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Improving Efficacy of Cancer Immunotherapy by Genetic Modification of Natural Killer Cells

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Abstract

Natural killer (NK) cells are members of the innate immune system that recognize target cells via activating and inhibitory signals received through cell receptors. Derived from the lymphoid lineage, NK cells are able to produce cytokines and exert a cytotoxic effect on viral infected and malignant cells. It is their unique ability to lyse target cells rapidly and without prior education that renders NK cells a promising effector cell for adoptive cell therapy. However, both viruses and tumors employ evasion strategies to avoid attack by NK cells, which represent biological challenges that need to be harnessed to fully exploit the cytolytic potential of NK cells. Using genetic modification, the function of NK cells can be enhanced to improve their homing, cytolytic activity, in vivo persistence and safety. Examples include gene modification to express chemokine, high affinity Fc receptor and chimeric antigen receptors, suicide genes and the forced expression of cytokines such as IL-2 and IL-15. Pre-clinical studies have clearly demonstrated that such approaches are effective in improving NK-cell function, homing and safety. In this review, we summarize the recent advances in the genetic manipulations of NK cells and their application for cellular immunotherapeutic strategies.

Key words: Natural Killer Cells; Gene transfer; Immunotherapy.

Introduction

Part of the innate immune system, natural killer (NK) cells play an important role in host responses against viral infections as well as against cancers, including leukemia. Although the complex biological networks at play in the anti-tumor activity of NK cells are not fully elucidated, the field of cellular immunotherapy is actively evaluating NK cells as a novel therapeutic for patients with cancer. However, we anticipate that genetic modification is necessary to fully exploit the potential of NK cells to treat patients with advanced malignancies where overcoming tumor immune evasion strategies are critical. The focus of this review is to summarize the recent progress in the gene modification of NK cells to optimize the tumor-specific NK-cell response to improve their in vivo persistence, homing and resistance to the immune suppressive tumor microenvironment.

NK-Cell Biology

Early reports of NK cells first identified a population of tumor-killing lymphocytes with fast-acting effects.[1] Later termed NK cells, these lymphocytes were found to lyse tumors in a manner entirely distinct from the antigen-specific mechanisms utilized by T cells. [2-5] Indeed, NK cells are members of the innate immune system that recognize target cells through activating and inhibitory receptors expressed on their surface and the balance between both signals will determine the NK-cell response against virally infected or malignant cells. NK cells are generated in the bone marrow and are derived from lymphoid progenitors which themselves develop from hematopoietic stem cells (HSC).[6] In the early stages of differentiation, NK progenitor cells are dependent on the expression of the transcription factor inhibition of DNA binding 2 (ID2) and the

IL2 receptor beta chain (CD122).[7] However, as they mature, NK cells are identified based on their variable expression of CD56, CD34, CD117, CD94, and CD16.[6] NK cell development is also dependent on high levels of interleukin-15 (IL-15), which interacts with components of the IL-2 receptor on the surface of the NK progenitor cell to elicit marked cell proliferation and activation of NK effector function.[8] As they fully develop, mature NK cells can be located in the bone marrow, lymph nodes, spleen, thymus, and tonsil, and can be further subdivided into three distinct subsets based on their expression of CD56.[9] CD56^{bright} cells have low cytolytic activity, and instead support an immune-regulatory environment by producing cytokines such as IFN- γ , TNF- α , GM-CSF, MCP-1, CCL3, CCL3, and CCL5.[2, 10-12] Along with expressing high levels of CD56, this subset also expresses CCR7 and CCR5, which contributes to their selective accumulation in secondary lymphoid organs.[13] The CD56^{dim} NK subset, in contrast, expresses CD16 (FC γ RIII) and has characteristically potent cytotoxic ability against target cells, as well as produces high levels of self-sustaining IFN- γ . [2, 14] CD56^{dim} cells uniquely express chemokines and factors involved in leukocyte trafficking such as CX3CR1, CXCR1, CXCR2, chemerin and the sphingosine 1-phosphate molecule (S1P), and can be located in both peripheral blood and lymphoid organs.[15, 16] Of note, both the CD56^{dim} and the CD56^{bright} subsets can express CXCR3, CXCR4, and CD62L which support transport into the peripheral circulation.[17] The third subset of CD56 negative NK cells are rare and represent a dysfunctional population of NK cells found in the setting of HIV viremic patients; they have impaired function and produce low levels of immune-modulatory cytokines.[18, 19] Although these three subsets represent a gradient of CD56 expression, it is mostly the CD56^{bright} and CD56^{dim} subsets that are

important in innate immunity and play a role in tumor lysis. As discussed in subsequent sections, the rapidly growing tumor micro-environment can introduce an altered chemokine and cytokine landscape. Because of their dependence on chemokine gradients and cytokines, the repertoire and distribution of NK cells can consequently be affected.[15]

As mentioned previously, unlike other cells of lymphoid origin, NK-cell recognition is not governed by high resolution antigen specificity but rather through a balance between activating and inhibitory signals received by receptors known as killer immunoglobulin-like receptors (KIR). KIRs on NK cells can be classified according to two haplotypes: A and B. NK cells of the A haplotype characteristically have multiple inhibitory receptors and only one activating receptor. NK cells of the B haplotype express a variety of both inhibitory and activating receptors.[20] NK cells can also express (i) receptors from the NKG2 family which can be either inhibitory or activating (NKG2A, B, C, D, E, F, and H), (ii) the activating receptor DNAX Accessory Molecule-1 (DNAM-1), (iii) the 2B4 family of receptors, and (iv) the natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 to recognize tumor antigens, viral ligands, and heat shock associated proteins.[21] The majority of NK cells express receptors of the NKG2 family, which uniquely bind to MHC class I molecules to initiate an inhibitory signal. Since MHC class I molecules, specifically HLA-E, are expressed on all nucleated cells, the inhibitory response following engagement with an NKG2 receptor serves as a recognition of “self”, which hinders lytic attack and thus prevents rampant NK-based autoimmunity.[22] NK cells recognize tumor cells by engaging their activating receptors with tumor antigens or viral proteins on the cell surface, while simultaneously not seeing ligands to engage their

inhibitory receptors. The lack of MHC on the surface of these foreign cells is known as the “missing self” hypothesis, by which the non-engagement of inhibitory receptors such as NKG2A leads to NK cell activation and tumor lysis.[23] Accordingly, the mimetic upregulation of MHC molecules illustrates a method of immune evasion employed by tumor cells preventing any consequent tumor cell lysis. NK cells exert their cytotoxic function directly through granzyme B and perforin-mediated apoptosis of target cells, or by promoting expression of Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) to signal death of the target cells.[24] Additionally, the crosslinking of CD16 on NK cells to the Fc portion on a target cell ligand can trigger antibody-dependent cellular cytotoxicity (ADCC), which can in turn lead to target cell lysis.[25, 26]

