Published in final edited form as:

Immunotherapy. 2012 March ; 4(3): 305–313. doi:10.2217/imt.11.174.

NK cell therapy: targeting disease relapse after hematopoietic stem cell transplantation

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Summary

As outcomes of hematopoietic stem cell transplantation have improved over time, disease relapse has emerged as the most important causes of treatment failure. Cellular therapy represents a promising therapeutic approach, not only to treat or prevent disease relapse after hematopoietic stem cell transplantation, but also conceivably to help patients achieve remission prior to transplantation or as consolidation therapy for high risk hematologic malignancies. Of the many cellular therapies available, NK cells infusion may be the most promising approach due to strong innate activity of NK cells *in vitro* and *in vivo* against malignant or virally infected cells. Published clinical data consists only of small feasibility studies of inadequate size to show clinical benefit. Here we review the current status of clinical investigation using NK cell therapy for patients with hematologic malignancies undergoing hematopoietic stem cell transplantation.

Keywords

NK cells; NK cell expansion; cellular therapy; haploidentical stem cell transplantation

Introduction

Human Natural Killer (NK) cells play a critical role in host defense against infections and malignant transformations [1]. They comprise of approximately 10–15% of all blood lymphocytes and are identified by the lack of CD3 and the presence of CD56 and/or CD16 expression on cell surface. There are two subsets of NK cells that differ phenotypically and functionally. The majority of circulating NK cells (90%) have CD16 and low CD56 surface expression, termed CD56^{dim}, and are important in direct and Antibody Dependent target Cell Cytotoxicity (ADCC) [2]. ADCC is mediated by CD16, the low-affinity Fc receptor which binds the Fc portion of IgG antibody [3]. These NK cells can release granules containing perforins, which form a pore in the cell membrane, and granzymes that enter target cells to mediate apoptosis [4]. They can also express death receptor ligands such as Fas ligand (FasL) and Tumor Necrosis Factor (TNF) Apoptosis-Inducing Ligand (TRAIL) [5].

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A minority of peripheral NK cells and 100% of NK cells in secondary lymphoid tissue (SLT) express high CD56 and low CD16, which are less cytotoxic but secrete more cytokines [6]. These CD56^{bright} NK cells have high levels of CD94/NKG2 family receptors with only very small fractions of Killer cell Immunoglobulin-like Receptors (KIRs) [7]. They express the high and intermediate affinity Interleukin 2 (IL-2) receptors and expand in vivo and in vitro in response to low doses of IL-2 [8]. Upon stimulation, they produce high levels of cytokines while cytotoxic activity and ADCC are minimal. However, cytotoxic activity can still be induced using IL-2 [9]. They also express the receptor tyrosine kinase ckit whose ligand augments IL-2 induced proliferation [8]. Since CD56^{bright} NK cells are dominant in SLT, it has been postulated that CD56^{bright} cells are less mature than CD56^{dim} cells. Activation of SLT CD56^{bright} NK cells increases expression of NK receptors that are characteristic of the CD56^{dim} such as CD16 and KIRs [10]. Recently, a novel subset of CD34^{dim} hematopoietic progenitor cells that reside in lymph nodes was identified and is capable of differentiating into CD56^{bright} NK cells through a four-stage model described by Freud and Caligiuri [11].

NK cell cytotoxicity is triggered when the balance of activating and inhibiting receptor signals is tipped towards activation. Inhibitory receptors primarily recognize Major Histocompatibility Complex(MHC) class I or class-I like molecules, preventing NK-mediated cell lysis of normal MHC-expressing cells. The main group of NK receptors, KIRs, have cytoplasmic domains comprised of a short immunoreceptor tyrosine-based activating motif (ITAM) or a long immunoreceptor tyrosine-based inhibition motif (ITIM) [12]. Fifteen KIR genes and two pseudogenes have been identified on chromosome 19 and are extremely polymorphic [101]. Other receptors include C-type Lectin Receptors and Natural Cytotoxic receptors (NCR). The NCR (NKp30, NKp44, NKp46 and NKp80) are activating receptors mostly restricted to expression on NK cells [13]. The C-type Lectin receptors in the NKG2 family are expressed as heterodimers with CD94 except NKG2D which is expressed as homodimer[12].

