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REVIEW

Cellular therapy of cancer with natural killer cells—where do we stand?

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Abstract

Although T-lymphocytes have received most of the attention in immunotherapy trials, new discoveries around natural killer (NK) cells suggest that they also should be suitable effector cells for cellular therapy of cancer. In addition to direct cytotoxicity, NK cells produce an array of immune-active cytokines, among them interferons and granulocyte-macrophage colony-stimulating factor, which places them at the crossroads of innate and adaptive immunity. They also augment monoclonal antibody activity through antibody-mediated cellular cytotoxicity and can be transfected with chimeric antigen receptors. One of the stumbling blocks for NK cell–based therapies has been the inability to predictably obtain and expand larger numbers from donors, but also to achieve sufficiently high transfection efficiency of target genes. The first clinical trials with NK cells suggest some benefit, but more definite evidence is needed to justify this relatively expensive treatment.

Cellular therapy is still the stepchild of medical therapeutics for cancer. Small molecules and monoclonal antibodies are dominating the space and biotech companies and big pharma are only slowly becoming interested in this treatment modality. Although approval by the Food and Drug Administration (FDA) of Dendreon's prostate cancer vaccine (Provenge, Seattle, WA, USA) provided a welcomed boost for cell-based therapies, the subsequent difficulties in getting it accepted in the medical oncology community as well as the surrounding reimbursement issues have not helped to create more momentum and enthusiasm for biological and cellular therapies (1). Cellular preparations are also heavily regulated and require highly specialized laboratories to prepare them. If not prepared "in house," which is the exception, the cells must be shipped to the point of service, where additional quality and release testing must be performed before they can be infused into a patient. Mesenchymal stromal cells are probably the most advanced in this regard because they can be expanded without the addition of cytokines, can be frozen for storage and shipping and can be given to patients without consideration of major histocompatibility (MHC) barriers (2,3). T cells are more problematic because they can cause graft-versus-host disease (GvHD) even if they come from a donor that is fully MHC-matched with the recipient (4,5). The patient's own (autologous) cytotoxic cells, on the other hand, are generally dysfunctional and unable to form proper immunological synapses with the malignant clone (6,7). Only when they are genetically engineered to express receptors that recognize specific tumor antigens, tumor-induced negative signals appear to be overcome (8–10).

Natural killer (NK) cells—defined as CD56+ and CD3− lymphocytes[1]—have gained some traction recently as we have learned to better understand how they encounter and lyse malignant target cells. Several receptor-ligand interactions have been identified, both activating and inhibitory (11–13). The killer cell immunoglobulin-like receptors (KIR) appear to dominate the receptor field. They send inhibitory signals to the inside of the NK cell when they encounter "self" MHC antigens. Hence, if tumor cells have lost or mutated MHC expression, they theoretically should be recognized by autologous NK cells; however, it is more complex. Among many lesser known factors, the tumor cells also must express ligands for activating NK cell receptors on their surface, which is not always the case (11–13).

[1]The CD56+/CD3+ cells are termed cytokine-induced killer cells that represent T cells with NK cell properties that can be expanded *in vitro* in the presence of interleukin-2 (rhIL-2) starting from peripheral blood mononuclear cells stimulated by anti-CD3 antibody. Because of their different biology, they are not included in this review.

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Recent data suggest that even in the "non-self" situation, NK cells may need some "licensing" before they can be fully activated (14–16). According to the licensing hypothesis, an NK cell that does not encounter a ligand for one of its KIR receptors during development is functionally deficient. There are other obstacles that apply to all types of cellular therapies: the cells must get to the tumor site and be able to leave the blood circulation and penetrate potentially fibrotic and necrotic tissue. Even if they are in close proximity to the tumor, molecules secreted by the tumor can downregulate transcription factors and "exhaust" the cells before they can kill the tumor cell (17). It is also known that the immediate tumor environment is acidic, which can "paralyze" NK cells (18).

The challenges of NK cell therapy

One of the major challenges in developing NK cell–based therapies is to consistently obtain sufficent numbers of cells for patient treatment—only approximately 10% of all lymphocytes in the blood are NK cells. Because infusions of autologous NK cells, even with concomitant administration of cytokines such as interleukin (IL)-2, are clinically ineffective (19–21), the focus has shifted to allogeneic NK cells obtained either from a relative or unrelated donor. Usually an apheresis is the starting point to obtain mononuclear cells. The majority of those peripheral blood mononuclear cells (PBMC), though, represent T cells that must be removed to prevent GvHD in the recipient. This can usually be achieved by negative CD3 depletion through the use of immunomagnetic antibody tagging with subsequent absorption of the cells over a magnetic column (ie, the Miltenyi CliniMACS system, Bergisch-Gladbach, Germany) (22,23).

