Cancer Immunology Research

Harnessing Natural Killer Cell Antitumor Immunity: From the Bench to Bedside

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Abstract

Natural killer (NK) cells are critical effector lymphocytes mediating tumor immune surveillance and clearance. They do so by direct tumor killing using cytolytic granules and death receptors, and by interfacing with and potentiating adaptive immune responses through the production of cytokines. From a therapeutic perspective, NK cells have been shown to exert graft-versus-leukemia activity in the context of hematopoietic stem cell transplantation and are important in the clinical efficacy of antibodies. Advances in basic and translational NK cell biology have led to multiple potential strategies to augment their *in vivo* activity to improve antitumor responses. Despite their potent effects, NK cells have been shown to be safe for adoptive cell therapy in both the autologous and allogeneic settings, with promising, but so far limited, clinical efficacy. This review will provide an overview of strategies being pursued to improve NK cell activity and efficacy, focusing on cell source, NK cell activation, and *in vivo* persistence.

Introduction

Cytotoxic lymphocytes are critical components of the antitumor defense system that exist within adaptive and innate arms of the immune system. Specifically, $CD8^+$ T cells require antigen presentation in the context of MHC class I and priming through antigen-presenting cells (APC). In contrast, natural killer (NK) cells are innate effectors that are not antigen-specific and can be active without immunologic priming. Tumors have been known to downregulate β2-microglobulin, leading to impaired MHC presentation, as a mechanism of T-cell escape (1), but these are better targets for NK cell killing because of their "missing self." NK cell activation is regulated by a complex balance of activating and inhibitory receptors (2). Briefly, the major inhibitory receptors include killer cell immunoglobulin–like receptors (KIR), which recognize classical HLA (human leukocyte antigens A/B/C), and the heterodimer CD94/NKG2A, which recognizes nonclassical HLA-E. Both NKG2A and NKG2C recognize HLA-E with different affinities leading to inhibition and activation, respectively (3). Other activating receptors include stress-induced ligands (e.g., NKG2D recognizing MICA/B, ULBP), antibodies (e.g., CD16 against the Fc portion of IgG1), and natural cytotoxicity receptors (e.g., NKp30, NKp44, and NKp46).

Due to their diverse targeting capabilities, NK cells are an attractive product for immunotherapy. In contrast to T cells, they have been shown to be safe without evidence of graft-versus-host disease in the allogeneic setting. This feature offers the potential for off-the-shelf application. NK cells were first tested against cancer in the 1980s in the form of lymphokine-activated killer cells, a mixture of T and NK cells expanded *ex vivo* with high-dose IL2 (4). Since that time, advances in the understanding of NK biology have improved the safety profile and efficacy of adoptive cellular therapies. Since the late 1990s, the feasibility and safety of NK cell adoptive transfer have been established by our group and others. The translational aspects arising from these important biological insights serve as the focus of this review. Specifically, attempts to improve NK cell efficacy can be broadly categorized into (i) developing an optimized NK cell source for adoptive cell immunotherapy, (ii) improving NK cell activity through priming, activation, targeting, and overcoming immunosuppressive mechanisms, and (iii) prolonging persistence (Fig. 1).

NK Cell Source

Identifying and developing an optimal source of NK cells is complex, but much has been learned in the context of hematopoietic transplantation, where NK cells are the first lymphocyte to reconstitute (5). The importance of promoting "missing self" through KIR/KIR-ligand mismatch serves as proof of concept for the efficacy of NK cell therapy (6–8). NK cell adoptive immunotherapy can be broadly divided into autologous and allogeneic approaches. Initial studies demonstrated safety of adoptively transferred autologous NK cells, but efficacy was disappointing, likely due to the presence of inhibitory receptor ligands, insufficient MHC downregulation in tumors, and the redundancy in the MHC system (9, 10). To overcome this limitation, we hypothesized that the use of allogeneic NK cells would allow at least some NK cells to persist from the donor product that would not be inhibited by host tumor residual MHC. Our initial study also compared various conditioning regimens and found that lymphodepletion was important for NK cell expansion and persistence, likely due to production of homeostatic cytokines including IL15. This initial study led to approximately 25% complete remissions in patients with refractory acute myeloid leukemia (AML) and served as proof of concept for this approach (11).

