Editorial

Engineered natural killer cells expressing chimeric antigen receptors (CAR)—a promising approach in tumor immunotherapy

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Cell gene therapy is a promising approach for the treatment of cancers resistant to conventional therapy with cytostatic drugs and monoclonal antibodies. In particular, the use of genetically engineered immune cells showed remarkable results in the treatment of patients with leukemia and lymphoma, but there are still undeniable limitations of this approach. In the paper "Human iPSC-derived natural killer cells engineered with chimeric antigen receptors enhance anti-tumor activity" (1). Li *et al*. provide interesting possibilities to enable improved anti-tumor activity and gives potentially concepts to overcome the limits especially in the field of solid tumors.

In recent years the adoptive cellular immunotherapy with chimeric antigen receptor (CAR) transduced T cells emerged as a promising concept for advanced cancers refractory to conventional therapy. The CAR is a genetically engineered protein complex, consisting of three parts: (I) an extracellular antigen-specific recognition domain derived from a single-chain variable fragment (scFv), (II) a transmembrane domain and (III) an intracellular signaling domain providing a T-cell activating signal. First-generation CARs contain only the tyrosine-based ξ-signal-transducing subunit from the T cell receptor (TCR)/CD3 receptor complex. In second generation CARs, additional costimulatory molecules such as CD28, DAP-12 or 4-1BB (CC137) were introduced thus mediating a superior activation, proliferation and *in vivo* persistence of CAR T cells when compared to first-generation CARs. Third-generation CARs include the combination of two costimulatory molecules. CARs combine the antigen-binding properties of a monoclonal antibody with the activating, effector and long-term function of a T cell (2). Therefore, the transduction of CAR vectors into T cells allows to redirect genetically engineered T cells toward specific antigens expressed on cancer cells. Furthermore, not only proteins but also carbohydrates, glycosylated proteins, and proteoglycans are suitable CAR antigens, thus expanding the repertoire of potential tumor antigens (3). An additional advantage of CARs is the ability to recognize unprocessed extracellular antigens. Thus, CAR T cells can act in a human leukocyte antigen (HLA)-unrestricted manner. This is particularly important in the light of the likely tumor escape via HLA downregulation.

In CAR T cell therapy, T cells collected from the peripheral blood of a patient via leukapheresis will be isolated, engineered to express CARs on their surface, expanded and subsequently transfused back into the patient, where they can attack and kill cancer cells expressing the target antigen. *In vitro* generation of this personalized product usually takes 2 weeks followed by quality control, i.e., microbiological sterility testing. Currently, there are 733 CAR T cell trials registered in "ClinicalTrails.gov", of whom 432 are in the status "recruiting" or "not yet recruiting". Also, our group inaugurated a clinical trial with third-generation CAR (scF.cD19.CD28.CD3ξ) which recruits patients with refractory or refractory CD19+

B-cell neoplasia, including acute lymphoblastic leukemia and non-Hodgkin's lymphoma (3). In particular, trials with the transfer of autologous CD19-CAR T cells in patient with hematological malignancies has been very successful and achieved impressive remission rates. Of note, last year the Food and Drug Administration (FDA) approved the first gene-modified CAR T cell therapy, Kymriah® (or tisagenlecleucel) for relapsed B-cell acute lymphoblastic leukemia (ALL) in children and young adults and the second gene-modified CAR T cell therapy, Yescarta® (or axicabtagene ciloleucel), for the treatment of adult patients with certain types of non-Hodgkin lymphoma (4).

Despite of the promising results has this personalized cell-based therapy with autologous CAR T cells several disadvantages and limitations:

(I) The production of the CAR T cells needs be performed under Good Manufacturing Practice (GMP) compliant conditions for clinical application. This implies a production which is a cumbersome, time-consuming, expensive and logistically challenging procedure.

(II) For the CAR T cell production, it can be difficult to get acceptable numbers of cells, because the patients are heavily pretreated (e.g., multiple chemotherapies). Furthermore, the production-time can be too long for patients with fast progression.

(III) The treatment with CAR T cells has high incidence of on-target/off-tumor toxicity and leads to adverse effects such as cytokine release syndrome (CRS) or neurotoxicity.

(IV) An allogeneic CAR T cell product would have the potential to overcome these limitations but carry a major risk of graft-versus-host disease (GVHD), even after HLA-matching.

(V) Loss or mutation in the target antigen for CAR T cells can result in the relapse of the underlying disease.

(VI) While CAR T cell treatment is clinically very effective in hematological malignancies, the results were less encouraging in patients with solid tumors.

