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Use of allogeneic NK cells for cancer immunotherapy

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Abstract

Controversy exists as to the role that the immune system plays in cancer therapy. While the immune system has been proposed to scavenge the body to prevent microscopic transformation from forming cancer, it has been difficult to mount its potential of shrinking established tumors. NK cells are components of the innate immune system. They can recognize targets without prior sensitization, making them ideal candidates to manipulate for therapeutic use against cancer. Initially, autologous NK cells were directed against tumors but it was realized that NK cells that recognize self cells are inhibited. More encouraging advances have been made with allogeneic NK cell therapy in clinical trials to overcome this limitation. In this article, we present developments in NK cell adoptive immunotherapy for hematologic and solid tumor malignancies.

Keywords

adoptive cell therapy; cancer immunotherapy; NK cell biology; NK cells

Basics of NK cells

Harnessing immune cells to treat malignancy has been a major goal over the last decades. Burnet and Thomas were the ones to formally introduce that the immune system can recognize and eliminate spontaneously arising tumor cells thereby protecting the host from cancer, the so-called ‘tumor immune surveillance theory’ [1–3]. The immune system is composed of multiple cell types that have distinct functions and distributions in the body. The cells within the innate portion of the immune system include granulocytes, monocytes/macrophage, dendritic cells and NK cells, whereas T and B cells are cells of the adaptive immune system. Human NK cells are a subset of the innate immune system made up of peripheral blood lymphocytes defined by the expression of CD56 and the absence of the T-cell receptor CD3 [4,5]. In 1975, NK cells were first characterized by two independent groups as large granular lymphocytes that could lyze virally infected and transformed targets without MHC restriction or prior sensitization [6–10]. They are found in multiple tissues, including the spleen, liver, lymph nodes, bone marrow, peripheral blood and comprise 10–15% of the lymphocyte pool in humans. There are two subsets of NK cells that can be distinguished by CD56 surface expression. The $CD56^{bright}$ subset comprises 10% of

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circulating NK cells, is more proliferative and produces more cytokines such as IFN-γ, TNF-β, GM-CSF, IL-10 and IL-13 in response to monokine stimulation [11]. The majority of human NK cells (90%) are of the $CD56^{dim}$ antibody-dependent cell-mediated cytotoxicity (ADCC) and produce negligible amounts of cytokines compared with $CD56^{bright}$ cells [12,13]. Resting $CD56^{dim}$ cells are more cytotoxic against NK-sensitive targets than $CD56^{bright}$ NK cells; however, after activation with IL-2 or IL-12, $CD56^{bright}$ cells show similar cytotoxicity against NK targets as seen with $CD56^{dim}$ cells [14]. The production of chemokines and cytokines by the $CD56^{bright}$ subset is stimulated by cytokine activation in contrast to the $CD56^{dim}$ population where significant production of chemokines and cytokines is triggered by target cell recognition [15]. When NK cells are exposed to cytokines, they show enhanced proliferation [16], augmented cytokine production, higher cytotoxicity against target cells [17], and upregulation of cytotoxic and adhesion molecules [18]. There are several common γ-chain interleukins that can activate NK cells such as IL-2, IL-15, IL-21 and the combination of IL-12 and IL-18 is especially potent to trigger IFN-γ [19–21]. IL-15 has been shown to be important for NK cell homeo-stasis and is most potent when encountered in a physiologic trans-presentation by other cells such as IL-15 receptor-α (IL-15Rα) expressed on dendritic cells [22].

NK cell cytotoxicity

NK cells have the ability to target foreign, damaged, malignant and virally infected cells. Most cytotoxic activity of NK cells is a result of direct killing that is mediated by perforin and granzyme release. Perforin is believed to allow perforation of the target cell membrane allowing apoptosis-inducing granules to enter the cell [23] and activate the caspase system in the intrinsic pathway [24]. Another mechanism of target killing is via interactions between death receptors expressed on target cells and the corresponding ligand expressed on NK cells. Fas–Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL)–TRAIL ligand induce apoptosis by activating caspase 8 and caspase 9 of the extrinsic pathway [23,25,26]. In addition, NK cells can mediate ADCC via CD16 (FcRγIII) [27]. It is now recognized that many of the most successful therapeutic antibodies work through ADCC and responses can be predicted by Fc receptor polymorphisms, which determine the strength of binding and signaling [28–30]. Cell–cell interactions involve both activating and inhibitory receptors to regulate the effector responses of NK cells to specific targets [31]. The net balance of activating and inhibitory receptors on the NK cell determines if lysis of the target cell will occur [32–34].

MHC & ‘loss of self’

The MHCs are antigen-presenting proteins essential for the discrimination of normal, altered-self cells, and nonself cells by regulating NK cell activity via interactions with NK cell receptors [32]. Human MHC molecules are called HLAs because they were first characterized on lymphocytes. HLA molecules can be found mapped on chromosome 6 but can be divided into HLA class I and HLA class II based on their differing structure, peptides presented and immune function [35]. There are two types of class I HLA molecules: a classical and nonclassical type. The classical HLA class I molecules (HLA-A, -B and -C) are expressed by almost all nucleated cells in the body. Infectious agents and some tumor cells can interfere with HLA class I processing of peptides and thereby avoid immune recognition [36–40]. The nonclassical HLA class I molecules are HLA-E, -F, -G and -H. HLA-E and HLA-G have been shown to be overexpressed by several malignancies [41–49]. NK cells can recognize malignant or virally transformed cells with decreased expression of class I MHC molecules, referred to as ‘loss of self’ [50,51]. The ‘missing-self hypothesis’ was first described in his doctoral thesis by Klas Kärre in 1981 and was published in 1985 [52,53].

