CAR-Expressing Natural Killer Cells for Cancer Retargeting

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Abstract

Since the approval in 2017 and the outstanding success of Kymriah® and Yescarta®, the number of clinical trials investigating the safety and efficacy of chimeric antigen receptor-modified autologous T cells has been constantly rising. Currently, more than 200 clinical trials are listed on clinicaltrial.gov. In contrast to CAR-T cells, natural killer (NK) cells can be used from allogeneic donors as an "off the shelf product" and provide alternative candidates for cancer retargeting. This review summarises preclinical results of CAR-engineered NK cells using both primary human NK cells and the cell line NK-92, and provides an overview about the first clinical CAR-NK cell studies targeting haematological malignancies and solid tumours, respectively.

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Introduction

As an essential part of the innate immune system, natural killer (NK) cells play a pivotal role as the body's first-line defence against virally infected and malignant cells. During the last 15 years, several clinical trials with donor NK cells have shown their safety and feasibility [1, 2], but limitations appeared due to tumour immune escape mechanisms (TIEM). To overcome TIEMs, NK cells can be modified by retroviral or lentiviral vector transduction to express antibody-derived single-chain variable fragments (scFv) for cancer retargeting. The backbone chimeric antigen receptor (CAR) design contains an antigen recognition domain derived from an scFv antibody connected to a hinge and transmembrane domain mediating an anchorage to the cell membrane and, in the case of first-generation CARs, a cluster of differentiation (CD) 3 zeta (ζ) chain-derived signalling domain responsible for effector cell activities. Additionally, second- and third-generation CARs include either one or two costimulatory domains, respectively [3]. In comparison to the outstanding clinical results with CAR-engineered T cells, CAR-modified NK cells can reveal some advantages by additional cancer-killing mechanisms, especially antibody-dependent cell-mediated cytotoxicity (ADCC). In terms of safety conditions, unwanted adverse effects are avoided by short-term persistence and the lack of antigen clonality of CAR-modified NK cells [4–6]. CAR-redirected NK cells appear to have a beneficial impact and improved effects in immunosurveillance. Similar to CAR-T cells, they were shown to mediate potent anti-tumour activity against CD19-positive cells in vitro and in a Raji lymphoma mouse model [7]. Currently, this leads to first immunotherapeutic approaches to cure both lymphomas of B

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Table 1. CAR-NK cells in clinical trials

Clinical trial identifier	Target	Condition/disease	Origin of NK cells	Phase	Status	Location
NCT03579927	CD19	Lymphoma and leukaemia	Umbilical cord blood	I/II	Not yet recruiting	Houston, TX, USA
NCT03056339	CD19	Lymphoma and leukaemia (relapsed/refractory B-cell malignancy)	Umbilical cord blood	I/II	Recruiting	Houston, TX, USA
NCT02892695	CD19	Lymphoma and leukaemia	NK-92	I/II	Recruiting	Suzhou, Jiangsu, China
NCT01974479	CD19	ALL	Haploidentical donor NK cells	I	Suspended	Singapore
NCT00995137	CD19	ALL	Expanded donor NK cells	I	Completed	Memphis, TN, USA
NCT02742727	CD7	Lymphoma and leukaemia	NK-92	I/II	Unknown	Suzhou, Jiangsu, China
NCT02944162	CD33	Acute myeloid leukaemia	NK-92	I/II	Unknown	Suzhou, Jiangsu, China
NCT02839954	MUC1	Solid tumours	Not specified	I/II	Unknown	Suzhou, Jiangsu, China
NCT03383978	HER2	Glioblastoma	NK-92	I	Recruiting	Frankfurt, Germany
NCT03415100	NKG2D ligands	Solid tumours	Autologous or allogeneic NK cells	I	Recruiting	Guangzhou, Guangdong, China
NCT03656705	–	Non-small cell lung cancer	Chimeric costimulatory converting receptor-modified NK-92 cells	I	Recruiting	Xinxiang, Henan, China

CAR, chimeric antigen receptor; NK, natural killer; MUC1, Mucin-1; HER2, human epidermal growth factor receptor 2; NKG2D, NK group 2, member D; ALL, acute lymphoid leukaemia.

