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Review

Generating natural killer cells for adoptive transfer: expanding horizons

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ABSTRACT

Natural killer (NK) cells are unique innate lymphoid cells that have therapeutic potential in adoptive cell transfer-based cancer immunotherapy that has been established across a range of early-phase clinical trials. NK cells for use in adoptive transfer therapies are obtained from various sources, including primary NK cells from peripheral blood or apheresis products (autologous or allogeneic) and umbilical cord blood. NK cells have also been generated from CD34+ hematopoietic progenitors, induced pluripotent stem cells, embryonic stem cells and malignant cell lines. Apheresis-derived NK cell products are often administered after brief cytokine-based *ex vivo* activation, ideally aiming for *in vivo* expansion and proliferation. NK cells from other sources or from smaller volumes of blood require a longer period of expansion prior to therapeutic use. Although *ex vivo* NK cell expansion introduces a concern for senescence and exhaustion, there is also an opportunity to achieve higher NK cell doses, modulate NK cell activation characteristics and apply genetic engineering approaches, ultimately generating potent effector cells from small volumes of readily available starting materials. Herein the authors review the field of clinical-grade NK cell expansion, explore the desirable features of an idealized NK cell expansion approach and focus on techniques used in recently published clinical trials.

Introduction

Natural killer (NK) cells are innate lymphoid cells that play an important role in the immune response to infection and cancer. Activated NK cells are capable of direct cytotoxic effects via perforin and granzyme release or through death receptor signaling and modulate other arms of the immune response via cytokine and chemokine production [1]. NK cell activation status reflects a balance of signaling through germline-encoded activating and inhibitory receptors, independent of HLA-restricted antigen presentation. The dominant NK cell inhibitory receptor ligands, major histocompatibility complex class I molecules, are recognized by NK cell killer immunoglobulin receptors (KIRs) and are frequently downregulated by cancer cells to evade T-cell anti-tumor immunity [2,3]. This is often accompanied by upregulation of "stress ligands" for NK cell-activating receptors, favoring NK cell activation [4]. Since the initial descriptions of their unique properties, harnessing and, more recently, augmenting the anti-cancer effect of NK cells have been a goal of immunotherapy research [5].

NK cells are increasingly implicated in the therapeutic benefit of established cancer therapies [6,7]. The alloreactive activity of NK cells within allogeneic stem cell transplant (SCT) products exerts a graft-versus-leukemia effect in acute myeloid leukemia (AML), likely enhanced by donor–recipient KIR-HLA mismatch [8]. Adoptive cell transfer (ACT) techniques using enriched, purified, expanded or manufactured NK cells have been investigated in hematological malignancies and some solid organ cancers, with many further clinical trials in progress [9]. Most clinical studies have applied autologous or allogeneic NK cells after either short-term activation aiming for *in vivo* expansion or longer-term *ex vivo* expansion aiming for higher infused doses. The approval of chimeric antigen receptor (CAR) T-cell therapies targeting CD19 in B-cell malignancies was a landmark in ACT immunotherapy and fueled investigation of genetically modified NK cell therapies. Emerging data support the safety and efficacy of allogeneic, umbilical cord blood (UCB)-derived CD19 CAR-NK cells [10]. Many investigators are seeking to leverage the advantages of NK cell-based therapies (reduced concern for graft-versus-host disease [GVHD], innate anti-tumor activity and viable routes to off-the-shelf application) while overcoming associated challenges (generating sufficient Good Manufacturing Practice (GMP)-grade cells, improving *in vivo* persistence and developing effective genetic engineering techniques) [11,12].

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With the availability of GMP-compliant closed manufacturing systems, academic cell therapy programs are expanding worldwide, with a focus on cost and logistical simplicity [13]. The rapidly evolving field of NK cell ACT now encompasses a range of cell sources, from cord or peripheral blood to induced pluripotent stem cell (iPSC)-derived NK cells, varied expansion and genetic engineering techniques and numerous disease applications in cancer and beyond [6,14]. Although overnight activation of freshly enriched apheresis-derived NK cell products allows rapid availability without NK cell exhaustion, longer-term expansion offers a greater window for *ex vivo* modulation and genetic engineering while producing higher doses from small amounts of starting materials. Herein the authors review current approaches to NK cell expansion for clinical application, with a focus on those applied in recent clinical trials. The authors also consider characteristics of an ideal expansion platform to aid in identifying areas for further research and in doing so, seek to build upon prior reviews of NK cell expansion for ACT and reference the related fields of iPSC-derived and genetically engineered NK cells [15–18].