NK cell receptors

NK cell activity is under the control of signals from inhibitory receptors [54] that most commonly bind MHC class I molecules [32,34]. MHC class I molecules are normally expressed on healthy cells of the body but upon viral or malignant transformation may be lost or downregulated [36–40]. Mature functional NK cells express at least one inhibitory killer immunoglobulin-like receptor (KIR) that recognizes Bw4 or HLA-C, or NKG2A (CD94) that is specific for HLA-E, expressed on chromosomes 19 and 12, respectively. However, there are also NK cells lacking expression of KIR. In most circumstances, inhibitory signals are thought to dominate overactivating signals in NK cells [55]. In instances of inflammation from viral infection or malignant transformation, activation signals may over-ride the inhibitory signals, especially when mediated by cytokines, which provide a strong stimulus. Activating NK cell receptors include the natural cytotoxicity receptors NKp30, NKp46 and NKp44 [56,57]. NKG2D is another dominant activating NK cell receptor constitutively expressed on all NK cells and recognizes the stress-induced molecules MHC class I-related MICA and MICB and the class I-like CMV homologous ULBP proteins, both of which are commonly unregulated on tumor or virally infected cells [58,59]. DNAM-1 receptor is also constitutively expressed on all NK cells and there are two ligands: CD155 (PVR) and CD112 (Nectin-2) [60]. Importantly, both ligands are expressed on some human tumors making them sensitive to NK cell-mediated killing.

One of the major challenges in NK cell biology is understanding the mechanisms by which NK cells acquire class I recognizing inhibitory receptors and explaining how these receptors interact with their cognate ligands and other activating signals to acquire the ability to kill targets. Human NK cells express KIRs, type I transmembrane molecules belonging to the immunoglobulin superfamily, all encoded on chromosome 19. KIRs are named based on the number of extracellular immunoglobulin domains (2D or 3D) and the length of the intracellular tail that determines whether they are stimulating (short) or inhibitory (long). Binding of higher affinity inhibitory KIR to their cognate, self class I HLA, suppresses NK cell effector responses including cell-mediated lysis and cytokine release [61]. The developmental mechanism by which NK cells acquire self-tolerance and alloreactivity has been referred to as licensing, calibration, arming or NK cell education [62–64]. This is an adaptive process that NK cells undergo in response to the MHC class I environment. Several models have been proposed to explain the integration of inhibitory receptor expression with the acquisition of effector functions. These concepts differ in their implied mechanisms and whether the process is one of activation or loss of function. ‘Disarming’ refers to the suppression of effector function in maturing NK cells that receive stimulatory signals unopposed by inhibitory signals via self-MHC receptors, analogous to the development of T-cell anergy [62,63]. ‘Licensing’ describes a terminal differentiation step by which NK cells become functionally competent only when they receive an appropriate signal via an inhibitory receptor ligating with self-MHC [65,66]. Elliott and colleagues showed that when performing adoptive transfer of peripheral, hyporesponsive NK cells from MHC class I-deficient donors to MHC class I-sufficient hosts, they could generate functional donor NK cells [67]. Others have shown that the functional activity of mature NK cells can be reset when the cells are exposed to a changed MHC environment [68,69]. More recently, the ‘rheostat’ model has been proposed suggesting that NK cell education is a continuous process where NK cells vary in their responsiveness to targets based on the strength of the inhibitory input received by the individual NK cell during education [70,71]. For example, the higher the tuning (the more exposure to self-MHC class I molecules or the higher the inhibitory input during education), the more likely that an NK cell will respond with degranulation and/or IFN-γ release when in a situation of stimulation. This model explains both the presence of ‘self-tolerance’ in the absence of a stimulating environment and the ability to optimize response to infection of healthy cells [68,70]. These findings are of

importance in NK cell immunotherapy as they suggest that donor NK cells 'unlicensed' by HLA alleles absent in the donor may become licensed by host HLA alleles, leading to activity of donor NK cells against host tumors lacking HLA expression. What is agreed upon between these and other models is that human NK cells lacking inhibitory receptors are hyporesponsive [72,73]. Therefore, rather than being autoreactive, they are self-tolerant. Although the exact mechanism remains unknown, self-tolerance may be the result of coordinated developmental pathways whereby mature NK function is synchronized with the acquisition of self-inhibitory receptors.

