Infusions of Allogeneic Natural Killer Cells as Cancer Therapy

Wing Leung1,2

Abstract
Natural killer (NK) cells are normal white blood cells capable of killing malignant cells without prior sensitization. Allogeneic NK cell infusions are attractive for cancer therapy because of non–cross-resistant mechanisms of action and minimal overlapping toxicities with standard cancer treatments. Although NK therapy is promising, many obstacles will need to be overcome, including insufficient cell numbers, failure of homing to tumor sites, effector dysfunction, exhaustion, and tumor cell evasion. Capitalizing on the wealth of knowledge generated by recent NK cell biology studies and the advancements in biotechnology, substantial progress has been made recently in improving therapeutic efficiency and reducing side effects. A multipronged strategy is essential, including immunogenetic-based donor selection, refined NK cell bioprocessing, and novel augmentation techniques, to improve NK function and to reduce tumor resistance. Although data from clinical trials are currently limited primarily to hematologic malignancies, broader applications to a wide spectrum of adult and pediatric cancers are under way. The unique properties of human NK cells open up a new arena of novel cell-based immunotherapy against cancers that are resistant to contemporary therapies. Clin Cancer Res; 20(13); 3390–400. ©2014 AACR.

Disclosure of Potential Conflicts of Interest
W. Leung is a co-inventor of a patent-pending diagnostic method assigned to St. Jude Children’s Research Hospital and licensed to Insight Genetics.

CME Staff Planners’ Disclosures
The members of the planning committee have no real or apparent conflict of interest to disclose.

Learning Objectives
Upon completion of this activity, the participant should have a better understanding of the biology of human natural killer (NK) cells relevant to cancer immunotherapy, the general steps of allogeneic NK-based treatments, and the avenues of current and future strategies being explored in NK cell therapy.

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Introduction
For many cancers, cure rates remain unacceptably low. Advances in genetics and immunology have led to biologic therapies with non–cross-resistant mechanisms of action and no overlapping toxicities. Cancer immunotherapy is possible with an array of effector mechanisms involving innate and adaptive immunity (1). Various cytokines, antibodies, and cellular therapies have been FDA approved or are in late-phase clinical trials.

Infusions of purified natural killer (NK) cells are late-comers in cellular immunotherapy, compared with dendritic cells (DC), lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes, and conventional cytotoxic T cells. Many investigators consider that the cytolytic activity of LAK cells commonly used in the 1980s actually represents the activity of IL2-activated NK cells, although the products contained polyclonal T cells. Biologically, murine NK cells have been known to kill malignant cells without T-cell assistance or prior sensitization since their initial description in 1975 (2–5); however, the therapeutic potential of human NK cells was not revealed until their biology of target cell recognition was elucidated in the 1990s and the antileukemia effect was observed in HLA-haploidentical hematopoietic cell transplantation (HCT) in the early 2000s (6, 7). This review covers NK cell biology relevant to cancer immunotherapy and outlines avenues being explored in clinical trials.
NK Cell Biology

NK cells were first named and characterized by Kiessling at the Karolinska Institute (Stockholm, Sweden) and by Herberman at the National Cancer Institute (Bethesda, MD; refs. 2–5). The term "natural" referred to their natural existence in rodents and "killer" to their spontaneous killing of lymphoma and leukemia cells in nonimmune animals. They were called "N-cells" or "K cells" by some investigators (3, 8). These cells were unique, as they lacked markers characteristic of B and T lymphocytes and were nonphagocytic, low-adhesive, and cytotoxic to IgG-coated targets.

NK cells are generated in bone marrow and develop normally in athymic mice and in humans with DiGeorge syndrome. Induction of cytototoxicity is possible in NK cells within minutes, in contrast with the slower conventional induction of the T-cell immune response (9). Approximately 5% to 15% of human blood lymphocytes are NK cells (CD56+, CD3/14/19-), 90% of which are CD56dim, with most of them being CD16+, and they are responsible for early innate immunity and antibody-dependent cell-mediated cytotoxicity (ADCC) against infection or cancer through IFNγ, perforin, granzyme, FasL, or TRAIL (10). Approximately 10% of human blood NK cells are CD56bright, and they participate in late (>16 hours) inflammatory response by secreting IFNγ, NK cells are essential for priming adaptive immunity. NK cells may also directly interact with T and B cells through CD40 ligand and OX40 (11, 12).

