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Targeting Cancer Stem Cells with Natural Killer Cell Immunotherapy

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Authors

Luna, Jesus I
Grossenbacher, Steven K
Murphy, William J
et al.

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Targeting Cancer Stem Cells with Natural Killer Cell Immunotherapy

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Article Highlights:

- Cancer stem cells are an important source of resistance to standard anti-cancer therapies and can seed relapse/metastasis.
- Natural killer cells are innate lymphoid cells which can kill tumor cells in an MHC-unrestricted fashion.
- Unlike cytotoxic cancer treatments, natural killer cells are able to target and eliminate quiescent/ non-proliferating cells such as cancer stem cells.
- Chemotherapy and radiation therapy eliminate non-cancer stem cells, leading to enrichment of cancer stem cells.
- Cancer stem cells upregulate natural killer cell ligands, such as MICA and MICB, after treatment which has been observed to increase natural killer cell targeting of these cells.
- Cancer stem cells also are able to utilize immune evasion strategies such as shedding of MICA and MICB to reduce natural killer cytotoxicity.
- Monoclonal antibodies specific for cancer stem cell epitopes can increase natural killer killing through antibody-dependent cellular cytotoxicity mechanisms.

ABSTRACT

Introduction: Standard cytoreductive cancer therapy, such as chemotherapy and radiotherapy, are frequently resisted by a small portion of cancer cells with “stem-cell” like properties including quiescence and repopulation. Immunotherapy represents a breakthrough modality for improving oncologic outcomes in cancer patients. Since the success of immunotherapy is not contingent on target cell proliferation, it may also be uniquely suited to address the problem of resistance and repopulation exerted by cancer stem cells (CSCs).

Areas covered: Natural killer (NK) cells have long been known for their ability to reject allogeneic hematopoietic stem cells, and there are increasing data demonstrating that NK cells can selectively identify and lyse CSCs. In this report, we review the current knowledge of CSCs and NK cells and highlight recent studies that support the concept that NK cells are capable of targeting CSC in solid tumors, especially in the context of combination therapy simultaneously targeting non-CSCs and CSCs.

Expert Opinion: Unlike cytotoxic cancer treatments, NK cells are able to target and eliminate quiescent/ non-proliferating cells such as CSCs, and these enigmatic cells are an important source of relapse and metastasis. NK targeting of CSCs represents a novel and potentially high impact method to capitalize on the intrinsic therapeutic potential of NK cells.

Introduction

Although still somewhat controversial, cancer stem cells (CSCs) have been proposed as an important mechanism of tumor initiation and/or repopulation after tumor debulking by radiotherapy and/or chemotherapy. In addition, CSCs have been increasingly identified as a source of tumor relapse and metastasis, even in cases of apparent complete response to systemic therapy.(1-3) Consequently, targeting of CSCs in a combination strategy which eliminates non-CSCs is hypothesized to translate to improved long term oncologic outcomes for cancer patients. NK cells represent a subset of cytotoxic lymphocytes with the ability to respond to and eradicate tumor cells. NK cells have also demonstrated the ability to detect and eradicate “stem-like” cells as shown by their ability to reject allogeneic hematopoietic stem cells. Therefore, NK-mediated killing is a promising candidate for targeting of CSCs, especially in the context of combination therapy where non-CSCs are eliminated by standard anti-proliferative therapies. In this review, we summarize recent studies that suggest that NK cell-based immunotherapy aimed at targeting CSCs can provide important therapeutic benefits in the combined modality treatment of solid malignancies.

Body

Cancer Stem Cells

CSCs are classically defined by their capacity to self-renew, differentiate into different lineages, and maintain homeostasis within the tumor, in principle making CSCs analogous to embryonic stem cells or pluripotent adult stem cells in their behavior (1, 4, 5). Key papers have demonstrated that CSCs are able to undergo symmetric cell division giving rise to “daughter” cells as a result of clonogenic expansion. Additionally, the progenitor CSC may divide and undergo asymmetric cell division generating multiple “daughter” cells having distinct

differentiation capacities in accordance with the anatomical site of origin and the hierarchical "stemness" position of the "mother" cell (6, 7). However, in contrast to these other stem cells, CSCs generally display neoplastic behavior such as perturbed growth properties. For example, unlike fully differentiated progeny cells derived from normal stem cells, differentiated or "bulk" tumor cells derived from CSCs have the potential to proliferate indefinitely (8, 9). Yet, despite the evidence in favor of the CSC model, it is important to note that a "stochastic" model has also been proposed as an alternative mechanism to explain tumor heterogeneity, and skeptics of the CSC hypothesis remain.(10, 11) The stochastic model asserts that all tumor cells are consider equipotent and only a fraction of tumor cells have high clonogenic potential to generate tumor growth and sub-clone formation. Defining the mode of tumor growth and maintenance of tumor heterogeneity represents a key hurdle to acquiring a better understanding of the involvement and contribution of CSCs to tumor maintenance and progression.

