EXPANSION AND ACTIVATION OF NATURAL KILLER CELLS FOR CANCER IMMUNOTHERAPY

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Abstract

Natural killer (NK) cells can kill a wide range of cancer cells and are a promising tool for cell therapy of cancer. NK cells cytotoxicity is regulated by a balance between stimulatory and inhibitory signals. Interleukin-2 is known to increase NK cell cytotoxicity. Although many cytokines have been studied in efforts to induce durable NK cell expansions, most reports indicate a rather modest effect and the requirement for additional stimuli. We found that contact with the K562 myeloid leukemia cell line, genetically modified to express a membrane-bound form of interleukin-15 and the ligand for the costimulatory molecule 4-1BB, induced vigorous expansion of NK cells from peripheral blood. Based on these findings, we developed a method for large-scale clinical-grade expansion of NK cells. This method is currently used to expand allogeneic NK cells for infusion in patients with leukemia and solid tumors. We here summarize methods for expansion and activation of NK cells from human peripheral blood mononuclear cells as well as clinical-scale methods to produce NK cells for immunotherapy under Current Good Manufacturing Practices (cGMP) conditions.

Keywords

NK cells; cell therapy; acute myeloid leukemia; acute lymphoblastic leukemia; chimeric receptors

INTRODUCTION

Natural killer (NK) cells comprise 5% to 20% of human peripheral blood lymphocytes and are derived from CD34+ hematopoietic progenitor cells [1]. The precise physiologic sites where NK cells mature and the mechanisms that drive the development of their functional characteristics have not yet been fully clarified but recent studies indicate that these occur in the bone marrow and the lymphnodes [2-4].

NK cells have the morphology of large granular lymphocytes, and are phenotypically defined by the expression of CD56 and the lack of CD3 and T-cell receptor molecules [3,4]. Approximately 10% of NK cells express very high levels of CD56 and also have dim expression of FcgRIII (CD16), a receptor that binds the Fc portion of IgG [5]. These cells mostly are believed to have primarily an immunoregulatory role exerted through the secretion of cytokines and chemokines [5]. Although less common in blood, bone marrow and spleen, this cell subset predominates in the secondary lymphoid tissue [6]. The remaining 90% of NK cells in blood express lower levels of CD56 and high levels of CD16 [5]. These cells appear to function predominantly in direct cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC)

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[5,7]. NK cells can directly induce apoptosis via the perforin-granzyme pathway, or by expressing death-receptor ligands on their cell surface. Such ligands include tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), or the Fas ligand, which directly trigger cell death via their respective receptors[8]

NK cells can kill target cells without the need for prior sensitization, an effect that is regulated by the balance of stimulatory and inhibitory signals [4,9,10]. Many of the signaling receptors on the surface of NK cells engage major histocompatibility complex (MHC) class I and MHC class I-like molecules. A well-described mechanism is the inhibition of NK activity by increasing expression of MHC or human leukocyte antigen (HLA) class I in target cells [11]. NK cells express killer immunoglobulin-like receptors (KIRs), most of which recognize specific corresponding HLA class I molecules on target cells, and deliver inhibitory signals [4,9,10],. These inhibitory signals from HLA can override activating signals and suppress the function of NK cells. A concept that has recently emerged is that interaction between NK cells and HLA molecules might also be important for their functional maturation and the generation of NK cells that are tolerant towards self molecules, a process which has been termed "licensing" [12].

This review summarizes methods for expansion and activation of NK cells from human peripheral blood mononuclear cells as well as clinical-scale methods to produce NK cells for immunotherapy under Current Good Manufacturing Practices (cGMP) conditions.

METHODS TO EXPAND NK CELLS

Cytokines and stimulants

Several protocols for NK-cell expansion have been reported (summarize in Table; see also www.nkcells.info/wiki/index.php/NK_cell_expansion). Many cytokines have been studied in efforts to induce durable NK cell expansion as well as increase their cytotoxicity [13]. Interleukin (IL)-2 can enhance the cytotoxicity of NK cells within 24 hours of incubation [14,15]. IL-2 can also stimulate their proliferation but only a minority of NK cells can maintain proliferation after the initial response [14,16,17]. IL-4, IL-7 and IL-12 also induced some proliferative stimuli but are overall less potent than IL-2 [18]. Likewise, IL-15 alone or in combination with IL-2 typically results in minimal NK cell expansion [8]. We found that IL-2 (1000 IU/mL) or IL-15 (10 ng/mL) did not induce significant expansion of NK cells [19]. It appears that cytokines may be necessary but not sufficient for optimal proliferation of NK cells.