The “missing-self hypothesis”, which postulates that NK cells recognize and destroy autologous cells with lost or altered self-MHC class I molecules [14], is an oversimplification of NK cell regulation, since MHC class I is not always required to protect from NK cytotoxicity nor is it always sufficient to prevent NK cytotoxicity[15]. Activation of NK cells with cytokines including IL-2 or IL-15 may be required to enhance the cytotoxicity and function of NK cells [15]. There have been several models used for predicting NK cell alloreactivity. Ruggeri et al. initially described the ligand-ligand mismatch model when there is incompatibility between the donor KIR ligand and recipient KIR ligand (i.e., ligand-ligand mismatch if the donor has a ligand that is absent in the recipient) [16]. However, we now recognize that KIR genotype has a profound effect on the KIR repertoire and that the expression of KIR genes is not entirely dependent on expression of their HLA ligands, but is controlled by promoter methylation [17]. In addition, it is important to note that possession of a KIR gene does not imply its expression on NK cells [18]. Thus, Leung et al. defined mismatch in the receptor-ligand model as incompatibility between the donor KIR and recipient KIR ligand on the basis of expression (i.e., receptor-ligand mismatch if the donor has an inhibitory receptor for which the cognate ligand is absent in the recipient) [19]. A third model was described by Gagne et al. in which

incompatibility was defined as a mismatch between the donor KIR and recipient KIR receptors (i.e., receptor-receptor mismatch if the donor has a receptor that is absent in the recipient) [20]. The last model can be considered the prototype for the subsequent 'KIR haplotype model' proposed by the Stanford group, which is founded on the notion that the more activating KIR genes the donor carries, the higher the potential for alloreactivity. The 'A' KIR haplotype has only one activating gene (KIR2DS4), whereas the 'B' KIR haplotype has more than one activating KIR gene, including KIR2DS1 and KIR2DS2. Because KIR 'B' haplotype carries more activating genes than the 'A' haplotype, transplants from donors with the 'B' haplotype are predicted to have more prominent NK cell-mediated anti-tumor effects [21].

NK cell expansion

Even with leukapheresis, a relatively small number of NK cells can be obtained from the peripheral blood of healthy donors, generally limiting adoptive immunotherapy to median cell doses of approximately 2×10^7 NK cells/kg body weight of the recipient [22]. Studies have shown that IL-2 increases the number of circulating NK cells; however giving high doses of IL-2 can be associated with significant side effects such as fever, hypotension, pulmonary edema, hepatic and renal toxicities, among others. Giving a low dose of IL-2 was found to have more effect on specific expansion of the CD56^{bright} NK cell subset, which may have a regulatory rather than cytotoxic role because of expressing the higher affinity IL2R α [8]. IL-2 may also affect T regulatory (T reg) cells which have the potential to counteract the antitumor effect of other immune effector cells including NK cells [23]. Increased levels of endogenous IL-15 are associated with in vivo expansion in human trials of haploidentical NK cell therapy [24, 25], but induction of sufficient IL-15 levels requires aggressive lymphodepleting chemotherapy. With this approach, persistence of NK cells adoptively transferred after lymphodepleting chemotherapy is detectable for 2–4 weeks in both the autologous [25], and haploidentical setting [24], with donor NK cells detectable at low levels in selected patients for up to 6 months.

Thus *ex vivo* expansion of NK cells is likely needed to maximize the therapeutic potential of this approach. Several expansion methods have been described for this purpose including cytokine support such as IL-2 [26] or anti-CD3 antibodies [27]. Data from these trials showed that ex-vivo purification and expansion of donor NK cells from leukapheresis products is technically feasible and safe. However, cytokines alone are not sufficient for optimal cellular proliferation. Additional signals from other cells are required, and support from mesenchymal stroma or genetically-modified artificial Antigen Presenting Cells (aAPCs) can also induce expansion [28]. Contact with K562 cells, a cell line derived from patient with myeloid blast crisis of chronic myeloid leukemia (CML) is known to induce modest proliferation of NK cells [29]. K562-based aAPC transduced with 4-1BBL (CD137L) and membrane-bound IL-15 (mIL-15) was used to achieve a mean expansion of 277-fold in 21 days; however, proliferation is limited by senescence attributed to telomere shortening [30]. We have found that membrane bound IL-21 (mIL-21) resulted in greater proliferation, longer telomere length, and less senescence than NK cells expanded with mIL-15, achieving a median 21,000-fold expansion in 21 days [31]. These expanded NK cells express high levels of NKG2D, NCRs and CD16. The high proliferation and activation