cell origin and acute lymphoid leukaemia (ALL). This includes human NK cells of different origins, such as the NK cell line NK-92, primary cord blood (CB)-derived as well as peripheral blood (PB) NK cells, which have recently entered clinical implementation testing the effectiveness in different phase I/II studies (Table 1) [4, 8–10]. Other than primary NK cells collected from CB or PB, the transformed cell line NK-92 originated from undifferentiated NK-cell precursors [11–13]. The malignant origin of NK-92 constituted an obstacle in the development of NK-92-based cell therapeutics which was conquered by irradiation [14, reviewed in 6]. The infusion of irradiated unmodified NK-92 cells was shown to be safe in patients suffering from "advanced, treatment-resistant malignancies" [15]. Furthermore, several groups impressively showed in vitro and in vivo that the cytotoxic activity of CAR-engineered NK-92 cells was preserved despite a proliferation-limiting irradiation [reviewed in 6]. Other than NK-92 cells, which can be expanded in cell culture, limiting amounts of primary NK cells challenged the development of NK cell-based therapeutic approaches. To solve this problem, a dose escalation phase I/II study with umbilical CB-derived CAR-engineered NK cells was authorised for patients with relapsed and refractory B-lymphoid malignancies (NCT03056339; Table 1) [4, 9]. In addition, the first clinical CAR-NK cell trials targeting antigens such as human epidermal growth factor receptor 2 (HER2), CD33, Mucin-1 (MUC1), CD7, and NK group 2, member D (NKG2D) ligands have been initiated (Table 1). In this respect, we discuss both the advantages and probable disadvantages of primary human CAR-NK cells and CAR-modified NK-92 as a meaningful alternative or complementary strategy for an immunotherapeutic approach towards CAR-modified T cells.

CAR-Modified NK Cells against Leukaemia and Lymphoma

In preclinical and clinical trials, CAR-engineered NK cells targeting several different antigens, such as CD19, CD20, CD33, CD138, CD319 (also known as signalling lymphocytic activation molecule family member 7, SLAMF7), CD3, CD5, and CD123, were investigated for their anti-tumour activity in vitro and in vivo [reviewed in 16]. Due to the tremendous therapeutic efficiency of CD19-directed CAR-modified T cells and their clinical use, data regarding the side effects and toxicity of CAR-T

cell therapy are routinely obtained. Since T cell infusion as a therapeutic approach can lead to a broad variety of severe side effects (cytokine release syndrome, febrile neutropenia, hypotension, decreased blood cell counts, neurological events, infections, tumour lysis syndrome, etc. [17]), other immune cell populations such as NK cells were discussed for CD19-directed CAR therapy. In 2004, Tonn and colleagues [18] presented data of a genetically modified NK cell line, NK-92, which harboured a first-generation CAR directed against CD19. Furthermore, in 2016, the group published in vitro data showing increased specific cytotoxic activity of CD19-directed CAR-NK-92 cells compared to unmodified NK-92 against cells expressing the target antigen [19]. The necessity for NK cell stimulation and activation by cytokines to improve their proliferation and persistence led to the development of optimised CAR constructs. CB-derived NK cells transduced with a construct harbouring coding sequences for a CD19-directed scFv, interleukin (IL)-15 (to enhance the cells' activity), and a suicide gene (inducible caspase-9; iC9) not only revealed anti-tumour activity but also showed extended persistence of the CAR-modified NK cells resulting in prolonged survival of mice using an aggressive Raji lymphoma mouse model [7]. To control their cytotoxic activity, iC9-bearing CAR-modified CB-derived NK cells could be efficiently eliminated upon rimiducid addition in vitro and in vivo [7].

