NK cells is the irradiated Epstein-Barr Virus-Immortalized Lymphoblastoid cell (EBV-LCL) line. Both CD3+ depleted/CD56+ selected PBMC NK cells[203] and TCR-α/β+/CD19+ depleted/CD56+ selected PBMC NK cells[192] have been expanded with IL-2 and EBV-LCL feeder cells (**Table 2**). Even though a similar protocol is followed to expand the differently depleted cells, CD3+ depleted NK cells only expand 510-fold in 19 days with a final NK cell purity of 95%,[203] TCR-α/β+/CD19+ depleted NK cells expand 850- to 1135-fold in 14 days, depending on the culture vessel, with a final NK cell purity greater than 99%.[192] This finding highlights the influence of all factors in a manufacturing protocol, in this case culture vessel, have on the final NK cell products (**Figure 2**). Additionally, since these are donor PBMC NK cells, there is donor-to-donor variability which will influence the final NK cell product. Again in these culture systems the expression of NKp46 does not change in the expanded NK cells, but in both the expression of TRAIL, NKG2D, NKG2A, relative to freshly isolated NK cells was observed.[192,203] Like autologous feeder cells, high

expansion rates can be observed using EBV-LCL feeder cells, with final cell products with greater cytotoxicity than freshly isolated NK cells (**Table 2**).

Lastly, genetically engineered cancer cell lines, manipulated to express cytokines and surface ligands supportive to NK cell expansion, are commonly used as feeder cells and can generate high numbers of potent NK cell in a quick expansion protocol. The most commonly used engineered cell lines are K562-mb15-41BBL (Clone 4.mbIL15)[152,228] and K562-mb21-41BBL (Clone 9.mbIL21)[180,188,235] (**Table 2**). The prior clone, Clone 4.mbIL15 cells, in combination with IL-2, can expand NK cells 90- to 209-fold in 7/8 days, but NK cell purities of between 70-83%.[152,228] To try to improve on these feeder cells, K562 cells were engineered to express mbIL-15, 41BBL, and MICA.[234] NK cells expanded with these feeder cells and IL-2 resulted in a 550-fold expansion in 24 days; NK cells expanded by these feeder cells expanded greater and were more cytotoxic than NK cells expanded with Clone 4.mbIL15 cells.[234] Unlike the previously mentioned studies, NK cells expanded with K562 cells expressing mbIL15, 41BBL, and MICA had upregulated NKp46 expression relative to NK cells expanded with Clone 4.mbIL15 cells.[234] To further improve on engineered feeder cells, the Clone 9.mbIL21 cells were developed with the goal of generating greater numbers of more cytotoxic NK cells, relative to Clone 4.mbIL15 expanded NK cells. Stimulating NK cell expansion with Clone 9.mbIL21 and soluble IL-2 can result in significant expansion rates of 2221-fold in 14 days[180,188] and 47,967-fold in 21 days.[235] NK cells expanded with Clone 9.mbIL21 or Clone 4.mbIL15 have increased expression of KIR2DL2/L3, slightly decreased expression of NKp30, and were significantly more cytotoxic to target cells, relative to freshly isolated NK cells.[235] While NK cells expanded with Clone 4.mbIL15 cells had decreased IFN-γ and TNF production in response to K562 target cells relative to fresh NK cells, NK cells expanded with Clone 9.mbIL21 had increased expression of these cytokines.[235] While these feeder cells result in significant expansion of NK cells, there is a potential for feeder cell contamination in the final cell product which poses additional patient risk and, consequently, additional burdens on the manufacturing and clinical translation processes to ensure safety.

As an alternative, plasma membrane vesicles from genetically engineered cell lines have been used to expanded PBMC-derived NK cells (**Table 2**). For example, plasma membrane vesicles from Clone 4.mbIL15 (PM15 particles) selectively expanded NK cells comparable to expanding NK cells with using Clone 4.mbIL15 cells.[200] Furthermore, NK cells expanded with PM15 particles[200] had comparable cytotoxicity to target cells as NK cells expanded with Clone 4.mbIL15 cells.[152,228] However, these cells expanded by both methods had characteristics of senescence after about 3 weeks in culture,[200] likely due to the IL-15 stimulation. To overcome this limitation, plasma membrane vesicles from Clone 9.mbIL21 cells (PM21 particles) were engineered.[201] The PM21 particles induced significantly greater NK cell expansion than the PM15 particles and expanded comparably to NK cells stimulated with Clone

9.mbIL21 cells.[201] Furthermore, the NK cells expanded by PM21 particles continued to expand until the end of the culture period of 28 days, whereas the NK cells expanded with the PM15 particles plateaued in expansion by day 22.[201] Interestingly, PM21 particles could also stimulate *in vivo* human NK cell expansion in NSG mice.[201] While further studies are needed, a combination therapy of PM21 particles and *ex vivo* expanded NK cells may be beneficial to patients, as the PM21 particles may stimulate the NK cell expansion *in vivo.* The advantages of using cell-derived particles rather than feeder cells to expand NK cells are they are an easily available culture reagent, since a batch can be frozen for some time before use,[200,201] and they eliminate the need for additional safety precautions associated with feeder cells.