Because of their unique biological properties, NK cells are relevant effector cells for adoptive immunotherapy. However, the use of NK cells in such therapeutic approaches comes with advantages and disadvantages, especially when compare to the better established T-cell effector population. The main disadvantage would be the poor in vivo persistence of adoptively transferred NK cells compare to T cells. On the other hand, NK cells are “serial killers” and therefore can efficiently kill multiple targets. Another advantage is the possibility of using an allogeneic source of NK cells which would help overcome the inhibitory signals provided by the MHC molecules, expressed on the tumor cells, without the risk of Graft versus Host Disease (GvHD).[27] Finally, the “cytokine storm” initiated by the pro-inflammatory cytokines release by T cells such as $\text{TNF}\alpha$, IL-1 and IL-6 would not be observed as NK cells produce different cytokines upon activation. To the best of our knowledge, no pre-clinical or clinical study directly compared NK cells and T cells as cytotoxic effector cells for the treatment of malignant diseases.

Although NK cells can demonstrate successful cytotoxic activity against tumor cells, lytic function in the tumor micro-environment is frequently impaired. The subsequent sections of the review will detail the many challenges facing NK-based adoptive cell therapy, and will highlight the advantages gained by genetic modification of NK cells in order to achieve a maximal therapeutic effect of adoptively transferred NK cells.

Immune escape from NK-Cell Detection and Cytotoxicity

Due to the emergence of their therapeutic potential as an off-the-shelf, renewable, highly cytotoxic agent, it has been of considerable recent interest to examine methods of cell-based adoptive immunotherapy by which NK cells themselves are administered to patients to exert a therapeutic effect against challenging malignancies. Unfortunately, many tumors have developed strategies to evade immune surveillance and detection by NK cells. **[Figure 1]** One immune evasion strategy is the down-regulation of endogenous tumor antigens and/or natural cytotoxicity receptors and/or ligands by the tumor cells.[28] Proteolytic shedding of the NKG2D ligands MICA/B has long been observed in tumor cells, thereby blocking NKG2D on CD8 T cells and NK cells, thus preventing recognition and destruction of the malignant cells.[29-31] Alternatively, a tumor can falsely up-regulate MHC class I molecules, (a phenomenon supported by common cancer-associated mutations such as p53 mutations), which in turn promotes the binding of inhibitory NKG2 receptors and suppression of NK activation and the effector response.[32-34] Moreover, the NK cells themselves can be affected by the tumor microenvironment in ways that prevent their cytotoxic activity against tumor cells. For example, NK cells within the tumor microenvironment can have defective activating

receptors,[35] and/or can have their activity suppressed by immunosuppressive cytokines such as TGF- β and IL-10 [36], as well as the immunosuppressive tyrosine converting enzyme indoleamine 2, 3-dioxygenase (IDO).[37] Tumor cells can also alter NK-cell function indirectly by interfering in the cross talk between dendritic cells (DCs) and NK cells. Indeed, prostaglandin E2 (PGE2), a major metabolite of COX-2 activity, has been identified as a potent inhibitor of NK-cell-DC cells crosstalk thus further hampering the anti-tumor activity of NK cells.[38, 39] IDO, PGE2 as well as reactive oxygen species (ROS) are also directly responsible for NCR down-regulation on NK cells.[37, 40] Finally, the tumor environment promotes the expansion of CD4+CD25+ regulatory T cells (Treg) which strongly modulate T- and NK-cell mediated immune responses for example via the effects of membrane bound TGF- β expressed on Tregs.[41] Ghiringhelli and colleagues demonstrated that metronomic cyclophosphamide treatment in advanced stage cancer patients curtails Tregs immunosuppression and restores T and NK cells effector functions. [42] Further, depletion of Tregs using an IL-2 diphtheria toxin fusion protein has shown to enhance the in vivo activity of adoptively transferred haplo-identical NK cells in patients with acute myeloid leukemia (AML).[43]

Genetically Engineered NK Cells

Cell-based therapy using genetically manipulated T cells has shown to be a powerful tool for the treatment of patients with cancer, in particular, hematologic malignancies.[44-46] The assertion is that similar genetic manipulations could improve the ability of NK cells to home to disease sites, enhance anti-tumor activity and increase in vivo persistence [**Figure.1**]. The development of efficient gene transfer and cell

expansion methods has been essential for the use of gene modified NK-cell protocols. [47][Kellner *et al.* 2016. Submitted and in press] Available methodologies for the transduction and the expansion of NK cells have been recently described in length elsewhere and therefore will not be discussed in this review. [48, 49] Clinical grade NK cells can be generated from diverse donor sources, with each having various clinical and logistical advantages. Examples include peripheral blood (from autologous or allogeneic donors), cord blood and NK cells derived from CD34⁺ hematopoietic progenitor cells.[50] NK-cell lines, such as the malignant Non-Hodgkin's Lymphoma NK-92 and NKG cell lines, large granular lymphocyte leukemia NKL and natural killer cell leukemia KHYG-1 cell lines, have also been evaluated in pre-clinical work with only the NK-92 cell line being investigated in clinically.[51] However, the mandatory irradiation of the NK-92 cell line prior infusion, to avoid any potential risk of allogeneic tumor engraftment in the patient, significantly impaired the long-term persistence and anti-tumor activity of the adoptively transferred NK-92 cells.