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Cancer Immunol Res 2019;7:1742–7

doi: 10.1158/2326-6066.CIR-19-0404

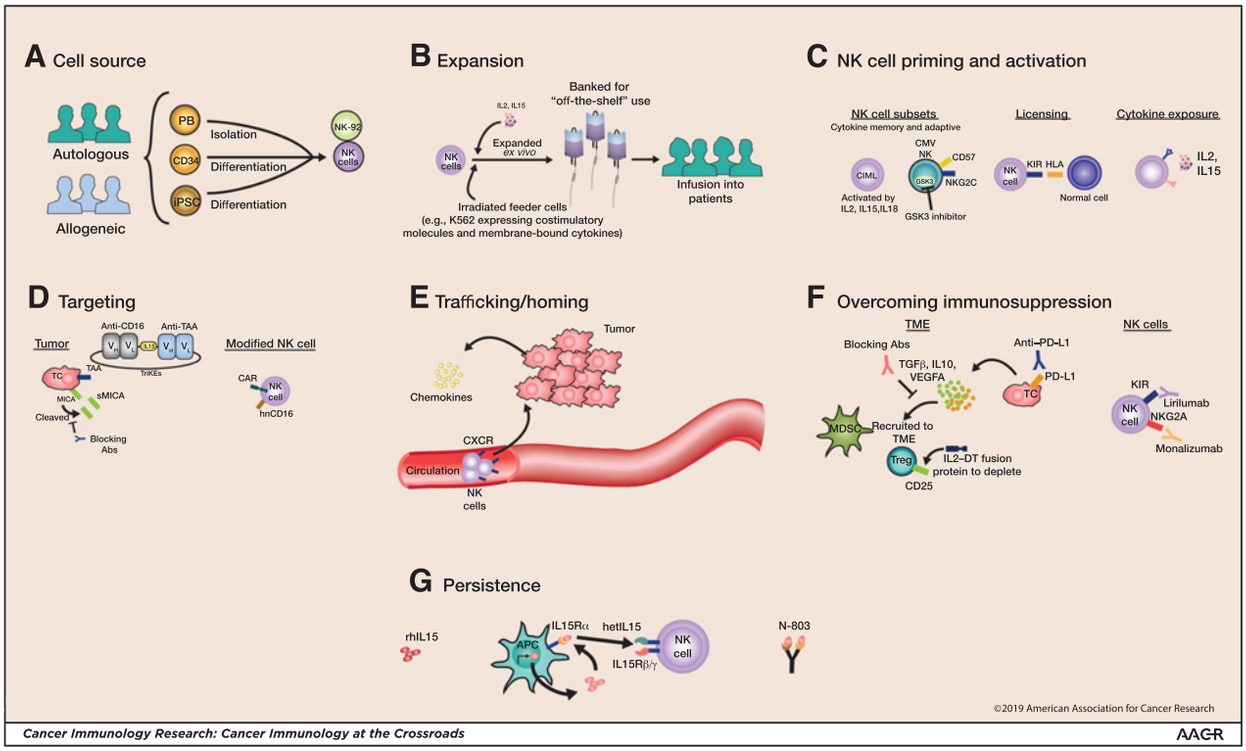

Figure 1.

