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Authors
Grossenbacher, Steven K
Aguilar, Ethan G
Murphy, William J

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Natural killer (NK) cells are potent antitumor effector cells of the innate immune system. Based on their ability to eradicate tumors *in vitro* and in animal models, significant enthusiasm surrounds the prospect of leveraging human NK cells as vehicles for cancer immunotherapy. While interest in manipulating the effector functions of NK cells has existed for over 30 years, there is renewed optimism for this approach today. Although T cells receive much of the clinical and preclinical attention when it comes to cancer immunotherapy, new strategies are utilizing adoptive NK-cell immunotherapy and monoclonal antibodies and engineered molecules which have been developed to specifically activate NK cells against tumors. Despite the numerous challenges associated with the preclinical and clinical development of NK cell-based therapies for cancer, NK cells possess many unique immunological properties and hold the potential to provide an effective means for cancer immunotherapy.

Steven K Grossenbacher[1], Ethan G Aguilar[1] & William J Murphy*,[1,2]
[1]Department of Dermatology, University of California Davis School of Medicine, CA 95817, USA
[2]Department of Internal Medicine, University of California Davis School of Medicine, CA 95817, USA
*Author for correspondence: wmjmurphy@ucdavis.edu

Natural killer cells

Natural killer (NK) cells are lymphocytes of the innate immune system capable of eliminating virally infected and malignantly transformed cells without prior immunization [1]. Based on their capacity to kill tumor cells, *in vitro* and *in vivo*, human NK cells are critical effectors of anticancer immunity [2]. Unlike T cells, NK-cell reactivity is not restricted to specific MHC-peptide sequences and NK cells, classically, do not undergo clonal expansion following an encounter with target cells. Phenotypically, NK cells do not express the T-cell receptor (TCR) or CD3, and are instead defined by the expression of CD56, in humans, with variable expression of the Fc receptor CD16 [3]. NK cells are capable of becoming activated against a variety of target cells regardless of the expression of a specific antigen; instead NK-cell activation and the subsequent release of cytotoxic perforin and granzymes, or cytokine secretion, is based on the balance of activating and inhibitory signals received upon target cell engagement [4,5]. This natural cytotoxicity and broad target-cell reactivity makes NK cells particularly attractive as vehicles for cancer therapy.

Originally, human NK cells were defined by their ability to spontaneously lyse tumor cells lacking the expression of MHC class I (HLA) without any prior immunization [6]. This phenomenon led to the formation of the 'missing-self hypothesis'. NK cells recognize self-HLA expression on target cells via their killer cell immunoglobulin-like inhibitory receptors (KIRs), allowing them to recognize target cells that have down-regulated or altered expression of self [7]. In general, KIR-HLA binding between an NK cell and its target cell leads to the induction of immune tolerance due to the dominant

negative signaling cascade initiated by the immuno-receptor tyrosine-based inhibition motif found on the intracellular stalk of the KIR protein [8]. However, not all of the proteins within the KIR family are created equal, with KIRs having differential affinity for self-HLA proteins. Additionally, some KIRs are activating instead of inhibitory. KIR expression is variable on a cell-to-cell basis, with KIR genes being expressed stochastically, meaning that within the same individual some NK cells will bind self-HLA with high affinity, while others will lack affinity for self [9]. These differences in KIR expression provide a means for NK-cell 'education' or 'licensing' to occur. NK cells expressing KIRs that are capable of binding self-HLA with high affinity represent the classic cytotoxic NK cells and are thought to be 'licensed to kill' target cells [10]. NK cells expressing KIRs that have lower affinity for self, or lacking expression of KIRs altogether are 'unlicensed' or 'uneducated to self' and are generally hyporesponsive in terms of cytotoxicity compared with their licensed counterpart [10]. This developmental paradigm stratifies NK cells along the spectrum of effector function and maturation, and evidence from human studies suggests that education can have a dramatic impact on NK-cell cytotoxicity toward tumor cells [11].