Improving homing

Although NK cells are lymphoid cells from the innate immune system capable of circulating through most of the tissues, developing strategies to improve homing of the adoptively transferred cells is desirable. Indeed, the expression profile of chemokine receptors on NK cells depends on the activation state of the cells which leads to different migration patterns. [52, 53] For instance, lymph node homing by activated NK cells would necessitate the expression of the chemokine receptor CCR7. Somanchi and colleagues have demonstrated that activated NK cells can be manipulated to express CCR7 and therefore can be redirected to lymph nodes. [54] However, transfer of the

chemokine was achieved using trogocytosis and not gene transfer. There is an unmet need for new and innovative strategies, which currently involve forced expression (i.e. by gene transfer) of chemokine receptors, such as CXCR3 and CXCR2 to improve homing to inflammation sites or tumors respectively, as already demonstrated for the chemokine receptor CXCR2 in tumor specific T cells.[55] To our knowledge, no pre-clinical or clinical study has yet utilized gene transfer to actively redirect the migration of NK cells to inflammation sites or tumors.

Enhancing Recognition and Killing

As a component of the innate immune system, NK cells can directly recognize and eliminate tumor cells. However, tumor cells have developed a wide array of mechanisms to evade NK-cell-mediated immune surveillance. To overcome the resistance of cancer cells from the NK cells recognition and therefore destruction, engraftment of a chimeric antigen receptor (CAR) to redirect the specificity of NK-cell represents an attractive alternative. Engineered CAR molecules provide the antigen specificity through the binding element or scFv (single chain variable fragment), which is derived from an antibody, and the functionality through the endodomain elements, which are derived from co-stimulatory molecules.[56, 57] In T cells, CARs with a cytoplasmic domain derived from the CD3 ζ , CD28, OX-40 or 4-1BB molecules, or a combination of the aforementioned, have been evaluated.[58, 59]. Because the signaling requirements are different between T cells and NK cells, NK-cell specific signaling endodomains such as the DNAX-activating protein 10 (DAP10), DAP12 and 2B4 (CD244) have been also evaluated for their inclusion in a more NK-specific CAR construct.[60-62] In the last 10 years, extensive pre-clinical studies of CAR-expressing NK cells against tumor-

associated antigens (TAA) have been reported and have demonstrated an efficacy of this approach in the pre-clinical setting [**Table 1**]. An appreciable number of studies have utilized CAR-expressing NK cells to target antigens such as CD19 and CD20 expressed by B-cell malignancies. Using the NK-92 cell line expressing a CD19-specific CAR, Boissel and colleagues demonstrated that the NK-cell-resistant acute lymphoblastic leukemia (ALL) cell lines SUP-B15 and TMD-5 as well as primary chronic lymphocytic leukemia (CLL) cells can be efficiently eliminated in vitro and in a xenograft mouse model.[63] More recently, Cho and colleagues have obtained similar results using peripheral blood derived NK cells.[64] Although similar results have been reported by both groups, it is important to acknowledge the differences between the two studies: the source of the NK cells (NK-92 vs. primary NK cells); the CAR engraftment methods (Lentivirus transduction vs. Trogocytosis); and the CAR endodomain constructs (CD3 ζ vs 4-1BB plus CD3 ζ). Because T cells expressing a CAR specific for CD19 have been used with great success in patients with B-cell malignancies [65], it is not a surprise that the only two clinical trials using NK cells engineered to express a CAR also target the antigen CD19 in patients with ALL (trial.gov NCT-00995137 and NCT-01974479). In pre-clinical investigations utilizing NK cells as effector cells, CARs specific for the following TAA have been evaluated: SLAMF7 and CD138 in Multiple Myeloma [66, 67]; CD33 in AML [68]; carcinoembryonic antigen (CEA) in a variety of carcinomas [69]; GD2 and GPA7 in Neuroblastoma and Melanoma [61, 70, 71]; EpCAM and HER-2 in breast and ovarian cancers [72-74]; EBNA3C in EBV positive T cell lymphomas [75]. In a field where most of the CARs have been targeting TAA, Chang and colleagues have developed an unusual and elegant strategy to enhance NK-cell cytotoxicity. Combined

with the endodomains DAP10 and CD3 ζ , primary NK cells were retrovirally transduced to express the activating receptor NKG2D. This approach has shown considerable anti-tumor activity against a large spectrum of tumor types in pre-clinical models. [60] Similarly, Kobayashi and colleagues, showed that targeting of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-receptor via the expression of TRAIL on NK cells resulted in efficient cytotoxicity in a broad range of tumor cells. [76] By definition, NK cells expressing a CAR are monospecific and therefore are limited in their application (i.e. single antigen specific). Therefore, another approach is the forced expression of the high affinity CD16-158V (HA-CD16) Fc receptor on NK cells which has the potential to improve the outcome of NK cells therapy when delivered in combination with any FDA approved immunoglobulin (IgG1). Enhancing the antibody-dependent cellular cytotoxicity (ADCC) by engrafting HA-CD16 on NK cells is highly relevant as only a minority of patients are homozygous for this high affinity receptor.[77] Several groups have already demonstrated that engraftment of HA-CD16 on either the NK-92 cell line or primary NK cells from donors which express a low affinity CD16-158F/F (LA-CD16) Fc receptor can substantially augments the ADCC against target cells coated with rituximab [78, 79].