Cancer Stem Cell Markers

In contrast to normal stem cells, CSCs generally lack a standard set of immunophenotypic markers that span across different types of tumors (9). Hence, in this review we provide a short list of some of the markers that our group and others have reported to be reproducibly associated with a tumor "stem-like" or CSC phenotype. As depicted in **Table 1**, CD133, CD44, and ALDH are some of the most widely used and characterized markers used in "defining" cell subpopulations with stem cell-like activity in solid tumors (1, 8, 12-14) including: prostate cancer (8, 12), breast cancer (15, 16), colorectal cancer (17), pancreatic adenocarcinomas (18), soft tissue sarcomas (19), and brain tumors/glioblastoma (20). Some of the aforementioned markers have been used individually, or in combination, with other markers associated with the particular tumor of interest and/or in accordance to the anatomical site of

origin of the target tumor. In addition, key pluripotent stem cell markers and transcription factors such as Notch and the wingless-related integration site (Wnt) gene family have been recognized as important phenotypic and functional markers of CSCs (21-23).

Aldehyde dehydrogenase 1 (ALDH 1) is a detoxifying enzyme involved in oxidation of intracellular aldehydes (24). It has been extensively reported that ALDH expression and activity is closely associated with drug resistance, cell proliferation, and response to stress-related stimuli such as the production of reactive oxygen species (12, 25, 26). Moreover, ALDH activity has become one of the most widely used phenotypic markers used to identify CSCs, especially in solid tumors. However, despite this extensive characterization of $ALDH^{bright}$ cells with a CSC phenotype, critics have highlighted the weaknesses of using ALDH as a CSC marker, including high intrinsic expression in non-neoplastic tissues (such as liver and pancreas) and subjective definitions of $ALDH^{bright}$ and $ALDH^{dim}$ cells when using immunohistochemical and flow cytometry-based readouts where a broad spectrum of enzyme activity is present rather than all-or-none expression (25, 27). As a result, critics contend that ALDH expression is not completely representative of cellular self-renewal characteristics, but instead also reflects other diverse cellular functions not completely associated with the CSC phenotype (26).

CD44 is a cell-surface glycoprotein involved with malignant tumor initiation (28). In addition, CD44 interacts multivalently with hyaluronan resulting in signal pathway activation of tyrosine kinases such as ErbB2, EGFR, and TGF-1(29). Consequently, targeting of CD44 has received increasing attention as a novel target in anti-cancer and anti-CSC strategies. However, similar to ALDH, CD44 has multiple paracrine and autocrine effects, including roles in multiple signaling cascades, so the precise mechanism by which it fosters the CSC phenotype is not definitively established. Prominin-1 (CD 133) is a transmembrane glycoprotein which remains

poorly characterized with respect to its precise biological functions and cognate interactions. Yet, CD133 has been extensively evaluated in multiple CSC studies and in multiple types of cancers (30-32). Numerous studies have validated the CSC phenotype in CD133+ cells, and an inverse relationship between CD133 expression and median disease-free survival has been shown in clinical-translational studies in colon cancer, among others (17).

The Wnt signaling pathway is involved in several key developmental and regenerative physiological processes. Importantly, however, this diverse signaling pathway (including both canonical and non-canonical networks) has also been associated with tumor oncogenesis including CSC recruitment, propagation, and cross-talk (21-23). A potential mechanism underlying the transition of Wnt from regulating homeostasis to driving oncogenesis/ CSC propagation has been explained by defects in the beta-catenin degradation complex, thereby resulting in redirection and accumulation of beta-catenin in the nucleus. Consequently, this accumulation triggers transcription of Wnt target genes and expression of the CSC phenotype (33).