Starting materials: sources of NK cells for therapeutic use

An overview of NK cell sources and expansion techniques applied to NK cell therapies is presented in Figure 1. Varied and innovative approaches have been taken to obtain NK cells for expansion, manipulation and administration. NK cells in humans can be found circulating in the peripheral blood (representing 2–18% of lymphocytes) and residing in the lymph nodes and tissues of many organs [1]. Initial approaches to NK cell ACT used primary NK cells isolated from peripheral blood mononuclear cells (PBMCs) derived from apheresis collection or whole blood [19]. NK cell therapies are traditionally used as freshly activated or expanded products, although recent reports suggest cryopreservation of mature NK cells may be feasible [20–22]. To obtain sufficient NK cells for short-term activation, an apheresis procedure or large volume of blood is required, whereas *ex vivo* expansion techniques have allowed therapeutic doses to be derived from as little as 40 mL of peripheral blood [21,23]. UCB is an alternative allogeneic NK cell source with the added benefit of non-invasive collection, existing banked material viable after cryopreservation and higher proportion of NK cells versus peripheral blood (up to 30%). Despite potential benefits of UCB over peripheral blood NK cells, a recent comparison found similar cytotoxic potential and expansion capacity when modified to express a CD19 CAR [24]. In choosing a source of allogeneic NK cells, most studies attempt to augment NK cell alloreactivity by selecting a donor with a favorable KIR-HLA mismatch in the recipient direction, although there is no standard approach. Further selection of NK cells from the mononuclear component is generally performed using clinical-grade immunomagnetic bead separation, such as the CliniMACS Plus system (Miltenyi Biotec), and involves either CD3 depletion alone or in combination with CD56 positive selection. Positive selection generally yields a greater NK cell purity at the expense of a lower final cell number, which may be a dose limitation in short-term activation protocols [25]. Some autologous protocols use unselected PBMCs expanding a predominantly NK cell population (>90%); however, T-cell depletion before administration would likely be required for allogeneic use, reducing the risk of GVHD [21].

NK cells can also be generated from multipotent progenitor cells and PSCs. Stem and progenitor cells can be cryopreserved and demonstrate higher transduction efficacy compared with mature NK cells. Although initial differentiation protocols relied upon fetal bovine serum content, serum-free, GMP-grade approaches are now established. Highly pure, functional NK cells have been generated from CD34+ hematopoietic stem cells isolated from UCB. This technique has been developed to conduct a phase 1 clinical trial delivering comparable doses to other early-phase NK cell adoptive transfer trials