Although the forced expression of CARs or activating receptors on effector cells such as NK cells can -undoubtedly- enhance their anti-tumor activity, the adoptively transferred NK cells must still withstand the immune-suppressive tumor micro-environment. TGF- β is the major culprit in the long list of cytokines and chemokines responsible for hindering the NK-cell anti-tumor immune response. The resultant effects include the down-regulation of activating receptors, such as DNAM1, NKG2D and

NKp30 and reduced of perforin and granzyme B expression.[80] Interestingly, it has been observed that cancer stem cells (CSCs) derived from patients with glioblastoma strongly suppressed the expression of NKG2D on CD8⁺ T cells and NK cells in a TGF- β -dependent manner.[81] Recently, Viel and colleagues provided evidence that the mammalian target of rapamycin (mTOR) was a critical target of TGF- β . [82] Our group and others have evaluated a strategy where a dominant negative receptor derived from the TGF- β receptor II (DNR β II) could potentially overcome the negative effect of TGF- β on ex vivo expanded NK cells. An identical approach has already been evaluated clinically using DNR β II transduced T cells for patients with EBV⁺ Nasopharyngeal Carcinoma (NCT 02065362) and in EBV⁺ patients with HER2⁺ tumors (NCT 00889954). Indeed, EBV-specific cytotoxic T cells genetically engineered to express the DNR β II receptor were resistant to TGF- β and displayed strong anti-tumor activity in vitro and in vivo against EBV⁺ lymphoma in the presence of TGF- β . [83, 84] Yang and colleagues applied this strategy by transforming the NK-92 cell line with a similar DNR β II transgene and observed an attenuated effect of TGF- β on the transformed NK-92 cells, resulting in superior anti-tumor activity in nude mice bearing lung anaplastic carcinoma [**Table 1**]. [85] Our group also successfully transformed primary NK cells derived from cord blood with the DNR β II transgene and expanded the NK cells to clinically relevant numbers using a K562-based feeder cells system and IL-2. Again, the NK cells obtained after engraftment of the DNR β II were insensitive to the effect of TGF- β by maintaining expression of activating receptors and Perforin/Granzyme B [Yvon E., Manuscript in preparation, May 2016]. This strategy could be readily implemented in clinical trials

where NK cells are utilized as effector cells to enhance their therapeutic function amidst an immune-suppressive environment.

Enhancing NK-cell Survival in vivo

To date, the in vivo survival and persistence of adoptively transferred NK cells has generally been poor when compared with adoptively transferred T cells. NK-cell stimulation and expansion is under the control of pro-inflammatory cytokines secreted by DCs as well as macrophages such as IL-2, IL-12, IL-15, IL-18, IL-21, IL-23 and the type I IFNs (IFN- α/β). [86] These cytokines produced by both NK cells and DCs are involved in multiple feedback loops, which regulate their survival. For example, danger signals provided by tumor cells or pathogen-infected cells in combination with cytokines such as IL-12 and IFN- α/β will trigger an NK cell mediated cytolytic response and secretion of IFN- γ and TNF- α . In turn, the inflammatory environment created by this NK-cell response will induce DCs maturation with increase of secretion of IL-12, IFN- α/β , and IL-15, the latter of which enhances NK-cell expansion. [87] Exogenous dependence of cytokines by NK cells remains a hurdle that may be overcome by combining the infusion of the NK cells with the administration of cytokines such as IL-2 or IL-15. In an early study by Rosenberg and colleagues, the combination of lymphokine activated killer cells (LAK cells) and IL-2 resulted in a 20% response rate in patients with melanoma or metastatic renal cell carcinoma. [88] However, the systemic administration of IL-2 is often associated with significant side effects that include neuropathy, capillary leak syndrome and renal failure. [89] Such severe toxicities however were not observed in patients with pleural mesothelioma who received locoregional administration of high dose IL-2 and for whom an increase in LAK cells activity was recorded. These studies

suggest that IL-2 can positively impact NK-cell function even in the absence of the systemic toxicities.[90, 91] Critical to NK-cell function and homeostasis, IL-15 represents an attractive alternative to IL-2 and has already been investigated in patients with metastatic melanoma and metastatic renal cell cancer. Indeed, the study by Conlon and colleagues reported that at a maximum-tolerated dose (MTD) of 0.3µg/kg per day, the administration of exogenous IL-15 can induce marked proliferation of NK cells and clinical responses with a limited toxicity. [92]

To improve the in vivo persistence of adoptively transferred NK cells, the genetic modification of the NK cells to express IL-2 or IL-15 represents an attractive alternative to the systemic cytokine administration. Nagashima and colleagues in 1998 demonstrated that NK-92 cells stably transduced with the IL-2 gene did propagate for a long period of time (5 months) independently of the addition of exogenous IL-2 in the culture. [93] More recently, Imamura and colleagues genetically modified NK-92 cells to express a membrane bound form of IL-15 (mbIL-15) and made similar observation. [94] Using a secreted form of the IL-15 cytokine, our group has observed that genetically engineered primary NK cells isolated from cord blood were able to survive in culture for at least 21 days without exogenous addition of IL-2 [Yvon E. Unpublished data]. In summary, this data suggests that NK cells engineered to express IL-2 or IL-15 have improved persistence in vitro as well as in vivo which is independent of exogenous cytokine administration [**Table 1**]. Finally, Sahm and colleagues have evaluated the combination of a CAR specific for the pancarcinoma antigen EpCAM with IL-15. [72] As expected, NK cells expressing both transgenes (i.e. the EpCAM-CAR and IL-15) displayed specific killing of EpCAM-expressing tumor cells and autonomous cell proliferation.

Optimizing Safety

The genetic manipulation of NK cells (as well as T cells) to improve their tumor in vivo can however raise safety concerns. Indeed, the risk of GvHD, off-target killing, cytokine release and tumor lysis syndrome should be kept in mind when such treatments are administered to patients.[95] Although severe adverse events have been the focus of CD19-CAR expressing T-cell protocols, the same concerns are valid for genetically modified NK cells. Since 1990, several suicide-gene approaches have been used for cellular therapies to control unwanted side effects. Indeed, Bonini and colleagues first developed a suicide-gene based on the herpes simplex thymidine kinase/Ganciclovir (HSV-tk/GCV) system. [96] While effective, the HSV-tk/GCV system has limitations that cannot be disregarded since only proliferative cells can be eliminated and because of its viral origin, HSV-tk-expressing cells can be rejected by the host's immune system.[97] Another suicide gene system based on the expression of CD20 may overcome the limitations of the HSV-tk/GCV system but has not been as widely utilized clinically.[98] In this system, gene transfer of the antigen CD20 to adoptively transferred therapeutic cells allows their targeted elimination by the administration of rituximab. This approach proved to be effective with the depletion of the CD20-expressing T cells in vivo. However, limitations of this suicide gene strategy include concerns related to the depletion of healthy B cells, and concerns related to the potentially more limited bio-distribution of the antibody which might prevent their access to sites where gene modified T cells or NK cells may traffic. Another promising suicide-gene system utilized the apoptotic signaling pathway by forcing the dimerization of an inducible caspase-9 (iCasp9) protein which in turn initiated the caspase-cascade leading to apoptosis [Table