Cancer immunotherapy

Immunotherapy strategies: autologous adoptive cell therapy

Evidence for NK cell-mediated killing of tumors was first shown *in vitro* [6,7,9,10]. Murine models have confirmed the support for NK cell-mediated killing of tumors *in vivo* [74]. More recently reported is the evidence for NK cell-mediated killing of freshly isolated human tumor cells from acute lymphocytic leukemia, multiple myeloma, neuroblastoma, ovarian, colon, renal and gastric cancers [75–83]. The first trials in the 1980s using adoptive cellular therapy to treat cancer however did not use NK cells specifically but were based on delivery of lymphokine-activated killer (LAK) cells [84–86]. The LAK cells were autologous peripheral blood mononuclear cells exogenously stimulated with IL-2 *in vitro* for 5–7 days to induce killer cells. The antitumor cytotoxicity was found to be mediated primarily by activated NK cells; however, despite a few objective responses, overall clinical benefit was minimal [85,87,88]. High-dose IL-2 was administered in an attempt to activate the autologous NK cells *in vivo*; however, significant toxicity owing to capillary leak syndrome occurred. Low-dose subcutaneous IL-2 with or without LAK cells also failed to show efficacy in patients with CML, lymphoma or breast cancer [89,90].

T-cell-based cellular therapy using expanded tumor-specific $CD8^+$ cytotoxic T lymphocytes from tumor-infiltrating lymphocytes (TILs) has been an attractive immunotherapeutic strategy. Adoptive transfer of TILs following lympho-depleting strategies has shown responses in over 50% of patients with metastatic melanoma [91–93] but some responses can be short lived [94]. Side effects from T-cell-based immunotherapy include vitiligo, ureitis and retinitis [91,95].

We now have an increased understanding of the *in vivo* environment necessary to allow for successful expansion of adoptively transferred lymphocytes. Many groups have shown that in order for expansion of adoptively transferred lymphocytes to occur, lymphodepletion to 'clear space' for the infused lymphocytes is necessary. Lymphodepletion is required so that the donor lymphocytes will not need to compete with recipient lymphocytes for growth factors and cytokines and so they are not immediately rejected [91]. Rosenberg and colleagues were the first to apply this to human therapy by inducing lymphopenia with high-dose cyclophosphamide (Hi-Cy; 60 mg/kg/day × 2 days) and fludarabine (Flu; 25 mg/m^2/day × 5 days) allowing for *in vivo* expansion of autologous adoptively transferred cytotoxic T lymphocytes in patients with melanoma, which led to enhanced clinical efficacy. The finding that 'space' must be created to allow for *in vivo* expansion of adoptively transferred cells was critical in the world of immunotherapy. In addition, new data suggested that failure of autologous LAK and NK cell therapies may be partially caused by the downregulation of NK cell killing by inhibitory KIR recognition of self MHC class I present on tumor cells [96]. The thought was that autologous LAK and NK cells may be suppressed by the physiological response resulting from NK cell recognition of self MHC molecules. Ruggeri and colleagues showed that stratifying patients by their KIR-ligand mismatch would select for patients with alloreactive NK cells that protect against acute myeloid leukemia (AML)

relapse [96]. This hypothesis led researchers to begin investigating the safety and therapeutic potential of using allogeneic cell therapy as opposed to autologous adoptive transfer.

Allogeneic NK cell therapy: moving beyond autologous transfer

Following the discovery of inhibitory KIR and the understanding that they play a role in preventing NK cell killing of self MHC I-expressing tumor cells, investigators began to research the possibility of using allogeneic donor cells as opposed to autologous cells. NK cells, as opposed to T cells, do not induce graft-versus-host disease (GVHD), therefore treatment-related toxicity owing to allogeneic donor NK cell administration is minimal. Alloreactive NK cells can be delivered either as adoptive immunotherapy or within the context of hematopoietic cell transplantation (HCT). There are three methods from which NK cell products for adoptive transfer can be prepared: from umbilical cord blood, cell lines and adult donor lymphapheresis products. The benefit of using adoptively transferred adult NK cells is that these cells are educated in healthy hosts and have the potential to have greater anti-tumor activity. Another benefit of adoptively transferred NK cells is based on the discovery by Ruggeri and colleagues who showed that KIR ligand mismatch between patients and their donors was associated with better outcomes in myeloid leukemia after T-cell-depleted haploidentical hematopoietic cell transplantation [96]. Methods have now been developed to select NK cell donors according to their KIR genotype or KIR ligand status to determine if improved outcomes can be observed in other tumor types.

Safety and efficacy of adoptive cellular transfer using alloreactive NK cells was established by Miller and colleagues in patients with meta-static melanoma, renal cell carcinoma, refractory Hodgkin's disease and refractory AML [97]. The necessity of a lymphodepleting preparative regimen to promote NK cell expansion was established in this trial, similar to conclusions reached with cytotoxic T-lymphocyte infusions. A total of three chemotherapy regimens were investigated; however, only patients receiving the Hi-Cy/Flu regimen, originally described by Rosenberg and colleagues, had successful NK cell expansion 14 days following NK cell infusion [91]. *In vivo* expansion of NK cells was assessed using a PCR-based chimerism assay. Only the Hi-Cy/Flu regimen produced pancytopenia as well as a surge in IL-15 levels (up to 100 pg) following chemotherapy induction. This is important as IL-15 is known to be a key cytokine in maintaining NK cell homeostasis and high levels in this trial correlated with successful NK cell expansion [98,99]. As the absolute lymphocyte count dropped, an inverse rise was seen in the IL-15 levels. A total of 10% of patients met the criteria for successful NK cell expansion (≥100 donor-derived NK cells/μl of whole blood at 12–14 days after NK cell infusion). Thirty percent of patients with poor prognosis AML achieved a complete remission and those achieving this status had a significantly higher proportion of circulating donor NK cells suggesting that persistence and expansion is required to see clinical efficacy. The donor NK cells in patients achieving remission were also more cytotoxic against K562 targets (the prototypical cell for NK cell killing), suggesting the observed clinical efficacy was owing at least in part to the allogeneic donor NK cells. Despite expectations, KIR-ligand mismatch status did not correlate with clinical efficacy in this trial [100]. This is not necessarily contradictory to other models as NK cells activated by endogenous IL-15 and IL-2 administration may act very differently from NK cells seen after allogeneic transplant. Detailed functional studies are underway to address this question.