Continued proliferation of NK cells *ex vivo* is limited by senescence of the cells over time. NK cell senescence can be evaluated by changes in NK cell shape, size, and proliferation and also the absence of cell-cycle entry.[237] Specifically, continuous IL-15 stimulation has been linked with telomere-induced senescence in NK cells.[235] IL-15 expanded NK cells have been found to have shorter telomeres than fresh NK cells and NK cells expanded with other cytokine stimulation, like IL-2 and IL-21.[235] As such, NK cells with shortened telomeres would have inhibited persistence and expansion in patients after infusion. NK cell senescence is an issue for all NK cell manufacturing protocols, whoever few studies have directly evaluated NK cell senescence during the expansion process. One study used Clone 9.mbIL21 cells that express IL-21 instead of IL-15, and were hypothesized to expand NK cells without evidence of senescence. Unlike NK cells expanded with Clone 4.mbIL15, NK cells expanded with Clone 9.mbIL-21 were cultured up to 6 weeks without evidence of senescence and had increased telomere length relative to fresh NK cells and Clone4.mbIL-15 expanded cells.[235] Other signs for senescent NK cells are being explored, such as phenotypic makers including CD57,[237] but still few studies look at the expression of this marker in NK cells during the expansion process (**Table 2**). It is important to evaluate markers for NK cell senescence when manufacturing NK cells, as senescent cells will be unable to function properly *in vivo.*

In summary, current manufacturing methods for *ex vivo* expanding NK cells have been able to generate great numbers of NK cells with greater cytotoxicity than freshly isolated NK cells (**Table 2**). However, the stimulation used to expand and activate the NK cells can greatly affect the end products' phenotype and function (**Figure 2, Table 2**). Protocols which lead to high proliferation rates and highly functional NK cells should be prioritized, but the regulatory considerations for establishing the protocol needs to be considered. Additionally, there are issues with high rates of donor-to-donor cell variability in the expansion rate, purity, phenotype, and function of the final NK cells. Intra-donor variability has also been seen in donor NK cells, as there was significant variability in phenotype between NK cells expanded from a donor isolated on multiple different occasions.[147] "Off-the-shelf" NK cell therapies could help

overcome these limitations, and optimized protocols for manufacturing NK cells from sources which permit "off-the-shelf" therapies need to be considered.

3.4 Culture Vessels for Expanding Natural Killer Cells

Many NK cell manufacturing protocols rely on discrete and open manual processing steps along the workflow, which makes them susceptible to operator-to-operator variability and contamination, and are not amendable to scale-up. Closed culture systems are encouraged since the risk of contamination is minimized, aseptic technique is improved, and for large-volume vessels less operator interaction is required due to less frequent medium and vessel change. Effort must be taken to establish protocols amendable to scale-up and wide spread adoption of the manufacturing process, to allow for more accessible treatment. Closed systems with limited operator interaction will help advance manufacturing protocols in this manner and allow for more consistent final products. In the various pre-clinical studies, culture vessels used to expand and manufacture NK cells include standard culture flasks,[193,194,196,197] medium-volume gas permeable bags (i.e. VueLife®),[151,153,154,161,162,195] large-volume gas permeable culture devices (i.e. G-Rex),[152,205,238] large-volume bioreactors (i.e. WAVE bioreactor™),[187,189,200,238] and special culture systems (i.e. CliniMACS Prodigy®)[190–192] (**Table 2**).

