

Utilizing chimeric antigen receptors to direct natural killer cell activity

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Natural killer (NK) cells represent an attractive lymphocyte population for cancer immunotherapy due to their ability to lyse tumor targets without prior sensitization and without need for human leukocyte antigens-matching. Chimeric antigen receptors (CARs) are able to enhance lymphocyte targeting and activation toward diverse malignancies. CARs consist of an external recognition domain (typically a small chain variable fragment) directed at a specific tumor antigen that is linked with one or more intracellular signaling domains that mediate lymphocyte activation. Most CAR studies have focused on their expression in T cells. However, use of CARs in NK cells is starting to gain traction because they provide a method to redirect these cells more specifically to target refractory cancers. CAR-mediated anti-tumor activity has been demonstrated using NK cell lines, as well as NK cells isolated from peripheral blood, and NK cells produced from human pluripotent stem cells. This review will outline the CAR constructs that have been reported in NK cells with a focus on comparing the use of different signaling domains in combination with other co-activating domains.

Keywords: chimeric antigen receptors, natural killer cells, cancer immunotherapy, NK-92 cells, induced pluripotent stem cells

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Specialty section:

This article was submitted to NK Cell
Biology, a section of the journal
Frontiers in Immunology

Received: 23 March 2015

Accepted: 08 April 2015

Published: 28 April 2015

Citation:

Hermanson DL and Kaufman DS
(2015) Utilizing chimeric antigen
receptors to direct natural
killer cell activity.
Front. Immunol. 6:195.
doi: 10.3389/fimmu.2015.00195

Introduction

Natural killer (NK) cells are an important component of the innate immune system due to their ability to lyse infected or malignant cells without prior sensitization and without human leukocyte antigens (HLA)-restriction (1). They play an important role in immune surveillance and early control of many malignancies. NK cells recognize infected or transformed cells through multiple cell surface receptors including NKG2D, CD16 [the receptor that mediates antibody-dependent cellular cytotoxicity (ADCC)], and natural cytotoxicity receptors (NCRs) such as NKp44, NKp46, and NKp30 (2). These receptors activate signaling adapter proteins such as DAP10, DAP12, and CD3 ζ , which contain immuno-tyrosine activation motifs (ITAMs) that initiate the release of cytolytic granules containing perforin and granzymes, as well as mediate production and release of cytokines and chemokines such as IFN- γ and TNF- α (3). Importantly, NK cell-mediated cytotoxicity does not rely on the presentation of self HLA. Therefore, NK cells hold significant clinical interest as a cell-based therapy for cancer because of their ability to be used in an allogeneic setting and potentially provide an off-the-shelf cellular product. Clinical trials using NK cells obtained from haploidentical donors demonstrate long-term remissions in patients with refractory acute myelogenous leukemia (4). Trials against solid tumors such as breast cancer and ovarian cancer have also demonstrated efficacy (5). NK cell lines (NK-92 cells) (6) and NK cells derived from umbilical cord blood (7) have also been tested in clinical trials (NCT01729091, NCT02280525).

Chimeric antigen receptors (CARs) are engineered proteins designed to activate lymphocytes, particularly T cells, upon target recognition. CARs contain a single-chain variable fragment (scFv) fused to a variety of possible intracellular signaling domain(s). The scFv is designed to target antigens either overexpressed or unique to tumor cells. The signaling domain initially tested was the ζ chain of the T cell receptor complex CD3 in first generation CARs (8). Second generation CARs employ co-activating proteins such as CD28, CD137 (4-1BB), or CD134 (OX40) in combination with CD3 ζ to increase T cell activation and proliferation (9, 10). Finally, CAR constructs incorporate multiple co-activation domains and CD3 ζ in the third generation CARs (11). The clinical success of anti-CD19 CAR-expressing T cells for treatment of B-cell malignancies has fueled the design and evaluation of CARs for T cell therapy toward other antigens and malignancies (12). While development of T cell-CAR-based therapies seems to be revolutionizing tumor immunotherapy, one major obstacle with this approach is the need to collect and utilize autologous cells. A second concern with the use of T cells is their long-term persistence, resulting in chronic on-target-off-tumor effects such as B-cell aplasia with the anti-CD19 CARs being used currently in clinical trials.