Based on the success observed in AML, we are currently testing adoptively transferred allogneneic NK cells in a solid tumor setting. We are using the Hi-Cy/Flu preparative chemotherapy regimen to investigate NK cell expansion and clinical efficacy in patients with recurrent meta-static breast and ovarian cancer [101]. Both of these diseases have been shown to be exquisitely sensitive to NK cell killing *in vitro* [82,102]. In our study, following haploidentical allogeneic NK cell transfer in 13 patients, although nine (69%) had detectable

donor chimerism measured using a standard short-tandem repeat assay at day +7 (mean: 47 ± 9%; range: 0–83%), none met the predefined definition for NK cell expansion (detection of ≥100 donor-derived NK cells/μl of whole blood at 14 days after NK cell infusion). Based on work of Rosenberg *et al.* [103,104] showing that the addition of 200–1200 cGy total-body irradiation (TBI) to a Hi-Cy/Flu preparative regimen promoted T-cell expansion and persistence in melanoma, and by the finding that 400 cGy TBI was associated with significantly better NK cell expansion in patients with AML [100], we investigated whether intensifying lymphodepletion by adding TBI 200 at cGy to our preparative regimen was necessary to achieve successful NK cell expansion. Unfortunately, we found a significantly greater time to hematopoietic recovery in our heavily pretreated population, but no improvement in rates of NK cell expansion in evaluable patients. It should be noted that the only patient found to successfully expand NK cells was not evaluable owing to having received high-dose steroids following NK cell infusion and an interruption in her IL-2 administration after four doses. These find-ings, however, provided proof of concept that allogeneic NK cells can expand in solid tumor patients. The question remains as to whether the addition of steroids is necessary to provide further immunosuppression to promote NK cell expansion, but this benefit can only be realized if the steroids do not have an adverse effect on function. Glucocorticoids have previously been reported to inhibit the functional activities of both T and NK cells primarily by interfering with transactivation of certain transcription factors such as NF-κB and AP-1 [105–107]. There are now data suggesting that hydrocortisone may assist in the proliferation and survival of activated NK cells. Hydrocortisone has been shown to advance NK cell development from $CD34^+$ hematopoietic stem cells [108]. Perez and colleagues demonstrated that in combination with IL-15, hydrocortisone expands peripheral blood derived $CD56^+$ cells, favoring the expansion of $CD56^+/CD3^-$ NK cells [109]. The $CD56^+$ cells exposed to both IL-15 and hydrocortisone not only maintained their cytotoxicity, but also increased cytokine production. Others have shown that the addition of hydrocortisone and stromal cells increases the frequency of progenitor cells that give rise to NK cells via the recruitment of myeloid precursors [110]. These data suggest that hydrocortisone may act as a costimulus for NK cell activation and expansion, and is the data from which stems our next planned clinical trial that will include steroid administration to patients.

NK cell lines as therapy

As described, autologous NK cells from cancer patients may be dysfunctional and may not recognize the malignant target. They also may be inhibited by self-HLA expression making allogeneic NK cells a potentially better NK cell product for immunotherapy. Another alternative is the NK-92 human NK-cytotoxic cell line that represents a pure allogeneic activated NK cell source [111]. NK-92 is IL-2 dependent, lacks KIRs and has been shown to be cytotoxic against a variety of hematologic and solid tumor cell lines [112]. Arai and colleagues published their Phase I trial using NK-92 in patients with advanced renal cell carcinoma or melanoma [111]. They showed that NK-92 cells successfully expanded on average 200-fold over 15–17 days with ≥80% viability. In this trial, the authors were able to determine the safety and efficacy of a large-scale NK-92 expansion and although efficacy was not measured, two patients experienced transient minor decreases in their tumor size. The ability to use NK cell lines as therapy offers a unique platform where the cell lines can be genetically altered to target specific tumor antigens such as CD20 [113] or ErbB2 [114] to increase cytotoxicity.