Recently, Alici E *et al* [20] used culture conditions using IL2 (500 IU/mL) with an anti-CD3 antibody (Orthoclone OKT-3; 10 ng/mL) and reported that the number of NK cells from the peripheral blood of 7 newly diagnosed, untreated patients with multiple myeloma had expanded on average 1,600-fold after 20 days of culture. This is in striking contrast with the 190-fold expansion obtained using NK cells from healthy individuals [21], implying that NK cells from myeloma patients may somehow be more susceptible to stimulation than NK cells from healthy individuals. It is also unclear how CD3 stimulation contributed to the expansion of CD3-NK cells in these studies.

It should be noted that the cell populations that results from the stimulation of peripheral blood mononuclear cells with IL-2 termed lymphokine-activated killer (LAK) cells are comprised of mostly of polyclonal T-cells and only a small fraction is CD56+ NK cells [22].

Expansion of NK cell with accessory cells

Most investigators believe that sustained proliferations of NK cells require additional signals [23,24], such as the presence of monocytes [23] or B-lymphoblastoid cells [16,25,26]. Miller et al. [23] reported an approximate 30-fold expansion of NK cells after 18 days of culture with

1000 IU/mL IL-2 and monocytes. Perussia et al. [27] found that contact with irradiated B-lymphoblastoid cells induced as high as a 25-fold expansion of NK cells after 2 weeks of stimulation. Harada et al [28] reported that HFWT, a Wilm's tumor-derived cell line, stimulates up to 400-fold NK expansion after 2 weeks. Other investigators have used allogeneic mononuclear cells[29], autologous lymphocytes[30], mitogen activated lymphocytes[25], and umbilical cord mesenchymal cells [31]

Contact with K562 cells, a cell line derived from a patient with myeloid blast crisis of chronoc myelogenous leukemia and bearing the *BCR-ABL1* translocation, is known to induce modest proliferations of NK cells [28,32], and augment NK cell proliferation in response to IL-15 [24]. We genetically-modified K562 cells to express two NK stimulatory molecules. One, the ligand for 4-1BB (4-1BBL), induces activation signals through 4-1BB (CD137), a costimulatory molecule expressed on the surface of NK cells [33]. The other, IL-15, is known to support NK-cell maturation and survival [34-37]. IL-15 has greater activity when bound to the cell membrane of stimulatory cells, rather than in its soluble form [38-42]; hence, we made a construct containing the human *IL-15* gene fused to the gene encoding the human CD8α transmembrane domain, and used it to transduce K562 cells. The resulting cell line (K562-mb15-41BBL) induced 21.6-fold expansion of CD56$^+$ CD3$^-$ NK cells from peripheral blood (range, 5.1–86.6-fold; n = 50) after 7 days, which was considerably superior to that produced by stimulation with interleukin (IL)-2, IL-12, IL-15 and/or IL-21 [19,43]. These cultures were performed using irradiated K562-mb15-41BBL cells and 10 IU/mL IL-2. We observed minimal or no proliferation of CD3$^+$ lymphocytes. NK cells could be further expanded with K562-mb15-41BBL cell stimulation by prolonging the cultures and adding 100 IU/mL IL-2 after day 7. Thus, median NK cell recovery increased to 152-fold after 14 days of culture, and 277-fold after 21 days [19,43]

NK cells expanded by K562-mb15-41BBL cells stimulation were significantly more potent than purified unstimulated or IL-2-stimulated NK cells against acute myeloid leukemia (AML) cells in vitro and could eradicate AML in murine models [43]. Preliminary studies indicate that these NK cells are also cytotoxic against cell lines derived from patients with Ewing sarcoma, rhabdomyosarcoma and neuroblastoma (D. Cho, D. Shook, D. Campana, unpublished results).

Although expanded NK cells acquired powerful cytotoxicity against a variety of cancer cell types, their capacity to kill acute lymphoblastic leukemia cells (ALL) remained overall limited. To overcome this resistance we transduced expanded NK cells with artificial receptors directed against CD19, a molecule expressed by ALL cells and cells of other B-cell malignancies. Anti-CD19 receptors linked to CD3ζ markedly enhanced NK-cell mediated killing of ALL cells, a result that was further improved by adding the 4-1BB costimulatory molecule to the chimeric anti-CD19-CD3ζ receptor [19]. Addition of 4-1BB was also associated with increased production of IFN-γ and GM-CSF [19].