was enabled in this system by IL-21R-mediated phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3) which upregulates NKG2D and telomerase expression (manuscript under review). If stimulatory cells are used, irradiation prior to co-culture is important to prevent their overgrowth and to ensure that no viable cells are infused with the cultured NK cells [32].

Infusion of NK cells after transplant

Several lines of evidence suggest that increased numbers of alloreactive NK cells would be of benefit in the transplant setting [33], despite the fact that retrospective results vary depending on patient population, underlying diseases, conditioning regimens, graft composition and cell dose, degree of T-cell depletion, post-transplant immunosuppressive regimens and differing methods of determining alloreactivity. Ruggeri et al. proposed the infusion of NK cells after transplant to target disease relapse based on a retrospective evaluation of NK alloreactivity in transplanted patients [15] and supportive NK models [16]. This group analyzed 92 patients with high risk leukemia who received a haplotype mismatched hematopoietic stem cell transplant (HSCT) and reported that NK cell alloreactivity improved engraftment, protected from graft-versus-host disease (GVHD) and reduced the rate of relapse. Using mouse xenograft models they demonstrated the role of alloreactive NK cells in eradicating human leukemia, improving engraftment by targeting host T lymphocytes and reducing GVHD by eliminating recipient-type dendritic cells [16]. In addition, the NK cell dose of the infusion product has been associated with better outcomes following matched sibling transplants [34].

There is little data on tissue migration of adoptively-transferred NK cells in humans. Meller et al reported migration of Indium-labeled NK cells to lung, liver, spleen, and bone marrow [35]. There are reports of successful PET-based imaging of NK cell lines in murine tumor models [36], suggesting that this modality may be applicable to human studies in the future. There is no definitive data regarding whether NK cells cross the blood brain barrier (BBB), although it is known that naive lymphocytes do not cross the BBB, while activated lymphocytes patrol the CNS freely and are actively recruited to sites of inflammation [37]. Whether this is also true for NK cells it is unknown.

A number of studies, listed in Table 1, have reported on the safety of NK cell infusion. While no dose-limiting toxicities have been described, none of the studies in the post-transplant setting were powered to assess the therapeutic effect of the NK cells. In a study by Koehl et. al, two pediatric patients with multiply-relapsed acute lymphoblastic leukemia (ALL) and one patient with acute myeloid leukemia (AML), age 6–16 years, were treated post haploidentical HSCT with repeated infusions of haploidentical KIR mismatched NK cells starting at day 1 post transplant then every 4–6 weeks after HSCT. Single doses of $3\text{--}34 \times 10^6$ CD56+CD3– cells/kg were infused. The AML patient received two infusions, while the first ALL patient received three infusions and the second ALL patient received one infusion. NK cell infusions were well tolerated without significant adverse events or GVHD [26]. Although all patients had active disease with blasts of 37–97% at the time of transplant, they achieved remission and complete donor chimerism by 4th week post transplant. The AML patient died of early relapse on day +80, while the ALL patients died