Therapeutic limitations of NK cells & future perspective

The therapeutic potential for adoptive transfer of allogeneic NK cells is limited by several entities, the number one being the failure of donor NK cells to expand *in vivo*. A question that remains is whether the proliferation and expansion of adoptively transferred NK cells

may be limited by host factors, including immune rejection by effector T cells, suppression by myeloid-derived suppressor cells, which have been widely studied for their suppressive properties in tumor models [115–117], and suppression by Tregs, known to maintain tolerance to self/tumor. In our breast and ovarian cancer study, all patients had low Treg ($CD4^+$, $CD25^+$ and CD127) profiles prior to starting chemotherapy; however, by day +14 as the percentage of circulating NK cells dropped, they were replaced by a corresponding increase in T cells. The T-cell profiles could be split into two clusters; one group with a significantly increased T-cell population of the Treg phenotype and the other with primarily $CD8^+$ T cells. We were unable to find any variable that predicted which patients would develop high Treg levels versus $CD8^+$ T cells. We are starting to realize that *in vivo* expansion may involve a complex network of competition between cells. While IL-2 was intended to potentiate *in vivo* expansion of NK cells, it is now well established that low-dose IL-2 is especially potent to expand Tregs, which express high-density CD25 IL-2 receptors [118]. These findings beg the question of whether Treg depletion will be needed for effective adoptive cellular therapy, especially those that utilize IL-2 as part of the therapy platform.

Combining NK cell-based immunotherapy with chemotherapy schemas other than Hi-Cy Flu is of potential interest. The rationale for this would be to abandon the myeloablative conditioning regimens that in turn would decrease the known toxicity associated with this therapy. Iliopoulou and colleagues did not use a myeloablative preparative regimen in their Phase I trial of adoptive transfer of allogeneic NK cells in advanced non-small-cell lung cancer [119]. Hydrocortisone and IL-15 were used for the *ex vivo* activation and expansion of allogeneic NK cells, which were administered by repetitive infusion. Patients were given up to four doses of NK cells during their prescribed chemotherapy, which provided an environment of relative lymphodepletion. This regimen was found to be safe, potentially clinically effective, and justifies further investigation into less toxic preparative regimens that could be used in NK cell-based immunotherapy.

Although we have shown that *in vivo* NK cell expansion is possible, the success and duration of expansion is unpredictable and is a major hurdle in patients with solid tumors. The success in AML and problems in solid tumors may be explained by the difference in immunologic health of these patient cohorts or the ability of different tumors to induce suppressive factors, cellular or soluble. In addition, the cytotoxicity of the expanded NK cell population remains an unknown. Future strategies to augment activating ligand expression on target cells to make them more susceptible to NK cell-mediated lysis is an active area of investigation. Improvements in techniques to make tumor cells more susceptible to the effects of NK cell cytotoxicity will ultimately increase the clinical efficacy of NK cell-based immunotherapy. Some of these techniques include selective depletion of Tregs, which have been shown to suppress NK cell proliferation and killing. Several *in vitro* and mouse models demonstrate that Tregs are able to profoundly inhibit NK cell proliferation and activation [120] and reduce the number of NK cells recruited to the tumor site [121]. Depletion of Tregs may improve the immune effector functions of the expanded NK cells [122]. In support of this, we have embarked on a clinical trial investigating the addition of cyclosporine (CsA) to our lymphodepleting regimen as a means of Treg depletion. CsA, a calcineurin inhibitor, inhibits Treg activity by blocking the induction of the nuclear factor of activated T cells, leading to a decrease in IL-2 and IL-2 receptor gene expression. The lack of IL-2 or its functional receptor blocks the generation of Tregs both in the thymus and in the periphery [123]. CsA has also been found to be significantly more immunosuppressive to T cells than NK cells [124] and may be a promising addition to our current regimen.

In addition, depletion of Treg, has shown to dramatically improve efficacy of adoptive cytotoxic T-cell immunotherapy in a murine AML model. Zhou *et al.* demonstrated that a

brief course of denileukin diftitox (DAB–IL2; Ontak®, Ligand Pharmaceuticals Inc., CA, USA) restored the proliferation of transferred cytotoxic T lymphocytes and reduced substantial pre-existing leukemia tumor burden, resulting in a significant increase in survival of leukemia-bearing mice [125]. DAB–IL2 is a recombinant DNA-derived cytotoxic protein composed of diphtheria toxin fragments A and B and the full-length IL-2 molecule. DAB–IL2 binds to CD25 (the α-chain of the IL-2 receptor) and, following internalization, inhibits protein synthesis, causing cell death within hours [126]. DAB–IL2 is US FDA-approved for the treatment of patients with persistent or recurrent cutaneous T-cell lymphoma whose malignant cells express CD25. Cutaneous T-cell lymphoma has been postulated to be a malignant proliferation of Tregs. DAB–IL2 intoxicates sensitive eukaryotic cells by CD25 receptor-mediated endocytosis and is 100-times more potent in killing cells bearing the high-affinity isoform (CD25) of the IL-2 receptor compare to low-affinity IL-2 receptors [127,128]. The relatively specific phenomenon of Treg depletion seen with DAB–IL2 may be owing to high surface expression levels of CD25 on Tregs. Administration of T-cell-depleting agents may prove useful in improving the therapeutic efficacy of allogeneic NK cell therapy in solid tumor patients and should be tested prospectively.