In addition to KIRs, a variety of other stimulatory and inhibitory cell surface proteins regulate human NK-cell responses and represent targets for NK cell-directed therapies. Well studied among these include the NK group 2 member proteins which are both activating (NKG2D and NKG2C) and inhibitory (NKG2A), the Fc receptor CD16, the natural cytotoxicity receptors (NKp30, NKp44, NKp46) and CD244 (2B4) [12]. With the clinical and preclinical development of new immunotherapies for cancer, several other immunoregulatory proteins have also been identified or suggested to influence the effector functions of NK cells [13]. Recent understanding of even more diverse NK-cell subsets has also led to potential changes in how NK cells could be targeted for therapeutic benefit.

Traditionally NK cells were thought to be short-lived effector lymphocytes of the innate immune system, however, recent investigations have identified NK-cell populations possessing qualities associated with adaptive immunity, such as long-term persistence and immunological memory [14]. Several groups have identified memory-like and/or adaptive NK cells expressing unique functional and phenotypic characteristics compared with conventional NK cells [15,16]. Memory-like and adaptive NK cells appear to be preferentially present in human cytomegalovirus sera-positive individuals and the increased presence of these cells following CMV reactivation in hematopoietic stem cell transplant (HSCT) patients correlates with improved outcomes [17,18]. Interestingly memory-like NK cells have been found to express PD-1, and thus may represent targets for anti-PD-1 checkpoint blockade [19]. The persistence and memory-like features of these subsets of memory-like and adaptive NK cells open up the possibility of isolating more refined populations of effector NK cells that could be exploited, under the right conditions, for cancer immunotherapy.

Despite the fact that in *in vitro* studies and preclinical models, NK cells are effectively capable of lysing malignant tumor cells; similar efficacy has yet to be fully realized in human clinical trials. However, significant enthusiasm remains over the development of NK cell-based immunotherapies and several new strategies are being pioneered which may improve the efficacy and application of these cells.

Strategies to engage endogenous NK cells

NK cells can be activated by a number of different cytokines, including IL-2, IL-12, IL-15, IL-18, IL-21 and type I interferons. IL-2 is the most common of these that has been used to date, with patients routinely receiving IL-2 in combination with NK-cell transfusions to promote cell expansion *in vivo* [20]. Despite this enhanced expansion, no clinical advantage of the addition of IL-2 therapy was detected in a cohort of patients undergoing autologous transplantation for the treatment of relapsed lymphoma or metastatic breast cancer [21]. A large limitation of IL-2 therapy is the concurrent activation and expansion of CD25+ T regulatory (Treg) cells. Tregs express the high affinity IL-2 receptor complex and have been shown to limit NK-cell function not only through IL-2 competition [22,23], but also through TGF-β-dependent mechanisms [24,25]. Therefore, other cytokines that can more selectively activate NK cells, and not Tregs, are being explored. IL-15 is an essential mediator of NK-cell generation and homeostasis, with previous studies showing that mice deficient in IL-15 or the IL-15 receptor α chain lack mature NK cells [26,27]. A number of recent studies report seeing improved clinical responses correlating with IL-15 treatment or elevated serum IL-15 [28–30]. Recombinant IL-15 administration improved the persistence and proliferation of adoptively transferred haploidentical NK cells in patients with acute myeloid leukemia (AML) [31]. IL-12 is another proinflammatory cytokine with known immunomodulatory properties for NK cells as well as other lymphoid cells. In preclinical models, NK cells have been shown to have enhanced migratory capacity and augmented function following IL-12 administration [32]. To date the use of IL-12 in the clinic has resulted in severe toxicity and limited efficacy, likely due to the pleiotropic nature of IL-12 [33]. Protein and antibody engineering

has also revealed new methods to improve the pharmacodynamic and pharmacokinetic properties of NK cell-directed therapies. For example, development of so-called 'super agonists' has improved the utility of cytokine-based therapy. The superagonist IL-15-IL-15Rα-Sushi-Fc fusion protein (ALT-803) has been shown to exhibit greater biological activity than native IL-15 and potently stimulate antimetastatic activity from NK cells. Clinical trials are underway to evaluate its safety and efficacy in patients (NCT02099539 and [34]). Combinatorial strategies offer an attractive alternative to these single modality approaches. The combined use of IL-12, IL-15 and IL-18 is one such strategy that has been explored, with data showing this combination induces a long lived cytokine-induced memory-like NK-cell population with enhanced effector functions [35,36].