Several different NK cell-based therapeutics targeting CD19 are in clinical trials by now, one of which is the aforementioned CB-NK cell therapeutic (iC9/CAR.19/IL-15-transduced CB-NK cells) adopted for use in humans and designed at the MD Anderson Cancer Center of the University of Texas (NCT03056339; Table 1). Additionally, a phase I/II clinical trial of a third-generation CAR (CD19-4-1BB-CD28-ζ)-transduced NK-92 cell line was enrolled in 2016 and is still ongoing (NCT02892695; https://clinicaltrials.gov; Table 1). In the manufacturing of CAR-T cells, lentiviral transduction is the method of choice since persistence is prolonged when memory T cell subsets, i.e., resting cells, are transduced [20, clinical use of lentiviral vectors summarised in 21]. In contrast, CAR-NK cell transduction rates are elevated due to optimised cultivation procedures opening an opportunity to use retroviral systems [22], whose good manufacturing practice (GMP)-compliant production is easier and cheaper [23]. Alpha-retroviral vectors hold attractive features for the manufacture of gene therapeutics regarding their integration pattern into the host genome. In 2004, a study was published where DNA integration of different retroviruses was compared, revealing that the avian sarcoma-leukosis virus (alpha-retrovirus) showed "the weakest bias toward integration in active genes and no favouring of integration near transcription start sites" [24]. A study from 2016 validated retroviral vector sys-

tems for the manufacture and modification of NK cells in order to obtain efficient transduction rates [22]. When alpha-retro-, gamma-retro-, and lentiviral vectors were compared, the designed alpha-retroviral vectors showed significantly increased transduction rates of over 90% when an NK cell line was used and up to 60% when primary NK cells were genetically modified [22]. Moreover, the expression profile and degranulation activity of alpha-retroviral-modified NK cells were unaffected, confirming the eligibility of this transduction tool in the manufacture of CAR-modified NK cells [22]. The authors argued that many factors influence transduction efficacy (e.g., transduction method and codon optimisation of the construct) and speculated that primary NK cells may exert specific responses against viral particles of certain pseudotypes [22]. Moreover, an advanced alpha-retroviral system was used in these works, which might tip the balance in favour of this approach [22, 25, 26]. However, it would be too early to speculate that different retroviral properties and elicited anti-viral responses correlate with the retrovirus-specific differences in transduction efficiencies, with RD114/TR pseudotyped alpha-retroviral particles, and with improved gene transfer in NK cells.

Besides the promising preclinical data obtained from CD19-directed CAR-NK cells, the cytotoxic effect of CD20-targeting CAR-NK-92 cells against primary chronic lymphocytic leukemia (CLL) cells was evaluated in vitro and in a Daudi mouse model [27]. When compared to rituximab- and ofatumumab-induced ADCC, anti-CD20 CAR-NK-92 cells showed increased anti-tumour activity against primary CLL cells in vitro [27]. Although CD20-directed NK cells – both stably transduced NK-92 and mRNA-transfected PB-NK cells – showed in vivo anti-tumour activity in localised tumour xenograft mouse models [27, 28], their transfer into clinical trials has not been successful yet. More recently, combination therapy of anti-CD20-CAR mRNA electroporated PB-NK cells and romidepsin was shown to induce synergistic anti-tumour effects both in vitro and in vivo using a localised Raji lymphoma model [28].

For the treatment of leukaemia and lymphoma, CD7 is under investigation regarding its potential for CAR-based therapeutic approaches (Table 1). Due to a stable expression of the antigen on leukaemic T cells [29], CAR-T cells were designed against CD7 for the treatment of T-ALL and T-cell lymphomas [30]. However, since CD7 is expressed by all T and NK cells including CAR-modified T cells, T cell expansion was precluded during manufacturing of CD7-directed CAR-T cells due to "substantial fratricide of T cells" [31]. To overcome the limitations of this therapeutic approach, several groups of scientists tested different methodical concepts. The first strategy was using clustered regularly interspaced short palin-