For example, Vermeulen et al. has shown that Wnt signaling activity is implicated in colon cancer CSCs and is regulated mainly by myofibroblasts surrounding the tumor microenvironment (34). This report also describes how non-CSCs can be reprogramed to express CSC markers and regain tumorigenic capacity in response to myofibroblast-derived factors which enhance Wnt signaling activity. In another important study, Howe et al. demonstrated Twist upregulation in murine mammary tumors in response to Wnt1 expression which then promoted epithelial-mesenchymal transition (EMT)-like processes in an autocrine and paracrine manner (35). EMT, the transitional process that allows polarized, immotile epithelial cells to transform to motile mesenchymal cells, has been linked to cancer invasion and metastasis

formation (36). In addition, important overlap has been established between EMT and the CSC phenotype (for example, overexpression of Twist and Snail induces EMT and induces the expression of CSC markers), reinforcing the premise that CSC targeting is anticipated to translate to meaningful clinical benefits in cancer patients (22, 23, 36). Ultimately, however, advances in understanding the biology of CSCs and methods to target them will benefit from the development of a more robust and clear-cut immunophenotypic characterization and classification of tumor subpopulations, especially those with CSC characteristics.

Cancer Stem Cell Validation

Identifying potential subsets of CSCs requires immunophenotyping of the bulk tumor population using cell sorting by flow cytometric approaches or using alternative cell separation techniques such as magnetic beads linked to antibodies or both. Once "CSC" subsets have been separated from the bulk tumor, validation is commonly performed by injecting cells into the non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice lacking a functional IL2RG chain (NSG or NOG) mice to confirm the ability of these cells to initiate tumors (7). Further assessment of the CSC phenotype can be characterized by the ability of the isolated cell population to closely mimic the tumorigenic characteristics of the tissue of origin. Even though *in vivo* models are the closest biological representation to elucidate tumor biology and function, these models may also be flawed because injecting CSCs into a new tissue location may fail to precisely recapitulate the environment of those tumor cells in the original tumor (37).

Tumor sphere formation and 3-D culture techniques are other *in vitro* assays commonly used in parallel with xenograft models to validate the tumorigenic capacity of CSCs (38). In both *in vitro* and *in vivo* models, cells are sorted from the overall tumor population based on individual markers or a panel of them, and CSCs are either transplanted into animal models at

different concentrations or allowed to aggregate as spheroids/organoids in low attachment culture conditions. Although immortalized cell lines grown in adherent culture conditions represent a useful tool to elucidate tumor formation either in *in vivo* or *in vitro* culture systems, a major disadvantage of using cell lines is the inherent use of long term culture conditions in which cells lines are maintained. These artificial conditions lack key microenvironmental factors found in the native tumor environment, thereby causing significant phenotypic differences in the cells in these systems. However, there still can be valuable information obtained from interrogating cell lines since they can allow for rigorous testing of possible pharmacological and/or physiological outcomes involved in primary tumors, recognizing that mechanistic data obtained from cell culture assays may not accurately or completely recapitulate the heterogeneity and/or epigenetic phenomenoma of other more complex tumor models.

Primary tumor/ short term culture lines (PL) may represent an improved alternative to using conventional cancer cell lines as PL are directly isolated from primary tumors and kept in culture for a shorter period compared to cell lines. The shortcoming of using PL is the potential for rapid phenotypic loss of epigenetic and microenvironmental expression patterns ascribed to *in vitro* culture conditions. Hence, maintaining PL in a congruent phenotype concomitant with the parent tumor may be a challenge, but the biological responses observed with PL, including the persistence of a CSC population, may be more relevant in these studies, especially when trying to extrapolate therapeutic effects of novel therapies. Moreover, primary tumor/ patient-derived xenografts (PDX) may help to further overcome the limitations associated with the use of cell lines and PL, especially with respect to studies of CSC biology and immune targeting (37).