Natural killer cells provide an alternative to the use of T cells for adoptive immunotherapy since they do not require HLA matching, so can be used as allogeneic effector cells (13). Clinical trials of adoptively transferred allogeneic NK cells demonstrate these cells can survive in patients for several weeks to months (4, 13). Additionally, expression of CARs in NK cells may allow these cells to more effectively kill solid tumors that are often resistant to NK cell-mediated activity compared to hematologic malignancies (especially acute myelogenous leukemia) that are typically more NK cell-sensitive (4, 5). As such, CAR-expressing NK cells have gained significant interest to provide a targeted, allogeneic, “universal” cell population for treatment of refractory malignancies. This review will focus on the CAR constructs and activating domains that have been reported to be used in NK cell lines (such as NK-92 cells) and peripheral blood (PB) NK cells. Additional work using NK cells produced from human pluripotent stem cells is also discussed.

Chimeric Antigen Receptors Used in NK Cell Lines

Natural killer cell lines have been utilized to evaluate CARs targeting several different antigens. By far, the most commonly studied NK cell line has been the NK-92 cell line, which has been previously (6) and is currently being used in clinical trials (NCT00900809 and NCT00990717). Other NK cell lines include NKG, YT, NK-YS, HANK-1, YTS cells, and NKL cells (14). Working with NK cell lines has several advantages such as providing a more homogeneous cell population compared to NK cells isolated from PB. In addition, the NK-92 cells have been well-defined and there is no need to perform any isolation of the NK cells from donors. However, NK cell lines also have distinct disadvantages. NK-92 cells lack the expression of several typical NK cell activating receptors such as CD16, NKp44, and NKp46 (15, 16). Also, NK-92 cells are tumor cell lines with multiple cytogenetic

TABLE 1 | CAR constructs utilized in NK cell lines (NK-92).

Reference	Target	CAR Construct	Method
(23)	ErbB2 (HER-2)	mCD8 α hinge/CD3 ζ	Amphotropic virus
(19)	CD20	mCD8 α hinge/CD3 ζ	Amphotropic virus
(22)	CD19	CD8 α TM/CD3 ζ	mRNA transfection (50%)
(30)	EpCAM	CD8 α hinge/CD28/CD3 ζ	Lentivirus along with IL-15
(31)	HLA-A2 EBNA3C	CD8 α TM/CD137/CD3 ζ	Retrovirus
(26)	GD2	mCD8 α hinge/CD3 ζ	Amphotropic virus
(20)	CD19/CD20	CD3 ζ	mRNA transfection (30–70%) and lentivirus
(28)	HLA-2 complex with melanoma-associated gp100 peptide	A2 TM/CD3 ζ	Transfection
(21)	CD19/CD20	CD3 ζ	Lentivirus
(32)	GS1	CD28 TM/CD28/CD3 ζ	Lentivirus
(27)	CD138	CD8 α hinge/CD3 ζ	Lentivirus
(24)	ErbB2 (HER-2)	CD8 α hinge/CD28/CD3 ζ	Transfection
(25)	ErbB2 (HER-2)	CD8 α hinge/CD3 ζ CD8 α hinge/CD28/CD3 ζ CD8 α hinge/CD137/CD3 ζ	Lentivirus
(34)	PSCA	CD28 hinge/CD28 TM/CD3 ζ DAP12 TM and signaling	Lentivirus in YTS NK cells and primary NK cell

abnormalities (17) and are latently infected with Epstein–Barr virus (18). Therefore, for safety purposes, these cells must be irradiated prior to infusion.

The majority of studies to express CARs in NK-92 cells have used first generation CAR constructs that contain CD3 ζ as their sole signaling domain. The scFvs of these CARs have targeted CD20 (19–21), CD19 (20–22), ErbB2 (HER2) (23–25), GD2 (26), and CD138 (27) (Table 1). In addition to directly targeting cell surface proteins, CARs can also recognize HLA-peptide complexes such as HLA-A2 expressing the melanoma-associated gp100 peptide (28). CARs directed toward CD19 and CD20 are designed to target B-cell malignancies and have also been studied extensively in T cells (29). The only other difference in the anti-CD19 or anti-CD20 CAR constructs used in NK-92 cells is the transmembrane region. One study used the CD3 ζ transmembrane sequence (19) while another used the CD8 transmembrane sequence (22). However, without a direct comparison it is unknown if one construct is superior. Another study used an HLA-A2 transmembrane region coupled to a CD3 ζ signaling domain (28), suggesting the transmembrane region may be easily altered without impacting CAR expression and functionality. Interestingly, comparison of CAR transfected NK-92 cells with ADCC function using NK-92 cells engineered to express CD16