dromic repeats and CRISPR-associated (CRISPR/Cas) 9 in order to knock out CD7 in those T cells which were used for subsequent CAR modification. The absence of CD7 ensured fratricide prevention and CD7-directed CAR-T cells showed cytotoxic activity against target antigen-expressing cells [30, 31]. In another approach, a protein expression blocker was used to downregulate CD7 expression by transducing CD7-specific endoplasmic reticulum/Golgi retention peptide-coding plasmids in T cells prior to transient CD7-directed CAR-modification [32]. The phenotype and cytokine profile of CD7-downregulated T cells were comparable to control-transduced T cells. Additionally, CAR-carrying and CD7-downregulated T cells showed potent cytotoxic activity against leukaemic cells in vitro and in xenograft T-ALL mouse models [32]. Eventually, a clinical trial was enrolled in 2016 investigating the safety and toxicity of CD7-directed CAR-NK cells (Table 1). Only the outcome of the clinical trial will give hints as to whether an NK cell-based CD7-directed cellular therapeutic can overcome the aforementioned hindrances.

Another potential target for T-cell lymphoma and T-ALL therapy is CD5. Approaches directed against this antigen face the same difficulties as CD7-directed approaches since CD5 is also a pan T-cell marker. Two studies showed that NK-92 expressing CD5-directed CARs were efficient in vitro against CD5-positive cell lines and primary tumour cells, and developed anti-tumour activity in vivo [33, 34]. Additionally, the use of anti-CD3 CAR-equipped NK-92 against primary peripheral T-cell lymphoma cells and T-cell leukaemia cell lines was investigated showing cytotoxic activity in vitro and against Jurkat cells in a xenogeneic mouse model [35].

Therapeutic NK Cells in the Treatment of AML

In 2017, gemtuzumab ozogamicin was re-approved by the FDA for the treatment of acute myeloid leukaemia [36]. Studies showing that CD33-binding antibodies were efficiently internalised upon antigen binding [37, 38] paved the way for therapeutic targeting of CD33. The humanised CD33-directed IgG4 antibody gemtuzumab ozogamicin is conjugated to a bacterial toxin, which binds to DNA, thereby mediating DNA damage in targeted cells [39]. CD33 is expressed on both healthy and malignant myeloid cells [39 and references therein]. For the targeting of CD33, several different therapeutic approaches are under current investigation, including antibody-drug conjugates and bispecific antibodies [reviewed in 40]. Additionally, CD33-directed CAR-modified T cells carrying an scFv derived from gemtuzumab ozogamicin were shown to mediate potent in vitro and in vivo anti-tumour activity [41]. Since these CAR-T

cells were produced by electroporation of in vitro transcribed mRNA, cytotoxic anti-tumour effects were induced only transiently to "[…] minimise the risk of long-term hematopoietic toxicity" [41]. In another study, the persistence and safety of CD33-directed CAR-T cells were compared between constructs harbouring different costimulatory domains within the CAR [42]. Regarding their efficacy, the examined anti-CD33-CAR-T cells were shown to induce similar anti-tumour activity in vitro as well as in a xenograft tumour mouse model [42]. Due to their shorter life span in comparison to T cells, CD33-directed CAR-modified NK-92 cells (second-generation CAR, CD28-CD3ζ) were designed and exerted potent cytotoxic activity against target cells in chromium-51-release assays [43]. Efficacy and safety data of allogeneic or haploidentical NK cell transplantations including NK-92 have been obtained from several clinical trials [reviewed in 44, 45]. However, a clinical phase I/II trial was enrolled in 2016 testing the safety and toxicity of CD33-directed NK-92 cell infusions (CD33-CD3ζ-CD28-4-1BB) in patients whose leukaemic disease relapsed (after stem cell transplantation and chemotherapy) or appeared refractory to treatment (NCT02944162; https://clinicaltrials.gov; Table 1). The outcome of this and other clinical trials of CAR-modified NK cells can shed light on toxicity, safety, and efficacy aspects of NK cell-based therapeutics.

Besides the therapeutic targeting of CD33, CD123 was investigated regarding its feasibility as an immuno-oncological target in the treatment of AML. Other than CD33, which is expressed on malignant cells and healthy haematopoietic stem cells [46], CD123 expression is increased in malignant haematological cells but rather low on haematopoietic stem cells [47]. Despite the favourable expression profile of CD123, T cells expressing CD123-guided CARs were shown to induce severe haematological off-target toxicities in AML mouse models [48, 49]. For further studies, strategies to either eradicate CAR-modified T cells from the periphery or to transiently express CARs are urgently needed. In the case of CD123, a CAR-NK cell-based concept would be a more practical approach. The GMP-compliant clinical-grade manufacturing of expanded and activated NK cells expressing CD123-directed CARs was proven feasible and the produced CAR-modified effector cells showed in vitro activity against both an AML cell line and patient samples [50].