More recently, there is evidence suggesting that NK cells play a role not only in the innate ability to kill tumor cells, but also as initiators of adaptive immune responses. This is of great importance as low numbers of NK cells normally circulating in peripheral blood provides an obstacle to NK cell-based immunotherapy, therefore methods to improve antitumor activity are needed. Krebs and colleagues have shown that injection of 10^4–10^5 antigen-expressing NK cell targets induces significant antigen-specific T- and B-cell responses. Their data suggest that the amplification of immune responses is dependent largely on MyD88/Trif signaling [129]. Salagianni and colleagues demonstrated that depletion of Tregs using DAB–IL2 in a murine lung cancer model enhanced the *in vivo* antitumor immunity induced by adoptively transferred NK cells. Interestingly, animals treated with DAB–IL2 plus adoptive transfer of hydrocortisone/IL-15-expanded NK cells that developed immunity against the lung tumors were rechallenged with the same lung cancer tumor in their flanks and had significant antitumor responses compared with the NK cell-only-treated groups. These data suggest that inhibiting the suppressive action of Tregs on $CD4^+$ and/or $CD8^+$ T cells with DAB–IL2 allows for adaptive antitumor immunity to occur [130]. The discovery that NK cells are able to activate the adaptive immune system may be important in future novel vaccines that could ideally target both the innate and the adaptive immune system.

Another possibility of improving the effects of NK cell cytotoxicity includes the use of agents that may sensitize cancer cells to NK cell-mediated killing. Bortezomib (PS-341, Millennium Pharmaceuticals Inc., MA, USA) has been shown to exert such a positive effect [131]. Bortezomib is the first of its class of proteasome inhibitors and is FDA approved for the treatment of multiple myeloma. Proteasome inhibition has been shown to directly inhibit growth of neoplastic cells as well as sensitize them to various chemotherapeutics and radiation [132]. Among the multiple receptors of the TNF group, the ligand of particular interest is TRAIL, for its property to induce apoptotic cell death in a variety of tumor cells by engaging the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), while sparing most normal cells [133,134]. NK cells lyze tumor targets directly primarily via the perforin–granzyme pathway; however, NK cells also express TRAIL. Sayers *et al.* reported that bortezomib could sensitize leukemic and renal tumor cells to TRAIL-mediated lysis [135]. Bortezomib has also been shown to sensitize leukemia cells to immune effector killing by upregulation of death receptor expression for Fas ligand and downregulation of MHC class I [136]. Understanding the role of MHC class I recognition by NK cells is rapidly evolving and stems from early observations that target sensitivity to NK lysis is inversely related to expression of MHC class I molecules on the target cell surface [137]. Therefore, decreasing

target cell expression of MHC class I molecules may expose the target cells to a higher killing percentage by NK cells. Upregulation of TRAIL and Fas death receptors on target cells and downregulation of MHC class I using a proteasome inhibitor may represent other potential pathways to exploit with NK cell immunotherapy.

Other methods to develop NK cells for adoptive transfer therapy include developing *ex vivo* expansion techniques and better strategies to expand NK cells *in vivo*. IL-15, which in humans is made by stroma and macrophages [138,139], plays an important role in NK cell development and the homeostasis of NK and $CD8^+$ T cells [99,140,141]. Although IL-2 is also important and is what is currently used to promote NK cell expansion following allogeneic NK cell delivery, IL-15 presented in trans by IL-15Rα delivers a unique and potent signal to promote NK cell proliferation. It has been shown in mice that the IL-15–IL-15Rα complexes induce greater *in vivo* expansion of NK cells and $CD8^+$ T cells than IL-15 alone [142] and has been used therapeutically in a murine model of melanoma [143]. The trans-presentation of IL-15Rα by dendritic cells is also critical to the survival of NK cells [144–146] and NK cell development. The use of IL-15 *in vivo* or *ex vivo* to improve NK cell expansion and function is in early clinical trials. It is hoped that IL-15 will stimulate Tregs significantly less than IL-2 based on *in vivo* administration into primates [147–149]. A study to test IL-15 with NK cell adoptive transfer is planned at our institution in patients with advanced AML. We anticipate that IL-15 will expand and promote NK cell activation *in vivo* safely allowing therapeutic strategies to maximize the antitumor effects of NK cells.

Another potential strategy to increase the effects of adoptively transfused NK cells in cancer patients is to increase the actual number of cells available for infusion following apheresis. Several methods for expansion and activation of NK cells *in vitro* have been investigated. These methods include overnight and long-term culture with cytokines [150,151], using peripheral blood mononuclear cells [152] and K562 cells [153], and Epstein–Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) as feeder cells [154,155]. Debate remains as to which method is best for enhancing the cytolytic activity of NK cells. To date, most clinical studies of adoptive NK cell transfer utilize short-term (12–16 h) IL-2-activated NK cells [97]. The number of IL-2-activated NK cells available using the short-term method may, however, limit the therapeutic potential of this approach. Recently, Berg and colleagues have used *ex vivo*-expanded human NK cells [97,156]. They showed large-scale production of *in vitro*-expanded NK cells using irradiated EBV-LCL feeder cells in a closed 'bag-based' culture system. These authors claim that *in vitro*-expanded NK cells have increased natural cytotoxicity receptors, TRAIL and NKG2D, expression, and superior tumor cytoxicity compared with short-term IL-2-activated NK cells [157]. These findings are important because, as mentioned above, bortezomib-treated tumors upregulate the death receptors for TRAIL. This leads to the question of whether *in vitro*-expanded NK cells can greatly enhance TRAIL-mediated cytotoxicity against bortezomib-treated tumors compared with other methods of NK cell expansion.