CSC Biology in Solid Tumors

The concept of the CSC niche in solid tumors has also been extensively modeled on parallel studies of non-neoplastic embryonic and pluripotent stem cells. It has been extensively documented that normal stem cells are contained within a “niche” that provides specific signaling through cell-to-cell contact, extracellular matrix, and paracrine/autocrine signals that maintain and protect stem cells. Similarly, it has been proposed that CSCs may be contained within a similar niche that nurtures CSCs and may also be responsible for promoting metastasis and tumor progression via cell-cell signaling and promotion of EMT (7). Importantly, there is evidence that a CSC-specific “niche” is also involved in promoting CSC quiescence, fostering immune evasion, and providing signals for therapeutic resistance (39). The foremost factors contributing to the tumor microenvironment as well as the CSC “niche” include the stroma, which is generally composed of a complex mixture of malignant and not malignant cells such as blood endothelial cells (BEC), lymphatic endothelial cells, fibroblasts, mesenchymal cells and other immune-associated cells (**Figure 1**). Other important factors having an effect on CSC and non-CSC behavior include biochemical signaling defined by hypoxic conditions and pH gradients within the tumor (40). These are all important issues to consider when evaluating novel treatments to target CSCs, such as natural killer (NK) cell immunotherapy, since BECs, for example, have been implicated in creating a suppressive environment for T cells via secretion of soluble CD137 (41) with resultant inhibitory effects on NK cells and macrophages (42).

Overview of Natural Killer cell biology

Natural killer (NK) cells are classically described as innate lymphoid cells with the ability to kill virally-infected or malignantly-transformed cells in an major histocompatibility complex (MHC)-I unrestricted manner.(43) However, an evolving picture of NK cells is emerging highlighted by diverse subsets with different physiology and effector functions

including cytotoxicity, cytokine production, and immune-editing(44). Key studies have demonstrated that NK cells are significantly more complex and more heterogeneous than the classic view of NK cells suggests (45). In fact, the plasticity and heterogeneity of NK cell biology likely underlies the difficulty to date of successfully translating the promise of NK immunotherapy to the clinic (46, 47). Moreover, despite the traditional view of NK cells as innate immune cells, there is increasing evidence to support the concept that NK cells have features of immunological memory, such as persistence, reactivation, and perhaps the ability to respond to specific antigenic stimuli (48). Similarly, Romee et al. have shown that short-term cytokine stimulation of human NK cells with combinations of IL-12, IL-15, and IL-18 engender "memory-like" NK cells with increased survival and effector functions, suggesting that this is a promising approach for enhanced NK efficacy in cancer immunotherapy (49, 50). Additionally, North and colleagues demonstrated that human NK cells can be "primed" by tumor cells rather than cytokines (51). Although the cytotoxicity of these "primed" NK cells was critically dependent on CD69 expression, their activity was propagated even in the presence of inhibitory signals, such as KIRs.

NK cells share important homology with T lymphocytes, particularly cytotoxic T lymphocytes. Morphologically, in an unactivated state, NK cells resemble other lymphocytes, but when they are activated, they enlarge and manifest intra-cellular granules. Although NK cells share a common hematopoietic precursor with T cells and share common features such as the release of perforin/granzyme for cytotoxic function, NK cells mature in the bone marrow and periphery rather than in the thymus. Importantly, NK cells also lack antigen specificity. Instead, NK cells express of an array of germline-encoded cell surface receptors, and these receptors

enable NK cells to bind many ligands and co-receptors which differentially affect their function. (52)

Human NK cells are typically characterized by the expression of CD56 (a glycoprotein important for cell-cell adhesion) and an absence of T cell markers, such as CD3 or the T-cell receptor.(53) NK subsets can be further classified into two broad categories, $CD56^{bright}$ and $CD56^{dim}$, based on the relative intensity/expression of this cell surface protein when analyzed by flow cytometry. $CD56^{dim}$ cells comprise the majority (approximately 90%) of circulating NK cells(54). Although these cells are highly cytotoxic to target cells, they produce negligible amounts of cytokines when compared to $CD56^{bright}$ cells (predominantly IFN-γ, TNF-β, IL-10, IL-13, and GM-CSF). In addition, $CD56^{dim}$ NK cells characteristically express killer-cell immunoglobulin-like receptors (KIR) as well as the Fcγ-receptor, CD16. The $CD56^{dim}$ CD16+ subset is considered a more cytotoxic NK subset, in part because of lower expression of inhibitory KIR receptors combined with sensitization by CD16 expression to cells bound with antibody. Conversely, $CD56^{bright}$ NK cells comprise approximately 10% of circulating NK cells in the blood, but are more abundant in lymph nodes and other solid tissues, such as the liver, spleen, and intestine (54). Due to their localization in lymph nodes combined with their high cytokine production, a growing consensus is emerging that $CD56^{bright}$ cells function as immune regulators, for example by modulating dendritic cell antigen presentation and by priming T cells toward a Th1 phenotype.(43)