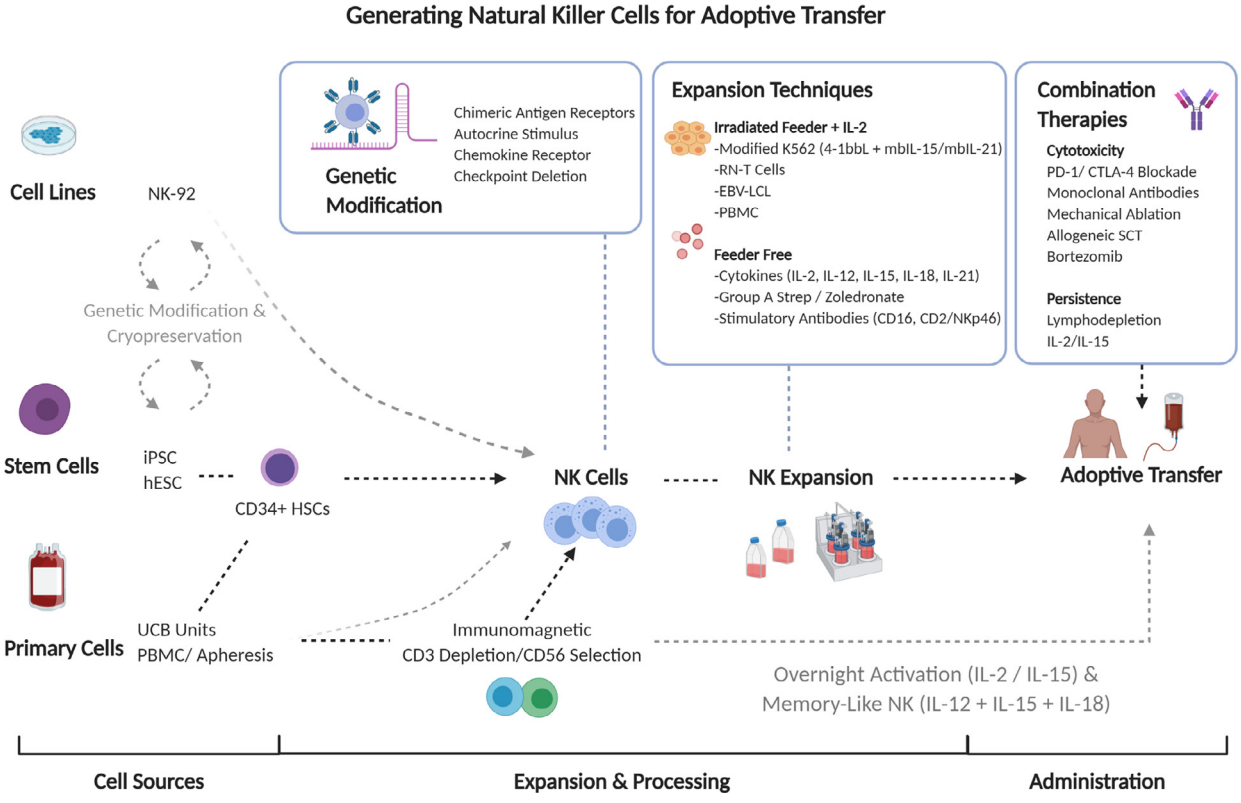

Figure 1. Generation of NK cells for adoptive transfer immunotherapy. EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid cell line; hESC, human ESC; HSCs, hematopoietic stem cells; RN-T, RetroNectin-stimulated T (cells); Strep, streptococcus. (Color version of figure is available online.)

[26,27]. Bone marrow harvesting of CD34+ progenitors is an invasive procedure, and peripheral blood apheresis after granulocyte colony-stimulating factor stimulation is an established alternative but may influence NK cell phenotype [28].

PSCs, either iPSCs or embryonic stem cells (ESCs), have the added advantage of easier long-term culture compared with CD34+ progenitors. This facilitates the incorporation of multiple genetic engineering steps from the outset, creating a master cell bank for repeated generation of a consistent, stably modified NK cell product. This is in contrast to a limited transduction efficacy in engineering primary NK cells during expansion and simplifies concerns around monitoring for off-target gene edits. The generation of iPSC NK and human ESC NK cells has been reviewed in detail [29]. In contrast to T cells, NK cell production via iPSCs is relatively simpler and, importantly, has been adapted to achieve clinical-grade expansion [30]. In addition, iPSC NK cells display cytotoxic effects comparable to primary expanded NK cells *in vitro* and in a xenograft model of ovarian cancer [32]. The opening of the first clinical trials of multigene-edited iPSC NK cells speaks to the promise of this approach (NCT:04245722) [31]. An emphasis on product over process as well as the feasibility of iPSC cryopreservation suggests that iPSC NK cells are close to realizing the off-the-shelf potential of NK cell therapy. Current differentiation and expansion protocols using iPSCs and CD34+ hematopoietic stem cells take 4–6 weeks, longer than that required for primary cell expansions (2–3 weeks), although, if successful, an ongoing manufacturing pipeline should overcome potential delays in treatment delivery.

Finally, transformed NK cell lines can be readily expanded, gene-edited and cryopreserved for off-the-shelf use, but a requirement for irradiation prior to administration limits *in vivo* expansion capacity and persistence. Experience suggests that primary NK cell persistence can be a determinant of clinical response after adoptive transfer, and a multiple, high-dose infusion strategy has been designed to overcome this using NK-92, the only NK cell line used clinically to date [33,34]. This also appears to be a safe approach, although the extent to which efficacy is restricted by the innate limitations of a cell line platform remains a concern.

An optimal NK cell expansion platform

NK cell expansion protocols generate high doses of NK cells generally over 10–21 days. In the absence of a regulatory authority-approved NK cell-based therapy, there is considerable heterogeneity across investigational expansion protocols. Clinical trials conducted to date have broadly supported the safety and feasibility of many techniques, with some notable efficacy signals [10,23,35]. Although NK expansion is one of many variables in the application of NK cell therapies, growing evidence suggests implications for the safety and efficacy of these products [36]. The authors will explore the desirable features of an expansion system (Table 1) before reviewing clinical-grade NK cell expansion techniques used in recent clinical trials.