A promising approach to overcome these limitations of CAR T cells constitutes in the use of natural killer (NK) cells transduced with a CAR vector. These "CAR NK cells" have several advantages over CAR T cells, which might lift the whole approach of CAR-based immunotherapy to a new level. NK cells are powerful effector lymphocytes of the innate immune system and competent to recognize and kill cancer cells. NK cells have a repertoire of activating and inhibitory receptors and the balance of these signaling receptors regulates NK cell-mediated cytotoxicity. NK-receptors are not rearranged, antigen-specific receptors, like the TCR of T cells, but germline-encoded. Physiological, non-malignant cells are protected from NK-mediated lysis by the recognition of "self"-HLA molecules on their surface through inhibitory NK receptors (5). In contrast, tumor cells often downregulate or lose their HLA molecules as an escape mechanism against T cells (5). This decreased expression of HLA molecules leads to a lack of inhibitory signals in NK cells (missing "self" recognition) and induces anti-tumor activity against the cancer cell, e.g., secretion of cytoplasmic granules containing perforins and granzymes.

Important activating receptors are NKG2D, NKp30, NKp44 and FasL, and important costimulatory receptors are LFA-1, CD244 (2B4) and 4-1BB. Moreover, NK cells express the activating receptor FcγRIIIa (CD16) which is able to recognize the Fc fragment of immunoglobulin G molecules, thus allowing NK cells to mediate antibody-dependent cell-mediated cytotoxicity (ADCC).

The transfer of CARs in NK cells combines (as well as in T cells) the powerful anti-tumor activity of these cells with the specific antigen-binding properties of an antibody in the CAR. Further advantages are:

(I) Mature NK cells have a relatively limited life-span and exert an effective anti-tumor activity (5).

(II) In case of a tumor microenvironment, selective pressure can lead to a loss or mutations of the CAR target antigen on cancer cells, to escape from the immune system. In case of CAR-NK cells, NK cells still maintain their intrinsic capacity to recognize and to target tumor cells through their native receptors (5).

(III) Trials using adoptive transfer of allogeneic NK cells showed a low risk of GvHD and minimal toxic effects like CRS. Therefor allogeneic NK cells constitute an interesting approach in adoptive cell therapy. Main sources for collecting or generating NK cells for CAR-engineering are peripheral blood (PB), umbilical cord blood (UCB), NK cell lines like NK92 and induced pluripotent stem cells (iPSC). This paves the avenue for a targeted "off-the-shelf" anti-cancer immunotherapy, where manufacturing of CAR NK cells can be standardized in a patient-independent manner and at large scale.

Currently, there are 14 clinical trials with CAR NK

Table 1 Ongoing clinical CAR NK cell trails (source: ClinicalTrails.gov)

Row	Clinical trial identifier	CAR target	Disease	Status	Phase	NK cell source	Study location
1	NCT03692767	CD22	Refractory B-cell lymphoma	N	I	U	Allife Medical Science and Technology Co., Ltd., Beijing, China
2	NCT03690310	CD19	Refractory B-cell lymphoma	N	I	U	Allife Medical Science and Technology Co., Ltd., Beijing, China
3	NCT03692663	PSMA	Prostate cancer	N	I	U	Allife Medical Science and Technology Co., Ltd., Beijing, China
4	NCT03692637	Mesothelin	Epithelial ovarian cancer	N	I	U	Allife Medical Science and Technology Co., Ltd., Beijing, China
5	NCT03415100	NKG2D ligands	Solid tumours	R	I	Auto/allo PBMCs	The Third Affiliated Hospital of Guangzhou Medical University, China
6	NCT02944162	CD33	Leukemias	U	I/II	NK92	PersonGen Bio Therapeutics Co., China
7	NCT02892695	CD19	Leukemia/lymphoma	R	I/II	NK92	PersonGen Bio Therapeutics Co., China
8	NCT03579929	CD19	Leukemia/lymphoma	N	I/II	CB	M.D. Anderson Cancer Center, USA
9	NCT03056339	CD19	Leukemia/lymphoma	R	I/II	CB	M.D. Anderson Cancer Center, USA
10	NCT03383978	5.28	Glioblastoma	R	II	NK92	Johann W. Goethe University, Germany
11	NCT02742727	CD7	Leukemia/lymphoma	R	I/II	NK92	PersonGen Bio Therapeutics Co., China
12	NCT02839954	MUC1	Solid tumours	R	I/II	U	PersonGen Bio Therapeutics Co., China
13	NCT03049449	CD30	Lymphomas	R	I	U	National Institutes of Health Clinical Center, USA
14	NCT02274584	CD30	Lymphomas	U	I/II	U	University of Florida, US & Peking University Cancer Hospital, China

CAR, chimeric antigen receptor; NK, natural killer; N, not yet recruiting; R, recruiting, U, unknown.