Strategies to improve NK cell immunotherapy. **A,** NK cells can be derived from autologous or allogeneic sources. Although most autologous NK cells are blood derived, allogeneic sources include PB NKs, and CD34- and iPSC-differentiated NK cells. CD34: $CD34^+$ hematopoietic stem cells; iPSC, induced pluripotent stem cell; PB NK, peripheral blood NKs. **B,** *Ex vivo* expansion is typically accomplished with cytokines such as IL2 or IL15, with many also incorporating irradiated feeder cells (typically using genetically modified K562 cells). The expanded NK cells can be used fresh or banked and frozen to be available on demand. To improve NK cell antitumor activity further, (**C**) cytokine-primed, viral, or small molecule–primed NK cells can be used, which include those with a memory phenotype, licensed subsets, and those generally exposed to gamma-chain cytokine-activating cytokines. CIML, cytokine-induced memory-like; CMV-exposed NK: NK cells from cytomegalovirus seropositive individuals; GSK3, glycogen synthase kinase 3. **D,** Tumor targeting can be accomplished through increasing tumor expression of activating ligands (e.g., MICA) via upregulation or preventing cleavage. Tumor-associated antigens (TAA) can also be targeted using therapeutic antibodies, engager molecules (e.g., trispecific killer engagers, TriKE), and chimeric antigen receptors (CAR). hnCD16, high-affinity, ADAM17 noncleavable CD16; sMICA, soluble MICA; TC, tumor cell. **E,** Expression of chemokine receptors (like CXCL4) on NK cells can improve homing to tumor sites. **F,** Strategies to overcome the immunosuppressive tumor microenvironment (TME) include blockade of inhibitory receptor interactions, interruption of negative immunoregulatory cytokines, and addressing suppressive immune cells such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) through targeted depletion. TC, tumor cell. **G,** Improving NK cell persistence utilizing prosurvival and proliferative cytokines that do not stimulate Tregs, such as IL15 or modified versions (e.g., hetIL15, N-803), may mimic physiologic IL15 transpresentation by APCs. rhIL15, recombinant human IL15.

In the allogeneic setting, multiple sources are being investigated (Fig. 1A). A frequent source of mature peripheral blood (PB) NK cells are haploidentical donors, which are half-matched for HLA from a sibling or child (11). NK cells can also be derived from $CD34^+$ hematopoietic cells, typically from umbilical cord blood (12), and also induced pluripotent stem cells (iPSC; ref. 13). NK cell lines, such as NK-92, derived from a patient with non-Hodgkin lymphoma are also being evaluated. One limitation of using NK-92 cells is that it is a transformed line that is aneuploidy. Therefore, for safety reasons, it requires irradiation prior to infusion to render them unable to proliferate (14). It has not yet been determined which cell source is best from a clinical standpoint. In terms of function, historically, PB NK cells have been the most potent in terms of antitumor activity (13). However, advances in *in vitro* NK cell differentiation, expansion, and activation have improved progenitor-derived NK cell function to rival that of PB NK cells (15). One additional advantage with progenitors to manufacture an NK cell product is the relative ease of gene modification (16) compared with PB NK cells (17) or cord blood–derived NK cells (18).

From a technical aspect, billions of NK cells are needed for adoptive transfer, requiring weeks of *ex vivo* expansion, depending on the starting cell source and number (Fig. 1B). An important advance in *ex vivo* expansion has been the use of irradiated feeder cells. One source of feeder cells that has been used both in bench research and for clinical trials is the erythroleukemic cell line K562, which can be transduced to express a variety of costimulatory molecules including 4-1BBL and membrane-bound cytokines including IL15 (19) and IL21 (20). Utilizing such irradiated feeder cell lines, clinically scalable expansions to over 1,000-fold have been achieved without evidence of NK cell exhaustion or senescence. To address potential safety concerns, feeder cell–free expansion systems are being developed, including the use of plasma

membrane particles derived from K562 cells, which can retain expression of stimulatory ligands and cytokines (21).