Despite advances in the expansion and activation of NK cells, NK cells do not respond to many tumors despite the loss of MHC class I surface expression in these tumors. In an attempt to improve tumor targeting, Kruschinski and colleagues genetically engineered primary human NK cells to specifically target $Her2^+$ tumors [158]. They showed that an antibody-based chimeric receptor-NK cell could elicit production of IFN-γ and IL-2 as well as degranulation and lysis when exposed to $Her2^+$ target cells in a murine model. This approach may represent an effective alternative for adoptive immunotherapy especially in tumors that lack MHC-I expression and/or are resistant to NK cell-mediated killing.

The recent development of anti-KIR blocking antibodies has led to important therapeutic implications in NK cell immunotherapy. 1-7F9 is a human monoclonal antibody that cross

reacts with KIR2DL-1, -2 and -3 and prevents their inhibitory signaling. Romagne and colleagues showed that 1-7F9 stably blocked KIRs and augmented NK cell-mediated killing of HLA-matched AML blasts *in vitro* and *in vivo* [159]. We have published our findings showing that dual blockade of NKG2A and leukocyte immunoglobulin-like receptor-1 (LIR-1) led to significant killing of targets by resting KIR-negative NK cells that usually exhibit tolerance to primary leukemia targets [160]. Our findings suggest that strategies to interrupt the inhibitory receptors NKG2A and LIR-1 in combination with an anti-KIR blockade may be important mechanisms to exploit to enhance cytotoxicity in NK cell immunotherapy.

In addition, investigation in immunotherapy has led to significant knowledge about the KIR gene family and how it influences the outcome of transplantation for AML. Polymorphic KIRs recognize polymorphic epitopes of HLA-A, -B, and -C, called KIR ligands [161–163]. KIR haplotypes can be divided into groups A and B according to gene content [162,164]. KIR A haplotypes have simple, fixed gene content. KIR B haplotypes have variable gene content and one or more of the B-specific genes: *KIR2DS-1*, *-2*, *-3*, *-5*, *KIR2DL2*, and *KIR2DL5*. All individuals can be assigned to the A/A genotype (homozygous for A haplotypes) or the *B/x* genotype (having one or two B). Cooley and colleagues KIR-genotyped donors from 1409 unrelated transplants for AML and acute lymphoblastic leukemia [165]. They found that donors with a *KIR B/x* haplotype protected against relapse and improved survival in the AML population. These findings suggest that KIR genotyping to identify favorable KIR gene content may result in superior outcomes for patients with AML. The relevance of the observed clinical benefit with KIR ligand mismatch in the AML population is unclear in the adoptive transfer setting. Whether similar relapse protection and survival benefit would be seen by identifying NK cell donors with favorable *KIR B* genotypes in a solid tumor population requires further study.

The above studies provide the basis for the development of further strategies to manipulate the NK cell product, host and targets with the ultimate goal of enhancing the therapeutic benefit of NK cell-based immunotherapy while minimizing the risks and toxicities (Box 1).

Box 1

NK cell-based immunotherapy

NK cell product

- Adult NK cells
  - Autologous/allogeneic cell lines (NK-92 and KHYG-1)
  - Umbilical cord blood/embryonic stem cells/induced pluripotent stem cells
  - Stem cells
- Processing
  - T-cell receptor $(CD3)^-$ alone
  - $CD3^-/CD56^+$
  - $CD3^-/CD19^-$
- *Ex vivo* manipulation
  - *Ex vivo* expansion
  - IL-2 or IL-15

- Anti-KIR, anti-NKG2A

Host

- Lymphoid/myeloid depletion
  - Cytokine sinks
  - Expansion space
- Tregs
  - Fludarabine/cytoxan
  - Total-body irradiation
  - Cyclosporine
  - Steroids
  - Ontak® (anti-CD25)
- Dendritic cell activation
  - Toll-like receptor agonists
  - Dendritic cell vaccines

Target

- Tumor/transformed/infected cells
  - Increase activating ligands
  - Decrease inhibitory ligands
  - Total-body irradiation/radiation
  - Chemotherapy
  - Bortezomib, histone deacetylase 1
  - Antibody targeting
- *In vivo* manipulation
  - IL-12 or IL-15
  - Anti-KIR, anti-NKG2A

Overview of current approaches to improve effectiveness of adoptive transfer of NK cells by manipulation of the NK cell product, the host and the target.

Conclusion

Investigators have demonstrated the role of allogeneic NK cell therapy in the treatment of various malignancies. Techniques for *ex vivo* expansion and refinement in the administration of lymphodepleting chemotherapy are currently under investigation. Improvements in techniques to augment *in vivo* NK cell persistence and expansion will increase the clinical efficacy of NK cell-based immunotherapy.