cells which are registered at the database ClinicalTrails.gov (*Table 1*). Nine of these evaluate the efficacy of CAR NK cell therapy in hematologic cancers. At least five of the trials use NK92 cells as basis for CAR-engineering and one trial employs NK cells from the peripheral blood. As far as indicated in the database, five trials imply CD28 as a costimulatory domain and three additionally 4-1BB. Furthermore, two trials at the M.D. Anderson Cancer Center, USA use primary NK cells from UCB as source of CAR-NK cells. The CAR-constructs contain the following genes: (I) a single chain fragment binding CD19 as target in patients with B cell lymphoid malignancies, (II) the known costimulatory domain CD28, (III) the gene to produce interleukin-15 (IL-15) ectopically and (IV) the gene of inducible caspase-9 (*iCasp9*) as a safety switch molecule. The cytokine IL-15 is crucial for NK-cell survival and proliferation. In murine model of lymphoma, the IL-15 gene dramatically increased the *in vivo* persistence and anti-tumor activity of CAR-NK cells. *iCasp9* is a suicide gene, which can be activated pharmacologically to eliminate CAR in case of toxicity (6).

Li *et al.* described in their paper "Human iPSC-derived natural killer cells engineered with chimeric antigen receptors enhance anti-tumor activity" (1) that NK cells, isolated from PB or UCB, show efficacy against leukemia but only less activity against solid tumors. Furthermore, primary NK cells were relatively less efficient to genetically modify compared with T cells. Consequently, the authors accentuated the need for a better approach to achieve a higher efficacy against solid tumors. Therefor the aim of the study was to find a method to improve the engineering of CAR-NK cells, which then induce increased anti-tumor activity in a solid tumor model.

The authors hypothesized that a CAR-construct for NK cells should be adapted to the need of these special effector cells. They supposed that engineering NK cell activation domains into CARs would improve the anti-tumor effectivity, especially against solid tumors that are

more resistant to NK cell-mediated killing. To find the optimal CAR-construct the researchers designed 9 different NK cell specific CARs consistent of (I) a single chain variable fragments, targeted to the tumor-associated antigen mesothelin (meso), (II) a transmembrane domain, (III) one or two costimulatory domains, (IV) a ξ-stimulatory domain, which is common to both the CD3 complex in T cells and CD16 in NK cells. They chose for the transmembrane domain the NK-activating receptors NKG2D, CD16, NKp44 and NKp46 and for the costimulatory domains 2B4, 4-1BB, DAP10 and DAP12, which are typically expressed in NK cells. As a control they also designed a classic third generation CAR-construct for T cells, consisted of the signaling domain CD3ξ and the costimulatory domains CD28 and 4-1BB, which was also redirected against meso (T-CAR).

To screen the CAR constructs, all constructs were transferred in NK92 cells. CAR-NK92 cells were then incubated with meso-negative/low and meso-positive/high K562-cells (cell-line) and ovarian cancer cells as target cells. After meso-specific stimulation, the lysis-level and the expression of CD17a (indicative of cytotoxic granule release) were analyzed. The CAR constructs NKG2D-2B4ξ, NKG2D-2B4-4-1BBξ and NKG2D-2B4-DAP10ξ displayed the highest cytotoxicity against positive targets, which was also distinctly higher as the cytotoxicity of the T-CAR construct. The authors chose the NK construct NKG2D-2B4ξ for their further investigation. The transmembrane receptor NKG2D is a key activator receptor of NK cells and its activation promotes the cytotoxic ability of NK cells. Ligation of NKG2D is associated with DAP10. The signaling, induced by binding of endogenous 2B4 with its ligand CD48 is required for optimal NK cell function. CD3ξ transmits in NK cells intracellular signaling which induces ADACC.

In further experiments the authors analyzed the role of each domain in the chosen NK-CAR construct by introducing site-specific mutations known to decrease the function of each domain. The study showed that NKG2- and 2B4-domains are powerful for anti-tumor activity, whereas mutations in the cytoplasmatic CD3ξ-domaine did not significantly weaken the cytotoxicity of the NK-CAR construct. Moreover, incorporation of additional costimulatory domains (DAP10, DAP12 or 4-1BB) in the NK-CAR construct did not improve CAR-mediated killing in this system. Li *et al.* also analyzed the effect of antigen-specific activation on the signaling pathway of the meso-NK-CAR-construct in comparison to the meso-T-CAR-construct. To this end, the phosphorylation of key NK cell signaling mediators was analyzed. NK-CAR induces in comparison of T-CAR significantly a potent upregulation in phosphorylated activation of PLC-γ, the Syk-vav1 Erk pathway, and also the NK-κB, which is sufficient to promote improved NK cell-mediated granulation, cytokine production, and cytotoxicity of antigen-expressing tumor cells. The authors also conclude that this activation leads to increased expansion and survival of NKG2D-2B4ξ-CAR NK cells, enabling improved anti-tumor activity.