1].[99] Di Stasi and colleagues have demonstrated the clinical potential of the iCasp9 system in recipients of haploidentical stem-cell transplants where iCasp9 gene modified T cells were given to patients to enhance immune reconstitution.[100] In the event of GvHD, elimination of the iCasp9-expressing T cells was achieved by the administration of a small molecule dimerizer drug (AP1903). The synthetic drug, also called chemical inducer of dimerization (CID) has the advantage of inducing apoptosis of the target cells independently of the cell cycle. Further, this agent has an optimal bio-distribution, and is able to eliminate iCasp9-expressing cells rapidly (i.e. within 30 minutes after administration). [100] Leboeuf and colleagues have also evaluated the use of allogeneic NK cells transduced with the iCasp9 suicide-gene in a HepatoCellular Carcinoma mouse model where anti-tumor activity by the NK cells was reversed after a single injection of CID.[101] More recently, our group has demonstrated that primary cord blood derived NK cells that are genetically modified to express the CD19-specific CAR, the IL-15 gene and the iCasp9 safety switch were able to kill the NK-resistant CD19 positive Raji cell line and were specifically eliminated when exposed to the CID drug [Yvon E. Unpublished data]. [102]

Concluding remarks

Because of their inherent anti-tumor activity, the therapeutic potential of NK cells for the treatment of advanced malignancies is obvious to many in the Adoptive Cell Therapy (ACT) field. However, our current knowledge of NK-cell and tumor biology indicates that the use of such effector cell populations has limitations. Based on the results from T-cell therapy studies, we assert that the genetic modification of NK cells

would unleash their full therapeutic potential. Pre-clinical studies evaluating gene modified NK cells demonstrate enhanced anti-tumor activity and persistence. Hence these results provide encouraging evidence that such strategies may have an impact clinically and it is clear that CAR-expressing NK cells, gamma-cytokine expressing NK cells or combination strategies are bound to enter clinical trials in a near future. Moreover, the incorporation of a safety switch -suicide gene- in modified NK cells is feasible and will serve to harness any possible toxicity that would appear during such immunotherapy interventions.

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References

1. Hellstrom, I., et al., *Cellular and humoral immunity to different types of human neoplasms*. Nature, 1968. **220**(5174): p. 1352-4.
2. Herberman, R.B., et al., *Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells*. Int J Cancer, 1975. **16**(2): p. 230-9.
3. Herberman, R.B., M.E. Nunn, and D.H. Lavrin, *Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity*. Int J Cancer, 1975. **16**(2): p. 216-29.
4. Kiessling, R., et al., *"Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell*. Eur J Immunol, 1975. **5**(2): p. 117-21.
5. Kiessling, R., E. Klein, and H. Wigzell, *"Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype*. Eur J Immunol, 1975. **5**(2): p. 112-7.
6. Carotta, S., et al., *Identification of the earliest NK-cell precursor in the mouse BM*. Blood, 2011. **117**(20): p. 5449-52.
7. Boos, M.D., et al., *Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity*. J Exp Med, 2007. **204**(5): p. 1119-30.

8. Carson, W.E., et al., *Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor*. J Exp Med, 1994. **180**(4): p. 1395-403.
9. Iannello, A., et al., *Antiviral NK cell responses in HIV infection: I. NK cell receptor genes as determinants of HIV resistance and progression to AIDS*. J Leukoc Biol, 2008. **84**(1): p. 1-26.
10. Dahlberg, C.I., et al., *Natural Killer Cell-Based Therapies Targeting Cancer: Possible Strategies to Gain and Sustain Anti-Tumor Activity*. Front Immunol, 2015. **6**: p. 605.
11. Fauriat, C., et al., *Regulation of human NK-cell cytokine and chemokine production by target cell recognition*. Blood, 2010. **115**(11): p. 2167-76.
12. Carrega, P., et al., *CD56(bright)perforin(low) noncytotoxic human NK cells are abundant in both healthy and neoplastic solid tissues and recirculate to secondary lymphoid organs via afferent lymph*. J Immunol, 2014. **192**(8): p. 3805-15.
13. Ferlazzo, G., et al., *The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic*. J Immunol, 2004. **172**(3): p. 1455-62.
14. De Maria, A., et al., *Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation*. Proc Natl Acad Sci U S A, 2011. **108**(2): p. 728-32.
15. Carrega, P. and G. Ferlazzo, *Natural killer cell distribution and trafficking in human tissues*. Front Immunol, 2012. **3**: p. 347.
16. Parolini, S., et al., *The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues*. Blood, 2007. **109**(9): p. 3625-32.
17. Juelke, K., et al., *CD62L expression identifies a unique subset of polyfunctional CD56dim NK cells*. Blood, 2010. **116**(8): p. 1299-307.
18. Mavilio, D., et al., *Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals*. Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2886-91.
19. Scott-Algara, D. and P. Paul, *NK cells and HIV infection: lessons from other viruses*. Curr Mol Med, 2002. **2**(8): p. 757-68.
20. Pyo, C.W., et al., *Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus*. PLoS One, 2010. **5**(12): p. e15115.
21. Koch, J., et al., *Activating natural cytotoxicity receptors of natural killer cells in cancer and infection*. Trends Immunol, 2013. **34**(4): p. 182-91.
22. Fauriat, C., et al., *Education of human natural killer cells by activating killer cell immunoglobulin-like receptors*. Blood, 2010. **115**(6): p. 1166-74.
23. Lodoen, M.B. and L.L. Lanier, *Viral modulation of NK cell immunity*. Nat Rev Microbiol, 2005. **3**(1): p. 59-69.
24. Wallin, R.P., et al., *Regulation of perforin-independent NK cell-mediated cytotoxicity*. Eur J Immunol, 2003. **33**(10): p. 2727-35.
25. Titus, J.A., et al., *Human K/natural killer cells targeted with hetero-cross-linked antibodies specifically lyse tumor cells in vitro and prevent tumor growth in vivo*. J Immunol, 1987. **139**(9): p. 3153-8.