NK cells are suspension cells, meaning there is limited adherence to tissue culture treated surfaces, and as such the density of the cells during culture and the total volume can affect their viability and proliferation. Employing tissue culture flasks for NK cell expansion has a high risk of contamination and extensive operator involvement, though this risk is lowered in GMP laboratory environments. In these vessels, a high amount of cells are not required to initiate culture which is beneficial if the starting number of NK cells is low. Additionally, larger cell numbers of $1.4x10^6$ NK cells in 14 days[193] and $2.16x10^9$ NK cells in 21 days[197] (**Table 2**) can be reached, but requires the use of multiple flasks. Culturing NK cells in gas-permeable small bags also supports lower cell seeding densities, but to reach higher cell numbers multiple culture bags will be required. In expansion protocols using culture bags, final NK cell numbers can reach $0.18\text{-}0.72x10^8$ cells in 19 days[154] and approximately $1.6x10^9$ cells in 6 weeks[187] (**Table 2**). In larger scale gas-permeable culture systems, like the G-Rex, the need for media exchange and cell manipulation is reduced, and was even eliminated in a study which expanded NK cells for up to 10 days.[152] In the G-Rex the media can be aspirated off the top during harvest without disrupting the cells, reducing the harvest volume of the cells.[152] This significantly reduces the time required for harvesting and the use of complex volume reducers or cell harvesters. Protocols utilizing the G-Rex culture flask for NK cell expansion resulted in $5\text{-}8.5x10^9$ NK cells in 8-9 days (**Table 2**).[152,238] In gas permeable bags and flasks the volume of medium is restricted by shape of the vessel and the surface for gas exchange. Therefore frequent medium changes, cell counting steps, and splitting of flasks/bags are typically required to maintain a sufficient environment for expansion. Culture vessels that reduce the need

for frequent, time-consuming cell manipulations reduce the risk of contamination and increase the consistency of NK cell products, making them ideal for large-scale expansion of NK cells.

Therefore, the use of closed system bioreactors, such as the WAVE bioreactor™ and CliniMACS Prodigy®, which require less cell manipulation and thus minimum operator involvement, are optimal for manufacturing NK cells. A limitation of current bioreactor systems is the requirement for a higher cell number to initiate culture, which can be limiting when few NK cells are available for culture initiation. To overcome the limitation of a high cell seeding density, some protocols utilize open system culture flasks for the first few days of culture, then transition to bioreactors when a certain cell number is reached.[189,238] However, this still requires operator involvement and increases the risk of contamination. Studies comparing closed and open systems have found opposing effects on NK cell expansion, with closed systems having lower[189,238] or higher[187] expansion relative to open systems, likely due to the specific expansion protocol (**Figure 2**). Still, with the different culture vessels comparable final NK cell numbers can be achieved, ranging from $2x10^9$ NK cells[187,192] to $9.8x10^9$ NK cells[189] (**Table 2**). Like opened systems, there is a need for multiple closed system devices to generate high dosages of NK cells (i.e. $1x10^8$ NK cells/kg) for an individual patient. Importantly, to transition NK cell protocols to closed systems, expanded NK cells must be at least as functional as NK cells expanded in open systems. NK cells expanded in the Wave bioreactor™ resulted in NK cells with no phenotypic or functional differences to those cultured in a manual system.[189] However, one study found NK cells cultured in CliniMACS Prodigy® were comparable in cytotoxicity to manually expanded NK cells, but had slight differences in expression of activating and inhibitory receptors.[192] An advantage of closed systems is the cells can be cultured in static or dynamic conditions at defined rates of movement. A dynamic culture condition can contribute to higher NK cell purity, relative to a static condition, because it creates a more homogenous culture environment.[189,238] However, the constant movement of the cells may disrupt the formation of cell-cell contacts that NK cells require for clustering and growth, resulting in relatively lower expansion rates. As such, protocols can utilize a combination of static conditions for culture initiation followed by dynamic conditions after a few days in culture (**Table 2**).[191,192] Novel features of the CliniMACS Prodigy® are the combination of NK cell purification and culture in a single closed, automated system and a low cell seeding density can be used. Overall, many studies found automation of the cultivation of PBMC-derived NK cells in closed systems is more practical than manual approaches.[187,192,238] Using automated, closed systems to culture NK cells, reduces the risk of contamination and operator-to-operatory variability and simplifies the manufacturing process, which generates more consistent final NK cell products.