Overall, the activity of NK cells is governed by the expression of diverse cell surface receptors which not only affect their effector functions but also their ability to migrate to sites of infection/tumor formation. As a general rule, NK cells express inhibitory receptors (KIRs) capable of binding self-MHC in order to become fully responsive, while NK cells not expressing

these markers remain hyporesponsive. However, this hyporesponsive state can be overridden upon activation, and as a result, unlicensed NK cells can contribute to anti-tumor effects under the proper conditions (such as in cancer immunotherapy when cultured NK cells are highly activated by pharmacologic doses of cytokines such as IL-2 and IL-15). KIR expression patterns in donor NK cells have also proven to be critical for hematological reconstitution and anti-tumor responses in the transplant setting.(55)

While current evidence suggests that the expression patterns of KIR inhibitory receptors are stochastic, other NK inhibitory receptors such as CD94/NKG2A and CD300a are frequently constitutively expressed (48). Similarly, activation receptors such as NKG2D, NKp30, NKp44, and NKp46 tend to be ubiquitously expressed, especially on $CD56^{dim}$ NK cells. In addition, other activation receptors including DNAM-1, NKG2C/CD94, and 2B4 have also been implicated in the activation of NK cells. The ligands for key NK activation receptors (such as NKG2D) are MHC-Ib molecules (e.g. MICA and MICB) which are upregulated during times of cellular stress, including rapid proliferation, viral infection, and cancer (56). For example, NKp30 has been shown to recognize the CMV pp65 protein, and both NKp44 and NKp46 have been observed to bind the influenza protein, hemagglutinin (57). It is important to recognize that there are many NK activating receptors whose ligands are not MHC-Ib molecules.

Perhaps the most potent stimulator of NK cells is the CD16 receptor, FcγRIIIA. Using CD16, NK cells are able to recognize IgG antibodies bound to target cells and lyse these antibody-coated cells through a process known as antibody-dependent cellular cytotoxicity (ADCC). In this way, NK cells, along with the complement system, can cooperate with the humoral immune response to eliminate pathogenic or diseased cells, highlighting a potential role for NK cells in cancer immunotherapy.

NK immunotherapy as cancer treatment

Although the role of NK cells in cancer surveillance has been debated, increasing data from both mouse and human studies suggest a significant role in this regard. For example, C57BL/6 mice depleted of both NK and NKT cells by administration of the NK1.1 monoclonal antibody are two to three times more susceptible to methylcholanthrene-induced tumor formation than non-depleted controls.(58) In addition, beige mice which are known to harbor NK cell deficiencies (among other immunological and biochemical lesions) demonstrate a significantly increased incidence of spontaneous and carcinogen-induced tumors compared with wildtype mice.(59, 60) Conversely, ectopic expression of Rae1, a mouse NKG2D ligand, promotes NK-cell-mediated rejection of tumors in other mouse tumor models.(61)

In humans, patients with immunodeficiencies related to NK cells have been shown to harbor an increased risk of malignancy, reinforcing the concept that NK cells contribute to the prevention and/or early phases of cancer elimination. For example, patients with Chediak-Higashi syndrome, an autosomal recessive disorder characterized by abnormal NK cytotoxic function similar to that in beige mice, have a 200-fold increased risk for developing cancer.(62) Similarly, biallelic mutations of the perforin gene have been associated with the development of lymphomas. In patients diagnosed with hepatitis C cirrhosis and end-stage liver disease, low NK cell activity (as measured by IFN-γ production and *ex vivo* cytotoxicity against K-562 cells) has been associated with an increased risk of developing hepatocellular carcinoma.(63) Moreover, reduced NK cell function in patients with established malignancies, especially hematologic ones, has been observed to correlate with an increased risk of recurrence after treatment and increased cancer-related mortality.(64-68) Altogether, these studies underscore a clinically-relevant role for NK cells in tumor immunosurveillance in human patients.