26. Garrido, M.A., et al., *Targeted cytotoxic cells in human peripheral blood lymphocytes*. J Immunol, 1990. **144**(8): p. 2891-8.
27. Lim, O., et al., *Present and Future of Allogeneic Natural Killer Cell Therapy*. Front Immunol, 2015. **6**: p. 286.
28. Costello, R.T., et al., *Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia*. Blood, 2002. **99**(10): p. 3661-7.
29. Sconocchia, G., et al., *The antileukemia effect of HLA-matched NK and NK-T cells in chronic myelogenous leukemia involves NKG2D-target-cell interactions*. Blood, 2005. **106**(10): p. 3666-72.
30. Salih, H.R., H.G. Rammensee, and A. Steinle, *Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding*. J Immunol, 2002. **169**(8): p. 4098-102.
31. Salih, H.R., D. Goehlsdorf, and A. Steinle, *Release of MICB molecules by tumor cells: mechanism and soluble MICB in sera of cancer patients*. Hum Immunol, 2006. **67**(3): p. 188-95.
32. Wang, B., et al., *p53 increases MHC class I expression by upregulating the endoplasmic reticulum aminopeptidase ERAP1*. Nat Commun, 2013. **4**: p. 2359.
33. Rouas-Freiss, N., et al., *HLA-G proteins in cancer: do they provide tumor cells with an escape mechanism?* Cancer Res, 2005. **65**(22): p. 10139-44.
34. Urosevic, M. and R. Dummer, *Human leukocyte antigen-G and cancer immunoediting*. Cancer Res, 2008. **68**(3): p. 627-30.
35. Veuillen, C., et al., *Primary B-CLL resistance to NK cell cytotoxicity can be overcome in vitro and in vivo by priming NK cells and monoclonal antibody therapy*. J Clin Immunol, 2012. **32**(3): p. 632-46.
36. Stringaris, K., et al., *Leukemia-induced phenotypic and functional defects in natural killer cells predict failure to achieve remission in acute myeloid leukemia*. Haematologica, 2014. **99**(5): p. 836-47.
37. Pietra, G., et al., *Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity*. Cancer Res, 2012. **72**(6): p. 1407-15.
38. Van Elssen, C.H., et al., *Inflammation-restraining effects of prostaglandin E2 on natural killer-dendritic cell (NK-DC) interaction are imprinted during DC maturation*. Blood, 2011. **118**(9): p. 2473-82.
39. Harizi, H., *Reciprocal crosstalk between dendritic cells and natural killer cells under the effects of PGE2 in immunity and immunopathology*. Cell Mol Immunol, 2013. **10**(3): p. 213-21.
40. Romero, A.I., et al., *NKp46 and NKG2D receptor expression in NK cells with CD56dim and CD56bright phenotype: regulation by histamine and reactive oxygen species*. Br J Haematol, 2006. **132**(1): p. 91-8.
41. Ghiringhelli, F., et al., *CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner*. J Exp Med, 2005. **202**(8): p. 1075-85.
42. Ghiringhelli, F., et al., *Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector*

- functions in end stage cancer patients. Cancer Immunol Immunother*, 2007. **56**(5): p. 641-8.
43. Bachanova, V., et al., *Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. Blood*, 2014. **123**(25): p. 3855-63.
 44. Gill, S. and C.H. June, *Going viral: chimeric antigen receptor T-cell therapy for hematological malignancies. Immunol Rev*, 2015. **263**(1): p. 68-89.
 45. Lee, D.W., et al., *T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet*, 2015. **385**(9967): p. 517-28.
 46. Pule, M.A., et al., *Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med*, 2008. **14**(11): p. 1264-70.
 47. Denman, C.J., et al., *Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS One*, 2012. **7**(1): p. e30264.
 48. Carlsten, M. and R.W. Childs, *Genetic Manipulation of NK Cells for Cancer Immunotherapy: Techniques and Clinical Implications. Front Immunol*, 2015. **6**: p. 266.
 49. Rezvani, K. and R.H. Rouse, *The Application of Natural Killer Cell Immunotherapy for the Treatment of Cancer. Front Immunol*, 2015. **6**: p. 578.
 50. Spanholtz, J., et al., *Clinical-grade generation of active NK cells from cord blood hematopoietic progenitor cells for immunotherapy using a closed-system culture process. PLoS One*, 2011. **6**(6): p. e20740.
 51. Tonn, T., et al., *Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy*, 2013. **15**(12): p. 1563-70.
 52. Inngjerdingen, M., B. Damaj, and A.A. Maghazachi, *Expression and regulation of chemokine receptors in human natural killer cells. Blood*, 2001. **97**(2): p. 367-75.
 53. Miller, J.S., et al., *Expansion and homing of adoptively transferred human natural killer cells in immunodeficient mice varies with product preparation and in vivo cytokine administration: implications for clinical therapy. Biol Blood Marrow Transplant*, 2014. **20**(8): p. 1252-7.
 54. Somanchi, S.S., et al., *Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. Blood*, 2012. **119**(22): p. 5164-72.
 55. Peng, W., et al., *Transduction of tumor-specific T cells with CXCR2 chemokine receptor improves migration to tumor and antitumor immune responses. Clin Cancer Res*, 2010. **16**(22): p. 5458-68.
 56. Pule, M., H. Finney, and A. Lawson, *Artificial T-cell receptors. Cytotherapy*, 2003. **5**(3): p. 211-26.
 57. Haynes, N.M., et al., *Single-chain antigen recognition receptors that costimulate potent rejection of established experimental tumors. Blood*, 2002. **100**(9): p. 3155-63.
 58. Pule, M.A., et al., *A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther*, 2005. **12**(5): p. 933-41.