In addition to cytokines, Toll agonists also play a significant role in NK-cell activation. Human NK cells are thought to express TLRs 1–9 and are responsive to viral and pathogen-associated molecular patterns. Thus the selected utilization of Toll receptor agonists could lead to improved antitumor efficacy via NK-cell activation [37]. Indeed recent experiments have shown that certain synthetic toll agonists are capable of demonstrating antitumor efficacy in murine models of metastatic cancer in an NK cell-dependent manner [38,39].

Several pharmaceutical agents are also potently capable of augmenting NK-cell reactivity toward tumors. The thalidomide derivative lenalidomid (Revlimid®), is approved in combination with dexamethasone for the treatment of multiple myeloma patients who have received at least one prior therapy. While the precise mechanism of action of lenalidomide is not completely clear, evidence suggests that significant NK-cell immune modulation occurs in response to treatment. For example, lenalidomide is known to increase IL-2 and IFNγ secretion from T cells and dendritic cells, aiding in NK-cell activation and function [40]. Lenalidomid treatment also leads to an increase in circulating NK-cell numbers in leukemia patients [41].

CD16 engagement of an antibody's Fc region is another primary method to activate NK-cell cytotoxicity. Many of the most commercially and clinically successful therapeutic monoclonal antibodies (mAbs), such as rituximab (Retuxin®), cetuximab (Erbitux®) and trastuzumab (Herceptin®) elicit their therapeutic effects, at least in part, by activating NK cells via antibody-dependent cell-mediated cytotoxicity (ADCC) [42,43]. Unlike other activating receptors, which generally require the coordinated engagement of multiple receptors in order to elicit a positive response; CD16 engagement alone is sufficient to provide a robust activation signal for NK cells [44]. For example, the efficacy of anti-GD2 antibody therapy for the treatment of neuroblastoma is achieved through ADCC mediated by NK cells [45]. The interest to generate ADCC-inducing mAbs has led to the development and engineering of tumor-specific antibodies capable of activating NK cells more robustly than conventional mAbs. Several clinical trials are under way to assess the efficacy of these antibody-based therapies, and new molecules are being designed to engage and/or target multiple stimulatory/inhibitory targets simultaneously [46].

Novel trispecific killer cell engagers and bispecific killer cell engagers have been developed to specifically trigger NK-cell activation by cross-linking tumor antigen-specific antibodies with cytokines such as IL-15, or with antibodies binding to alternative epitopes of CD16 [47–49]. These engineered molecules elicit higher cytotoxic potential compared with conventional antibodies targeting the same antigen [46]. Additionally, CD16 is known to be lost or cleaved from the surface of NK cells rendering them hyporesponsive to ADCC [50]. Based on this finding, strategies are being developed to prevent the cleavage of CD16 through the inhibition of disintegrins and metalloproteases such as ADAM17 [50]. For example, the addition of ADAM17 inhibitors has been found to improve the cytotoxicity of a CD16xCD33 bispecific killer-cell engager against CD33+ AML cells *in vitro*, and a clinical trial is underway assessing the combination of rituximab with an ADAM17 inhibitor following autologous HSCT for patients with diffuse large B-cell lymphoma (NCT02141451).

Additionally, monoclonal antibodies designed to prevent NK-cell inhibition are in various stages of clinical development. Even though T cells remain the major focus of checkpoint blockade immunotherapy, NK cells have also been found to respond to this approach. NK cells can express PD-1 and a variety of other proteins associated with co-stimulation or inhibition such as TIGIT, Tim-3, CD96, CD137, OX40, GITR and CD27 [51]. The PD-1-PD-L1 pathway is a critical immune regulator limiting the antitumor immune responses of T cells and NK cells. PD-1 is known to be expressed on activated T cells and was recently found to be expressed on NK cells from patients with multiple myeloma [52]. Interestingly these PD-1+NK cells were capable of killing autologous tumor cells *ex vivo*, in the presence of an anti-PD-1 antibody, suggesting that PD-1 blockade may augment endogenous NK-cell responses by breaking tumor-induced tolerance. It was also recently established that a subset of fully mature, memory-like NK cells expressing PD-1, can have their effector functions augmented in responses

to anti-PD-1 antibody blockade [19]. In addition to these approaches, KIR blockade has also been evaluated as a method of NK cell-specific checkpoint blockade. Interestingly recent clinical trials have proven the use of a novel anti-KIR2D antagonist (IPH2101) to be ineffective as a monotherapy. In fact IPH2101 treatment led to a rapid reduction in KIR2D expression and rendered NK cells hyporesponsive which may have been due to the antibody blocking the KIRs associated with the education process of NK cells during their development [53]. This study raises concern over the use of conventional antibody-based methods aimed at augmenting endogenous NK-cell responses, particularly through KIR-blockade as this may have unforeseen effects on NK cell development.