Regulatory receptors

Human NK cells are regulated by a sophisticated network of surface receptors (Fig. 1), allowing them to distinguish normal from abnormal cells within seconds. Target cell lysis occurs when the activating signal dominates the inhibitory signal (Fig. 2). Early studies revealed similarities in interactions between murine NK cells and cells responsible for so-called "hybrid resistance" to parental hematopoietic grafts (13–14). The proposal that the resistance was related to receptors recognizing the absence of MHC class I arose from observations that murine lymphoma cells that were low in MHC expression were NK-susceptible (15). This led to the "missing-self" hypothesis in the 1980s (16). Breakthroughs in human NK receptor biology were seen in the early 1990s, when the KIR gene family was found to recognize HLA class I (17–19). Identification of other NK receptors, such as NCRs, NKG2D, DNAM, 2B4, and CD94/NKG2A, collectively points to a sophisticated network that controls human NK activity.

Step 1: KIR Typing for Donor Selection and Prognostication

Three general steps in NK-based therapy are summarized in Fig. 3. KIR typing is a critical first step because KIR is highly polymorphic. KIR typing is not only useful for allogeneic donor selection but also for prognostication in autologous NK therapy (20). Among known NK receptors, the KIR receptor family is one of the primary determinants of NK response. Subsets of T cells also expressed KIR (21). Clinical KIR typing includes genotyping for gene content and A/B haplotype categorization, phenotyping for gene expression and number of KIR+ cells, and allelotyping for functional strength.

The first level of KIR diversity is gene content (22, 23). Only approximately 5% of people have all 15 gene family members; others lack one or more genes. The diversity of gene content is based primarily on diversity in B haplotypes, which typically contain more activating KIRs. The two hallmark genes for A haplotypes are KIR2DL3 and KIR3DL1. They are segregated with KIR2DL2 and KIR3DS1 as alleles in the centromeric (Cen) and telomeric (Tel) motifs, respectively (Fig. 4A). On the basis of this pattern, a simplified typing and scoring method for B haplotype content can be derived (Fig. 4B), viz., KIR2DL3+/KIR2DL2-, Cen-A/A; KIR2DL3+/KIR2DL2-, Cen-A/B; KIR2DL3+/KIR2DL2-, Cen-B/B; KIR3DL1+/KIR3DS1-, Tel-A/A; KIR3DL1+/KIR3DS1-, Tel-B/B; and KIR3DL1+/KIR3DS1-, Tel-B/B. Because KIRs are encoded in chromosome 19 and ligand HLAs are in chromosome 6, these gene families are segregated independently; thus, HLA genotypes cannot be used to predict KIR content, and autologous KIR-HLA mismatch is possible.

The second level of diversity is gene expression (24). By real-time PCR and flow cytometry, more than 10-fold variability in expression has been observed among donors. Nowadays, the easiest method to quantify NK cells expressing only one inhibitory KIR gene (i.e., negative for all other MHC inhibitory receptors) is multicolor flow cytometry (24–26). The number of single KIR+ cells is in direct proportion to activity against target cells without the ligand (receptor–ligand mismatched). Although the number of single KIR+ cells correlates with the presence of self-ligand (25), the variability among donors renders predictions based on the ligand alone unacceptable for clinical use.

The third level of diversity is allele polymorphism, which has been observed in all inhibitory KIR genes (22, 23). For example, in the KIR2DL1 family, 25 alleles have been observed, and each has different strengths in inhibiting NK cells and different durability of surface expression after interaction with ligands (27). These differences correlate with the intensity of inhibitory signaling through SHP2 and β arrestin-2, resulting in differences in immune synapse formation. Mechanistically, the arginine residue at position 245 in the transmembrane domain is important in inhibition. Alleles with arginine in position 245 are stronger than those with cysteine in that position (27). On the basis of this molecular determinant, single-nucleotide polymorphism assays have been developed for rapid, high-throughput clinical typing (28). The same assay can be simultaneously used for KIR ligand typing. Notably, this assay requires less than 0.1 μg of DNA and can be performed within 1 day. Using this assay, investigations showed that patients who received a donor graft containing the stronger KIR allele had...
fewer relapses, better survival, and less transplant-related mortality (29). These effects were observed regardless of primary disease [acute myelogenous leukemia (AML) vs. acute lymphoblastic leukemia (ALL)], total body irradiation, T-cell depletion, and donor type. Statistically, there is an interaction effect with HLA-C receptor–ligand mismatch:

Figure 1. Surface receptors and their ligands. Cytokine receptors are shown on top of a human NK cell. Other receptors are broadly classified and color-coded on the basis of their primary function (inhibitory receptors in red, activating receptors in green, inhibitory coreceptors in red-black stripes, and activating coreceptors in green-black stripes). Their ligands are shown within parentheses. Many other known receptors are not shown, including chemotactic receptors (CCR-2, -5, -7, CXCR-1, -3, -4, -6, CX3CR1; and Chem23R), adhesion receptors (CD2 and β1 and β2 integrins), and activating coreceptors (CD96, CS1, and TLR).

Figure 2. Dynamic equilibrium. After cell-to-cell contact, NK cell integrates signals from its surface receptors in seconds, resulting in either target-cell attack or no response and continual immunosurveillance of other cells. A, healthy cells express normal amount of MHC class I ligands with no activating “stress” ligands. B, downregulation or absence of MHC ligand for cognate inhibitory receptors is insufficient to trigger NK cells. This happens in the physiologic setting with red blood cells and autologous KIR receptor–ligand mismatch cells, and in pathologic conditions with adult lymphoblastic leukemia. C, sufficient activating ligands must be expressed on target cells to induce NK cell activity. D, if self-MHC ligands are expressed in normal amount, the reactivity of the NK cell is ultimately dependent on the balance of activating and inhibitory signals. Successful NK cell therapy relies on clinical strategies that optimize activation and avoid inhibition by cancer cells.
Patients with the best survival were those with a stronger KIR allele and receptor–ligand mismatch from donors. The second-best group was those with a stronger KIR allele but no receptor–ligand mismatch. The worst survival was in those with the weaker allele, regardless of mismatch.

Donor KIR typing is not necessary in some KIR mismatch models (Fig. 4C). The first model was proposed by the Perugia group (7); the ligand mismatch model requires typing HLA in both donor and recipient. A donor is mismatched if a ligand is present in the donor but absent in the recipient. This ligand mismatch model does not require donor or recipient KIR typing. The second model was proposed by the Nantes group (30); in this model, KIR is typed in both donor and recipient. If a receptor is present in the donor but absent in the recipient, the donor is mismatched. Because B haplotypes contain more genes than A haplotypes, mismatch typically signifies a B haplotype in the donor. The third model is the receptor–ligand mismatch model proposed by the Memphis group (31). This model requires donor KIR typing and recipient HLA typing. A donor is mismatched if an inhibitory receptor is present in the donor but the ligand is absent in the recipient. Notably, the receptor–ligand mismatch model is the only model applicable to autologous transplantation and antibody therapy; studies have shown that the model is useful for predicting relapse in both therapeutic settings (32–34). Not all potential donors require all three levels of KIR typing (Fig. 4D).
**A**

Centromeric (Cen A) and Telomeric (Tel A) regions showing KIR2DL and KIR3DL alleles.

**B**

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**C**

<table>
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<th>Mismatch model</th>
<th>Relationship</th>
<th>Test ordered</th>
<th>Definition of mismatch</th>
<th>Example</th>
<th>Applicable to autologous cell therapy</th>
<th>Applicable to antibody therapy</th>
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<td>Ligand mismatch</td>
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<td>Donor HLA recipient HLA</td>
<td>Missing KIRL in a recipient for a KIRL that is present in donor</td>
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<td>No</td>
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<td>Receptor mismatch</td>
<td>Donor KIR vs. recipient KIR</td>
<td>Donor KIR recipient KIR</td>
<td>Missing KIR in a recipient for a KIR that is present in donor</td>
<td>Donor: C2, Recipient: C2</td>
<td>No</td>
<td>No</td>
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<td>Receptor-ligand mismatch</td>
<td>Donor KIR vs. recipient KIRL</td>
<td>Donor KIR recipient HLA</td>
<td>Missing KIRL in a recipient for a KIR that is present in donor</td>
<td>Donor: C2, Recipient: C2</td>
<td>Yes</td>
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**D**