In search of a suitable source for the expression of their NK-CAR construct, Li *et al.* chose human inducible pluripotent stem cells (iPSC), as (I) previous studies demonstrated that human iPSCs can be differentiated into NK cells with phenotypic and functional similarities to NK cells isolated from PB-NK cells, (II) this system was efficient and well-defined to produce homogeneous and well-characterized human NK cells from iPSCs, and (III) iPSC-derived NK cells could be produced on a large scale as a standardized cell product. The authors demonstrated that iPSC-NK cells engineered to express the optimized NKG2-2B4ξ CAR-construct maintain a typical NK-cell phenotype and NK-cell-mediated cytolytic machinery (i.e., antigen-specific lysis, CD107a-expression, interleukin-γ (IFN-γ). In comparison to NK-CAR-iPSC-NK cells, T-CAR-iPSC-NK cells showed a lower capacity to kill meso-expressing target cells. However, expression of the "T-construct" versus "NK-construct" in CD3+ T-cells respectively demonstrated that T-CAR T-cells exhibit a better cytolytic activity against meso-expressing targets.

In the next step, the author evaluated the activity of the NK-CAR NK cells *in vivo* by evaluating the killing of meso-expressing ovarian cancer cells in a mouse xenograft model. In these experiments they compared NK-CAR-iSPC-NK cells, T-CAR-iSPC-NK cells, non-CAR-expressing iPSC-derived NK cells (iPSC-NK cells) and non-CAR-expressing PB-derived NK cells (PB-NK cells). Mice received a single injection of 1.5×10^7 NK cells. Additionally, IL-2 and IL-15 were administered to the mice for 21 days to promote *in vivo* NK survival and expansion. Mice were monitored at days 7,10, 21, 28, 35 after injection. All NK cells showed a significant reduction in tumor after 7 days, but NK-CAR-iPSC-NK cells markedly improved anti-tumor activity and survival when compared to T-CAR-iPSC-NK cells, iPSC-NK-cells and PB-NK cells. Investigation on the *in vivo* persistence of NK cells in blood, spleen and peritoneal fluid was for NK-iSPC-NK cells significantly increased on day 10 (compared to PB-NK cells and iPSC-NK cells), but

their level returned to the same levels as in PB-NK cells and iPSC NK cells, when exogenous cytokine administration was discontinued.

In further experiments, the authors compared the anti-tumor activity of NK-CAR-iSPC-NK cells to third-generation T-CAR-expressed primary T cells. The same ovarian-tumor bearing mouse xenograft model was employed. Mice were inoculated with a single dose of 1.0×10^7 NK or T cells. Both T-CAR T cells and NK-CAR-iSPC NK cells demonstrated a significant *in vivo* persistence post-injection, as well as an antitumor activity and reduction of the tumor burden. However, T-CAR-expressing T cell-treated mice developed a significant body weight loss and/or severe visceral hemorrhage and ischemia, leading to death. These mice showed enlarged spleens and pathogenic damages in liver, lung, kidney, and gut. Of note, NK-CAR-iPSC-NK cell-treated mice did not demonstrate weight loss or early non-tumor-mediated death. Evaluation of production of human IFN-γ (hIFN-γ), human tumor necrosis factor-α (hTNF-α), and human IL-6 (hIL-6) in the plasma demonstrated that both NK and T cells induced an increase of hIFN-γ and hTNF-α. But in case of NK-CAR-iPSC-NK cells, the level of both cytokines returned to baseline level. However, T cells led to a more enduring increase of hIFN-γ, hTNF-α and hIL-6 levels, what is in case of hTNF-α and hIL-6 tightly associated with CRS in clinical practice.

In conclusion, Li *et al.* identified a promising CAR-construct, which was adjusted especially for NK cells. In iPSC-derived NK cells, this CAR-construct enables these NK cells to induce significant tumor activity without toxicity in an *in vivo* solid tumor environment. Therefore, the authors provide a potential strategy for an "off the shelf" production of targeted allogeneic lymphocytes suitable to treat refractory malignancies. Moreover, this work gives more insights about the mode of action of NK-cell receptors, NK-cell costimulatory molecules and their signaling pathways. This will help in the design of new CAR-constructs with additional properties like inducible apoptosis by substantial toxicity. Moreover, these CAR-NK cells open new avenues towards the use in chronic infectious diseases like HIV.

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Footnote

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