The one-step depletion of CD3+ cells results in approximately 20–40% enrichment of CD56+ cells, depending on the donor. If this is followed by a positive selection for CD56+ cells, generally a purity of >90% of NK cells can be obtained (24). However, this two-step depletion/selection process can result in significant cell loss, and some concern has been raised that the anti-tumor effect with a highly enriched CD56+ cell product may be compromised. NK cells also appear to need the presence of accessory cells such as monocytes to become optimally activated (25). On the other hand, the additional CD56+ enrichment has the advantage of removing B-lymphocytes that are still present in a product after CD3+ depletion. Those residual B cells can potentially cause problems. A passenger lymphocyte syndrome with hemolysis and an Epstein-Barr virus (EBV)–driven B-cell lymphoma have been reported (23,26). Those complications can also be prevented by depletion of CD19+ B cells, a procedure that, however, requires a second immunomagnetic column that adds to the costs of the cell separation. Alternatively, those B-cell–driven complications can be prevented or treated by monoclonal antibodies (ie, rituximab), although this potentially leads to other complications related to a long-lasting B-cell deficiency (27).

Expanding NK cells to obtain sufficient numbers for clinical use

Because it is currently unresolved how many NK cells are necessary to obtain an anti-tumor response in patients, investigators generally assume that NK cell–based immunotherapy will be most efficacious when the number of infused cells can produce a high effector to target (E:T) ratio. Various methods of NK cell expansion have beeg explored (22). When evaluating clinical protocols for NK cell infusions, only those should be considered conclusive that contain a high percentage of NK cells to ensure that toxicities and efficacy are truly caused by NK cells. Another important quality control assay before release is the cytotoxicity against K562 (or any other NK cell–sensitive target cell line) at a given E:T ratio (ie, 20:1 with at least 20% killing) to avoid infusing NK cells that are just expanded to large numbers but have no functional activity.

Sutlu *et al.* (28) placed unseparated PBMC in different expansion devices (flasks, bags, Wave bioreactor) for 3 weeks, after which the lymphoid cell population had expanded a median 770-fold in biorectors and 77-fold in flasks. Although this system obviates the need to isolate NK cells before initiating the culture, the expanded cells consisted predominanly of CD3+/CD56− T cells and CD3+/CD56+ NK-like T cells. To enrich for NK cells, T depletion is required, an expensive procedure because of the large number of T cells that expanded.

Other centers have reported expansion numbers of 190-fold in healthy individuals (29) and even 1600-fold after a 3-week culture in IL-2 and anti-CD3 stimulation for cells from patients with myeloma (30). As impressive as these numbers are, the NK cells probably have insufficient *in vivo* cytototoxicity, because their killing of K562 at the end of the culture period was <10% at 1:1 E:T ratio. The anti-CD3 antibody in those expansions induces a cytokine release from T cells that further supports NK cell expansion. However, for allogeneic NK cell infusions, these cultures must be T-cell depleted to prevent GvHD. Such a manipulation after expansion not only results in some cell loss but is also more costly because of the higher T-cell number requiring additional immunomagnetic separation.

The group at St Jude's, Memphis, TN, USA (31), has developed a clincal-grade expansion system whereby the upfront CD3-depleted PBMC are expanded in the presence of (irradiated) K562 cells

as a feeder layer that had been transduced with genes for the adhesion molecule 4-1BB-ligand and for IL-15 (termed K562-mb15-41BBL, for which a master cell bank has been established). Although this method seems to be reasonably effective for blood NK cell expansion, there is significant donor-to-donor variation as to the yield of NK cells. Furthermore, the procedure also requires removal of T cells either upfront or after 1 week in culture to prevent their overgrowth in the culture system. Before the expanded NK cells can be infused into patients, tests also must confirm that they do not contain any viable K562 tumor cells.