Adoptive NK-cell therapy

The use of unmodified cells either from healthy donors (allogeneic) or cells sourced from the patient themselves (autologous) has been explored for the treatment of a variety of hematological and solid tumor types with varying results [54]. NK cells are well known to be one of the first cell types to reconstitute following HSCT and appear to be a critical mediator of the antitumor efficacy of HSCT [55]. Importantly, patients with quicker NK-cell reconstitution post-transplant have better outcomes [56]. The strongest evidence supporting the efficacy of donor-derived NK cells comes from studies in patients with AML. In the seminal study, patients receiving HLA-haplotype mismatched bone marrow cells with KIR ligand incompatibility were protected from graft rejection, GVHD, and AML relapse when compared with patients who received KIR-ligand compatible cells [57]. Follow-up studies have found similar efficacy for other patients with AML. In the non-HSCT setting, haploidentical NK cells were found to expand *in vivo*, and led to remission for a subset of high-risk AML patients, with best responses observed in patients who received KIR-mismatched cells [20]. Interestingly, the transfer of purified autologous NK cells into patients with either stage IV renal cell carcinoma or metastatic melanoma led to no meaningful clinical benefits. Despite the lack of clinical benefit in these trials, the transferred NK cells did appear to persist in the patients' circulation for several months, in some cases. It is important to note that the recovered cells expressed lower levels of the activating receptor NKG2D and could not lyse tumor targets *in vitro* without restimulation with IL-2 [58]. However, it was found that the persistent NK cells could mediate ADCC *in vitro*, suggesting that combination strategies with monoclonal antibodies may allow for enhanced efficacy of adoptive NK-cell therapy. Additionally, the differences in efficacy of NK-cell therapy between

hematological and solid malignancies suggest that approaches which sensitize tumor cells from solid tissues to NK-cell killing, could have significant clinical benefit.

In addition to its direct antitumor effects, ionizing radiotherapy is a potent modulator of tumor cell susceptibility to NK-cell cytotoxicity. The DNA damage response induced by the formation of free radicals in cells exposed to radiotherapy is well known to initiate the transcription of genes associated with cellular stress, such as NKG2D ligands and death receptors, which are recognized by NK cells [59]. Perhaps as a method to restrain the malignant transformation and growth of genotoxically stressed cells, NK cells are intrinsically armed with the ability to identify and lyse stressed targets through their NKG2D activating receptor. In fact, radiotherapy is known to upregulate NKG2D ligands on a variety of cancer cell types *in vitro* and *in vivo* leading to increased sensitization to NK-cell-mediated killing [60,61].