59. Long, A.H., et al., *4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors*. Nat Med, 2015. **21**(6): p. 581-90.
60. Chang, Y.H., et al., *A chimeric receptor with NKG2D specificity enhances natural killer cell activation and killing of tumor cells*. Cancer Res, 2013. **73**(6): p. 1777-86.
61. Altwater, B., et al., *2B4 (CD244) signaling by recombinant antigen-specific chimeric receptors costimulates natural killer cell activation to leukemia and neuroblastoma cells*. Clin Cancer Res, 2009. **15**(15): p. 4857-66.
62. Topfer, K., et al., *DAP12-based activating chimeric antigen receptor for NK cell tumor immunotherapy*. J Immunol, 2015. **194**(7): p. 3201-12.
63. Boissel, L., et al., *Retargeting NK-92 cells by means of CD19- and CD20-specific chimeric antigen receptors compares favorably with antibody-dependent cellular cytotoxicity*. Oncoimmunology, 2013. **2**(10): p. e26527.
64. Cho, F.N., et al., *Enhanced cytotoxicity of natural killer cells following the acquisition of chimeric antigen receptors through trogocytosis*. PLoS One, 2014. **9**(10): p. e109352.
65. Gill, S. and D.L. Porter, *CAR-modified anti-CD19 T cells for the treatment of B-cell malignancies: rules of the road*. Expert Opin Biol Ther, 2014. **14**(1): p. 37-49.
66. Chu, J., et al., *CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance in vitro and in vivo antitumor activity against human multiple myeloma*. Leukemia, 2014. **28**(4): p. 917-27.
67. Jiang, H., et al., *Transfection of chimeric anti-CD138 gene enhances natural killer cell activation and killing of multiple myeloma cells*. Mol Oncol, 2014. **8**(2): p. 297-310.
68. Schirrmann, T. and G. Pecher, *Specific targeting of CD33(+) leukemia cells by a natural killer cell line modified with a chimeric receptor*. Leuk Res, 2005. **29**(3): p. 301-6.
69. Schirrmann, T. and G. Pecher, *Human natural killer cell line modified with a chimeric immunoglobulin T-cell receptor gene leads to tumor growth inhibition in vivo*. Cancer Gene Ther, 2002. **9**(4): p. 390-8.
70. Esser, R., et al., *NK cells engineered to express a GD2 -specific antigen receptor display built-in ADCC-like activity against tumour cells of neuroectodermal origin*. J Cell Mol Med, 2012. **16**(3): p. 569-81.
71. Zhang, G., et al., *Retargeting NK-92 for anti-melanoma activity by a TCR-like single-domain antibody*. Immunol Cell Biol, 2013. **91**(10): p. 615-24.
72. Sahm, C., K. Schonfeld, and W.S. Wels, *Expression of IL-15 in NK cells results in rapid enrichment and selective cytotoxicity of gene-modified effectors that carry a tumor-specific antigen receptor*. Cancer Immunol Immunother, 2012. **61**(9): p. 1451-61.
73. Kruschinski, A., et al., *Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas*. Proc Natl Acad Sci U S A, 2008. **105**(45): p. 17481-6.
74. Schonfeld, K., et al., *Selective inhibition of tumor growth by clonal NK cells expressing an ErbB2/HER2-specific chimeric antigen receptor*. Mol Ther, 2015. **23**(2): p. 330-8.

75. Tassev, D.V., M. Cheng, and N.K. Cheung, *Retargeting NK92 cells using an HLA-A2-restricted, EBNA3C-specific chimeric antigen receptor*. *Cancer Gene Ther*, 2012. **19**(2): p. 84-100.
76. Kobayashi, E., et al., *A chimeric antigen receptor for TRAIL-receptor 1 induces apoptosis in various types of tumor cells*. *Biochem Biophys Res Commun*, 2014. **453**(4): p. 798-803.
77. Wu, J., et al., *A novel polymorphism of FcγRIIIa (CD16) alters receptor function and predisposes to autoimmune disease*. *J Clin Invest*, 1997. **100**(5): p. 1059-70.
78. Binyamin, L., et al., *Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy*. *J Immunol*, 2008. **180**(9): p. 6392-401.
79. Weng, W.K. and R. Levy, *Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma*. *J Clin Oncol*, 2003. **21**(21): p. 3940-7.
80. Wilson, E.B., et al., *Human tumour immune evasion via TGF-β blocks NK cell activation but not survival allowing therapeutic restoration of anti-tumour activity*. *PLoS One*, 2011. **6**(9): p. e22842.
81. Beier, C.P., et al., *The cancer stem cell subtype determines immune infiltration of glioblastoma*. *Stem Cells Dev*, 2012. **21**(15): p. 2753-61.
82. Viel, S., et al., *TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway*. *Sci Signal*, 2016. **9**(415): p. ra19.
83. Bollard, C.M., et al., *Adapting a transforming growth factor β-related tumor protection strategy to enhance antitumor immunity*. *Blood*, 2002. **99**(9): p. 3179-87.
84. Foster, A.E., et al., *Antitumor activity of EBV-specific T lymphocytes transduced with a dominant negative TGF-β receptor*. *J Immunother*, 2008. **31**(5): p. 500-5.
85. Yang, B., et al., *Blocking transforming growth factor-β signaling pathway augments antitumor effect of adoptive NK-92 cell therapy*. *Int Immunopharmacol*, 2013. **17**(2): p. 198-204.
86. Zwirner, N.W., et al., *Cytokine-driven regulation of NK cell functions in tumor immunity: role of the MICA-NKG2D system*. *Cytokine Growth Factor Rev*, 2007. **18**(1-2): p. 159-70.
87. Mattei, F., et al., *IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation*. *J Immunol*, 2001. **167**(3): p. 1179-87.
88. Rosenberg, S.A., et al., *Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer*. *N Engl J Med*, 1985. **313**(23): p. 1485-92.
89. Rosenberg, S.A., et al., *Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients*. *Ann Surg*, 1989. **210**(4): p. 474-84; discussion 484-5.
90. Astoul, P., et al., *Intrapleural recombinant IL-2 in passive immunotherapy for malignant pleural effusion*. *Chest*, 1993. **103**(1): p. 209-13.