The expansion capacity of the technique should consistently produce the desired cell quantities for ACT. The optimal dose of NK cells for adoptive transfer is an area of active investigation and will likely vary between products and disease applications. Varied approaches to lymphodepleting chemotherapy and exogenous cytokine support introduce further variables when interpreting dose data. Some protocols aim to deliver multiple doses requiring larger volume expansions or multiple expansions. Published studies can inform expected minimum outputs of an ideal expansion approach. Doses of 2×10^7 cells/kg appear safe with short-term activated NK cells in a process also limited by apheresis volume, whereas 1×10^7 cells/kg was the maximum dose applied in a phase 1 study using UCB-derived CD19 CAR-NK cells modified to express IL-15, supporting persistence, again with promising safety features. A higher mean dose of 5×10^9 total cells was utilized safely with irradiated CAR NK-92 cells, reflecting the lack of capacity for *in vivo* expansion [34], and 4.7×10^{10} mean total autologous expanded NK cells were safely infused in a phase 1 trial treating solid tumors, representing the highest single dose recorded [37]. Although the lack of clinical response in the latter study emphasizes the importance of other variables, inconsistency in expansion capacity, particularly with donor-specific products, could result in an inability to treat eligible patients.

Table 1
Desirable features of an optimal NK cell expansion platform.

Expansion capacity	Consistently producing sufficient cells for the desired treatment protocol. Potential to produce multiple doses from a single small-volume starting product without apheresis.
NK cell characteristics	Avoidance of exhaustion phenotypes and senescence. Favorable expression patterns for inhibitory, activating, chemokine and death receptor pathways. Favorable metabolic profile. Cytotoxic potential confirmed via *in vitro* functional assessment.
Gene editing	Compatible with gene editing approaches, including multiple gene editing steps.
Safety	GMP-compliant process. Minimizing emergent clinical adverse effects. Pure NK cell populations without contamination.
Simplicity	Ease of application to enable rapid establishment at new facilities. Adaptable to closed systems, existing infrastructure. Compatible with readily available starting materials, with ease of collection.
Cost	Cost-effective and competitive to establish and maintain.
Cryopreservation	Compatible with cryopreservation solutions to realize off-the-shelf potential.
Rapidity	Where products will be manufactured per patient, rapid expansion and availability are required.
Acceptability	Ethically sourced starting materials. Acceptability of genetic engineering approaches and off-target effects. Acceptable clinical adverse effect profile.

Beyond cell quantity, NK cell characteristics are also influenced by specific expansion stimuli, with potentially defining implications for clinical efficacy. Although there is no accepted optimal phenotype, investigators have demonstrated increased expression of activating receptors (e.gNKG2D, CD16), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and chemokine receptors to support cytotoxicity and homing potential [21,38]. Downregulation of inhibitory receptors (e.g., PD-1, TIGIT) and maintenance of telomere length may suggest enhanced cytotoxicity and *in vivo* expansion potential, respectively [39]. Functional assays confirming *in vitro* cytotoxicity and cytokine release are also frequently performed. Although the relative importance of these observations to clinical outcomes remains unclear, recent observations on the mechanisms underlying NK cell diversity within and between individuals may refine these assessments. Pfefferle *et al.* [40] have described intra-lineage plasticity that maintains the relative frequency of NK cell subpopulations in humans and confirmed that the functional potential of NK cells reflects their acquired phenotypes. Interestingly, cells that acquired phenotypes of the greatest proliferative potential were least cytotoxic and most prone to apoptosis. Understanding the impact of such finely balanced homeostatic mechanisms in the artificial environment of *ex vivo* NK cell expansion may ultimately redefine an “ideal” donor and expanded cell composition [41]. “Adaptive” or “memory-like” NK cells are a subset of therapeutic interest because of their improved survival and cytolytic capabilities [42]. Although recent studies seeking to harness these properties applied transient cytokine exposure during activation, efforts to selectively expand these cells represent a step toward exploiting NK cell functional diversity [44–46].

An additional approach to augmenting NK cell activity is through gene editing. Potential gene targets have been reviewed previously

but include introducing antigen-specific CARs, removing inhibitory checkpoints and receptors, reducing effector cell fratricide, enhancing persistence and homing and improving metabolic fitness [46]. An ideal approach to expansion should be compatible with the varied genetic engineering tools currently in use. The safety and quality of the final product are of paramount importance, and although sign-out criteria vary, they generally seek to ensure sterility, NK cell purity and viability and exclude T-cell, B-cell or viable feeder cell contamination. The persistence of T cells in allogeneic products risks GVHD, which has not generally been encountered with alloreactive NK cells. Published quality criteria have permitted up to 1×10^6 CD3+ cells/kg in infused haploidentical products, although this risk is also likely modulated by other features, including lymphodepletion regimens and systemic IL-2 use [47,48].