Other *ex vivo* expansion techniques for NK cells use a third-party irradiated EBV transformed lymphoblastic cell line as feeder layer (32). Starting with an apheresis product, T cells are removed and the product is selected for NK cells, which are then frozen in aliquots to be used for expansion when needed. The investigators report an approximately 180-fold expansion of NK cells over a 2-week culture. The relevant aspect of this study is that it showed that NK cells from a cryopreserved "stock" of selected NK cells can be expanded on demand. Unfortunately, the double upfront manipulation step will make this expansion technology expensive—each column costing approximately $6000 plus labor.

NK cells from umbilical cord blood have sparked some interest because they are more readily available without any donor risks and inconvenience. Frozen cord blood cells can serve as the starting material and after thawing are depleted of CD3+ cells if this has not already been done before cryopreservation. Because of the immaturity of the NK cells in cord blood, they must be exposed to a cocktail of cytokines, and a culture time of several weeks is usually required to arrive at acceptable NK cell numbers. Our group has used a combination of IL-2, IL-15, Flt-3L and IL-3 on positively selected cord blood NK cells that were placed on a feeder layer of mesenchymal stromal cells (33). Although expansion of NK cells was at median 60-fold, the low starting number in any given cord blood unit would make it necessary to pool several units if used for treatment.

Another expansion protocol for cord blood NK cells resulted in the generation of approximately 5×10^9 CD56+CD3− NK cells, which is roughly the number of NK cells that can be obtained by one leukapheresis from peripheral blood without expansion (34). In addition to an initial CD34+ selection, the expansion process also required a total of eight different cytokines over a culture period of 6 weeks. Unfortunately, most of the cytokines are not available in Good Manufacturing Practice (GMP) grade quality for use in humans. Together with the length of culture, it will be difficult to see this or similar cord blood expansion protocols implemented clinically.

Ex vivo expansion of peripheral blood or cord blood−derived NK cells is not only cumbersome and costly, but it also restricts the process to specialized laboratories that are compliant with GMP. The *ex vivo* expansion can either be performed in gas-permeable bags, in flasks or in bioreactors that expand larger cell numbers on a smaller footprint. From a cost perspective, any manipulation such as T-cell depletion ought to be performed before culture expansion because their numbers will go up substantially, adding material costs and staff time to the procedure. The bag sytem allows work in a closed system, applying various sterile connections and bags for transfer. Milteny's Prodigy (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) is a new device awaiting approval in the United States that has cell expansion automated in a closed system with constant medium and cytokine feed as per program. The G-Rex biorecator (Wilson Wolf Manufacturing, New Brighton, MN, USA) expands NK cells well and medium change is simplified as the cells collect at the bottom of the flask (35).

NK cells are known to lose their replicative potential after 10−15 population doublings and die (36). There is also some concern that long-term cultured NK cells will acquire genetic abnormalities, both of which are good reasons to collect fresh cells from different donors. In addition to obtaining sufficient and predictable numbers of NK cells for treatment, there are additional challenges: *ex vivo* stimulation of NK cells with cytokines can lead to their apoptosis when they are removed from the ongoing stimulation, and hence the cells may not persist or even expand *in vivo* (37). Moreover, cultured cells can assume a different shape, and it is unclear how this affects their migration to the cancer site. In general, we do not understand the homing pattern of infused NK cells. Murine data indicate that after intravenous injection, they home, not unexpectedly, first to the lung, from which they then disappear into the circulation. They can be found in small numbers in spleen, liver and bone marrow (Klingemann, unpublished observation).

Although the life span of blood NK cells can be prolonged after transfection with the gene for telomerase reverse transcriptase (36), it has not been possible to immortilize NK cells suitable for treatment. Largely experimental at this point is the generation of NK cells from embryonic stem cells or induced pluripotent stem cells (38). As an alternative to blood NK cells, clonal NK cell lines with the ability to lyse a wide spectrum of malignant target cells can be considered (39,40). Most of them, however, have low and inconsistent cytotoxicity against cancer targets. The only cell line with

predictable high anti-tumor cytotoxicty and good expansion kinetics (in bags and bioreactors) is NK-92 (Neukoplast, Conkwest, San Diego, CA, USA), which has been well charcterized in *in vitro* and severe combined immunodeficiency (SCID) mouse xenotransplant assays to have broad anti-tumor activity (41–45). A master cell bank has been established, and NK-92 has finished two phase I studies in patients with advanced cancers (46–48). A third trial at Princess Margeret Hospital, Toronto, Canada, in lymphoid malignancies is close to completion (A. Keating, personal communication).