3.5 Genetic Engineering Expanded Natural Killer Cells

While there are many reviews which focus on CAR NK cells and associated challenges,[176–178] the following focuses on considerations for genetically engineering NK cells directly relevant to expanding NK cells. Engineering NK cells, for expression of CARs or other factors, can be challenging, due to variable transduction efficiencies and cell death after modification. While transduction efficiency of retroviruses for T cells is consistent and high, for NK cells the efficiency is lower and highly variable - 15-97% in NK cell lines,[179,239] 6-50% in freshly isolated NK cells,[180,239] and 6-96% in *ex vivo* expanded NK cells.[180,239] Methods to improve transfection, such as electroporation, are not commonly used and have their own limitations.[239] Furthermore, the time during expansion at which the NK cells are transduced can have an effect on the transfection efficiency and cytotoxicity of the final cell product. After initiation of culture, during the first 3 to 6 days there is a loss of primary NK cells, after which point the cells begin to expand at variable rates, depending on the culture conditions.[147,154,191,240] As such, the state of the cells is highly variable during the expansion timeline and will greatly influence the transduction efficiency. For example, the transduction efficiency of alpharetroviral vectors, encoding for an anti-CD123 CAR in PBMC-derived NK cells was greatest when cells were transduced on day 14 of expansion (approximately 7%) and was lowest on day 2/3 of expansion (**Table 2**).[191] However, the highest cytotoxicity to target cells was observed by CAR NK cells transduced on day 8 and 14.[191] Additionally, in the pre-clinical and clinical study of cord blood derived CAR NK cells, cells were transduced with retroviral vectors encoding for an anti-CD19 CAR, IL-15 production, and a suicide gene on day 4 or 6 of expansion, and cultured for a total of 14 days.[180,188] On day 14 of expansion, the transfection efficiency was 66% and 49% of NK cells transduced on day 4 or 6, respectively. [180,188] Higher transfection efficiencies have been observed with NK-92MI cells of 93% prior to cell expansion, after transfection with a lentiviral vector encoding for an anti-CD33 CAR prior to expansion.[179] Further CAR NK cell research is needed to determine the optimal NK cell source and the optimal CAR constructs for NK cells in regards to both intracellular and extracellular domains.[176–178] However, the studies presented here highlight the influence of timing of transfection during NK cell *ex vivo* expansion on the transfection efficiency of CAR encoding constructs, and the cytotoxicity of the final cell product.

3.6 Cryopreservation of Expanded Natural Killer Cells

A significant amount of coordination goes into the manufacturing of fresh NK cell products and the patients' clinical care. There are major challenges associated with this therapy plan, including the limited time available for quality control testing, the product failing to meet quality standards and the resulting effects, and the high cost. Cryopreservation of NK cells would overcome some of these limitations and ensure patients can receive a sufficient number of potent NK cells for multiple dosages. Cryopreservation of NK cells after large-scale expansion is a practical option since the expanded NK cells

have a short expiry period, after which their proliferation and cytotoxicity can decrease. Additionally, for "off-the-shelf" NK cell therapies cryopreservation is a critical step, since a frozen master bank of readily available cells would need to be established. However, NK cells are highly sensitive to cryoprotectants, and the freezing and thawing processes.[238,241,242]

Cryopreservation media typically used to freeze NK cells commonly contains a combination of medium, serum, and 10-20% dimethyl sulfoxide (DMSO), a cryoprotectant. However, DMSO has a negative effect on NK cell viability and cytotoxicity. There are a number of studies demonstrating that after thawing frozen NK cells immediate viability is high, but cytotoxic function against target cells is impaired (**Table 2**).[152,153,155,203] However, short term culture of thawed NK cells with stimulatory cytokines has been shown to rescue NK cell function (**Table 2**).[152,153,155,191,203] A decline in viability upon short term culture in IL-2 has been reported,[155,229,243] although a modest improvement was observed with IL-2, IL-21 (initial), and IL-15 (**Table 2**).[191] In addition to the thawing procedure itself, another consideration is the time during expansion at which the cells are frozen. Freezing NK cells on different days of the expansion protocol (day 0, 6, 8, 10, and 14) affected their expansion after thawing, with the cells frozen on day 10 having the best ability to expand (**Table 2**).[191] One pre-clinical study in mice showed infusions of NK cells either immediately after thawing or after overnight IL-2 culture had significantly smaller numbers of circulating NK cells, relative to infusions of fresh NK cells.[229] Few studies have explored frozen NK cell products in clinical trials, but in one study peripheral blood NK cells counts were lower in multiple myeloma patients who received cryopreserved *ex vivo* expanded NK cells than those who received freshly expanded NK cells.[155] Additionally, the recovery of the cryopreserved NK cells immediately after thawing was extremely poor.[155] These findings demonstrate that NK cells are highly sensitive to the stress of freezing and thawing. Overall, the viability of cells immediately after thawing is not an accurate representation of their potency and proliferative capacity over time.

Indeed, it has been found that NK cells are susceptible to cryopreservation-induced phenotypic changes. Cryopreserved and thawed NK cells were found to have lower surface expression of TRAIL and NKG2D,[203] and were more likely to have a $CD16^{dim/-}$ phenotype.[203,244] Importantly for solid tumors, chemokine receptors and adhesion receptors may be internalized after cryopreservation.[245] The expression of CXCR4 on cryopreserved NK cells was significantly downregulated compared to fresh NK cells; culturing the cells after thawing could restore some CXCR4 expression, but not to the levels of fresh NK cells (**Table 2**).[125] In another study, culturing thawed NK cells for 48 hours in IL-2 upregulated the expression of CXCR3 and CCR7, but CXCR4 expression remained the same, relative to immediately thawed NK cells (**Table 2**).[190] Furthermore, immediately after thawing, the NK cells lacked CD62L expression but did express LFA-I and VLA-4, stimulating the cells with IL-2 led to an increase in these adhesion receptors (**Table 2**).[190] Furthermore, on cryopreserved CD34+ cells, the expression of