CLINICAL LARGE-SCALE NK CELL ACTIVATION AND EXPANSION

Methodologic considerations

It is now feasible to obtain clinical-grade purified functional NK cells for infusion [44]. NK cells can be obtained from different sources: from the patient (autologous), the patient's human leukocyte antigen (HLA)-matched siblings, or haploidentical family members or unrelated donors. It might be convenient if NK cells could be collected in advance, cryopreserved and thawed before infusion. Unstimulated cryopreserved and thawed NK cells have phenotype and cytotoxicity that resembles that of fresh cells[45]. Whether this is also the case for activated and expanded NK cells remains to be established.

For clinical-grade NK cell activation and expansion, cells need to be cultured for a period of time that varies between less than 24 hours to several weeks [20,46]. Several tissue culture media have proven effective including stem cell growth medium (SCGM) (CellGenix, Freiburg, Germany) [20,43] and X-VIVO serum-free media (BioWhittaker, Verviers, Belgium) [47]. The media can be supplemented with fetal bovine serum (from certified sources) or human serum from AB blood donor [47] is used for clinical applications. NK cells can be cultured in flasks or in bags such as Teflon (FEP) bags[29,48] and Baxter Lifecell bags [8]. If stimulatory cells are used, it is important to prevent their overgrowth and to ensure than no viable cells are infused with the cultured NK cells. Irradiation, at doses of 30 Gy [49], 50 Gy [28], 70 Gy [29] or 100 Gy [19] is a safe and effective method.

Our clinical protocol used cGMP guidelines to process apheresis products and obtain expanded activated NK cells [43] (Figure). Mononuclear cells are Ficoll-separated and placed in culture in SCGM medium supplemented with 10% FBS, gentamicin sulfate (50 mg/L), and 10 IU/mL human IL-2 at a concentration of 0.5×10^5 CD56$^+$ CD3$^-$ cells/mL. Irradiated (100 Gy) K562-mb15-41BBL cells (from a Master Cell Bank) are added at a ratio of 1 CD56$^+$ CD3$^-$ cell: 10 K562-mb15-41BBL cells. Cultures are performed in a closed VueLife bags system (American Fluoroseal, Gaithersburg, MD). Cells are fed after 2 and 5 days, and harvested after 7 days of culture. The cell product is then depleted of residual T cells using the CliniMACS System (Miltenyi). Finally, cells are washed and resuspended in PlasmaLyte-148 (Baxter, Deerfield, IL) with 0.5% human serum albumin. Under these conditions, we obtained a median 90.5-fold expansion of CD56$^+$ CD3$^-$ NK cells (n = 12) after 7 days of culture [43]. The expansion in these large-scale cultures was higher than that of observed in small-scale experiments, most likely because of to the use of SCGM tissue culture medium instead of RPMI-1640 (SCGM appears to be well suited to support NK cell growth [20]. We therefore estimate that it should be feasible to obtain the number of NK cells planned for infusion in our protocol (maximum dose, 5×10^7 NK cells/kg) and more from a leukapheresis product. For example, considering that an average apheresis from a normal adult donor gives us about 100×10^8 nucleated cells and the average percentage of NK cells is 7.0%, a 90-fold expansion would result in 6.3×10^{10} NK cells. If necessary, larger numbers could be obtained by prolonging the cultures beyond 7 days.

An alternative to expand NK cells is represented by the continuously growing NK cell lines NK-92, derived from a patient with non-Hodgkin lymphoma that is cytotoxic against a wide range of malignant cancer cells [50]. These cells have practical appeal, but irradiation is mandatory before infusion in patients, which may limit their efficacy in vivo.

Therapeutic applications of NK cells

In the setting of hematopoietic stem-cell transplantation, donor NK cells may exert an anti-leukemia effect if they do not express KIRs reacting with the HLA class I epitope expressed by the patient's leukemia cells. In animal models, donor NK cells killed host leukemic cells and lympho-hematopoietic cells without affecting non-hematopoietic tissues [51], suggesting the possibility of an NK-mediated graft-versus-leukemia (GVL) effect without systemic disease. Therefore, it is now a common practice at some clinical centers to select donors with an HLA and KIR type that facilitates NK-cell activation [52-54].