Because *ex vivo* expansion of peripheral and cord blood NK cells is technically challenging, could NK cell numbers be increased in the recipient? Not much is known, but some results indicate that expansion of NK cells in the recipient may correlate with IL-15 serum levels (49), which, in contrast to IL-2, does not appear to support the emergence of T-regulatory cells that potentially can suppress NK cell function (50). However, infusions of IL-15 into macaques did not increase the number of adoptively transferred, *ex vivo*–expanded NK cells (51). In those animals, the IL-15 adiministration was also associated with a significant expansion of T-regulatory cells.

If allogeneic NK cells are infused, recipients require pre-infusion immunosuppression for several reasons: a relatively intact immune system of the recipient will reject the MHC-incompatible cells. Second, NK cells also must expand in the host to have some benefit, which requires the presence of cytokines. The patient's own immune cells compete with cytokines, a phenomenon that is known as "cytokine sink" (52). There is a third reason why the immune system of the recipient must be knocked down. The emergence and presence of T-regulatory cells can silence NK cells (50).

An effective and widely used combination of drugs given before cytotoxic cell infusion is cyclophosphamide and fludarabine. After receiving this regimen, donor NK cells can be detected in the recipient for approximately 1 week after infusion (49). NK cells may persist longer when low-gade (ie, 200 cGy) total body radiation is added to the preparative regimen (53).

There are a few reports that NK cell numbers and their function *in vivo* can be affected by certain drugs. Aside from needing confirmation in clinical trials, these observations alert us to the some possible interactions: Desatinib can increase NK cell numbers *in vitro* and *in vivo* (54). Imatinib can activate NK cell function through dendritic cells (55). NK cells expanded in the presence of the histone deacetylase inhibitor depsipeptide display enhanced expression of the activating receptor NKG2D as well as TRAIL receptor, which, in addition to the perforin/granzyme system, can cause tumor cell apoptosis (56). Trail receptors on NK cells can also be upregulated by bortezomib (57). Inhibition of JAK1 and JAK2 can increase the susceptibility of tumors to NK cell killing (58). Lenalidomide also increases NK cell function, an effect that is abrogated by concurrent dexamethasone treatment (59,60). Some studies also focus on making cancer cells more sensitive to killing by improving synapse formation between NK cells and tumor cells (6). On the other hand, NK cell function is inhibited by Sorafenib as a consequence of impaired phosphorylation of PI3K and ERK, which directly control NK cell reactivity (61).

Engineering NK cells to target tumors

Autologous T-lymphocytes have been successfully engineered to make them cytotoxic to specific tumor-associated antigens (8–10,62–64). Introducing such chimeric antigen receptors (CARs) can override any inhibitor signals on lymphocytes. CARs consist of a single-chain Fv antibody fragment that is fused through a flexible hinge region to the CD3 zeta chain as the cytoplasmatic signaling moiety that can activate the cytolytic machinery of the cytotoxic cells. Several phase I trials with CAR-transfected T cells have been completed (62,63). First-generation CARs contained some murine components in the Fv region that triggered an immune response against the transfected cells, and humanized CARs are now preferred. Most CARs also include co-stimulatory molecules (ie, CD28, OX40 and 4-1BB) incorporated into their endodomain, which improves activation and expansion of T cells. These "third-generation CARs," however, can potentially cause an overshooting immune activation with cytokine release. In fact, two cases of fatal side effects have occurred after infusion of CAR-engineered T cells believed to be caused by a cross-reaction against antigens on nontarget tissues and subsequent massive release of cytokines (65,66). This "on-target but off-organ" reaction can occur when normal body cells also express the tumor-associated antigen.

Blood derived NK cells are clearly lagging behind T cells in the scientific and translational development of CAR engineering for two reasons: (i) the inability to generate predictably sufficient numbers for treatment and (ii) the challenge of achieving consistently high transfection efficiency of the target gene. Another reason is that NK cells are believed to be short-lived with no "memory." It has been suggested that disease control after CAR T-cell treatment correlated with persistence of memory T cells (8). However, some recent data indicate that NK cells may be able to mount a memory response and are able to initiate adaptive (T-cell) immunity through a "cross-talk" with dendritic cells (67,68).