CD62L[246–248] CD29 (integrin β1),[248] CD49d (integrin α4),[248] CD49e (integrin α5),[247] CD49f (integrin α6),[247] CCR5,[245] CXCR4[247] is significantly downregulated compared to fresh cells. The decreased CD62L expression has been associated with lower *in vitro* migration of thawed CD34+ cells.[246] However, incubating the cells in a cytokine cocktail for 16 hours increased the expression of certain adhesion molecules.[248] There have been limited studies specific to NK cells on the expression of chemokine receptors, protease, and adhesion receptors after cryopreservation. However, from the studies mentioned here there is strong evidence there will be downregulation of key factors involved in NK cell homing and migration after cryopreservation. This needs to be taken into account when developing an adoptive NK cell therapy for solid tumors.

Recently, work into replacing the conventional cryoprotectants with nontoxic bioinspired cryoprotectants has been explored.[242,249] Utilizing a combination of dextran, carboxylated poly-l-lysine, and ectoine as cryoprotectants maintained the viability, morphology, proliferative capacity, and cytotoxic activity of NK-92 cells following short- and long-term (up to 2 months) cryopreservation.[242,249] Importantly, these DMSO-free freeze medium's may help improve the viability and function of NK cells after thawing, which would greatly progress manufacturing protocols. In addition to removing DMSO altogether, a study explored the effects of lowering the concentration to 5% and included 25% dextran in the freeze medium.[198] Immediately after thawing, the NK cells had a high viability (92%) and their phenotype and functional activity *in vitro* was maintained relative to fresh NK cells.[198] Additionally, the frozen and thawed NK cell's anti-tumor efficacy *in vivo* after infusion into mice with subcutaneous human hepatocellular carcinoma tumors was only slightly reduced relative to fresh NK cell.[198] These alternatives are promising for NK cell viability and function, however their effect on factors related to NK cell migration and homing also needs to be explored for use in treating solid tumors.

Clinical efficiency of cryopreserved NK cells depends on many factors including, time at which expanded NK cells are frozen, freezing temperature, length of cryopreservation, composition and chemical nature of the cryoprotectant, and thawing rate. Cryopreservation is necessary for the development of an "off-the-shelf" therapy and can make the therapies cheaper and more readily available, but there are major concerns about the cell viability, cytotoxicity, and migratory ability after thawing which need to be addressed. To help overcome these issues, a short term culture of the thawed NK cells may be necessary prior to infusions, but the duration and stimulations need to be further studied to ensure NK cell persistence and function are not significantly compromised.

3.7 Important Considerations for Natural Killer Cell Therapy for Solid Tumors

Given that solid tumors pose unique challenges for NK cells to overcome compared to hematological cancers, additional considerations should be taken when manufacturing NK cells for the treatment of solid tumors to retain their homing and migratory functions. However, there have been

limited studies on the expression of chemokine receptors, adhesion receptors, and proteases in expanded NK cells (**Table 2**). Of the studies that have examined these proteins, there appears to be compelling evidence that their expression is in fact affected during the NK cell expansion process (**Table 2**). The expression of chemokine receptors has received the most attention due to their critical role in NK cell response to inflammation. Most commonly, CXCR3 expression is increased in expanded NK cells, relative to freshly isolated NK cells (**Table 2**).[122,123,190,198] However, NK cells expanded with OKT3, IL-2, and irradiated autologous PBMC feeder cells in one study had no change in the expression of CXCR3 relative to freshly isolated NK cells.[195] Often, expanded NK cells have decreased expression of CXCR4, relative to freshly isolated NK cells[125,199] (**Table 2**), and the expanded NK cells are less responsive to SDF-1α induced migration than freshly isolated NK cells.[125] However, NK cells expanded with OKT3, IL-2, and irradiated autologous PBMC feeder cells in one study had increased expression of CXCR4,[195] while in another study had no change in the percent of CXCR4+ or CXCR5+ NK cells,[198] relative to freshly isolated NK cells (**Table 2**). Other chemokine receptors which have been found to be upregulated in expanded NK cells, relative to freshly isolated NK cells, are CXCR6,[199] CCR7,[190] CCR5,[192] CXCR6,[192] CCR2,[192] and CXCR1.[192] Specifically, one study found NK cells expanded with irradiated EBV-LCLs and IL-2 had increased CXCR3 expression, resulting in increased migration to different chemokines, relative to freshly isolated NK cells.[122] Conversely, chemokine receptors which were downregulated in expanded NK cells, relative to freshly isolated NK cells, have been CXCR1,[123] CX3CR1,[192] and CCR7.[192] These findings highlight that the starting donor PBMC cells and different expansion methods have different effects on the expression of chemokine receptors (**Figure 2**). In addition, the chemokine receptor profile of the starting cell source may affect the final NK cell product, as cord blood-derived NK cells have decreased expression of CXCR1 but increased expression of CXCR4, relative to adult peripheral blood NK cells.[250] Utilizing expansion methods which result in decreased chemokine receptor expression could undermine the homing ability of the NK cells *in vivo.* Given that different solid tumors express different chemokine profiles, to the extent possible, it may be of interest to design manufacturing protocols which generate NK cells with specific chemokine receptors for a tumor type of interest. Expansion protocols which result in a final NK cell product with comparable, if not increased, expression of chemokine receptors should be prioritized when the NK cells are intended to treat solid tumors.