of thrombotic-thrombocytopenic purpura and atypical viral pneumonia on days +45 and +152, respectively [26]. In a second study by the same authors, seven pediatric patients (1 AML, 4 ALL, 1 Hodgkin's disease (HD), 1 rhabdomyosarcoma) were treated with repeated infusions of NK cells at days +3, +40, +100 after haploidentical HSCT [38]. The patients received $6.6\text{--}32.3 \times 10^6$ CD56⁺CD3[−] cells/kg and $0.5\text{--}53.3 \times 10^3$ CD3⁺ cells/kg from the parental donors. No immediate adverse reactions were observed after the infusion. Three patients developed GVHD, one had grade III GVHD because of a high CD3⁺ cell dose associated with the NK cell infusion of 53.3×10^3 CD3⁺ cells/kg, and the other two patients because of a T-cell add back of 1 and 1.5×10^5 CD3⁺ cells/kg, respectively. The remaining 4 patients who received $<20 \times 10^3$ CD3⁺ cells/kg had no signs of GVHD. Six of the 7 patients who received a median of 11.5×10^6 ($7\text{--}29.6 \times 10^6$) CD34⁺ cells/kg showed moderate or fast immune reconstitution with full donor chimerism, while one patient transplanted with 13.6×10^6 CD34⁺ cells/kg had graft rejection. Five patients were alive in complete remission with a short follow up (4–13 months) post haploidentical HSCT [38]. Passweg *et al.* infused NK cells up to 14×10^6 cells/kg 3–12 months following T-cell depleted haploidentical HSCT to five patients (4 AML, 1 CML), ages 3–25 years. Indications were incomplete engraftment, early relapse, and graft failure. NK alloreactivity was present in 3 of 5 patients. All patients except one received at least 2 infusions. No GVHD or infections were observed. Two patients had an increase in donor chimerism, (one of these two received an additional stem cell dose after NK), one had stable mixed chimerism, one had decreasing chimerism and another patient experienced relapsed AML. Four of the five patients are alive and in continuous remission 8–18 months after the first NK infusion (median follow-up 12 months) [39]. In an update, Passweg reported that 3 of 5 treated patients were alive and in complete remission 18–36 months after NK infusion [40]. Slavin *et al.* used IL-2-activated NK cells in 8 patients with median age of 25 years (range 4–63), with relapsed hematological malignancies after HSCT. Patients received positively selected CD56⁺ cells prepared from rIL-2 activated donor lymphocytes. Donors were haploidentically mismatched (3 patients), matched siblings (4 patients) and matched unrelated (1 patient). Disease categories included acute leukemia and myelodysplastic syndrome (MDS) (n=5) patients and lymphoma (n=3) patients. Positively selected cells were median 39% CD56⁺ cells (range 30–71%), with a median of 3% contaminating CD3⁺ cells (range 2–21%). The median number of CD56⁺ cells infused was 120×10^6 (range $10\text{--}600 \times 10^6$). Cell infusion was uneventful and no GVHD was observed in all 8 treated patients [41]. One patient with relapsed ALL achieved remission after receiving 25×10^6 /kg, but died 8 months later due to pulmonary aspergillosis, present on admission for HSCT. A second recipient of haploidentical HSCT from KIR non-alloreactive mother treated for refractory MDS was in complete remission more than 8 months post-transplant with no GVHD, after infusion of 2.5×10^6 NK cells 3 months post SCT. Five patients are alive, two with disease 18 and 20 months post transplantation and three with no evidence of disease after 8, 12 and 20 months post transplant [41]. Barkholt *et al.* evaluated the infusion of long-term *ex vivo* expanded allogeneic NK and NK-like T-cells following allogeneic HSCT in five patients ages 48–67 years (4 with solid cancers, 1 with chronic lymphocytic leukemia) after disease progression [27]. These patients received NK cell infusions at a median of 15 months post transplant. No major adverse effects or signs of acute GVHD were observed [27]. The CLL patient in this study received a stem cell boost for disease relapse post HSCT 11 months before NK