- Donor KIR2DL1 allelotyping: C245 R245
- Recipient HLA typing: BW^4 C^1 C^2
- Donor KIR genotyping: Select donor with highest B score
- Donor KIR phenotyping by flow cytometry: Receptor-ligand mismatch
- Select donor with largest amount of receptor-ligand mismatched cells

Total B score = Centromeric B score + Telomeric B score = (_____)

*Total B scores range from 0–4.*
Step 2: NK Cell Processing and Quality Assurance

NK cell purification

The second step for therapy is NK cell processing and preinfusion quality assessment, including cell count, viability, sterility, phenotype, function, and purity (35). Studies in the 1980s with autologous LAK cells consisted primarily of expanded polyclonal T cells with low NK percentages (36). The easiest way to select highly purified NK cells is immunomagnetic cell separation (37). After separation, the product typically contains more than 90% NK cells with very few T cells, B cells, and monocytes. Thus, the risks of GVHD, regulatory T cell (Treg) suppression, cytokine competition, Epstein-Barr virus–lymphoproliferative disease, passenger lymphocyte syndrome, cytokine storm, and suppression by myeloid suppressor cells or blood DCs are minimized (38–40). The NK phenotype is unaltered, and cells retain extensive proliferative capacity in vivo with potent antitumor response (41). Importantly, purified NK cells can be infused in small volumes, and studies have shown that purified cells are safe, do not cause GVHD, and are detectable in the recipient’s blood for approximately 2 to 4 weeks after infusion with preferential expansion of KIR-mismatched NK cells (42).

NK cell bioprocessing ex vivo

Donor NK cells can be manipulated ex vivo using a combination of strategies to optimize efficacy and specificity (Fig. 5). One challenge has been NK exhaustion, as adoptively transferred cells rapidly lose activating receptor expression and IFNγ production capability through downregulation of transcription factors T-bet and eomesodermin (43). Furthermore, a highly acidic tumor environment may render NK cells nonfunctional (44). A common strategy to prime and activate cells is using soluble factors such as IL-2, -12, -15, -18, and -21 or type I IFN (45). Studies have shown that IFNα and IL2 synergistically augment NK cells against solid tumors. Similarly, IL2 with either IL2 or IL18 can be used to overcome resistance (31). In cytokine-based culture systems, massive expansion of mature NK cells is possible. In a study of 7 patients with newly diagnosed, untreated multiple myeloma, the number of NK cells expanded on average by 1,600-fold after 20 days of culture with cytokine competition, Epstein-Barr virus–lymphoproliferative disease, passenger lymphocyte syndrome, cytokine storm, and suppression by myeloid suppressor cells or blood DCs are minimized (38–40). The NK phenotype is unaltered, and cells retain extensive proliferative capacity in vivo with potent antitumor response (41). Importantly, purified NK cells can be infused in small volumes, and studies have shown that purified cells are safe, do not cause GVHD, and are detectable in the recipient’s blood for approximately 2 to 4 weeks after infusion with preferential expansion of KIR-mismatched NK cells (42).

Another way to increase NK potency is using chimeric antigen receptor (CAR). Receptor insertion can be mediated using retroviral, lentiviral, mRNA, or Sleeping Beauty transposon/transposase systems (49–51). Investigations have shown that NK cells transduced with anti-CD19 CAR or NKG2D are potent against B-lineage and osteosarcoma cells in vivo and in vitro (49, 52). CAR+ cells have been generated against GD2 in neuroblastoma and CD33/CD123 in myeloid leukemia (51, 53, 54). Clinical trials are ongoing, including NCT00995137 and NCT01974479, for B-lineage hematologic malignancies.

For centers without gene modification capabilities, NK cells could be activated by culturing in the presence of unmodified CD56+ cells and stimulating cytokines. Recently, investigators showed that this method expanded and activated NK cells from donors and patients with neuroblastoma (55). These cells have great efficacy toward neuroblastoma in vitro and in vivo through NCR, DNAM-1, perforin, and granzyme B without risk of GVHD.