In addition to efficacy and safety, logistical simplicity and cost considerations are likely to be dominant factors in determining the long-term success of ACT approaches. An ideal NK cell expansion platform would require small blood volumes, reducing the expense associated with apheresis procedures. Logistical simplicity would enable rapid establishment at new institutions [13]. For example, expansion techniques adaptable to commercially available closed systems, minimizing manual intervention, will reduce cost and support scalability [49,50]. NK cell research remains a subset of a wider ACT field dominated by T-cell therapies, and the ability to adapt existing infrastructure and processes to NK cell approaches will be beneficial [12]. The ability to generate a cost-effective product is aided by the off-the-shelf potential of NK cell therapies, now being realized through the cryopreservation of iPSCs, accumulation of mature NK cryopreservation experience and generation of hundreds of therapeutic doses from a single UCB unit [51]. Expansion protocols are fundamental to optimizing each of these domains of NK cell therapy. The rapidity of manufacturing, reducing the time between treatment decision and product administration, has been recognized as a priority in the next generation of cell therapies. Finally, the acceptability of cell therapy products to patients will also influence which modalities endure. There may be concerns around the safety of cell line-based therapy and ethical concerns around ESC-derived products.

NK expansion techniques

Practically, expansion protocols use combinations of serum-free media, supplements, recombinant cytokines, stimulatory antibodies, small molecules, medicinal products and irradiated feeder cells. Clinical-grade protocols avoid the use of animal-based serum products, and earlier protocols involving tissue culture flasks have evolved into bioreactor or culture bag systems conducted in dedicated GMP facilities with increasing degrees of automation. Cytokine stimulation with IL-2 or IL-15 is required but insufficient for clinical-grade NK cell expansion, resulting in modest NK expansion capacity when used alone. Co-stimulatory signals are vital to augmenting NK expansion to clinical scale. Feeder cell layers provide these signals via cell-to-cell contact, and the development of engineered cell lines expressing specific co-stimulatory ligands has allowed a greater understanding of key pathways involved. A variety of feeder cell approaches have been developed for clinical use, with recently published examples explored in the following sections.

Feeder-based NK cell expansion

K562 and derivatives

K562 is an erythroleukemia cell line that lacks HLA antigen expression. K562 cells have been shown to provide a cell contact-dependent co-stimulatory signal, enhancing the expansion of NK cells [52]. Combined with IL-2 and IL-15, up to 300-fold expansions have been reported [53]. It was with the enforced expression of specific co-stimulatory molecules that a remarkable increase in fold expansion was achieved. Imai *et al.* [54] described the striking and synergistic increase in NK expansion (mean 1089-fold) achieved when both 4-1BBL and a gene encoding IL-15 combined with the transmembrane domain of CD8α were expressed in K562 cells. Importantly, this occurs without inducing T-cell expansion. The phenotype of the expanded cells is in keeping with NK cell activation, with upregulation of CD56, KIRs and the natural cytotoxicity receptors NKp30, NKp44 and NKp46. Indeed, upregulation in natural cytotoxicity receptor expression was implicated in the cytolytic capacity of NK cells expanded under this stimulus [55]. Further studies reported the application of this approach to apheresis-derived autologous PBMCs and demonstrated greater potency relative to resting or short-term IL-2-activated NK cells *in vitro* [56]. The researchers also derived a GMP-grade master K562-mb15-4-1BBL cell bank for clinical use.

K562-mb15-4-1BBL-expanded NK cells have been the basis of a variety of clinical trials. When cells expanded via this technique were used as an NK cell donor lymphocyte infusion post HLA-matched (T-cell-depleted) allogeneic SCT in pediatric patients with solid tumors, increased acute GVHD was observed [57]. The extent to which this expansion technique contributed continues to be debated, and other examples of NK cell infusions leading to generalized immune activation have been reported [35]. Other examples of NK cell donor lymphocyte infusion have not triggered GVHD, however, and other applications of K562-mb15-4-1BBL-expanded NK cells appear safe [23]. Starting with unselected PBMCs from 250 mL of blood from (KIR-mismatched) haploidentical donors, Vela *et al.* [47] reported a phase 1 clinical trial using K562-mb15-4-1BBL-expanded NK cells to treat a cohort of pediatric patients with relapsed acute lymphoblastic leukemia or AML outside the setting of allogeneic SCT. Notably, there were up to 11×10^6 T cells/kg in the infused products, with a median NK cell purity of 85%, although some products were T-cell-depleted. Although clinical outcomes in this pediatric cohort were favorable, the contribution of the haploidentical NK cell infusions was unclear, and the protocol applied was well tolerated, without GVHD. Use of systemic IL-2 administration post NK infusion as well as lymphodepleting chemotherapy may also influence the differences in GVHD risk noted between these trials. K562-mb15-4-1BBL-expanded NK cell infusions are also being investigated as consolidation therapy for lower risk AML patients in first complete remission not proceeding to allogeneic SCT (NCT02763475). Another early-phase clinical trial conducted in multiple myeloma using this expansion process generated a relatively pure (median 90%) NK cell population from autologous PBMCs without initial immunomagnetic selection. Multiple expansions were used to deliver repeated dosing, although at doses lower than other NK cell clinical trials (7.5×10^6 cells/kg) [58].