Despite their intrinsic ability to kill tumor cells, manipulating NK cells in therapeutic settings has proved challenging (69). Adoptive transfer of lymphocyte activated killer cells, or LAK cells, was first pioneered by Rosenberg et al. in the 1980s.(70, 71) In-depth characterization revealed that NK cells were a dominant component of LAKs, laying important groundwork for subsequent cell-based studies focusing more exclusively on NK cells. Important work by Rosenberg and others, such as Miller et al., subsequently established key principles for clinical studies of NK immunotherapy, including the need for *in vivo* cytokine support (such as IL2 with risk of toxicity) to support function and the need for lymphodepleting chemotherapy in order to allow for *in vivo* expansion of donor cells.(72, 73)

As NK cells are found primarily in the blood and rarely infiltrate solid tissue tumors, NK immunotherapies have been most successful in hematopoietic malignancies.(74) Early phase clinical trials have evaluated the efficacy and response rates of allogeneic NK cells in diverse solid tumors, including breast, ovarian, and bronchogenic carcinoma. Although these trials have shown NK adoptive therapy to be relatively safe, the results in these trials have been overall disappointing.(75) However, the dramatic responses observed using NK therapy in hematologic malignancies, including complete remission in 26% of AML patients, underscores the potential therapeutic benefit of NK transfer in solid malignancies.(73)

Additionally, significant experimental evidence exists in support of a role for NK cells in suppression of metastasis formation. In a landmark paper, Kim et al. evaluated the contribution of NK cells to *in vivo* tumor formation in transgenic mice which could be selectively depleted of NK cells but developed functionally normal B, T, and NK/T cells.(76) The authors observed significantly greater liver and lung metastases in animals lacking NK cells, and this effect was only partially rescued by NK/T and T cells, suggesting a fundamental role for NK cells in

prevention of metastasis. More recently, Lopez-Soto et al. demonstrated a provocative link between the acquisition of EMT characteristics in human colon cancer cell lines and archived human colon cancer samples and the upregulation of activating NK ligands, including MHC class I chain-related molecules A and B (MICA/B), providing further evidence in support of NK-mediated immune surveillance of pre-metastatic tumors with possible preferential targeting of CSCs.(77)

Furthermore, it is important to recognize that there are several key advantages to using NK cells for cancer immunotherapy. First, NK cells do not require the expression of a specific antigen expressed on a given HLA allotype. Cancer therapies which target a specific antigen, such as monoclonal antibodies or vaccines, are dependent on the presence of that antigen. Paradoxically, while these therapies may be highly effective and achieve long-term responses when that antigen is present, antigen-shedding and tumor escape variants may lead to eventual resistance to therapy and tumor progression.(78) Second, NK cells can be easily isolated and expanded *ex vivo* which allows for their use in adoptive or autologous cell therapies. Third, since NK cells in the majority of cases have a shorter lifespan than clonally-expanded T cells, concerns about over-activity, “off-tumor” effects, and need suicide vectors are minimized.(79)

In addition, NK cells have been long known for their unique ability to reject allogeneic hematopoietic stem cells, and this observation has proved fruitful in studies on both graft-versus-leukemia and graft-versus-host disease in the setting of bone marrow transplantation.(80, 81) Interestingly, recent data by Perez-Cunningham et al. have demonstrated that NK cells can also reject embryonic stem cells in an NKG2D-dependent manner.(82) Given the previously mentioned similarities between embryonic stem cells and CSCs, data such as these have led our

group and others to an underlying hypothesis that NK cells can selectively identify and lyse CSCs (**Figure 2**).(83, 84)

NK targeting of CSCs

Although previous studies have suggested that CSCs are less immunogenic than non-CSCs as evidenced by down-regulation of MHC class-I (MHC I) expression (85, 86), increasing reports have demonstrated that CSCs may be preferentially susceptible to NK cell targeting. Tallerico et al., for example, demonstrated that in models of colorectal cancer, CSCs showed increased susceptibility to NK killing (87). The vulnerability of CSCs to NK attack was associated with upregulation of the activating natural cytotoxicity receptors, particularly NKp30 and NKp44. In addition, Tallerico and colleagues observed lower levels of MHC class I expression on CSCs compared to non-CSCs, indirectly suggesting that the differences observed in MHC class I expression were linked to effective targeting of CSCs by NK cells.