Radiotherapy has also been found to enrich for stem-like tumor cells, which can mimic stem cells in their ability to undergo asymmetric differentiation, and have a reduced replicative capacity. Human tumors, especially those arising from solid tissues, are known to have cell-to-cell differences in genetic programs and mutational loads leading to marked intratumoral heterogeneity [62]. This heterogeneity poses a serious problem for the use of conventional chemotherapy, radiotherapy and small molecule drugs aimed at targeting certain genetic drivers and oncogenic pathways within a tumor. So-called 'cancer stem cells' or 'tumor-initiating cells' have the ability to undergo asymmetric cell divisions and are thought to be able to seed relapse and metastasis following conventional therapy [63]. Recent studies have shown that allogeneic and autologous NK cells are inherently capable of recognizing and killing these cancer stem cells *in vitro* and *in vivo*. In mouse models of pancreatic cancer, glioblastoma and others, it has been discovered that these stem-like tumor cells express elevated levels of several proteins including MICA, PVR-1 and DR5 that bind to the activating proteins, NKG2D and DNAM-1, and the apoptosis-inducing protein, TRAIL, respectively, present on the surface of NK cells, compared with tumor cells with a more differentiated phenotype [64,65]. Similar studies show that radiotherapy, when applied in a rational combination prior to the administration of activated allogeneic NK cell transfer could lead to a synergistic and near complete eradication of cancer stem cells (CSCs) *in vivo* [60]. This combination strategy could be applied clinically as a way to reduce patient relapse rates and improve the utility of both NK-cell transfer and radiotherapy (Figure 1). NK cells have also been
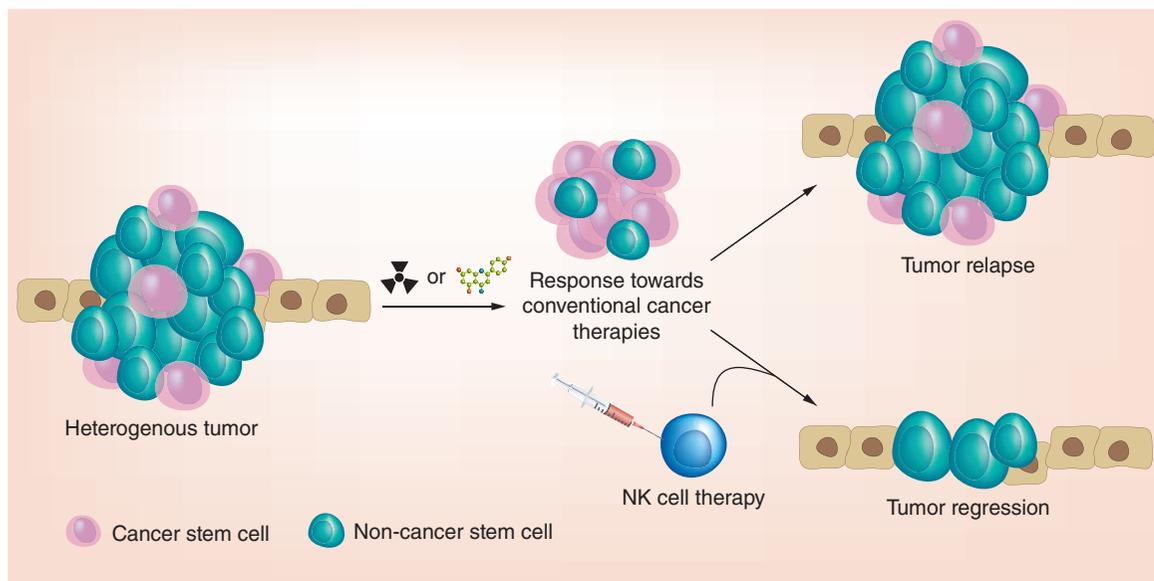
Figure 1. Schematic of natural killer-cell targeting of cancer stem cells following radio/chemotherapy. Populations of cancer stem cells can withstand conventional radiotherapy and/or chemotherapy. In the absence of a cancer stem cell targeting approach, the residual cancer stem cells can seed relapse of the primary tumor. NK cells are capable of killing cancer stem cells and reducing the onset of relapse.
NK: Natural killer.

found to synergize with chemotherapy to target CSCs in preclinical models of nonmuscle invasive bladder cancer [66]. While the role of the cancer stem cell populations in human cancer progression remains controversial, the ability of NK cells to target cancer stem cells may offer a paradigm shift in cancer treatment approaches.

Additionally, the proteasome inhibitor bortezomib (Velcade®) is known to sensitize tumor cells to NK-cell cytotoxicity by inducing the expression of apoptosis-associated death receptors, such as DR5, and by reducing the generation of peptides normally bound for the HLA complex, leading to a decrease in HLA expression [67–69]. In fact, recent preclinical studies demonstrated that bortezomib is capable of sensitizing tumor cells treated with the oncolytic virus, oHSV-1, to adoptive NK-cell killing [70]. In this study, bortezomib and oHSV-1 synergized to induce the necroptotic cell death of orthotopic glioblastoma xenografts. The addition of intracranially infused NK cells to the combination therapy significantly enhanced tumor cell death leading to enhanced survival of tumor-bearing animals.