Enhancing NK Cell Activity

Priming/activation

Multiple factors have been identified that impact NK cell activation. Although NK cells do not require immunologic priming, certain conditions have been shown to lower their threshold of activation and enhance antitumor immunity. These include cytokine stimulation, viral infection, and the paradox of functional acquisition through inhibitory receptors (i.e., licensing; Fig. 1C). Not all activating receptors are equally potent. Using agonist antibodies to trigger single-receptor signaling, only anti-CD16 was able to activate resting NK cells. However, culturing NK cells with cytokines lowers the threshold for activation, whereby single-receptor triggering beyond CD16 is now sufficient (22). Cytokines such as IL2 and IL15 provide not only a proliferative signal to NK cells, but also enhance function. IL15 is more selective for NK cell proliferation and is presented *in trans* by APCs. In an attempt to improve IL15 stability and transpresentation, natural IL15/IL15Rα heterodimers (hetIL15) and IL15/IL15Rα-Fc complexes (N-803) have been tested (23). Monomeric rhIL15 has been tested alone (24) and in combination with haploidentical NK cell infusions, resulting in 35% complete responses in refractory AML. Unique toxicities were seen with this combination, including cytokine-release syndrome and neurotoxicity, explained by subcutaneous administration leading to IL15 accumulation from decreased elimination as a result of lymphodepletion (25). These various formulations are undergoing further fine-tuning to minimize toxicity and avoid NK cell exhaustion (26). Also, when comparing various feeder cell phenotypes, chronic stimulation by K562 cells expressing membrane-bound IL15 induced senescence, which is not observed with membrane-bound IL21 (20).

Cytokines have also been shown to induce memory-like NK cells. A brief 16-hour exposure to a combination of IL12, IL15, and IL18 is able to induce long-lived, functional changes in NK cells (27). These cytokine-induced memory-like NK cells have been tested clinically in relapsed/refractory AML, leading to responses in 5 of 9 treated patients (28). NK cell memory is also induced by cytomegalovirus (CMV), where a distinct subset of NK cells has been identified. These cells express the maturation marker CD57 and the activating receptor NKG2C (with a reciprocal downregulation of NKG2A) and an epigenetic signature that is reminiscent of memory $CD8^+$ T cells. Decreased expressions of signaling molecules FcεR1γ, SYK, EAT-2, and the transcription factor PLZF are part of this signature (29). These cells have been termed "adaptive NK cells," given that they are long-lived, expand further with CMV reactivation, and are also more broadly primed against cancer targets. In a first clinical link to these cells and clinical outcomes, reconstitution of $CD57^+NKG2C^+$ NK cells following transplantation in those with CMV reactivation was associated with improved disease-free survival due to relapse protection (30, 31). Efforts to selectively expand or enrich these adaptive cells led to the identification of the small molecule glycogen synthase kinase 3 inhibitor, which when combined with IL15 and NK cells from CMV seropositive donors results in highly activated mature NK cells with enhanced cytotoxicity *in vitro* across a panel of solid tumor cell lines (lung, ovarian, and pancreas; ref. 32). These findings have been translated into an ongoing phase I clinical trial (NCT03081780).

NK cell licensing is another factor that contributes to the activation state. In animal models, germline knockout of MHC abrogates NK cell function, indicating that NK cell education via exposure to self-MHC ligands is important in their development (33). NK cells that cannot undergo education are termed unlicensed, comprising up to 50% of NK cells in mice and over 20% in humans. Metabolically, unlicensed NK cells are dependent on oxidative phosphorylation, whereas licensed NK cells are able to utilize glycolysis and glutaminolysis (reminiscent of central memory $CD8^+$ T cells; ref. 34). Therefore, licensed NK cells are better able to sustain activation. However, in the context of self-MHC–expressing infected and transformed cells, unlicensed NK cells may have some advantages compared with licensed NK cells (33). Ultimately, whether licensing will affect clinical outcomes in adoptive NK cell immunotherapy has not been determined.