Similarly, Castriconi et al. reported that glioblastoma-derived CSCs were susceptible to NK cell cytotoxicity.(88) The authors used fresh tumor specimens from glioblastoma patients to establish PL which displayed classical neural stem cell features. Interestingly, these neural-CSCs were resistant to unactivated NK cells, but were highly susceptible to both allogeneic and autologous NK killing in co-culture models after pre-treatment with IL-2 and IL-15. The authors also demonstrated low or absent expression of MHC class I molecules on their glioblastoma-derived CSCs, further supporting downregulation of these NK-inhibitory ligands in the mechanism of NK targeting of CSCs. Additionally, increased expression levels of DNAM-1 ligands poliovirus receptor (PVR/CD155) and Nectin-2 were observed on the tumor-derived CSCs grown in PL, although the lack of non-CSCs or mixed cell populations for comparison of the CSC-specific NK effects was a limitation of this study (88).

In another pre-clinical study, Pietra et al. reported that when melanoma cell lines were exposed to IL-2 activated allogeneic NK cells, both CD133- and CD133+ populations showed sensitivity to NK cell cytotoxicity possibly mediated by the DNAM-1 ligands Nestin-2 and PVR (89). Yin et al. reported that breast cancer CSCs showed sensitivity to IL-2 and IL-15 activated NK cells, and these preferential effects were likely mediated by increased expression of the NKG2D ligands ULBP1, ULBP2 and MICA on $CD44^{+}CD24^{-}$ human breast CSCs since blocking assays inhibited these effects (90).

Another important component of NK targeting of CSCs occurs following pre-treatment of bulk tumor with cytotoxic therapies to eliminate non-CSCs. In our laboratory, we have observed an increased frequency of CSCs post radiation (RT) and targeted therapy in cell line and xenograft models, as well as with primary breast, pancreas, and sarcomas analyzed immediately after surgical resection.(19, 91, 92) The enrichment in CSCs following anti-proliferative therapies was mirrored by an increased expression of the NKG2D stress ligands MICA/B on surviving CSCs, suggesting that cytotoxic therapy (especially RT) also sensitizes CSCs to NK attack. In addition, we observed that pretreatment of tumor-bearing mice with local RT prior to NK transfer resulted in significantly longer survival (92). These reports support the hypothesis that NK cells could potentially be aimed to specifically target CSCs upon strategic combination with other cytoreductive therapies. However, it would be pivotal to take into consideration immunosuppressive factors found in the tumor microenvironment that are known hinder or neutralize the cytotoxicity effect of NK cells such as TGF-□, IL-6, IL-8, or IFN-□.

Despite the CSC targeting capability of NK cells, the complexity of the tumor microenvironment where CSCs reside is an important barrier to overcome in order to develop effective therapies targeting CSCs. Kozlowska et al., for example, demonstrated that increased

secretion of the proinflamatory cytokines IL-6 and IL-8 in the presence of decreased secretion of IFN-□ inhibited tumor growth and blocked NK cell mediated lysis of glioblastoma multiforme CSCs (93). Furthermore, Wang et al. observed evasion of immunosurveillance and reduction of NK killing of breast cancer CSCs through shedding of MICA and MICB by CSCs and apparent CSC recruitment of regulatory T cells to promote an immune privileged state.(94, 95) Kryczek et al. observed that IL-22 promoted a CSC phenotype in both pre-clinical and patient-derived models and that higher IL-22 levels were associated with worse survival outcomes.(96) Importantly, IL-22 is produced by NK and T cells, suggesting that CSCs may have multiple mechanisms for inducing immune evasion.

Another important strategy to improve the targeting capabilities of NK cells in cancer therapy, especially toward refractory CSCs, is antibody-dependent cellular cytotoxicity (ADCC). Over the last 30 years, intensive research and development have resulted in the generation of several monoclonal antibodies that may be used to target CSCs. Some of these antibodies are being designed to target CSCs based on previously validated phenotypic markers including CD44, CD24, CD133, and ALDH-1 (97). Although NK immunotherapy represents an exciting approach to target CSCs, to date there are no clinical trials in human patients which are currently testing this hypothesis either alone or as part of a combination approach. At our institution, we are collaborating with the School of Veterinary Medicine to conduct a phase 2 canine clinical trial evaluating intratumoral injection of NK cells following palliative RT for dogs with appendicular osteosarcoma (**Figure 3**). Although canine NK cells have been incompletely characterized in the literature, and few studies have utilized adoptive NK transfer in canines, expanded canine NK-like populations have been isolated using immunomagnetic negative

selection with anti-CD5 antibody to isolate a $CD5^{dim}$/CD3+/CD8+/TCRαβ/γδ population (98, 99).

