

Are natural killer cells superior CAR drivers?

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T lymphocytes engineered to express a chimeric antigen receptor (CAR) are being celebrated as a major breakthrough of anticancer immunotherapy. Natural killer cells have not received similar attention as CAR effectors, although the use of these relatively short-lived cytotoxic cells is associated with several advantages.

The latter American Society of Hematology (ASH) meeting, which took place last December in New Orleans, was dominated by the enthusiasm on the anti-leukemia effects of T lymphocytes engineered to express a CD19-targeting chimeric antigen receptor (CAR).¹ Fourteen out of 16 pediatric patients with relapsed or advanced acute lymphoblastic leukemia (ALL) entered a remission in response to the adoptive transfer of these cells. A similar outcome was documented for adults with chronic lymphocytic leukemia.

CAR-expressing T cells are usually generated from autologous T cells, but T lymphocytes from allogeneic donors are also being explored in this sense, especially upon relapse after stem cell transplantation.² CAR-bearing T cells are usually activated with anti-CD3/CD28 beads and expanded in culture flasks (such as the Wave^R system) in the presence of interleukin (IL)-2. CARs against an expanding array of cell surface-exposed tumor-associated antigens (TAAs) have been and continue to be engineered.³ Since the majority of these TAAs are not tumor specific, CAR-expressing T cells can cross-react with healthy cells, mediating an “on-target/off-tumor” side effect. For example, T cells expressing a CD19-targeting CAR can cause a profound and long-lasting

B-cell deficiency as they eliminate normal B cells.⁴ T lymphocytes bearing a CAR specific for interleukin 3 receptor, α (ILR3A, also known as CD123) kill not only leukemic cells but also bone marrow cells that express the same receptor, leading to prolonged and profound marrow suppression.⁵ In some cases this on-target/off-tumor side effect can be fatal, as it happened in a patient with metastatic colon carcinoma who received T cells engineered to express a HER2 targeting CAR. In this case, the side effects of CAR-expressing T cells on low level HER2 expressing lung epithelium led to fatal pulmonary complications combined with a massive cytokine release.

It has been suggested that the antineoplastic activity of CAR-expressing T cells is related to and dependent on their persistence in the patient circulation and malignant tissue. If this were indeed the case, the on-target/off-tumor effects would also persist. For CD19-redirecetd T cells, this would entail a prolonged depletion of normal B cells and hence long-term defects in humoral immunity.

As recent clinical trials have suggested, antigen loss cancer variants can emerge as a result of the selective pressure imposed by immunotherapeutic interventions, often driving disease relapse.¹ In this setting, TAA-specific T cells would continue to mediate on-target/off tumor effects, such as the suppression of normal B cells or bone marrow precursors. A potential solution to this issue is provided by the transduction of T cells with CAR-coding mRNAs, usually resulting in the loss of expression over a few days.⁷ Indeed, most CAR-expressing T cells currently tested in clinical trials are obtained with lentiviral constructs, which integrate into

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Table 1. Comparison of CAR- expressing T, natural killer and NK-92 cells

Parameter	T cells	NK cells	NK-92 cells
Collection	Leukopheresis	Leukopheresis	Continuously growing cell line consisting of “pure” (100%) activated NK cells
Preparation	Activation of cells with anti-CD3/CD28 beads Allogeneic donor: MHC match required	NK cells represent only 10% of all lymphocytes. Autologous: Enrichment needed (selection for CD56+ cells). Allogeneic donor: MHC-matched donor or depletion of alloreactive T-cells to prevent GvH reactions	No processing necessary prior to CAR engineering
Expansion	Flasks, bags or WaveR expansion system	Requires engineered feeders (example: K562 cells expressing IL-15 and TNFSF9) plus IL-2 (in flasks, bags or bioreactors)	Expansion in serum free-medium without feeders, but IL-2 only (in flasks, bags, or bioreactors)
Transduction	Lentiviral systems transduce about 1/3 of T cells	Low transfection efficiency even with viral vectors	Transfection efficiency of about 50%, compatible with sorting
Cytotoxic mechanisms	CAR-restricted killing In case of antigen loss on tumors, CAR-expressing T cells become ineffective	Multiple receptors can trigger CAR-independent and FcR-dependent cytotoxicity	Multiple receptors can trigger CAR-independent and FcR-dependent cytotoxicity
Adverse events	Can cause “off target” effects Survive for prolonged periods in the patient circulation Can induce a storm of pro-inflammatory cytokines	Limited life span in patients No concern about persisting CAR-associated side effects	Limited life span in patients No concern about persisting CAR-associated side effects
Miscellaneous	Suicide genes are required to control life span in vivo	No need for suicide gene	No need for suicide gene
Clinical results	Phase I studies have shown clinical benefit	Proof of clinical benefit pending	Proof of clinical benefit pending
Off-the-shelf CAR-specific cellular product?	Autologous cells, required preparation on a per patient basis	Possible to have donor NK cells cryopreserved, but recovery is poor after upon thawing	Possible to have NK-92 cryopreserved and expanded upon thawing (before infusion)