To introduce CARs into NK cells, retrovirus- or lentivirus-based vectors can be used. The transfection efficiency of fresh or expanded blood NK cells is approximately 30–60% for retrovirus (69,70) and 20–60% for lentivirus (71). To avoid the potential risk of insertional mutagenesis and to ease the regulatory process, electroporation of plasmid DNA or messenger RNA (mRNA) are being explored. Electroporation is harsh on cells, and transfection of plasmid DNA into blood NK cells affects their viability, resulting in <5% transfection efficiency (72). Even electroporation of plasmid DNA into the NK-92 cell line resulted in high cell death rate. However, since NK-92 are IL-2 dependent, a bicistronic vector with an IL-2 gene allows for selection of surviving cells with subsequent expansion.

Because mRNA must only get into the cytoplasma, this results in higher transfection rate, although protein expression usually is limited to a few days. Transfection of mRNA coding for specific CARs has been tested *in vitro* for enriched fresh and culture expanded NK cells with a transfection efficiency of 30–60% (73–75). Despite the rather favorable transfection rate, NK cell numbers decreased to approximately 50% of the input cells 24 h after electroporation. Similar transfection efficiency was seen with NK-92 cells transfected with mRNA for anti-CD19 CAR with the use of the Bio-Rad (Waltham, MA, USA) electroporator (74). Compared with blood NK cells, NK-92 maintained good viability (>95%), and NK cell–resistant B-lymphoblastic cell lines and primary chronic lymphocytic leukemia cells were readily killed by anti-CD19 CAR mRNA-transfected NK-92.

In adddition to anti-CD19 CAR (74,75), NK-92 cells have been engineered to express CARs for Her-2 neu (76), CD20 (77,78), GD2 (79), EpCAM (80) as well as EBNA (81). For most of these CARs, retrovirus- or lentivirus-based constructs were used. On average, 50% transduction efficiency can be achieved with fresh NK-92 cells. This percentage, though, can be increased to 100% after cell sorting (72).

Another way of targeting NK cells to cancer cells is to combine the infusion of NK cells with monoclonal antibodies (mAbs). NK cells are effector cells for antibody-mediated cellular cytotoxicity (ADCC). The Fc receptor (FcγRIIIa) CD16 on the surface of NK cells captures the Fc portion of the IgG1 mAb that will trigger release of perforin/granzyme fom NK cells. The ADCC pathway is used by many clinically efficacious mAbs such as rituximab, ofatumumab, herceptin and cetuximab (82). In fact, patients with follicular lymphoma and Waldenstrom disease had better clinical responses to rituximab when their NK cells expressed the high-affinity FcγRIII (83,84). Some *in vitro* observations suggest that even autologous NK cells from patients with cancer may be suitable effector cells because they maintain ADCC (21). It will be interesting to see how CAR-directed NK cell therapy will compare with NK cell–augmented ADCC (19).

Clinical trials with NK cells

Autologous NK cells

Early studies by investigators at the National Cancer Institute with lymphokine-activated killer (LAK) cells essentially consisted of infusions of expanded polyclonal T cells only containing a small fraction of NK cells (85). A number of uncontrolled trials reported infusion of selected autologous NK cells usually combined with higher doses of IL-2 (20,21). Although no side effects (except those expected from IL-2) were seen, no clear benefit of the NK cell infusions was noted. The group at the National Heart, Lung and Blood Institute treated patients with various malignancies with escalating doses of autologous expanded NK cells (after CD3 depletion and CD56 enrichment) with the use of EBV-transformed feeder cells (86). Before the infusion, patients received immunosuppression with pentostatin and a single dose of bortezomib to sensitize the patient's tumor cells to TRAIL. After infusion, IL-2 (2 Mill units/m^2) was given twice daily for 1 week. Except for the typical side effects of IL-2 (constitutional symptoms, thyroiditis), the infusions were well tolerated. No clear responses were noted in this phase I study.

In a recent study (19), autologous PBMC were depleted of CD3 cells and expanded on a feeder layer of autologous PBMC in the presence of IL-2. Seven patients with progressive, advanced melanoma or renal cancer received the cells after a lymphodepleting chemotherapy (Cytoxan/Fludarabine), followed by administration of a high dose of recombinant IL-2 (720,000 IU/kg every 8 h). No clinical responses were noted, but the adoptively transferred NK cells persisted in the patients' circulation and were still capable of mediating ADCC with rituximab or anti-HER2/neu antibody. The study is relevant because it shows that even a fairly elaborate treatment protocol involving lymphodepleting chemotherapy, extensive *ex vivo* NK cell expansion and high doses of IL-2 after infusion will not necessarily result in a clinically meaningful response to infusion of unmanipulated autologous NK cells. Also, detecting NK cells in the blood circulation after infusion does not necessarily mean that they provide anti-tumor activity.