Additionally, histone deacytlase inhibition was recently shown to lead to heightened NKG2D expression on pancreatic cancer cells, suggesting that several rational combinations could be increasingly applied with cellular immunotherapy to improve patient outcomes [71,72]. Similar strategies are now being examined to enhance the specificity of adoptive NK-cell therapy through genetic manipulation of NK-cell surface receptors.

Chimeric antigen receptor engineering has transformed the nature of adoptive T-cell therapy. CD19-directed T cells have demonstrated tremendous efficacy in juvenile patients with acute lymphoblastic leukemia [73]. Trials are now underway to assess the efficacy and tolerability of CAR-engineered T cells in a variety of hematological and solid tumors [74]. Despite the interest and clinical success of CAR engineering, CAR-engineered NK cells have yet to make the same imprint on the cancer immunotherapy community. While there are some key advantages for T-cell immunotherapy, CAR-directed NK cells offer an alternative treatment modality that could overcome some of the challenges associated with T-cell CARs, most prominent among them is the manifestation of life-threatening toxicities which have led to the termination of clinical trials and drug development programs [75].

Clinical trials are currently underway investigating the use of CD19-directed NK cells in the treatment of acute lymphoblastic leukemia (Table 1) (NCT00995137 and NCT01974479). The fact that, unlike T cells, NK cells are generally perceived to be short-lived effector cells, which do not undergo clonal expansion, may allow CAR-directed NK cells to be utilized with less fear for the development of autoimmunity or adverse toxicities associated with rampant T-cell expansion that can occur in patients receiving CAR-T. Another concern with the expanded use of CAR-T is the need for the continued and robust expression of a specific antigen. Tumor cells are known to evade immune detection by altering HLA expression

NCT number	Phase	Disease	Treatment
Table 1. Ongoing clinical trials of natural killer cell adoptive therapy.			
Non-engineered			
NCT02809092	I/II	AML	Membrane-bound IL21 (mbIL21)-expanded haploidentical NK cells
NCT02280525	I	AML, ALL, CML	Umbilical cord blood-derived NK cells
NCT01823198	I/II	AML, MDS, CML	KIR-mismatch haplo-donors/KIR-mismatched cord blood derived/matched sibling donors
NCT02854839	II	Hepatocellular carcinoma	*Ex vivo* expanded allogeneic NK cells
NCT02573896	I	Neuroblastoma	Autologous expanded NK cells + anti-GD2 antibody
NCT01729091	I/II	Multiple myeloma	Umbilical cord blood-derived NK cells
NCT02100891	II	Sarcoma, neuroblastoma, CNS tumors	HLA-haploidentical NK cells
NCT02734524	II	NSCLC	Autologous NK cells + taxol + carboplatin
NCT02650648	I	Neuroblastoma	HLA-haploidenitcal NK cells + anti-GD2 antibody
NCT02725996	II	Hepatocellular carcinoma	Autologous NK cells following curative surgical resection
NCT01898793	I	AML	Cytokine induced memory-like NK cells
NCT00720785	I	Lung cancer, prostate cancer, colon cancer, kidney cancer, pancreatic cancer, leukemia, melanoma	*Ex vivo* expanded autologous NK cell-administered following bortezomib treatment
NCT02118415	II	NSCLC	Hsp70-peptide TKD/IL-2 activated, autologous NK cells
CAR-engineered			
NCT03056339	I/II	ALL, CLL, NHL	CD19-CD28-ζ-2A-iCasp9-IL15-transduced cord blood NK cells
NCT01974479	II	B-ALL	Activated NK cells bearing anti-CD19-BB-ζ receptors
NK-92			
NCT02944162	I/II	AML	Chimeric CD33 receptor modified NK92 cells
NCT02892695	I/II	Leukemia/lymphoma	CD19-directed NK92 cells
NCT02742727	I/II	Leukemia/lymphoma	CD7-directed NK92 cells
NCT02839954	I/II	Solid tumors	MUC-1-directed NK92 cells
NCT03027128	I	Solid tumors	NK-92 engineered to express the high affinity variant of CD16

ALL: Acute lymphocytic leukemia; AML: Acute myeloid leukemia; CLL: Chronic lymphocytic leukemia; CML: Chronic myeloid leukemia; MDS: Myeloid dysplastic syndrome; NHL: Non-Hodgkin's lymphoma; NK: Natural killer; NSCLC: Non-small-cell lung cancer.

or through tumor heterogeneity and the generation of antigen loss variants [76,77]. Unlike T cells, NK cells kill in an antigen-independent manner and would, therefore, retain mechanisms independent of the CAR to target and kill those tumor cells that would otherwise evade T-cell recognition. Despite the potential benefits of CAR-NK cells, several challenges remain concerning the fact that NK cells can be difficult to manufacture and that different activation and expansion protocols have been found to lead to drastic differences in NK-cell function.