One obstacle in NK therapy is insufficient homing to tumor sites. Recently, investigators used CCR7+ cells to transfer CCR7+ cells onto NK cells via trogocytosis, resulting in better homing of NK cells to the tumor site in a mouse model and in Transwell migration experiments (56). Another way to facilitate adhesion of NK cells to tumor cells is using immunocytokines such as antibody against GD2 conjugated to IL2 (57). This chimeric protein attaches to GD2 on neuroblastoma cells on one side and to IL2 receptor on NK cells on the other.

In addition to mature NK cells, human embryonic stem cells (hESC), induced pluripotent stem cells, and NK leukemia cell lines may be used as starting cells (58–60). hESC-derived NK cells can carry the CD94+CD117low phenotype, which has potent antitumor activity (58). Cytokine-based culture systems, in particular, enhance the expansion of NKG2D/NCR+ NK cells from umbilical cord blood (59).

Because of the technical difficulty in producing cellular products at treatment centers, the National Heart, Lung, and Blood Institute (NHLBI; Bethesda, MD) sponsored the Production Assistance for Cellular Therapies program (PACT; ref. 61). Using this approach, apheresis cells have been sent to remote processing centers and purified, and activated NK cells were sent to the transplant centers for infusion into patients (62).

Step 3: NK Augmentation In Vivo

Combination with therapeutic antibody or chimeric proteins

One mechanism of antibodies in cancer therapy is ADCC. NK cells carry high-affinity Fc receptors that mediate this function. Conceptually, to overcome resistance, it would be

Figure 4. KIR haplotype map, B scoring, mismatch model, and typing algorithm. A, simplified genomic maps of A and B haplotypes. KIR genes that are present in some but not all cen-B motifs are color-coded in green and the two loci that are used for simplified B-scoring are in red. B, calculation of total B scores. C, all three mismatch models are biologically sound but are fundamentally different in principles and in clinical usages, including the relationship in consideration, the blood tests required, the definition of mismatches, and the applicability to antibody therapy and autologous settings. D, donor typing and selection algorithms.
attractive to administer NK therapy concurrently with antibodies such as those against CD19, CD20, CD22, CD33, CD123, HER2, EGFR, and GD2 (63, 64). Typing for recipient and donor FcgRIII 158V/F polymorphism is important in optimizing ADCC (65). Stimulating NK cells with an agonistic monoclonal antibody specific for CD137 or with blocking antibodies against KIR/NKG2A may increase cell killing (66–68). Thus, using a second antibody that activates donor NK cells may improve the efficacy of antibodies against tumor-associated antigens.

Another method to activate CD16 is a bispecific or tri-specific killer cell engager (BiKE or TriKE). A fully humanized CD16-CD33 BiKE has been created that strongly activates NK cells against CD33⁺ AML blasts (69). Pretreatment with ADAM17 inhibitor prevented CD16 shedding and overcame inhibition of class I MHC-recognizing inhibitory receptors. Incubating cancer cells with BiKE and TriKE increased NK cytolytic activity against tumor targets and production of IFNγ, TNFα, GM-CSF, IL8, MIP-1α, and RANTES (70).

Combination with supplemental medication
After NK infusion, many regimens include low-dose IL2 injections. Newer agents are being investigated. Lenalidomide enhances ADCC in rituximab treatment of non-Hodgkin lymphoma and B-cell chronic lymphocytic leukemia through enhanced granzyme B and FasL expression (71). The effects of lenalidomide are partly related to CD4⁺ T-cell production of IL2 (72). Imatinib triggers DC-mediated NK activation (73), whereas dasatinib enhances NK expansion via eomesodermin (74). Ligation of CD86 on NK cells with CTLA4-Ig fusion protein or blockade of A2A adenosine receptor interaction with CD73 increases tumor killing (75, 76).

During NK therapy, concurrent suppressive medications should be avoided, including agents such as azacytidine and sorafenib, which substantially impair PI3K and ERK phosphorylation and hamper NK reactivity (77, 78). Sunitinib does not inhibit NK cells, and decitabine may augment NK reactivity (78).
Combination with tumor cell modulation