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Executive summary

Basics of NK cells

- NK cells
  - Human NK cells are a subset of peripheral blood lymphocytes defined by the expression of CD56 or CD16 and the absence of the T-cell receptor CD3.
  - NK cells comprise of 10–15% of the lymphocyte pool in humans.
  - NK cells that have been exposed to cytokines show higher cytotoxicity against target cells and produce more cytokines.
- NK cell cytotoxicity
  - Most cytotoxic activity of NK cells is a result of direct killing mediated by perforin and granzyme release.
  - The other mechanism of target cell killing is via interaction between death receptors expressed on target cells and the corresponding ligand on NK cells.
  - Engaging Fas–Fas ligand and TNF-related apoptosis inducing ligand (TRAIL)–TRAIL ligand interactions are two methods by which NK cells can induce lysis of target cells.
  - NK cells can mediate antibody-dependent cell-mediated cytotoxicity via CD16 (FcRγIII).
- MHC and ‘loss of self’
  - MHC class I (MHC-I) are antigen-presenting proteins essential for the discrimination of normal, altered self and nonself by regulating NK cell activity via interactions with NK cell receptors.
  - NK cells can recognize malignant or virally transformed cells with decreased expression of MHC-I molecules; this is referred to ‘loss of self’.
- NK cell receptors
  - NK cell activity is under the control of signals from inhibitory and activating receptors, in most circumstances, inhibitory signals dominate over activating signals.
  - Mature functional NK cells express at least one inhibitory receptor either killer immunoglobulin-like receptor (KIR) or NKG2A (CD94), which are specific for a self-MHC-I ligand that is normally expressed on all healthy cells of the body.
  - When there is viral or malignant transformation of a target cell, activating signals often over-ride inhibitory signals.
  - NK cell education is the process by which NK cells gain function through inhibitory receptor engagement of MHC-I molecules.
  - NK cells can also gain function through other mechanisms, such as cytokine activation that probably signals differently.

Cancer immunotherapy

- Immunotherapy strategies: autologous adoptive cell therapy
  - The first trials in the 1980s using adoptive cellular therapy to treat cancer were based on delivery of lymphokine-activated killer cells that are autologous peripheral mononuclear cells exogenously stimulated with IL-2 *in vitro* to induce killer cells. The antitumor activity was mediated primarily by activated NK cells.
  - High- and low-dose IL-2 was administered to activate autologous NK cells *in vivo*; however, no efficacy was seen in patients with chronic myeloid leukemia, lymphoma or breast cancer.
  - Nonmyeloablative lymphodepleting chemotherapy using high-dose cyclophosphamide and fludarabine has been shown to be necessary to 'clear space' for infused autologous lymphocytes in order to decrease competition with recipient lymphocytes for growth factors and cytokines.
  - Failure of autologous lymphokine-activated killer and NK cell therapy thought to be partially owing to the downregulation of NK cell killing by inhibitory KIR recognition of self-MHC-I present on tumor cells. This notion was supported by haploidentical T-cell-depleted transplantation studies suggesting that KIR mismatch with tumor MHC (i.e., KIR ligand) may lead to greater tumor kill by increasing the frequency of alloreactive NK cells.
- Allogeneic NK cell therapy: moving beyond autologous transfer
  - Hypothesis that autologous NK cells may be suppressed by physiologic response resulting from NK cell recognition of self-MHC molecules led to allogeneic NK cell infusions.
  - Safety and efficacy of adoptive cellular transfer using alloreactive NK cells was established in patients with metastatic melanoma, renal cell carcinoma, refractory Hodgkin's disease and acute myeloid leukemia. Successful expansion of adoptively transferred NK cells required a lymphodepleting preparative regimen of high-dose cyclophosphamide and fludarabine.
  - Allogeneic NK cell therapy remains an investigational and developmental therapy in other solid tumors such as ovarian and breast cancer.
- NK cell lines as therapy
  - NK-92 is a human NK-cytotoxic cell line that represents a pure allogeneic activated NK cell source.
  - NK-92 is IL-2 dependent, lacks KIRs, and has been shown to be cytotoxic against a variety of hematologic and solid tumor cell lines.
  - The ability to use NK cell lines as therapy offers a unique platform where the cell lines can be genetically altered to target specific tumor antigens.

Therapeutic limitations of NK cells & future perspective

- Proliferation and expansion of adoptively transferred NK cells may be limited by host factors such as immune rejection by myeloid-derived suppressor cells and Tregs.

- There is ongoing investigation into methods (cyclosporine or denileukin diftitox [Ontak®]) of suppressing Treg and T-effector cells that may be inhibiting donor NK cell expansion *in vivo*.
- Further investigation is ongoing into the use of agents (proteasome inhibitors) to improve NK cell cytotoxicity by increasing death receptors on tumor cells and downregulating MHC-I.
- Development of *ex vivo* expansion techniques is a promising avenue for NK cell allogeneic therapy. IL-15 presented in trans by IL-15 receptor-α has been shown to deliver a potent signal to promote NK cell proliferation and may offer a clinical alternative to IL-2 therapy in adoptive cellular therapy.
- Anti-KIR blocking antibodies has led to important therapeutic implications in NK cell immunotherapy. 1-7F9 is a human monoclonal antibody that cross reacts with KIR2DL-1, -2, -3 and prevents their inhibitory signaling.
- 1-7F9 has been shown to stably block KIR and augment NK cell-mediated killing of HLA-matched acute myeloid leukemia blasts *in vitro* and *in vivo*.
- New data suggest that donors with a *KIR B/x* haplotype are better than *KIR A/A* haplotype donors in protecting against acute myeloid leukemia relapse. The role of these donor factors in the adoptive transfer setting and whether it is of relevance in patients with solid tumors needs further study.