As an intermediate between murine studies and a human clinical trial, canines offer an excellent translational model for human clinical trials because their tumors closely resemble those in humans, they have intact immune systems, and they present many of the same challenges faced in "scaling up" a cell-based therapeutic system. In addition, there are added benefits of reduced time, expense, and regulatory hurdles of performing novel immunotherapy trials in humans.,

Comparable to NK cells, γδ T lymphocytes represent another MHC-independent immunotherapy approach which has demonstrated preferential CSC targeting activity in solid tumor models. Numerous parallels exist between NK cells and γδ T cells, including potent MHC-unrestricted cytotoxicity and recognition of NKG2D- target ligands. For example, Todaro et al. demonstrated that the Vγ9Vδ2 subset of γδ T cells could be efficiently sensitized to CSC killing using zoledronate in *ex vivo* models of colon cancer, supporting further investigation of γδ T cells in novel combination immunotherapy protocols (100).

Expert Opinion

It is increasingly clear that tumor heterogeneity is a major barrier in the successful treatment of solid tumors, and subpopulations of cells are present which are genetically and epigenetically diverse. The cancer stem cell hypothesis postulates that CSCs are responsible for a substantial component of this tumor heterogeneity, and CSCs has been identified in nearly all human and mouse malignancies. In addition, CSC sub-populations have been linked to metastasis and resistance to conventional cytoreductive therapies, especially after initial responses to treatment.

The ability of the immune system to recognize and eradicate transformed cells is the central rationale behind the application of immunotherapy for cancer. Although major recent advances have prominently established cancer immunotherapy as the “fourth arm” in cancer treatment, obstacles still remain, including both innate and acquired resistance to immunotherapy. Pre-clinical studies suggest that NK immunotherapy holds promise in attacking the heterogeneity of cancers, including CSCs. Decades of research has highlighted the potential of NK cells to eradicate transformed cells, but successful translation of this therapy to the clinic has been slow. Barriers to successful NK immunotherapy include limited NK persistence/longevity *in vivo*, inadequate homing to tumor sites, and hyporesponsiveness/dysfunction of NK cells, especially autologous NK cells, in the setting of malignancy.(69) Targeting of CSCs with NK immunotherapy represents a novel approach to circumvent many of these barriers.

Autologous and allogeneic cytokine-activated NK cells are capable of targeting stem-like tumor cells both *in vitro* and *in vivo*, but successful translation of these laboratory observations to the clinic has yet to be realized. Future studies will need to evaluate the immunological impact of NK cell killing of stem-like cells as well as the nature of these tumor-host interactions in immunocompetent models. Should targeting of CSCs by NK immunotherapy prove to be feasible for even a subset of solid malignancies patients, then this approach will have significant clinical impact. Therapeutic trials in immunocompetent large animal models, such as dogs, is an innovative platform to speed translation of novel NK immunotherapy approaches to the clinic.

Moreover, although unproven, it is reasonable to hypothesize that NK targeting of CSCs in a combination approach treating both CSCs and non-CSCs could lead to sustained therapeutic results. In fact, it may be possible to use NK targeting of CSCs to initiate and amplify adaptive T

cell-mediated responses. While the results of NK cell monotherapy in solid tumors to date has been modest, ongoing studies point to a novel application of NK cells in combination with other, more traditional therapies. As our understanding on the nature of the stem-like or CSC subpopulations continues to evolve, so too will our ability to apply immunotherapy more effectively.

Ultimately, the development and use of NK cell based treatments aimed at targeting residual disease, and promoting anti-tumor immunity is advancing from preclinical proof-in-concept to clinical reality. While previous clinical trials have focused on the NK cell as a sole effector, new trials are being designed to combine NK cell effector functions with traditional treatments in order to combat the therapeutic resistance that is a hallmark of CSCs. These studies, and others like it, will serve as key translational steps allowing clinicians and researchers to gain valuable insights into how NK cells can be used to target CSCs in animal models which more closely mirror the human condition, a factor which is critical for understanding the behavior of CSCs. Indeed, as our understanding of NK cell and CSC biology improves, so too will our ability to identify vulnerabilities inherent to CSC biology which can be exploited by the effector functions of NK and other cell-based immunotherapies.

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