During homing, NK cells need adhesion receptors to roll and adhere to the endothelium for transendothelium migration. This function could be greatly inhibited in expanded NK cells, as it is consistently reported that *ex vivo* expanded NK cells have significantly decrease the expression of CD62L (L-selectin), relative to freshly isolated NK cells (**Table 2**).[189,192,199,200,229,243] However, there was one report of NK cells with increased CD62L expression, relative to freshly isolated NK cells, when expanded with OKT3, IL-2 and PBMC feeder cells.[195] Similarly, using nicotinamide (NAM) and EBV-LCL feeder

cells to expand NK cells resulted in increased expression of CD62L and an improved homing ability in NSG mice, compared to NK cells expanded without NAM.[243] Additionally, the expression of adhesion receptors on the NK cell source needs to be considered. iPSC-derived and cord blood NK cells express lower levels of CD62L,[206,218] which may suggest these cells have a decreased homing ability compared to PBMC NK cells. Focusing on other adhesion receptors, NK cells expanded with KL-1 cells and IL-2 had upregulated expression of ICAM-1 (CD54), LFA-1 (CD11a), CD48, LFA-2 (CD2), VLA-4 (CD49d), and LFA-3 (CD58), relative to freshly isolated NK cells (**Table 2**).[202] Similarly, NK cells expanded with IL-2 and OCI-AML3 cells expressing membrane bound IL-21 had upregulated expression of ICAM-1 (CD54) and LFA-1 (CD11a), relative to freshly isolated NK cells (**Table 2**).[199] Generally IL-2 stimulation on NK cells has been shown to upregulate the expression of integrins in primary NK cells,[106,251] but the effect of long term expansion on integrins has not been studied. While CD62L is largely downregulated during NK cell expansion, many other adhesion receptors and integrins may be upregulated during NK cell expansion with IL-2. Analyzing the expression of adhesion receptors in expanded NK cells is less common than chemokine receptors, but their expression is as important to NK cell homing and migration.

While it remains unclear if NK cells rely more on mesenchymal or amoeboid migration, there is evidence that human NK cells use proteases to degrade matrices *in vitro*.[105–108] Further understanding of the mechanisms of NK cell migration and infiltration into tumors will enable the development of manufacturing protocols which conserve, if not increase, the ability of NK cells to migrate *in vivo*. Still, some information can be gathered from IL-2 stimulation on primary NK cells, which increases MMP expression, enabling them to migrate on protein coated surfaces in 2D and through protein matrices in 3D.[107,108,252] However, it is currently unknown how the long term expansion of NK cells affects protease expression, which would affect their migratory ability. There is indication from T cell expansion, that certain proteases may be decreased after long term NK cell expansion. *Ex vivo* expanded T cells, stimulated with IL-2 and, OKT3, and CD28-specific antibodies, or antigen-presenting cells had downregulated heparanase expression, which degrades heparin sulfate proteoglycans, a main component of solid tumors.[253] Expanded T cells also had a reduced invasion capacity through Matrigel than freshly isolated NK cells.[253] As heparanase is important for murine NK cell migration,[254] studies should explore the effect of *ex vivo* expansion on its expression in expanded human NK cells. These studies demonstrate the expansion process will have an effect on proteins related to homing and migratory functions, and need to be considered when expanding NK cell for treatment of solid tumors.

3.8 Summary of Manufacturing Methods

Overall, it is important to note the complexity of manufacturing NK cells, and that each factor during the process will affect the outcome of the final NK cell product in terms of purity, viability, phenotype, and function (**Figure 2**). As many NK cell manufacturing protocols have been established to

produce high numbers of potent NK cells, all final NK cell products have different qualities. Protocols which require the least amount of operator involvement but result in the expansion to a significant number of high functioning NK cells should be prioritized. Additionally, it is important to consider the effect of *ex vivo* expansion on NK cells' homing and migratory ability when the cells are intended for solid tumor therapy, as these functions are likely critical to the success of the therapy As such, protocols which maintain, or increase, the expression of chemokine receptors, adhesion receptors, and proteases, should be prioritized. Furthermore, work into cryopreservation of NK cells would enable the development of "off-the-shelf" therapies and allow more patients to have access to quicker therapies.