Allogeneic NK cells

When it became more widely known that NK cells become activated and kill target antigens when they recognize "non-self," attention turned to the infusion of allogeneic instead of autologous NK cells. In

support of these preclinical observations were those that came from the bone marrow transplant team in Perugia (Italy) that performed MHC haplotype–mismatched stem cell transplants for acute leukemia (87,88). To avoid GvHD, the donor graft was aggressively depleted of T-lymphocytes, but NK cells remained. Results showed that patients who had received KIR-ligand–mismatched NK cells as part of the stem cell transplant for acute myeloid leukemia (AML; but not for acute lymphoblastic leukemia [ALL]), had a lower relapse rate. These observations triggered a flurry of retrospective data analyses to determine whether KIR mismatching contributes to relapse reduction in matched related or unrelated stem cell transplant recipients (89–92). The results are somewhat inconsistent. Although in theory it makes sense to mismatch for KIRs and their ligands, other NK receptors (activating and inhibitory) and ligands/receptors on tumor cells are of relevance and contribute to the delicate balance of activating and inhibitory receptor/ligand interactions. In this context, it remains to be seen how effective the anti-KIR antibody 1-7F9 will be that is currently in clinical trials for myeloma and leukemia (93,94).

A number of clinical trials with infusion of allogeneic NK cells in patients with malignant diseases have shown feasibility with no significant side effects (22). Most of the studies have infused non–culture-expanded, freshly isolated and CD3-depleted leukapheresis collection. Because of the phase I and the uncontrolled design of these studies, conclusions about efficacy are limited. However, some observations are noteworthy:

Miller *et al.* (49) "conditioned" patients with renal cell cancer or leukemia with an "immunoablative" combination of variable doses of cyclophosphamide and fludarabine before the infusion of CD3-depleted lymphocytes from an MHC haplotype–mismatched relative, followed by daily IL-2 injections (1.75×10^6 IU/m^2 for 3 weeks or 10 MU three times per week). The cell product contained between 20–42% CD56+ NK cells. None of the patients with renal cancer had a response, but five of 19 patients with acute myeloid leukemia obtained a remission. Those patients who responded showed persistence of their NK cells in blood for at least 2 weeks, which appears to correlate with the intensity of the preparative regimen—the combination of cyclophosphamide 60 mg/kg × two infusions followed by Fludarabine 25 mg/kg × 5 days being the most effective.

The same group treated 20 patients with recurrent ovarian ($n = 14$) or breast cancer ($n = 6$) with a similar protocol (26). No tumor responses were seen in those patients, and again the infusions were well tolerated except for the occasional rigors and fever. Of note is that two patients had development of a passenger lymphocyte syndrome and a B-lymphocyte–driven lymphoproliferative disease, respectively. Despite the fact that the lymphodepleting chemotherapy given before infusion was augmented with 200 cGy of total body irradiation for a subgroup of seven patients, no increased rate of donor NK cell chimerism or significantly higher levels of IL-15 were observed. All patients had emergence of T-regulatory cells by 2 weeks, which suggests that the preparative regimen is not sufficient to suppress those cells longer-term. In contrast to the study in patients with AML who showed a correlation of a higher number of circulating donor NK cells with response, this correlation was not confimed in this study. It may simply be that AML cells are more susceptible to NK cell cytotoxicity, as has initially been reported by the Perugia group in patients after stem cell transplant (95).

Further supporting the assertion that response to NK cell infusions may be disease-dependent is the study by Rubnitz *et al.* (96), who treated 10 children with good/intermediate prognosis AML after induction chemotherapy with KIR-HLA (histocompatibility leukocyte antigen)–mismatched allogeneic NK cell infusions. The leukapheresis product was T-cell depleted and CD56 selected and infused without further expansion after patients had finished a preparative regimen of fludarabine/cyclophosphamide. No adverse events occurred, and all patients remained in remission for at least 2 years. Although this outcome is encouraging, any conclusions with respect to efficacy are difficult to draw from this trial, considering the small number of patients and a possible selection bias. The center is now conducting a phase II study. Even so, the results of the above studies (Perugia, Minnesota, St Jude's) appear to suggest that allogeneic NK cell infusions could have some effect on AML cells and suggest that KIR-HLA mismatching conveys a benefit.