A similar expansion protocol using genetically modified K562 as feeder cells, but substituting membrane-bound IL-21 for IL-15, has also been evaluated [39]. During initial studies, K562 cells were transduced with CD64, CD86 (B7-2), 4-1BBL and truncated CD19 and labeled clone 9. These were further modified to express either membrane-bound IL-15 or IL-21. Clone9.mbIL-21 feeder cells generated highly cytotoxic expanded NK cells from patient-derived PBMCs and drove a greater fold expansion than that seen with mbIL-15. Clone9.mbIL-21-expanded NK cells also displayed similar immunophenotype and gene expression profiles, longer telomeres (suggesting a greater *in vivo* expansion potential), greater cytokine secretion and enhanced antibody-dependent cellular cytotoxicity. A similar membrane-bound IL-21-based expansion process demonstrated an enhanced NK cell metabolic profile [59]. The clone9.mbIL-21 expansion process was used in a phase 1 clinical trial of NK cells administered pre- and post-haploidentical allogeneic SCT in a cohort of patients with high-risk myeloid malignancies [23]. All planned expansions achieved the dose target, and the highest dose level was

1×10^8 cells/kg. NK cells were administered on day −7, day +7 and day +28, with the later doses administered after cryopreservation. The starting product was CD3-depleted PBMCs from 500 mL of donor peripheral blood collected on day −16. No dose-limiting toxicity was noted, there was no excess of GVHD and rates of cytomegalovirus and BK virus reactivation were low. NK reconstitution and function were improved, and clinical outcomes were positive relative to a historical cohort, although the contribution of the NK cells versus other components of the treatment regimen is hard to assess. The authors also acknowledged the importance of cost, which was reported to be a fraction of that associated with SCT.

The clone9.mbIL-21-based expansion process has also been adapted to expand UCB-derived NK cells. Shah *et al.* [60] reported the GMP-grade expansion of UCB NK cells using clone9.mbIL-21 administered as part of a phase 1 clinical trial in combination with autologous SCT in multiple myeloma. The approach met all dose targets (up to 1×10^8 cells/kg) and sign-out criteria and was safely applied, proceeding to a phase 2 trial. Notably, logistical and cost implications informed the maximal dose chosen, but the authors believed a further log scale expansion would be possible. This approach was also used to support a ground-breaking clinical trial developing a CD19 CAR-NK cell therapy at MD Anderson Cancer Center [10]. The manufacturing process for UCB CAR-NK production uses thawed UCB, and after a negative selection step, a 15-day expansion process is begun based on irradiated clone9.mbIL-21 feeder cells. A retroviral transduction step to express a transgene, including CD19 CAR, IL-15 (supporting persistence) and inducible caspase 9, was performed during expansion. A median 2222-fold expansion was achieved, with acceptable transduction efficacy (47–87%), and doses up to 1×10^7 cells/kg were administered without cytokine release syndrome, neurotoxicity or GVHD. Objective clinical responses were seen, and non-HLA-matched CAR-NK cells persisted for up to a year post infusion, potentially supported by autocrine IL-15 stimulation. The scale of NK cell expansion achieved would permit up to 100 doses of CAR-NK cells from a single UCB unit, a transformative cell therapy if longer-term clinical outcomes are comparable to CAR T-cell therapies [51]. K562 cells have also been modified to express a different member of the tumor necrosis factor superfamily than 4-1BBL—OX40 ligand—which also enhanced expansion capacity (mean 1000-fold expansion) relative to K562 alone and without qualitative differences in NK cell phenotype [53]. The addition of a short IL-21 exposure had a synergistic effect on expansion using this technique.