In addition to their use in the context of allogeneic stem cell transplantation, allogeneic NK cells can be directly infused in non-myeloablated patients. Miller et al. [46] first demonstrated the potential utility of this approach. These investigators treated 19 adult patients with high risk AML with cyclophosphamide (60 mg/kg for 1 or 2 doses), fludarabine (25 mg/m^2 daily for 5 doses), IL-2 (10 million units per dose for 6 to 9 doses), and an infusion of 2×10^7/kg CD3-depleted NK-cell product (NK cells enriched to approximately 40%) that was activated for 18 hours with 1000 IU/mL IL-2. Eight of 15 AML patients showed at least 1% engraftment

at day 7 or later after the infusion, and 5 patients achieved a complete remission. Interestingly, lymphodepletion induced higher levels of IL-15 which in turn might have been important in prolonging the survival of the infused NK cells [46]. Our current protocol using NK cells expanded by stimulation with the K562-mb15-41BBL cell line uses a framework identical to that described by Miller et al. [46]. Thus, patients are treated for 7 days with cyclophosphamide and fludarabine and receive subcutaneous IL-2 after infusion of the expanded NK cells.

If cancer cells present a tumor-specific antigen in the HLA context they can be recognized and lysed by cytotoxic T lymphocytes (CTL) specific for the antigen. For example, expanded T lymphocytes specific for Epstein-Barr virus (EBV)-associated molecules have been applied for the treatment and prophylaxis of EBV-associated lymphoproliferative disease and lymphoma [55]. Other EBV-associated tumors may also be amenable to this form of therapy [56,57]. However, most cancers lack identifiable virus-associated antigens [58], although other molecules, such as WT1 and Pr3, are overexpressed in some cancer cells and are possible targets for adoptive T-cell therapy [59,60] NK cells offer some potential advantage over CTL therapy. First, a wide range of cancer cells appear to be sensitive to NK cell cytoxicity. In addition to AML, NK-sensitive malignancies include soft-tissue sarcomas (D. Cho, D.Shook, D. Campana, unpublished), neuroblastoma [59,60] and malignant glioma [59]. Second, they can be used in an allogeneic setting without the risk of graft versus-host-disease [44]. Thirdly, with the method described in this review, large numbers of cytotoxic NK cells can be reliably obtained in a relatively short period of time.

For malignancies that are relatively resistant to NK cells, strategies that can be explored include genetic modification with artificial receptors, such as the one that we reported using anti-CD19 receptors for the treatment of B-cell neoplasias [19]. To this end, the overall strategy that we have described is not limited to CD19^{+} leukemia and lymphoma cells but is also applicable to many other molecules express by cancer cells and can be implemented by replacing the anti-CDc19 scFv with the scFv of another antibody [61,62] .