Work is now underway to expand second- and third-generation CAR platforms to include genes which can elicit IL-15 production as well as caspase-controlled suicide vectors in an effort to improve CAR effectiveness and safety [78]. Additionally, the transfection efficiency of NK cells remains fairly low and new methods are being developed in order for CAR-NK technologies to scale-up to the level of CAR-T [79]. An interesting alternative to the use of primary human NK cells is the use of the NK-cell line, NK-92. NK-92 was originally derived from an NK-cell lymphoma and over the years it has consistently shown the ability to be expanded and activated to lyse tumor cell targets since its isolation more than 20 years ago. Because of its ease of use and consistent activity, NK-92 is an attractive platform for CAR development. Preclinical NK-92 cells have been engineered to express a variety of antigen receptors and

surface proteins including receptors to CD19, HER2 and EGFR [80–82]. Additionally, because NK-92 cells do not express CD16, groups are now working to engineer high affinity CD16 molecules into NK-92 cells as a means to improve their effector functions [83]. As a whole, several clinical trials are underway assessing the efficacy of genetically manipulated autologous NK, allogeneic NK and NK-92 cells for the treatment of hematological and solid malignancies (Table 1). While these strategies have given rise to promising CAR-NK platforms, a key limitation for the use of NK cells for solid tumors is the failure of adoptively transferred NK cells to traffic into tumor sites.

The ability to influence the trafficking and intratumoral homing of a transferred NK-cell population is a significant hurdle for the advancement of adoptive NK-cell therapy. The presence of NK cells in pretreatment tumor biopsies correlates with improved outcomes for patients with solid malignancies [84]; however, the ability of adoptively transferred NK cells to traffic into tumors is severely limited [85]. A fact that may also explain the varied outcomes observed from NK-cell trials in hematological versus solid malignancies. Several strategies are being evaluated which may improve the efficacy of NK-cell therapy by increasing the homing potential of transferred cells into both the tumor site and the draining lymphoid tissues, which may harbor metastatic tumor cells. CCR7-modified NK cells were shown to migrate toward CCL19 and CCL21 *in vitro* and demonstrated improved lymph node homing in nude mice [86]. Interestingly in these experiments, CCR7 was transferred to the NK cells through the process of trogocytosis. Trogocytosis, the acquisition of cell surface proteins via direct cell–cell contact, has been identified as a means to overcome the challenges associated with viral transfection of primary NK cells. It has been proven as a means to impart functional antigen receptors recognizing CD19 and HER2, as well as chemokine receptors such as CCR7 [87,88]. Despite these increases in NK-cell homing, some have reported that trogocytosis can render immune cells hyporesponsive and act as a method of tumor immune escape [89]. In addition, there are many logistical challenges that would need to be overcome to make the large-scale production of trogocytosis-engineered NK cells feasible. More studies are needed to understand how to overcome the limited ability of NK cells to traffic into solid tumor sites.

A final factor that heavily influences the efficacy, utility and reproducibility of primary NK cells as adoptive therapy is the method of stimulation and expansion. Cytokines such as IL-2, IL-12, IL-15 and IL-18 have been used to induce the activation and expansion of human NK cells *in vitro* [2]. While cytokine-based activation techniques can yield impressive cytotoxicity in *in vitro* and *in vivo* assays, the NK cells become cytokine addicted and quickly lose viability once transferred into the systemic circulation. In addition to cytokines, the use of engineered 'feeder' cell lines has been developed as a method to generate robust NK-cell expansion and activation necessary to meet the needs of clinical utilization. Engineering of cell lines, such as the human K562 cell line with a variety of co-stimulatory proteins including CD137 and membrane forms of IL-15 or IL-21, has been demonstrated to induce greater than 1000-fold expansion of human primary NK cells over a 3- to 4-week period [90]. Continued experimentation is needed to optimize the precise method and cocktail of cytokines and feeder cells capable of maximizing NK cells for clinical use.