Targeting

For NK cells to kill a tumor target, engagement of an activating receptor(s) is required. This can be achieved through endogenous receptor-ligand binding or redirected killing through engager molecules and chimeric antigen receptors (CAR; Fig. 1D). NKG2D is an activating receptor that typically recognizes stress-induced ligands such as MICA/B. To improve tumor clearance, stress-induced ligand expression can be upregulated by treatment with hypomethylating agents such as decitabine (35). However, tumor cells have developed strategies to cleave MICA from their surface to avoid NK cell recognition. Antibodies targeting the proteolytic cleavage site can prevent MICA clipping, restoring sensitivity to NK cell killing (Fig. 1D). Treatment with this antibody can skew the NK cell phenotype within the tumor microenvironment (TME) toward activation with increased expression of eomesodermin, granzyme B, and perforin (36). MICA shedding can also be antagonized by small molecule inhibitors of the metalloprotease ADAM17 (37). CD16 is an important activating receptor on NK cells, mediating specificity through antibody targeting. Strategies to augment CD16 NK cell responses include exploiting high-affinity CD16 polymorphisms (38, 39) and antibody Fc modifications to improve antibody specificity for CD16A, the dominant isoform on NK cells, and avoid triggering inhibitory Fc receptors (40). CD16 is also subject to proteolytic cleavage by ADAM17 upon NK cell activation (41), and inhibition of ADAM17 with antibodies, small molecule inhibitors, or genetic modification of the cleavage site can maintain CD16 expression and should augment antibody-dependent cellular cytotoxicity (42).

Another approach to (re)direct NK cell killing utilizes "engager" molecules. These small molecules combine two different antibody-targeting domains (e.g., variable fragments). One of these domains targets an activating receptor of choice, such as CD16/CD16A, whereas the other targets a tumor antigen or stress ligand (43–46). Therefore, these engagers can improve the immunologic synapse and cell activation, at least in part by engendering higher NK receptor binding affinity and specificity (Fig. 1D). These engagers can be further modified with the addition of IL15 between the two targeting domains, providing additional costimulation. A variety of trispecific killer engagers (TriKE) have been tested *in vitro* including against chronic lymphocytic leukemia (CD19; ref. 46) and

myeloid neoplasms (CD33; ref. 45). In each of these settings, TriKEs selectively induced NK cell proliferation and augmented antitumor toxicity better than constructs without IL15. Genetic modifications to NK cells have the potential to improve their specificity and reactivity. For example, expressions of the high-affinity and/or cleavage-resistant variants of CD16A have been transduced on the NK-92 cell line, as well as progenitor-derived NK cells, both of which have been shown to express low or absent CD16 natively (41). CARs have been successfully expressed on NK cells with an optimal signaling domain of NKG2D and 2B4 (refs. 18, 47; Fig. 1D).

NK cells are infrequently found within the TME, but increased numbers have been associated with improved outcomes in solid tumors including lung, esophageal, and colon (48). Various strategies to enhance NK cell homing have focused on chemokines and chemokine receptors (Fig. 1E). For example, forced expression of CXCR4 improves NK cell trafficking into and control of glioblastoma tumors, which secrete CXCL12/SDF-1α (49). For tumors that do not express chemokines, strategies to artificially create a chemotaxis gradient have been developed, including targeting mesothelin expressed by pancreatic cancer with an antibody–chemokine conjugate containing a cleavable linker to the chemokine CXCL16 (50). These and other approaches to enhance homing could improve NK cell antitumor efficacy.

Dampening immune suppression

A significant hurdle in antitumor immunity is the immunosuppressive TME. Tumors themselves can directly secrete suppressive cytokines such as TGFβ, IL10, and VEGFA, as well as express inhibitory ligands including PD-L1. Tumor-derived factors can skew APCs toward a more immunosuppressive subtype and recruit immunoregulatory cells including regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC). With respect to NK cell–specific mechanisms, various blocking antibodies have been developed to counter inhibitory signals (Fig. 1F). Lirilumab is an antibody that targets KIRs (specifically KIR2DL1/2/3), and monalizumab targets NKG2A. These antibodies, as well as those targeting TIGIT, TIM-3, and LAG3, are all checkpoint inhibitors in various phases of clinical development (2). Combinations are also being tested. For example, targeting multiple mechanisms with dual checkpoint blockade against NKG2A/HLA-E and PD-1/PDL-1 axes (e.g., monalizumab plus durvalumab in NCT02671435) or checkpoint blockade plus therapeutic antibody (e.g., monalizumab plus an anti-EGFR antibody in NCT02643550) are being tested (51, 52).