Acknowledgments

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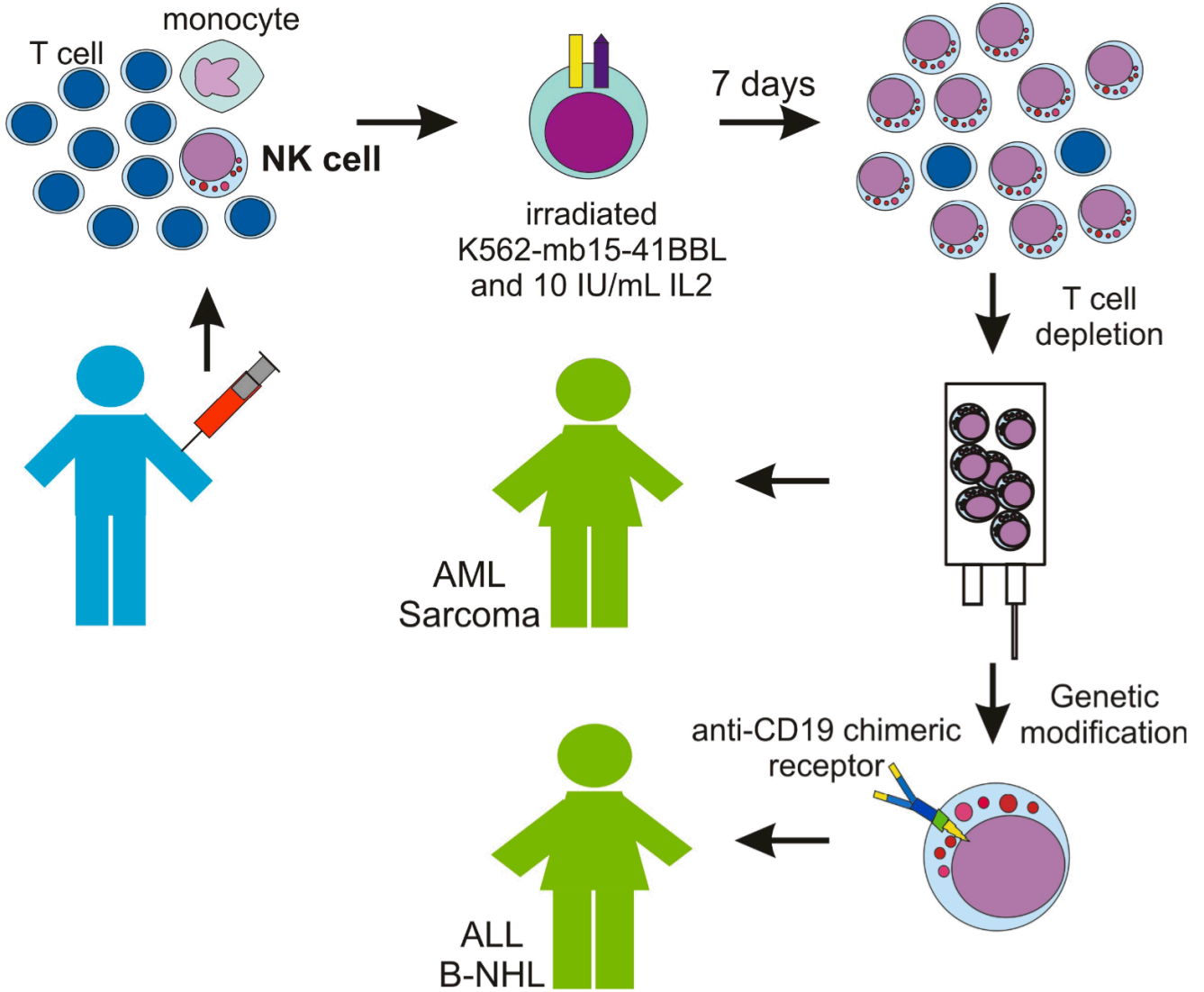
Figure. Schematic representation of protocols using expanded NK cells at St Jude Children's Research Hospital
The leukapheresis product obtained from a haploidentical donor is mixed with irradiated K562-mb15-41BBL cells. After 7 days of culture, most cells recovered are activated NK cells. After T-cell depletion using the CliniMACS system, NK cells are infused in patients with NK-sensitive malignancies such as acute myeloid leukemia (AML), Ewing sarcoma or rhabdomyosarcoma. For patients whose neoplasia is less sensitive to NK cytotoxicity, such as B-lineage acute lymphoblastic leukemia (ALL) or B-cell non-Hodgkin lymphoma (B-NHL), expanded NK cells are transduced with an anti-CD19 chimeric receptor before infusion.

Table

Selected protocols for expansion and activation of NK cells

Protocol	Median fold expansion after culture (% of CD56+CD3- cells)			Reference
	Day 7	Day 14	Day 21	
PBMC from donor, OKT3 (10ng/mL), IL2 (500 IU/mL)	-	-	193 (55)	[21]
PBMC from myeloma, OKT3 (10ng/mL), IL2 (500 IU/mL)	-	-	1625[a] (65)	[20]
Non-adherent PBMC from ALL, RPMI8866, IL2 + IL15	-	40[b] (62–95)	-	[49]
PBMC from donor, HFWT, IL2 (200IU/mL)	-	58–401[c] (77.4–85.6)	-	[28]
NK cell enriched from donor, IL2 (100IU/mL) + IL15 (10IU/mL) + PHA (100ug/mL) + ionomycin (1μM/mL)	-	80–200	-	[29]
PBMC from donor, K562-mIL15–41BBL, IL2 (10–100 IU/mL)	21.6 (62.9)	152 (90)	277 (96.8)	[43]

Abbreviations: PBMC, peripheral blood mononuclear cells; ALL, acute lymphoblastic leukemia

[a] Measured on day 20

[b] day 10

[c] day 10–21