4. Future Directions

4.1 Engineering CAR-Chemokine Receptor Natural Killer Cells

Engineering effective NK cells for the treatment of solid tumors may require increasing their homing/migratory ability in addition to increasing their cytotoxic potential. There has been strong evidence that engineering NK cells to express higher levels of chemokine receptors, such as CCR7, CXCR1, CXCR2, and CXCR4, leads to better directed migration *in vitro* and better tumor and bone marrow homing and infiltration *in vivo*, compared to naïve NK cells.[123–125,250,255] Still, increasing NK cells cytotoxic function is as important, and as such investigators have explored generating CAR NK cells with increased chemokine receptor expression, as this is also being explored for T cells in pre-clinical and clinical trials (NCT04153799 and NCT03602157). NK cells engineered to co-express CXCR4 with an anti-EGFRvIII CAR had greater migration toward CXCL12 secreting glioblastoma cells *in vitro*, relative to naïve NK cells.[124] After infusion of the NK cells into CXCL12 secreting glioblastoma tumor bearing mice, there was increased tumor infiltrating NK cells and the overall survival increased, compared to mice receiving control NK cells.[124] However, it should be acknowledged that the engineered NK cells only had increased migration and infiltration toward glioblastoma cells engineered to secrete CXCL2, not the naïve cancer cells.[124] This demonstrates the importance of high chemokine levels produced by a tumor for this therapy to be effective. Another group developed NKG2D CAR NK cells expressing CXCR1 (CXCR1+) which had increased migration *in vitro* to conditioned media derived from an ovarian cancer cell line and maintained increased cytotoxicity toward the same cell line, relative to NKG2D CAR NK cells (CXCR1-).[123] After infusion of CAR NK cells into ovarian tumor bearing mice, those treated with CXCR1+ CAR NK cells had less tumor burden and increase survival relative to those treated with CXCR1- CAR NK cells.[123] Importantly, the intended tumor type needs to considered when genetically engineering NK cells with chemokine receptors, as all tumors have different production profiles of chemokines. As such, NK cells can be engineered for specific tumor types with a specific CAR and chemokine receptor for a potent anti-tumor effect.

4.2 Patient-Specific or "Off-the-Shelf" Natural Killer Cells

There are two options for NK cell therapies – a specific donor for each patient (patient-specific) or "off-the-shelf" NK cells. The majority of the studies in **Table 1** were patient specific studies, with one donor selected for each patient, with the exception of the few using cord blood NK cells and NK-92 cells. To develop more potent patient-specific NK cell therapies, NK cells could be manufacturing in a manner which increases the expression of activating receptors[196] or chemokine receptors specific for patients' tumor. Furthermore, NK cells could be engineered with CAR specificity and chemokine receptors for the specific solid tumor type, which may be more beneficial than naïve NK cells. However, patient-specific NK cell therapies require a specific manufacturing protocol required at the time which the patient is suitable for therapy. As such, a tight coordination between the isolation of donor NK cell in the clinic, the manufacturing process in the laboratory, and preparation of the recipient in the clinic is required. Additionally, the therapy would not be widely available due to the high cost; NK cells would be manufactured in a manner comparable to the current FDA approved CAR T cell therapies, which cost $373,000 (FY 2019) per infusion.[11,12] Furthermore, each batch of NK cells may differ in yield, purity, phenotype, and function, due to the vast degree of donor-to-donor and intra-donor variability seen with all expansion methods. Additionally, the use of complex and specific manufacturing protocols may limit the adoptability of the process to many locations, which limits the range of clinics at which patients can be treated with fresh NK cell products. The ability to cryopreserve patient-specific NK cells would help increase the availability of this therapy, and reduce delay time of therapy initiation and the total cost of the therapy. Yet, existing cryopreservation methods compromise the final NK cell product and thus are not suitable for NK cell therapies. While patient-specific NK cell may be more potent and effective for treating patients, there are drawbacks with this platform and their widespread use may be limited.