After CD16, NKG2D is the second most potent activating receptor expressed on NK cells. Unfortunately, NKG2D ligand expression in most cancer cells is low (79). Recently, various methods have been used to upregulate NKG2D ligand expression or prevent its shedding from the cell surface, including epigenetic modulation with histone deacetylase (HDAC) inhibitors (which may induce glycogen synthase-3 activity; ref. 80), proteosome inhibitors, and demethylating agents. These agents may also work together to induce expression of Fas and TRAIL-R2 (DR5; refs. 81, 82). Recently, high-throughput screening of 5,600 bioactive compounds revealed spironolactone as a novel RXRγ agonist that activates the ATM–CHK2 pathway to upregulate transcription of all classes of NKG2D ligands for cancer prevention and control (83). In another study using a lentiviral shRNA library targeting more than 10,000 human genes, silencing of JAK1 and JAK2 increased bioactive compounds revealed spironolactone as a novel RXRγ agonist that activates the ATM–CHK2 pathway to upregulate transcription of all classes of NKG2D ligands for cancer prevention and control (83). In another study using a lentiviral shRNA library targeting more than 10,000 human genes, silencing of JAK1 and JAK2 increased the susceptibility of a variety of tumor cell types to NK-mediated lysis (84).

Clinical Considerations

NK cell therapy is applicable to various forms of cancer (85, 86), but its current use has been focused primarily in hematologic malignancies (20, 63, 87, 88). NK cells can be used for patients with refractory leukemia and can be given before conventional HCT for induction of remission, after HCT as consolidation, or in place of HCT.

In an early feasibility study, IL2-stimulated NK cells were infused post-HCT into 3 pediatric patients who had persistent leukemia blasts at the time of transplant (89). No significant toxicity was observed, and complete remission and complete donor chimerism were observed within 1 month after HCT. In a subsequent prospective phase II study, preemptive immunotherapy with purified NK cells after haploidentical HCT was investigated in 16 patients (90). The patients received 29 NK infusions 3, 40, and 100 days after transplantation. The median dose of NK cells per product was $1.21 \times 10^7$/kg. With a median follow-up of 5.8 years, 4 of the 16 patients with high-risk leukemia remained alive.

In 10 patients with advanced multiple myeloma receiving autologous HCT, haploidentical T cell–depleted, KIR ligand–mismatched NK cells were infused (91). No GVHD or failure of autologous stem cell reconstitution was observed. Encouragingly, 50% of the patients achieved near complete remission. These results set the stage for future studies of KIR ligand–mismatched NK therapy in the autologous setting.

NK therapy without HCT

For allogeneic NK therapy in a nontransplant setting, transient suppression of the host immune system is required to allow time for donor-derived NK expansion and therapeutic effect. Even in the autologous setting, induction of transient leukopenia may promote homogeneous expansion of NK cells and reduce suppressive effects from Tregs and myeloid suppressor cells (32, 33, 92). One common outpatient regimen includes fludarabine and low-dose cyclophosphamide, and this regimen is well tolerated even in children, with 10 of 10 patients surviving free of leukemia (42). The rare occurrence of complications results in the cost of fresh NK therapy being less than 10% of that for conventional HCT. Infusions of purified NK cells were feasible after similar conditioning in 13 elderly patients including septuagenarians (93).

In a study of haploidentical related-donor NK infusion, a high-intensity inpatient regimen of high-dose cyclophosphamide and fludarabine resulted in a marked increase in endogenous IL15, expansion of donor NK cells, and induction of complete hematologic remission in 5 of 19 patients with poor-prognosis AML (94). In a subsequent phase II study for recurrent ovarian and breast cancer, 20 patients underwent the regimen of fludarabine and cyclophosphamide with or without low-dose total body irradiation (95). After cell infusion, IL2 was administered subcutaneously for 2 weeks. With a mean NK cell dose of $2.16 \times 10^7$/cells/kg, 9 of 13 (69%) patients without irradiation and 6 of 7 with irradiation had donor DNA detectable after NK cell infusions. In another study of 6 patients with advanced B-cell non-Hodgkin lymphoma, 4 patients showed an objective clinical response (96). Unfortunately, Tregs also expanded in vivo in these two studies; therefore, future strategies should include novel techniques to suppress the expansion of Tregs.

Conclusions

In summary, NK cells are promising in cancer therapy, considering their speed of action, potency in cancer cell killing, applicability to many types of cancer, lack of adverse effects, ease of preparation and administration, availability, permissibility of donor selection, affordability, and complimentary actions with other therapies. The unique properties of NK cells open a new arena of novel cell-based immunotherapy against cancers that are resistant to contemporary therapies.

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