Conclusion

NK cells have proven to be significantly more diverse than originally thought. These stereotypically short-lived effector cells, capable of indiscriminately eliminating tumor cells, are now known to have features of adaptive immunity and may have potential to improve the efficacy of conventional therapies and represent an ideal partner for combinatorial immunotherapy. NK cells could represent a method of immunotherapy that, unlike T cells, could be more readily fine-tuned to prevent treatment-associated toxicity and immune-mediated adverse events. The development of inexpensive genetic sequencing approaches will allow for further investigation and understanding of how NK-cell education and HLA-KIR interactions affect tumor formation and response to therapy. While murine studies have been the predominant means of examining NK-cell biology, these models are still limited. Inbred mice lack the genetic complexity needed to fully examine the effects of HLA-KIR (MHCI-Ly49 in mouse) binding on tumor progression and immune cell function. As a result, new, more complex models are being investigated for the study of immunotherapy, including the use of companion canines bearing spontaneous malignancies, which more closely match the human cancer scenario. The continued genetic manipulation of NK cells into antigen-specific effector cells could lead to improved responses of unmodified cells, however, key obstacles must be considered if NK cell-based immunotherapies are to meet their perceived potential. These obstacles include the ability to traffic into solid tumor sites and avoid tumor immunosuppression, the resistance to current gene transfection techniques, and the development of culture and expansion techniques that promote the reproducible growth and expansion of NK cells for therapy.

Future perspective

The clinical success of recent cancer immunotherapies targeting immune checkpoints and utilizing chimeric antigen receptor engineered T cells has ushered in a new age for the treatment of cancer. Immunotherapy now stands as the fourth leg in the clinical arsenal, joining surgery, chemotherapy and radiotherapy, to treat a variety of hematological and solid malignancies. The key for the continued success of cancer immunotherapy will be to understand how different combinatorial strategies can be harnessed and tailored to meet the needs of certain patient populations. An in-depth understanding of the antitumor efficacy and function of NK cells will allow future clinicians and scientists to better leverage the innate killing abilities of these cells. While it is unlikely that unmodified NK cells themselves can be completely efficacious as a monotherapy, it is likely that certain tumor types will respond favorably to NK-cell treatment in combination with other therapies. Those tumors harboring more undifferentiated cell types, and expressing higher levels of stress-associated and/or stem-like proteins, would be particular good candidates for NK-cell therapy in combination with conventional chemotherapy and/or radiotherapy. Last, as we develop a better understanding of both the physiological relationship between CMV exposure and NK-cell functions, and the immunological consequences of NK-cell education, tumor immunologists will be able to shed light on how early tumorigenesis occurs in spite of the innate recognition and killing functions that are inherent to NK cells. Therapeutic strategies designed to leverage NK cells will continue to be part of the cancer immunotherapy paradigm for years to come.

Executive summary

- Natural killer (NK) cells are innate lymphocytes found in the peripheral circulation and in lymphoid tissues. NK cells are capable of lysing virally infected and malignantly transformed cells without prior immunization or exposure. NK-cell effector functions are regulated by the balance of activating and inhibitory signals received from cell surface receptors and proteins upon encountering a target cell.
- NK cells exist as diverse populations with different phenotypes and propensities for cytotoxicity and cytokine function. This diversity is the result of CMV exposure and influences NK-cell cytotoxicity of tumor cells.
- NK cells are known to express a variety of co-stimulatory molecules which represent targets for novel immunotherapies. Recent clinical trials investigating the use of inhibitory KIR blockade proved ineffective as a monotherapy.
- NK cells are particularly effective at recognizing and killing cancer stem cells. Following conventional therapy, such as radiotherapy and/or chemotherapy, NK cells are able to identify residual tumor cells and lyse them via NKG2D and other activating receptors.
- CAR-NK-cell therapy may be more advantageous than T-cell CARs due to limited clonal expansion, leading to reduced toxicity. However, the transfection techniques and methods of isolation and expansion of cells *ex vivo* can affect the final cell therapy dramatically.
- All of these data suggest that combinatorial strategies may be beneficial for leveraging NK cells for cancer immunotherapy.

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