Abbreviations: CAR, chimeric antigen receptor; FcR, Fc receptor; GvH, graft-vs.-host; IL, interleukin; NK, natural killer; TNFSF9, tumor necrosis factor superfamily, member 9.

the genome and hence ensure persistent transgene expression.

Natural killer (NK) cells may represent alternative cytotoxic effectors for CAR-driven cytotoxicity. Allogeneic NK cells are expected to induce an immune response and be rejected after a few days, and even autologous NK cells should disappear relatively rapidly from the circulation, owing to their limited lifespan. NK cells have additional advantages over T cells (Table 1). In particular, while T lymphocytes only kill their targets by a CAR-specific mechanism, NK cells are endowed with spontaneous cytotoxic activity and can trigger the demise of target cells in a TAA-unrestricted manner via specific natural cytotoxicity receptors (NCRs), including NCR3 (also known as NKp30), NCR2 (also known as NKp44), NCR1 (also known as NKp46), and

killer cell lectin-like receptor subfamily K, member 1 (KLRK1, best known as NKG2D). NK cells also express the Fc fragment of IgG, low affinity III, receptor (FcγRIII), that binds the Fc fragment of antibodies to elicit antibody-dependent cell-mediated cytotoxicity (ADCC). This specific feature of NK cells would enable the combination of 2 targeted therapies recognizing different (or the same) TAA(s), namely CAR-expressing NK cells and a TAA-specific monoclonal antibody.

Additional features of NK cells could make them better and potentially safer CAR drivers than T cells. For instance, NK cells produce a host of cytokines that are different from those produced by T cells, including interferon γ (IFN γ) and granulocyte macrophage colony-stimulating factor (GM-CSF). The cytokine storm initiated by the infusion of

CAR-expressing T cells is indeed largely mediated by their pro-inflammatory cytokines such as tumor necrosis factor α (TNF α), IL-1, and IL-6. It is also known that NK cells are “serial killers.” Thus, time-lapse videomicroscopy studies have shown that NK as well as NK-92 cells (a continuously-growing, highly-active, NK cell-derived cell line) diligently move from one target to the next one, killing on as many as 7–10 cells.⁸ Evidence for such a serial killing by T cells is lacking at this point.

Nonetheless, there are some obstacles for the use of circulating NK cells for CAR-based immunotherapy. Like T lymphocytes, NK cells are obtained (from patients or donors) by leukopheresis, which can be time-consuming, costly and occasionally requiring central venous access. Since only about 10% of circulating

lymphocytes are NK cells, some extent of selection/enrichment is necessary, which is generally performed by positive CD56-based, magnetic immunoselection. In case of allogeneic donors, peripheral blood mononuclear cells also must be depleted of T cells to prevent graft-vs.-host reactions. Moreover, a feeder layer and additional cytokines are required to maximize the expansion of circulating NK cells in vitro. A feeder layer consisting of the leukemia K562 cells engineered to express tumor necrosis factor superfamily, member 9 (TNFSF9, best known as 4-1BBL) and IL-15 seems to be very effective, but a product testing before infusion must ensure that all malignant cells have been completely removed.⁹

Finally, the transfection efficiency of circulating NK cells is variable and generally not very high, even when viral vectors are employed.¹⁰

Although the challenge of introducing CAR-coding genes into sufficient numbers of circulating NK cells may be overcome at some point, NK-92 cells present an open cellular platform for CAR-based immunotherapy.¹¹ The transfection efficiency of NK-92 cells is about 50%, even with non-viral methods.¹⁰ Besides