infusion followed by three doses of non-KIR alloreactive NK cells ($1, 9.6$ and $17.3 \times 10^6/\text{kg}$). IL-2 in dose of $6 \times 10^6 \text{ IU}/\text{m}^2/\text{day}$ was given for three consecutive days starting on the day of the first cell infusion. Unfortunately the patient relapsed and remained alive six months after the last infusion. Uharek et al. reported on 25 patients (16 AML, 5 ALL, 2 CML, 1 Hodgkin's disease and 1 MDS patient) who received a haploidentical HSCT followed by infusion of purified CD56+CD3–NK cells on day +2. Preparatory regimen consisted of total body irradiation, thiotepe, fludarabine, and OKT3 [42]. NK cells were isolated from the CD34– fraction using an automated two-step procedure of CD3+ depletion and subsequent CD56+ selection. No other immunosuppression was routinely given. Patients received a mean of 9.8×10^6 CD56+CD3– NK cells/kg (range $1.61\text{--}32.2$) with $2.89 \times 10^4/\text{kg}$ ($0.95\text{--}7.4$) CD3+ cells. Most of the patients developed early GVHD of the skin (median onset 12 days), which promptly resolved after a short-term treatment with steroids and cyclosporine. The main cause of death consisted of infections ($n=10$), chronic GVHD ($n=2$), and relapse ($n=4$). After a median follow-up of 1442 days (3.9 years) (range 0.1 to 7.1 years) 9 of 25 patients are alive and in complete remission resulting in a 2-year overall survival of 29%. Out of 16 high-risk patients with AML, 10 had refractory or active disease prior to transplantation. In a matched pair analysis with EBMT database patients, the 16 AML patients treated with haploidentical HSCT plus NK cell infusions had superior 2-year overall survival (40 vs. 11%, $p=0.02$) [42]. Rizzieri et al. recently reported on a total of 51 NK cell-enriched donor lymphocyte infusions (DLIs) to 30 patients following a 3–6/6 HLA matched T-cell-depleted nonmyeloablative allogeneic transplant with alemtuzumab [43]. Eight weeks following transplantation, donor NK cell-enriched DLIs were processed using a CD56+ selecting column with up to 3 fresh infusions allowed. Fourteen matched and 16 mismatched transplanted patients received a total of 51 NK cell-enriched DLIs. The median number of CD3– CD56+ NK cells infused was 10.6×10^6 cells/kg and 9.21×10^6 cells/kg for matched and mismatched, respectively. The median number of contaminating CD3+CD56– T-cells infused was 0.53×10^6 and 0.27×10^6 in the matched and mismatched setting, respectively. All but 2 subjects had donor engraftment accounting for >80% of their hematopoiesis at the time of first infusion. After a median follow-up of 12 months for matched sibling donor transplants and 27 months for the haploidentical transplants, 1 year overall survival was 43% and 42%, respectively. Evaluating outcomes by disease, 1-year survival was 50% for the 19 patients with myeloid diseases and 29% for patients with lymphoid diseases. Only one patient each in the matched ($n = 14$) or mismatched ($n = 16$) groups experienced severe aGVHD with little other toxicity attributable to the infusions. Long-term responders had improved T-cell phenotypic recovery and improved duration of responses and overall survival after multiple NK cell-enriched infusions [43]. All of these trials are small pilot studies in diverse populations of lymphoid and myeloid malignancies. However, these studies have shown that infusion of NK cells is safe and feasible with potential for future larger studies. In a few of these studies some patients developed GVHD; however, since the infusions were given early after HSCT it is impossible to ascertain whether GVHD occurring in these patients was due to the transplant or the NK cell infusion. In some instances, the development of GVHD has been associated with less efficient T-cell depletion. For clinical application most of these trials used a large scale purification methods allowing automated, efficient and relatively rapid isolation of human NK cells.

We currently have an ongoing study to assess the safety of infusing alloreactive NK cells from a haploidentical relative and to determine the maximum tolerated dose of these cells given in combination with busulfan, fludarabine, antithymocyte globulin and allogeneic transplantation from a separate HLA matched donor for treatment of AML/MDS and to assess the rate of engraftment, GVHD, immune reconstitution and survival after infusion of alloreactive haploidentical NK cells effects.

Conclusions

Relapse remains the major cause of treatment failure after allogeneic stem cell transplantation. Alloreactive NK cells mediate a potent antileukemic effect and may enhance engraftment and reduce GVHD. Aforementioned studies demonstrated that the infusion of haploidentical alloreactive NK cells is safe and does not increase the incidence of GVHD. There are still many unanswered questions regarding the type of NK cells infused, optimal dose of NK cells and timing of infusions. Future studies will assess the effectiveness of increased doses of NK cells, made possible by *ex vivo* expansion, to treat or prevent disease relapse post-transplant and to explore the infusion of NK cells in non-transplant disease-specific trials,

Acknowledgments

This study was supported in part by a MD Anderson Cancer Center Institutional Research Grant to S.O.C.