found that the anti-CD20 CAR engineered cells lysed primary CLL cells more effectively than NK-92 cells acting through ADCC using rituximab (21). This study suggests that even first generation CARs may be an improvement over ADCC-mediated anti-tumor activity by NK-92 cells. It is important to note that NK-92 cells require transfection of CD16 in order to perform ADCC. This leaves open the possibility that PB-NK cells may still be better equipped to perform ADCC better than NK-92 cells.

Solid tumor antigens can also be targeted by first generation CAR constructs expressed in NK-92 cells. An anti-ErbB2 CAR construct against HER2-positive breast, ovarian, and squamous cell carcinoma cell lines mediated improved killing ability of NK-92 cells (23). Additionally, this study showed a reduction in tumor growth using ErbB2-expressing NIH 3.3 cells mixed with NK-92s in a subcutaneous mouse model (23). An anti-GD2 CAR using just the CD3 ζ transmembrane and signaling domains was able to target primary glioblastoma cells as well as GD2-positive melanoma and breast carcinomas (26). NK-92 cells can also be targeted against multiple myeloma (MM) using an anti-CD138 CAR with only CD3 ζ as a signaling domain (27). Notably, mice bearing a subcutaneous tumor treated with CAR-expressing NK-92 cells survived significantly longer than NK-92 cell alone in a CD138-positive tumor model; whereas, when a CD138-negative MM tumor was used no difference was detected (27). These data clearly demonstrate that first generation CARs are an effective means to induce target cell lysis in NK-92 cells both *in vitro* and in mouse models; however, many of the tumor models are subcutaneous, which may fail to properly recapitulate the complete tumor environment or NK cell trafficking issues.

Second generation CARs expressing a second signaling domain in conjunction with CD3 ζ vastly improves the overall activity CAR-expressing T cells (9). This has generated interest in using second generation CARs in NK cells. Similar to first generation CARs, several different scFvs have been used with second generation CARs including EpCAM for multiple carcinomas including breast and ovarian cancer (30), an HLA-A2 EBNA3C complex for Epstein–Barr virus (31), CS1 for MM (32), and ErbB2 for HER2 positive cancers (24, 25). The most common second generation CAR utilized in NK-92 cells pairs the CD28 intracellular domain with CD3 ζ (Table 1). Notably, NK cells do not naturally express CD28 (35); therefore, the effect that this domain has in NK cells is unclear. Other second generation CARs combine CD137 (4-1BB) intracellular domain with CD3 ζ . Similar to first generation CARs, all of the constructs lead to antigen specific killing of target cells, displaying the diverse set of tumor antigens CARs can target. Comparison of an ErbB2 scFv fused with CD3 ζ alone, CD28/CD3 ζ , or CD137/CD3 ζ tested head-to-head against breast cancer cells found that both of the second generation constructs improved killing compared to the first generation CARs (25). Specifically, the CD28/CD3 ζ had 65% target lysis in ErbB2-positive MDA-MB453 while the CD137/CD3 ζ lysed 62% and CD3 ζ alone killed 51% (25). Another modification in their construct design was the modification of a cysteine to a serine in the CD8 α signaling peptide used, which the authors suggest improves surface expression of the CAR in NK-92 cells. Finally, CD28/CD3 ζ was compared to DAP12 alone using an anti-PSCA CAR in YTS NK cells for prostate cancer (34). In 293T

cell lines engineered to express PSCA, a significant increase in cell killing was observed with the DAP12 containing CAR compared to the CD28/CD3 ζ CAR, suggesting DAP12 may provide a better signaling domain than CD3 ζ (34).