References

- Grupp SA, Frey NV, Aplenc R, Barrett DM, Chew A, Kalos M, Levine BL, Litchman M, Maude SL, Rheingold SR, et al. T Cells engineered with a Chimeric Antigen Receptor (CAR) targeting CD19 (CTL019) produce significant in vivo proliferation, complete responses and long-term persistence without GvHd in children and adults with relapsed, refractory ALL. *Blood* 2013; 122:67
- Kochenderfer JN, Dudley ME, Carpenter RO, Kassim SH, Rose JJ, Telford WG, Hakim FT, Halverson DC, Fowler DH, Hardy NM, et al. Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. *Blood* 2013; 122:4129-39; PMID:24055823; <http://dx.doi.org/10.1182/blood-2013-08-519413>
- Sadelain M, Brentjens R, Rivière I. The promise and potential pitfalls of chimeric antigen receptors. *Curr Opin Immunol* 2009; 21:215-23; PMID:19327974; <http://dx.doi.org/10.1016/j.coi.2009.02.009>
- Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 2011; 3:95ra73; PMID:21832238; <http://dx.doi.org/10.1126/scitranslmed.3002842>

Table 2. CAR-coding genes transfected/transduced so far into natural killer cells

Target	Indication(s)	Blood NK cells	NK-92 cells	Refs.
CD19	Lymphoid malignancies	X	X	10,12-15
CD20	Lymphoid malignancies	X	X	16-18
CD38	Multiple myeloma		X	19
ERBB2	Breast carcinoma Head and neck cancer Ovarian carcinoma Glioblastoma	X	X	11,20
GD2	Neuroblastoma		X	21
EPCAM	Breast carcinoma Pancreatic cancer		X	22
EBNA3C	EBV infections		X	23
CS1	Multiple myeloma		X	24
LMAN1	Melanoma Neuroblastoma		X	25

Abbreviations: EBNA3C, Epstein-Barr nuclear antigen 3C; ERBB2, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2; EPCAM, epithelial cell adhesion molecule; GD2, ganglioside GD2; LMAN1, lectin, mannose-binding, 1

being technically more simple and under less-constraining regulations, avoiding viral vectors eliminates the risks of oncogene activation and insertional mutagenesis. Upon sorting, CAR-expressing NK-92 cells can be enriched to obtain a population near-to-exclusively composed of NK-92 carrying the CAR of interest.

So far, NK-92 cells have been efficiently transduced with a number of different CAR-coding constructs (Table 2) and pre-clinical studies in xenotransplanted immunodeficient mice have demonstrated

the potential therapeutic effects of this approach. Several centers are gearing up to test whether CAR-expressing NK cells can keep up with their T-cell counterparts. Eventually, we might even discover that both these cell types have their place in the multimodal approach that is required to eliminate cancer and control its recurrence.

Disclosure of Potential Conflicts of Interest

H.K. is Founder of Conkwest Inc. and its Chief Medical and Scientific Officer.

- Gill S, Tasian SK, Ruella M, Shestova O, Li Y, Porter DL, Carroll M, Danet-Desnoyers G, Scholler J, Grupp SA, et al. Anti-CD123 Chimeric Antigen Receptor T cells (CART-123) provide a novel myeloablative conditioning regimen that eradicates human acute myeloid leukemia in preclinical models. *Blood* 2013; 122:143
- Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010; 18:843-51; PMID:20179677; <http://dx.doi.org/10.1038/mt.2010.24>
- Zhao Y, Moon E, Carpenito C, Paulos CM, Liu X, Brennan AL, Chew A, Carroll RG, Scholler J, Levine BL, et al. Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. *Cancer Res* 2010; 70:9053-61; PMID:20926399; <http://dx.doi.org/10.1158/0008-5472.CAN-10-2880>
- Bhat R, Watzl C. Serial killing of tumor cells by human natural killer cells--enhancement by therapeutic antibodies. *PLoS One* 2007; 2:e326; PMID:17389917; <http://dx.doi.org/10.1371/journal.pone.0000326>
- Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockett T, Eldridge P, Leung WH, Campana D. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res* 2009; 69:4010-7; PMID:19383914; <http://dx.doi.org/10.1158/0008-5472.CAN-08-3712>
- Boissel L, Betancur M, Wels WS, Tuncer H, Klingemann H. Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. *Leuk Res* 2009; 33:1255-9; PMID:19147228; <http://dx.doi.org/10.1016/j.leukres.2008.11.024>
- Uherek C, Tonn T, Uherek B, Becker S, Schnierle B, Klingemann HG, Wels W. Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. *Blood* 2002; 100:1265-73; PMID:12149207
- Li L, Liu LN, Feller S, Allen C, Shivakumar R, Fratantoni J, Wolfrum LA, Fujisaki H, Campana D, Chopas N, et al. Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. *Cancer Gene Ther* 2010; 17:147-54; PMID:19745843; <http://dx.doi.org/10.1038/cgt.2009.61>