Recombinant human fibronectin fragment CH296 (RetroNectin)-stimulated T cells

Fibronectin is an extracellular matrix protein and signaling molecule capable of immune cell stimulation. Recombinant human fibronectin fragment CH296 (FN-CH296) (RetroNectin) was initially used to drive T-cell proliferation and later adapted to NK cell expansion. Two early-phase clinical trials have used autologous NK cells expanded with RetroNectin-stimulated T (RN-T) cells [21,61]. The process involves an initial generation of RN-T cells from autologous PBMCs and later expansion of NK cells from PBMCs using IL-2, RN-T cells, GT-T507α medium, autologous plasma (heat-inactivated) and OK-432 (a lyophilized mixture of group A streptococcus). The trials were conducted in patients with gastrointestinal cancers, establishing the safety of monotherapy and combination with monoclonal antibody therapy. Infused products were delivered at up to 2×10^9 total cells per infusion, with a median 92% NK cells, after 3-week culture. Notably, the RN-T cells are prepared from a patient's PBMCs over 2 weeks, prior to irradiation and feeder layer use. Although this involves a considerable potential delay to treatment, the cells were cryopreserved with acceptable viability (>80%), albeit with some decline in CD16 expression upon recovery. Both monotherapy and combination therapy appear safe, although the best clinical response observed was stable disease. This promising technique may require some modification to be applied as an allogeneic solution.

Irradiated PBMCs

Early studies using PBMCs as feeder cells in combination with IL-2 found a 20-fold increase in NK cell numbers as opposed to a 3-fold increase when cultured with IL-2 alone [62]. The CD14+ monocyte fraction was considered a key determinant of the added benefit of the PBMC layer. Modifications of PBMC-based expansions have been used clinically. Parkhurst *et al.* [37] describe an expansion protocol capable of achieving the highest NK cell doses used in adoptive transfer trials, albeit in a labor-intensive protocol using leukapheresis-derived autologous PBMCs as a starting material. Their approach cultured CD3-depleted PBMCs with irradiated autologous PBMCs, AIM V media, 10% heat-inactivated AB serum, IL-2 and the anti-CD3 monoclonal antibody OKT3 in flasks and cell culture bags. When used in this setting, OKT3 leads to T-cell activation and cytokine secretion, enhancing NK cell expansion. These autologous high-dose NK cells were well tolerated, and peripheral NK counts remained increased after 7 days. However, their functional status was questioned by a lack of clinical response in patients with melanoma or renal cell carcinoma, low NKG2D expression and requirement for IL-2 reactivation to achieve *in vitro* cytotoxicity at 7 days. Autologous PBMCs overcome concerns for persistence of viable malignant feeder cells in the NK cell product. The addition of an anti-CD16 monoclonal antibody to irradiated PBMCs also maintains this benefit and allows production of 2×10^9 NK cells from a small quantity of peripheral blood [63].

Epstein-Barr virus lymphoblastoid cell line

Granzin *et al.* [50] reported the adaptation of an effective NK cell expansion technique using an Epstein-Barr virus-transformed lymphoblastoid cell line as a feeder layer in an automated GMP-compliant protocol suitable for standardization. The resulting protocol uses the CliniMACS Prodigy system (Miltenyi Biotec), incorporating T- and B-cell depletion and CD56 positive selection of PBMCs, prior to a 14-day expansion in a closed system using these feeder cells and IL-2. The program includes all media additions and mixing and facilitates sterile sampling. This method produces cytotoxic NK cells with maintained telomere length, which is reassuring for clinical applications. Manual approaches using this feeder cell line have been shown to produce highly pure (99.7%) NK cell expansions, providing multiple doses of up to 1×10^9 cells from a single autologous apheresis product. This expansion approach is the basis of an ongoing clinical trial, in combination with bortezomib, acting as a TRAIL -based NK cell sensitizing agent (NCT00720785) [16,64]. The group subsequently reported the modification of this process to include IL-21, achieving a greater fold expansion of highly cytotoxic NK cells [65].

Feeder-free NK cell expansion

Although feeder cell-based expansion systems are the basis of many recent clinical trials, driven by the capability to robustly expand NK cells with favorable *in vitro* characteristics, feeder-free approaches have several potential advantages. They may be easier to adapt to a GMP-compliant process, without concern for the infusion of viable feeder cells, and may facilitate easier licensing as an advanced therapy medicinal product. Cytokine stimulation has generally been insufficient for clinical expansion, and the correct sequencing and duration of cytokine exposure continue to be investigated [36,66]. The inclusion of a variety of non-cell-based activating supplements is increasingly showing the promise of a feeder-free system.