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Table 1

Clinical studies using NK cell infusion post hematopoietic stem cell transplantation.

Author	Disease and # of treated patients	Age of patients	Type of SCT	Time of NK infusion post SCT	Nk selection/preparation	dose	Phase of clinical trial	aGVHD Y/N	Response/outcome	Ref #
Koehl et al.	ALL (2), AML (1)	6–16 years	Haplo	D+1, wk4–6, wk8–12	unstimulated leukaphereses, CD3 depletion, CD56 selection then NK were IL2 activated	Repeated infusions. Single dose 3–34×10 ⁶ CD56+CD3–/Kg. Total per patient ranged 20–47.4/kg	I	N	Although all patients showed blast at time of SCT, 3/3 CR and complete donor chimerism within 1 month postSCT.	26
Koehl et al.	AML (1), ALL (4), HD (1), RMS (1)	unknown	Haplo	D+3, +40, +100	unstimulated leukaphereses CD3 depletion, CD56 selection	Single dose: 6.6–32.3×10 ⁶ CD56+CD3– cells/kg	I	Y (3 pts developed grade III GVHD, 1 who had NK with 53.3×10 ⁶ 3 CD3+/Kg and 2 post T cell add back.	5/7 patients were alive in CR 4–13 months post SCT	38
Passweg et al.	AML (4), CML (1) With mixed chimerism or impending relapse	3–25 (median 16) years	Haplo	Several wks to months post sct	unstimulated leukaphereses CD3 depletion, CD56 selection	0.69–1.4×10 ⁷ /kg (med 0.21)	I	N	3/5 pts were alive and CR 18–36 months after NK infusion 2/5 had increased donor chimerism	39, 40
Slavin et al.	MDS (2), AML (1), ALL (1), biphenotypic (1), HD (1); NHL (2)	4–63 (median 25) years	3 Haplo 4 MRD 1 MUD	unknown	Positively selected CD56+ rIL2 activated donor lymphocytes	10–600×10 ⁶ (median 120×10 ⁶)	I	N	One pt with relapsed ALL and one with MDS had CR. 4 pts alive, 3 disease free 9–22 month post SCT.	41
Barkholt et al.	Solid tumors (4), CLL (1)	48–67 years	MRD	Time from last DLI 2–26 months	expanded NK and NK-like T cells	Escalating doses of at 1–10 ⁶ and a median of 13.2 (range from 8.1 to 40.3) × 10 ⁶ NK and NK-like T cells/kg. the last 3/6 got subcutaneous IL-2 injections.	I	N	CLL patient progressed	27

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Uharek et al.	AML (16), ALL (5), CML (2), HD (1), MDS (1)	unknown	Haplo	D-2	NK cells isolated from the CD34- fraction. CD3 depletion, CD56 selection	of 9.8×10^6 CD56+CD3- NK cells/kg (range 1.61-32.2)	I/II	Most developed early GVHD of the skin which promptly resolved after short term treatment with steroids and CSA.	9/25 patients alive and in CR, with 2-year OS of 29%	42
Rizzieri et al.	30 patients with myeloid and lymphoid malignancies	unknown	14 matched 16 mismatched SCT	1.5-3 months	unstimulated leukaphereses were enriched for NK cells using a CD56 antibody	median infused dose of 10.60×10^6 cells/kg	I/II	Y (14 pts, 6/14 had grade I skin aGVHD)	Total of 16 patients in CR with 1 year OS of 43% and 42% of matched and mismatched respectively	43

Figure legend: AML – acute myeloid leukemia, ALL – acute lymphoid leukemia, MDS myelodysplastic syndrome, HD – Hodgkin's disease, NHL – non-Hodgkin's lymphoma, CLL – chronic lymphocytic leukemia, CML – chronic myeloid leukemia, OS – overall survival, RMS – rhabdomyosarcoma, CR – complete remission, SCT – stem cell transplantation, pts – patients, **MRD** – **Matched Related Donor**, **MUD** – **Matched Unrelated Donor**