Chimeric Antigen Receptor use in Peripheral Blood NK Cells

Chimeric antigen receptors have also been evaluated in PB-NK cells, which can be isolated from donors through simple blood draws or by apheresis if larger numbers of cells are needed. In contrast to NK-92 cells, activated PB-NK cells express a wider range of activating receptors, such as CD16, NKp44, and NKp46 as well as KIRs, which play an important role in NK cell licensing (36). In addition, PB-NK cells can be given without irradiating the cells so have the ability to expand *in vivo*, which has been correlated with effectiveness in trials involving AML (4). A greater variety of CAR constructs have been used and directly compared in PB-NK cells targeting CD19 (37–39), CD20 (33), or ErbB2 (40, 41) (Table 2). Imai et al. describe the use of two first generation anti-CD19 CARs, CD3 ζ , or DAP10 signaling, and one second generation CAR with CD137 and CD3 ζ . Compared to CD3 ζ , DAP10 induced a much weaker response in PB-NK cells, and addition of the CD137 domain to the CAR resulted in augmented killing of RS4:11 and 380 (ALL) cell lines (37). Another study compared CD3 ζ or 2B4 alone, 2B4 combined with CD3 ζ , and a CD137/CD3 ζ anti-CD19 CAR and tested them against the leukemic cell line REH. *In vitro* studies demonstrated the 2B4 alone CAR was slightly less active compared to CD3 ζ alone. Comparing the second generation CARs, both were significantly better than CD3 ζ alone while similar activity was observed in the 2B4/CD3 ζ and CD137/CD3 ζ CARs (38). When this work was extended to an anti-GD2 CAR for neuroblastoma with just the CD3 ζ and 2B4/CD3 ζ endodomains, again the 2B4/CD3 ζ was significantly better than CD3 ζ alone (38). Another study compared CD3 ζ alone with a CD28/CD3 ζ CAR using ErbB2 as a target.

TABLE 2 | CAR constructs utilized in PB-NK cells.

Reference	Target	CAR construct	Method
(37)	CD19	CD8 α TM/CD3 ζ CD8 α TM/DAP10 CD8 α TM/CD137/CD3 ζ	Retrovirus (mean 69%)
(40)	ErbB2 (HER-2)	CD28/CD3 ζ	Retrovirus (mean 55%)
(38)	CD19/GD2	CD3 ζ or 2B4 alone 2B4/CD3 ζ CD8 TM/CD137/CD3 ζ	Retrovirus (13–24%)
(39)	CD19	CD137/CD3 ζ	Transfection (mRNA) (mean 58%)
(42)	NKG2D ligands	NKG2D/CD3 ζ co-expressed with DAP10	Retrovirus and mRNA transfection
(41)	ErbB2 (HER-2)	CD3 ζ alone CD28/CD3 ζ	Retrovirus (40–50%)
(33)	CD20	CD137/CD3 ζ	Transfection (mRNA) (50–95%)

While no direct lysis experiment was performed, similar levels of INF- γ production were observed in PB-NK cells engineered with just CD3 ζ or CD28/CD3 ζ (41). While different measures were used, the finding that CD28/CD3 ζ does not improve activity in PB-NK cells whereas the same construct was found to be more active in NK-92 suggests there may be differences in CAR activation of PB-NK and NK-92 cells.

One unique approach to CAR creation was to use the ectodomain of NKG2D, an NK cell activation receptor, and link it directly to CD3 ζ (42). This approach utilizes natural NKG2D ligands commonly overexpressed on malignant cells to activate the CAR. Further, NKG2D associates with DAP10 providing a secondary signaling molecule. Indeed, co-expression of DAP10 with the NKG2D/CD3 ζ CAR increased surface expression. This CAR was tested against multiple cell lines derived from several malignancies with the best responses demonstrated against ALL, osteosarcoma, prostate carcinoma, and rhabdomyosarcoma (42).

A third source of NK cells suitable for CAR expression are NK cells derived from human pluripotent stem cells – both induced pluripotent stem cells (iPSCs) or human embryonic stem cells (hESCs) (43–47). These NK cells display a similar phenotype to PB-NK cells (43, 44, 48), and hESC/iPSC-NK cells can be grown on a clinical scale (48). iPSC-derived NK cells engineered with a CD4/CD3 ζ CAR are able to inhibit HIV replication (49). In these studies, the CAR was expressed in the iPSC cells, which were then differentiated into CAR-expressing iPSC-NK cells. The CD4/CD3 ζ iPSC-NK cells were shown to suppress the *in vitro* replication of HIV, providing a platform from which to work for the further development of CAR positive iPSC-NK cells. iPSC-derived NK cells combine the best of PB-NK and NK-92 cells since the cells express NKP44, NKP46, and KIRs, are a homogeneous population with no evidence of undifferentiated iPSCs or T cells in the expanded NK cell population. Additionally, CARs can be easily expressed in hESC and/or iPSC-derived NK cells using non-viral gene transfer methods (49, 50). This is in contrast to PB-NK cells that are much more challenging to achieve high levels of stable CAR expression.