The development of the alternative "off-the-shelf" NK cell therapy would generate more consistent final cell products, while reducing the cost and increasing the availability of the therapy. While this approach has been used in clinical trials, improvements to the cryopreservation technique and function of these therapies need to be made to improve clinical efficacy. "Off-the-shelf" NK cells would reduce the extensive donor selection process in the clinic setting, as screening could be done to determine the optimal donor NK cell source based on the expansion rate, and the final NK cell phenotype, cytotoxicity, and migratory abilities prior to their use clinically. Furthermore, frozen cell products would allow for the shipment of the product to various sites for patient infusion, which would greatly expand the number and demographic of patients who could receive treatment. As previously mentioned, the candidates for "off-the-shelf" NK cells are hESC-derived[204] and iPSC-derived NK cells,[205,207] the NK-92/NK-92MI cell lines,[161,162,179] and cord blood NK cells.[180,256] However, there may be limitations with decreased cell homing[157–159,206,218] and cytotoxicity potential[157–159] which would likely need to be

overcome to increase the efficacy of these therapies. While “off-the-shelf” NK cell products are appealing for a number of reasons, the clinical efficacy needs to be determined of cryopreserved NK cells, using optimal techniques, and compared with fresh NK cell products in patients. Further, since the expression of activating and inhibitory receptors and chemokine receptors on the NK cells would not be tailored for the specific tumor type, the efficacy of “off-the-shelf” NK cells needs to be compared to patient-specific NK cells to determine the optimal method for NK cell therapies. While “off-the-shelf” NK cell therapies have many advantages, the final cell product needs to be effective for its wide-spread use in patients with solid tumors.

4.3 Standardizing the Manufacturing Process and Release Criteria

Manufacturing clinical-grade NK cells on a patient-specific basis, for multiple patients, is complex, operator-intensive, time consuming, and expensive. Reducing these limitations of manufacturing NK cells should be a priority of future protocols, since they make scaling out NK cell production to multiple locations and treating many patients , in a timely manner, challenging. Therefore, simplifying NK cell manufacturing protocols and focusing on fully automated systems for NK cell purification and expansion is needed. This will ensure a well-defined, highly standardized, and operator-independent manufacturing procedure is followed to result in reproducible final NK cell products. This will also allow for the procedure to be run at many different locations, without effects of operator-to-operator variability. While the CliniMACS Prodigy® may be an optimal culture vessel to address this need, it has yet to become a standard for manufacturing NK cells. There exist many protocols which generate high numbers of NK cells in 2 weeks, resulting in NK cells with increased cytotoxicity relative to freshly isolated NK cells, which could be applied in the CliniMACS Prodigy®. Additionally, selecting the methods which conserve, or improve, NK cell homing and migration, needs to be prioritized when determining an optimal manufacturing method. Standardizing the manufacturing process will allow for a widely available and cheaper NK cell therapy, with more uniform final cell products.

Furthermore, the final NK cell product must undergo strict testing to ensure that the product is safe and to determine the identity, strength, potency, and purity prior to its release for patient infusion. A set of release criteria for the final product is established and each batch of cells is tested against these criteria to determine if the batch of cells meets the minimum specifications. Required release testing for early stage clinical trials of NK cells include sterility (i.e. viral, bacterial, as well as other), mollicutes contamination (i.e. mycoplasma and ureaplasma), endotoxin levels, NK cell purity and viability, T cell contamination, and removal of residual substances (i.e. feeder cells). However, it is common to perform other testing including NKT cell, monocyte, and B cell contamination, activating and inhibitory receptor expression, and cytotoxicity and degranulation. Including these tests in the release criteria for NK cells help identify batches of NK cells with greater potency and provide rationale for clinical efficacy after

patient infusion. Standardizing the specific quality control testing method (i.e. for cytotoxicity and degranulation) and the minimum release criteria would help harmonize and improve compatibility between NK cell immunotherapies.

5. Concluding Remarks

NK cells offer great potential for solid tumor immunotherapy and have unique advantages over existing cell based therapies, in terms of safety and function. To develop better NK cell therapies for the treatment of solid tumors, the cells need to retain, or improve, their ability to extravasate from the blood stream, migrate through the tumor ECM, lyse target cells, stimulate other immune cells, and persist in the blood stream (**Figure 1**). As such, engineered NK cells with increased homing and cytotoxicity potential may have a significant impact on solid tumors. Engineered NK cells can help to elucidate if solid tumors respond better to lower cell dosages of highly functioning NK cells or just high cell dosages of naïve NK cells. In turn this information will impact how NK cells are manufactured for clinical trials. The process to manufacture NK cells is complex, with each parameter influencing the final product (**Figure 2**). Work toward standardizing the manufacturing NK cells and the release criteria are needed to advance this field. With further streamlining of manufacturing protocols, and improving cryopreservation, genetic engineering, and homing and migratory ability of NK cells, the full potential of NK cell therapies for the treatment of solid tumors can be reached.

Declaration of Competing Interest

The authors have no conflicts of interest to report.

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