13. Imai C, Mihara K, Andreansky M, Nicholson IC, Pui C-H, Geiger TL, Campana D. Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia* 2004; 18:676-84; PMID:14961035; <http://dx.doi.org/10.1038/sj.leu.2403302>
14. Shimasaki N, Fujisaki H, Cho D, Masselli M, Lockey T, Eldridge P, Leung W, Campana D. A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. *Cytotherapy* 2012; 14:830-40; PMID:22458956; <http://dx.doi.org/10.3109/14653249.2012.671519>
15. Romanski A, Uherek C, Bug G, Muller T, Rossig C, Kampfmann M, Krossok N, Hoelzer D, Seifried E, Wels W, et al. Re-targeting of an NK cell line (NK-92) with specificity for CD19 efficiently kills human B-precursor leukemic cells. *Blood* 2004; 104:751a
16. Chu Y, Yahr A, Ayello J, van de Ven C, Zhou X, Cairo MS. Anti-CD20 Chimeric Antigen Receptor (CAR) modified expanded Natural Killer (NK) cells significantly mediate Burkitt Lymphoma (BL) regression and improve survival in human BL xenografted NSG mice. *Blood* 2013; 122:3263
17. Boissel L, Betancur-Boissel M, Lu W, Krause DS, Van Etten RA, Wels WS, Klingemann H. Retargeting NK-92 cells by means of CD19- and CD20-specific chimeric antigen receptors compares favorably with antibody-dependent cellular cytotoxicity. *Oncoimmunology* 2013; 2:e26527; PMID:24404423; <http://dx.doi.org/10.4161/onci.26527>
18. Müller T, Uherek C, Maki G, Chow KU, Schimpf A, Klingemann HG, Tonn T, Wels WS. 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; 57:411-23; PMID:17717662; <http://dx.doi.org/10.1007/s00262-007-0383-3>
19. Yang S, Xin A, Brown RD, Ho J, Gibson J, Joshua DE, Wels W, Sze, DM. Development of retargeted CD38-specific NK-92 cell line for potential anti-myeloma immunotherapy. [abstr]. *Blood* 2005; 106:5104
20. Kruschinski A, Moosmann A, Poschke I, Norell H, Chmielewski M, Seliger B, Kiessling R, Blankenstein T, Abken H, Charo J. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proc Natl Acad Sci U S A* 2008; 105:17481-6; PMID:18987320; <http://dx.doi.org/10.1073/pnas.0804788105>
21. Esser R, Müller T, Stefes D, Kloess S, Seidel D, Gillies SD, Aperlo-Iffland C, Huston JS, Uherek C, Schönfeld K, 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; 16:569-81; PMID:21595822; <http://dx.doi.org/10.1111/j.1582-4934.2011.01343.x>
22. Sahn C, Schönfeld K, Wels WS. 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; 61:1451-61; PMID:22310931; <http://dx.doi.org/10.1007/s00262-012-1212-x>
23. Tassef DV, Cheng M, Cheung NK. Retargeting NK92 cells using an HLA-A2-restricted, EBNA3C-specific chimeric antigen receptor. *Cancer Gene Ther* 2012; 19:84-100; PMID:21979579; <http://dx.doi.org/10.1038/cgt.2011.66>
24. Chu J, Deng Y, Benson DM, He S, Hughes T, Zhang J, Peng Y, Mao H, Yi L, Ghoshal K, 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* 2013; (Forthcoming); PMID:24067492; <http://dx.doi.org/10.1038/leu.2013.279>
25. Koehne G, Guo HF, Trivedi D, Williams RY, O'Reilly RO, Cheung V. Redirecting NK-cell cytolytic activity to solid tumors using chimeric scFv receptor gene-modified adoptive immunotherapy. *ASCO Proc* 2001; 22: 175a.