In an attempt to further decrease the high relapse rate after autologous hematopoietic stem cell transplant, our group—in collaboration with the PACT (Production Assistance for Cellular Therapies) (97) center at the University of Minnesota—infused CD3-depleted, NK-enriched lymphocytes from an MHC haplotype–mismatched relative into recipients early after an autologous stem cell transplant to determine whether such a maneuver is feasible and whether GvHD or marrow suppression would occur (98). Patients did well, with only the occasional mild rigor and fever during the infusion. The same trial also confirmed that cell collection from the donor (Boston), cell processing (Minnesota) and cell infusion into the patient (Boston) can be performed at distant sites without compromising NK cell viability and function (99). The cell product was placed in IL-2 for "in flight activation," and the

cells were washed in Boston before infusion. The processing release criteria for this study called for CD3+ lymphocytes to be $<5 \times 10^5$/kg in this haplotype-mismatched setting, which is higher than stated in the FDA guidelines for transfusion products. However, no GvHD occurred in recipients, which is in line with observations by the Minnesota group in 49 patients who received MHC haplotype−mismatched, NK-enriched lymphocytes after an immunoablative conditioning regimen (24,25). The number of T-lymphocytes infused with those products ranged from 9.79×10^3 to 6.62×10^5 cells/kg (100). The lack of GvHD is reassuring because it is known that fresh cells and products from a related donor increase the risk of tansfusion induced GvHD. Somewhat in contrast is a recent European study (101) in which patients after a haploidentical stem cell transplant were given CD3-depleted, CD56-selected donor lymphocyte infusions. Several patients had GvHD when the CD3 number was $>0.5 \times 10^5$/kg, in particular when the cells were given within the first week after the transplant.

Rizzieri *et al.* (102) infused allogeneic stem cells transplant recipients with NK cell−enriched lymphocytes from the donor who was either a fully HLA-matched or a haploidentical family member. Donor cells were collected by one leukapheresis, selected for NK cells with the use of the CliniMACS device and freshly infused approximately 8 weeks after the tranpslant for up to three infusions in those patients with high-risk disease. The infusate contained $<0.5 \times 10^6$/kg CD3+ cells for the mismatched and 1×10^6/kg in the matched setting. Only one patient each in the matched or mismatched setting had severe GvHD. Because no upfront T-cell depletion was performed, the infusate contained approximately 10% CD56+/CD3+ CIK cells or γ-δ T cells. Again, because of the phase I nature of this study, proof of efficacy may be circumstantial, but the investigators suggest that those patients who had prolonged survival had a notable increase in the recovery of T cells and NK cells.

NK-92 cells have shown anti-tumor effects in a number of human xenotransplant SCID mouse models (42,43). The fact that they lack almost all known KIRs may acount for their broad anti-tumor activity. After extensive preclinical safety testing, NK-92 cells have completed phase I trials in humans (48). As with other cell-based studies, it is difficult to define a "true" maximum tolerated dose, and the highest therapeutic dose is often guided by the technical limitations to grow sufficient numbers of cells in a given time period. In the trial by Arai *et al.* (47), three infusions of $3 \times 10^9/m^2$ NK-92 cells were given 48 h apart to patients with advanced cancers. Additional studies by Tonn *et al.* (48) and in Toronto (A. Keating, ongoing) have confirmed the safety of NK-92. Some tumor control in patients with melanoma, lung cancer and lymphoma was seen. NK-92 cells have been further engineered to secrete IL-2 (103) or to express CARs against known tumor antigens (76−81). They have also been engineered to express a high-affinity FcγRIII receptor (104) to augment Fc-receptor−mediated ADCC of monoclonal antibodies. Because they express the activating receptor NKG2D, NK cells could also be used as effector cells for the new class of bispecific antibodies that link tumor recognition molecules with receptors on cytotoxic effector cells (105).

To get acceptance of NK cell−based cellular therapy, the clinical community must see some convincing trial results. Although AML appears to be the disease with some responsiveness, studies in lung cancer could make use of the "first passage" of NK cells after infusion. It also needs simplified ways of enriching and expanding blood NK cells. In addition to transfections with CARs, NK cells and NK-92 can be used as effector cells to augment monoclonal and bispecific antibody therapy. NK cells hence represent a great opportunity for cell engineereing, and they have the potential to step out of the shadow of T cells.

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