Although most clinical studies utilize overnight-activated or feeder cell-expanded NK cells, there are selective examples of feeder-free

systems that have been applied clinically. Notably, these utilize apheresis products, ensuring high numbers of PBMCs or NK cells at the outset, reflecting the lower expansion capacity of these approaches in their current iterations. Li *et al.* [67] reported the use of expanded autologous NK cells in combination with chemotherapy as an adjuvant therapy post resection of colorectal cancer. The NK expansion began with apheresis PBMCs, followed by culture in an anti-CD16-coated flask with AIM V media, 5% autologous plasma and 700 IU/mL IL-2. For the initial 24 h only, OK-432 was included in the expansion media. After 2–3 weeks, doses of up to 4×10^9 total NK cells were administered, and multiple aphereses and expansions permitted NK cell infusions with each cycle of chemotherapy. The mean purity of NK cells was 95.4%, and there was no emergent NK cell-related toxicity.

In an innovative study using UCB-derived CD34+ hematopoietic progenitor cells, Dolstra *et al.* [26,] describe a 6-week process in which NK cells were differentiated, expanded and administered as consolidation therapy to 10 older patients with AML who were not eligible for allogeneic SCT. The GMP-compliant process initially uses static cell culture bags to expand UCB CD34+ cells, followed by NK cell differentiation in a bioreactor in the presence of differentiating media containing stem cell factor, IL-7, IL-15 and IL-2 [68]. The main adverse effects encountered in the clinical trial related to lymphodepleting chemotherapy with doses escalating to 3×10^7 NK cells/kg. Median NK cell purity was 75%, although the T-cell content was 0.03% of the total at day 42, and the remaining cells were likely monocytic. The expanded NK cells also demonstrated an activated phenotype expressing NKG2D and natural cytotoxicity receptors. Notably, two patients relapsed prior to the therapy being administered, and one product was not released due to CD3 content $>0.1 \times 10^5$ cells/kg. This trial confirmed the feasibility and safety of this differentiation and expansion approach. Ongoing efforts to adapt this and similar protocols to include cryopreservation or reduce the time to product availability will be beneficial in the future.

Additional approaches to feeder-free expansion have incorporated alternative supplements with NK cell expansion capability. Peled *et al.* [69] reported the development of an expansion process using nicotinamide, the water-soluble amide form of vitamin B3. Starting with CD3-depleted PBMCs isolated from an apheresis product, NK cells accounted for 97% of the expanded product at the end of a 14-day culture. IL-15 outperformed IL-2 in this setting for NK cell purity, and a GMP-compliant process is being evaluated in a phase 1 trial treating refractory lymphoma and myeloma in combination with monoclonal antibody therapy, delivering up to 2×10^8 cells/kg [70]. A recent report describes the use of IL-2, zoledronate (a Food and Drug Administration-approved bisphosphonate medication used in the treatment of osteoporosis and bony metastases) and a group A streptococcus preparation as an activating cocktail, resulting in NK expansion from UCB to numbers suitable for clinical use [71]. Zoledronate and group A streptococcus displayed synergistic benefits with regard to the degree and purity (>90%) of NK cell expansion. Although optimization is ongoing, this expansion protocol would also have the benefit of using products licensed for clinical use as well as favorable cost and logistical simplicity. Antibody-coated beads targeting the NK activating pathways CD2 and NKp46 (CD335) are commercially available for pre-clinical research requiring NK activation and expansion (Miltenyi Biotec) [72]. A similar principle, but using dissolvable microspheres (Cloudz expansion kit; Bio-Techne), has been developed, enhancing NK expansion while simplifying cell harvesting, representing another interesting model for future clinical approaches [73].

Priorities for further research

In light of the findings provided by the present review, the authors recommend the following priorities for further research: (i) optimization of NK cell cryopreservation, enabling off-the-shelf applications of expanded and gene-modified NK cells; (ii) improvement of clinical-grade, feeder-free expansion capacity from UCB or small-volume peripheral blood donations, avoiding apheresis; and (iii) harmonization of release criteria, dosing definitions and *in vitro* function assessments to permit better comparison between approaches.

Conclusion

The past decade has seen advancements in our understanding of NK cell biology applied to ACT techniques. Because of reliable and robust expansion of NK cells, feeder cell-based expansion protocols have dominated recent trials utilizing expanded NK cells. Considerable efforts are ongoing to improve existing and develop new GMP-grade expansion protocols that approach the features of an ideal NK expansion protocol. The process of NK cell expansion remains fundamental to many clinical applications of NK ACT, with implications for dosing, safety, efficacy and cost of these investigational cancer immunotherapies.

Declaration of Competing Interest

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Author Contributions

Conception and design of the study: MO. Acquisition of data: SK and MG. Drafting or revising the manuscript: SK, MG and MO. All authors have approved the final article.

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