91. Goey, S.H., et al., *Intrapleural administration of interleukin 2 in pleural mesothelioma: a phase I-II study*. Br J Cancer, 1995. **72**(5): p. 1283-8.
92. Conlon, K.C., et al., *Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer*. J Clin Oncol, 2015. **33**(1): p. 74-82.
93. Nagashima, S., et al., *Stable transduction of the interleukin-2 gene into human natural killer cell lines and their phenotypic and functional characterization in vitro and in vivo*. Blood, 1998. **91**(10): p. 3850-61.
94. Imamura, M., et al., *Autonomous growth and increased cytotoxicity of natural killer cells expressing membrane-bound interleukin-15*. Blood, 2014. **124**(7): p. 1081-8.
95. Maus, M.V., et al., *Antibody-modified T cells: CARs take the front seat for hematologic malignancies*. Blood, 2014. **123**(17): p. 2625-35.
96. Bonini, C., et al., *HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia*. Science, 1997. **276**(5319): p. 1719-24.
97. Bonini, C. and C. Bordignon, *Potential and limitations of HSV-TK-transduced donor peripheral blood lymphocytes after allo-BMT*. Hematol Cell Ther, 1997. **39**(5): p. 273-4.
98. Vogler, I., et al., *An improved bicistronic CD20/tCD34 vector for efficient purification and in vivo depletion of gene-modified T cells for adoptive immunotherapy*. Mol Ther, 2010. **18**(7): p. 1330-8.
99. Straathof, K.C., et al., *An inducible caspase 9 safety switch for T-cell therapy*. Blood, 2005. **105**(11): p. 4247-54.
100. Di Stasi, A., et al., *Inducible apoptosis as a safety switch for adoptive cell therapy*. N Engl J Med, 2011. **365**(18): p. 1673-83.
101. Leboeuf, C., et al., *In vivo proof of concept of adoptive immunotherapy for hepatocellular carcinoma using allogeneic suicide gene-modified killer cells*. Mol Ther, 2014. **22**(3): p. 634-44.
102. Hoyos, V., et al., *Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety*. Leukemia, 2010. **24**(6): p. 1160-70.
103. Boissel, L., et al., *Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells*. Leuk Res, 2009. **33**(9): p. 1255-9.
104. Muller, T., et al., *Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells*. Cancer Immunol Immunother, 2008. **57**(3): p. 411-23.
105. Li, L., et al., *Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method*. Cancer Gene Ther, 2010. **17**(3): p. 147-54.
106. Shimasaki, N., et al., *A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies*. Cytotherapy, 2012. **14**(7): p. 830-40.

107. Boissel, L., et al., *Comparison of mRNA and lentiviral based transfection of natural killer cells with chimeric antigen receptors recognizing lymphoid antigens*. Leuk Lymphoma, 2012. **53**(5): p. 958-65.
108. Chu, Y., et al., *Targeting CD20+ Aggressive B-cell Non-Hodgkin Lymphoma by Anti-CD20 CAR mRNA-Modified Expanded Natural Killer Cells In Vitro and in NSG Mice*. Cancer Immunol Res, 2015. **3**(4): p. 333-44.
109. Lee, J.M., et al., *Direct and indirect antitumor effects by human peripheral blood lymphocytes expressing both chimeric immune receptor and interleukin-2 in ovarian cancer xenograft model*. Cancer Gene Ther, 2010. **17**(10): p. 742-50.
110. Jiang, W., et al., *hIL-15 gene-modified human natural killer cells (NKL-IL15) augments the anti-human hepatocellular carcinoma effect in vivo*. Immunobiology, 2014. **219**(7): p. 547-53.

Immunosuppressive tumor microenvironment

Figure 1: Gene modification of NK cells to improve the efficacy of adoptive immunotherapy. New gene therapy methods to restore or protect NK-cell function against the immune-suppressive tumor microenvironment have been developed and are under investigation in pre-clinical and clinical trials. The aims of such strategies are: (i) to enhance NK-cell tumor specificity using chimeric antigen receptors (CARs), (ii) to augment tumor recognition and killing using NKG2D and TRAIL engrafted on NK cells, (iii) to increase ADCC using NK cells modified to express a high affinity Fc receptor (HA-CD16), (iv) to protect NK cells from the effect of TGF-β using a dominant negative receptor II for TGF-β, and (v) to improve in vivo survival by forced expression of the cytokines IL-2 or IL-15 by NK cells.

CAR-expressing NK cells		
<i>Target</i>	<i>Malignancies</i>	<i>References</i>
CD19	B-ALL, CLL	[63, 103-106]
CD20	B-ALL, CLL	[63, 104, 107, 108]
CD33	AML	[68]
SLAMF7	Multiple Myeloma	[66]
CD138	Multiple Myeloma	[67]
GD2	Neuroblastoma	[61, 70]
HER2/neu	Breast, ovarian and Renal cell carcinoma,	[73, 74, 109]
GPA7	Melanoma	[71]
EpCAM	Breast carcinoma	[72]
EBNA3C	EBV+ T-cell Lymphoma	[75]
CEA	Colon carcinoma	[69]
<i>Clinical trials</i>		
CD19	B-ALL	NCT 00995137 (<i>Completed</i>)
	B-ALL	NCT 01974479 (<i>Recruiting</i>)
Enhancing NK cells function		
<i>Target</i>	<i>System</i>	<i>References</i>
TGF- β	Dominant Negative Receptor II	[85]
NKG2D Ligands	NKG2D	[60]
TRAIL-R1	TRAIL	[76]
Monoclonal Abs	HA-CD16	[78, 79]
Improving NK cells persistence		
<i>Target</i>	<i>System</i>	<i>References</i>
γ c cytokine	IL-2	[93, 109]
	IL-15	[72, 94, 110]
Optimizing NK cells safety		
<i>Target</i>	<i>System</i>	<i>References</i>
Caspase	Inducible Caspase 9	[101]

Table 1: Preclinical and clinical trials utilizing gene modified NK cells including primary NK cells and NK-cell lines.