Other innovative strategies to address immunosuppression have used fusion proteins such as diphtheria toxin conjugated to IL2 (IL2–DT; Fig. 1F). This molecule preferentially binds to Tregs, which express the high-affinity IL2Rα (CD25). IL2–DT has been shown to partially deplete Tregs leading to improved NK cell persistence and antileukemia responses (53). Another approach is the expression of a dominant-negative TGFβ receptor on NK cells, which makes them resistant to TGFβ-mediated suppression, evidenced by maintained expression of activating receptors NKG2D and DNAM1, and preservation of antitumor activity *in vitro* (54). It is also important to highlight that adaptive NK cells have been shown to be relatively resistant to the suppressive effects of Tregs and MDSCs, which may have important clinical implications (55, 56).

Persistence

We and others have shown that NK cell persistence correlates with clinical outcomes. In the setting of adoptive cell therapy with haploidentical NK cells, persistence greater than 7 days is associated with improved disease control (11, 53, 57). The importance of persistence has also been observed in CAR T-cell trials (58). The loss of the NK cell persistence is mediated by two primary mechanisms: (i) insufficient survival/proliferation signals and (ii) allorejection. Lymphodepletion helps by increasing endogenous levels of homeostatic cytokines such as IL15. Exogenous cytokine supplementation with IL2 or IL15 may also promote NK cell maintenance, but this is not without limitations as IL2 can stimulate Tregs cells. One could hypothesize that endogenous production of cytokines through genetic modification of NK cells or APCs to mimic IL15 transpresentation could be optimal (ref. 18; Fig. 1G), including, as discussed earlier, hetIL15 and IL15/IL15Rα-Fc complexes (N-803; ref. 23).

Allorejection is another concern, and degrees of HLA-mismatch or specific HLA alleles may increase the clearance of allogeneic NK cell infusion. Attempts at immunologic stealth are ongoing in the sphere of progenitor stem cells, which requires evasion from multiple arms of the immune system. For example, knocking out HLA A/B/C and class II, overexpressing PD-L1 to avoid T-cell clearance, and overexpressing HLA-G and CD47 to avoid NK cell and macrophage clearance, respectively, result in hypoimmune pluripotent stem cells resistant to allorejection (59). It would be interesting to see whether a similar strategy would improve persistence of the various allogeneic NK cell platforms. These strategies could be important for proposed "universal" off-the-shelf options for multidosing, but this remains to be tested.

Conclusions

NK cells are potent mediators of antitumor immunity. Their use in cellular therapy has been shown to be safe, with minimal toxicities both in preclinical murine models and in clinical trials. Although results from NK cell trials have been promising, responses will need to be more durable. It is likely a multipronged approach that addresses current limitations to NK cell therapy will be optimal, including addressing issues of potency, specificity, and persistence balanced with safety. We are in important and exciting times for cell-based immunotherapy, and NK cell–targeted approaches may rival those already seen in the T-cell field. This remains to be tested and will be the focus of ongoing and future clinical trials.

Disclosure of Potential Conflicts of Interest

J.S. Miller is consultant for Fate Therapeutics and GT Biopharma, and a scientific advisory board member for Onkimmune; reports receiving commercial research grants from Fate Therapeutics and GT Biopharma; and has an ownership interest (including patents) in Fate Therapeutics and GT Biopharma. No potential conflicts of interest were disclosed by the other author.

Acknowledgments

K.V. Woan was supported by an NIH National Heart, Lung, and Blood Institute (NHLBI) Hematology in Research training grant (T32HL007062-42). J.S. Miller was supported by NIH NCI grants R35CA197292, P01CA111412, and P01CA065493.

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