Outlook

As the interest in using CARs in not only T cells (10) but also in NK cells continues to grow, there are still a number of questions that remain to be answered. Perhaps most important is what CAR constructs mediate optimal anti-tumor (or anti-viral) activity. Limited studies in NK-92 cells and in PB-NK cells directly compare first and second generation CARs. Second generation CARs in PB-NK cells are generally more active than first generation CARs. Additionally, the use of CD3 ζ seems better than DAP10 as the signaling domain (37, 38). In NK-92 cells, DAP10 outperformed a CD28/CD3 ζ CAR, but it remains unclear if NK-92 cells provide a good model for how CARs may function in PB-NK cells or hESC/iPSC-derived NK cells. Since NK cells do not naturally express CD28 (35, 51), it is not clear if CD28 is functioning in CAR-expressing NK cells. Different CAR constructs may be required to provide optimal NK cell activation depending on the tumor type or target antigen. More direct comparisons using various intracellular signaling domains and scFvs are needed to best resolve these questions.

Additional research is also needed to determine whether use of an NK cell line (such as NK-92 cells), PB-NK cells, or iPSC-NK cells will provide the best overall benefit. Both 4-1BBL/IL-15 (52) and mbIL-21 (53) artificial antigen presenting cells (aAPCs) can be used to expand PB-NK or iPSC-NK cells (48). Therefore, production of enough NK cells from these sources for clinical use is not a problem. However, it remains to be determined if one aAPC leads to an improved population for adoptive transfer, and the methods to engineer PB-NK cells still need to be further improved. iPSC-NK cells represent an attractive population of cells for NK-CAR therapy because once engineered the iPSC line can be maintained indefinitely and provide an almost limitless supply of NK cells. In addition, careful monitoring of the insertion site of the CAR can be achieved. Finally, NK cell lines provide another alternative but in general express fewer natural NK cell receptors and must be irradiated prior to infusion, which limits *in vivo* expansion and persistence of NK cells.

The method for CAR incorporation provides another important consideration. To get stable expression of CARs, retro- and lentivirus methods have dominated. However, following transduction of NK-92 cells a selection step is usually required to get a pure CAR-expressing population. In PB-NK cells, the efficiencies of gene transfer were at best 69% (37) and ranged as low as 13–24% (38) with most reporting around a 50% transduction efficiency. One way around this issue is the possibility of expressing the CAR in iPSCs and subsequent differentiation into mature NK cells (49), which is done via nucleofection with transposon and avoids the hazards of viral methods. Another consideration is whether the use of suicide systems, such as Cas9 or thymidine kinase (TK), will need to be put in place if unexpected toxicities arise despite the expectation that CAR-expressing NK cells will only circulate for a few weeks (14).

Despite the questions that remain, the ability to engineer NK cells with CARs holds great promise as a novel cellular immunotherapy against refractory malignancies and potentially chronic infectious diseases. The success of T cell-CARs in cases of ALL and CLL has revolutionized the prospects for cell-based immunotherapy. CAR-NK cells can build upon this success to provide important benefits as CAR-based therapy expands. Notably, NK cells can provide a homogenous, off-the-shelf, standardized product that can be used in as an allogeneic product to treat patients. Therefore, this process does not need to be done on a patient-specific basis, as with current T cell-CAR-based therapies. The ability to more potently direct NK cell-mediated cytotoxicity against refractory tumors through the expression of CARs can continue to revolutionize cancer treatment.

Author Contributions

DH performed the review of the literature and wrote the manuscript. DK wrote and edited the manuscript.

Acknowledgments

DH Ph.D., would like to acknowledge the Cancer Research Institute for support through the Irvington Postdoctoral Fellowship award.

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