Other CARs targeting AML, yet focussing on CAR-T therapy, are directed against folate receptor-β (FRβ), C-type lectin-like molecule-1 (CLL1, also known as CLEC12A), fms-related tyrosine kinase 3 (FLT3, CD135), B7H6, NKG2D, and Lewis Y antigen (LeY), and some have already been transferred to clinical trials [reviewed in 44].

CAR-NK Cells in Multiple Myeloma

As the progression of multiple myeloma (MM) is connected to NK cell impairment by tumour escape mechanisms [51–54] and the biomolecular action of anti-myeloma agents involves NK-cell responses [also reviewed in 55], enhancing NK cell-mediated anti-tumour activity is an apparent strategy in cellular MM therapy. Although some data indicate that many primary MM tumours are insensitive to NK-92-mediated killing [54], the NK-92 cell line was investigated in a phase I clinical trial showing "some evidence of efficacy" [56]. Upgrading this approach, two CARs against CD319 (also known as CS1 or SLAMF7) and CD138 were inserted into NK-92 and NK-92MI (derived from NK-92, IL-2-independent), respectively, and were successfully tested in vitro and in vivo against cell lines and primary MM cancer cells [57, 58]. CD138 is the main diagnostic MM marker [reviewed in 59]. The safety of SLAMF7 as an MM target is well supported since it is targeted by a therapeutic antibody (elotuzumab, previously known as HuLuc63), which mediates NK-cell-dependent ADCC against cells expressing the target antigen [60, 61], as well as by CAR-T and CAR-NK cells [57, 62]. Besides CD138 and SLAMF7, preclinical studies with CAR-T cells for the treatment of MM concentrated on antigens such as CD38, CD44v6, B-cell maturation antigen (BCMA, CD269), and κ-light chains [reviewed in 55], thereby holding potential for further CAR-NK cell candidates. Until now, CAR-equipped NK cells targeting MM have not entered clinical trials.

Recently, SLAMF7-directed CAR-NK cells were combined with the therapeutic CD38-directed antibody daratumumab for the treatment of relapsed MM [63]. The authors hypothesised synergistic effects on tumour eradication only in the presence of both SLAMF7-directed CD38-negative CAR-NK cells and daratumumab, indicating the combination of conventional with novel treatment options as an appealing approach [63].

Effectiveness of CAR-Engineered Primary NK Cells against Solid Tumours

Primary human CAR-NK cells have a high potential for use as both autologous or allogeneic cytotoxic CAR effector cells in immunotherapeutic approaches [8]. However, compared to vulnerable/sensitive lymphomas with stable overexpression of B-cell antigens, most antigens used for solid tumour therapy show alternating surface expression levels with heterogeneity even within the tumour or between primary and metastatic lesions [64, reviewed in 65–67]. In preclinical studies, it could be shown that disialoganglioside- (GD$_2$; expressed on tumours of neuroectodermal origin [68]) positive Ewing

sarcoma (ES) cells could be used for the engineering of a second- and third-generation anti-GD$_2$-CAR. This led to an improvement of the NK cell-driven cytotoxicity against several cancer cell lines in vitro and resulted in effective counteraction against ES tumour resistance [69, 70]. However, adoptive transfer of anti-GD$_2$-CAR-NK cells in GD$_2$-expressing ES xenograft models could not confirm previous in vitro experiments [70]. Furthermore, the diminished anti-tumour response of those CAR-modified NK cells was connected to increased expression levels of immunosuppressive human leukocyte antigen G (HLA-G) on ES cells, thereby mediating tumour immune escape [71]. The authors concluded that HLA-G represents a potential checkpoint, which would have to be inhibited before initiating therapies based on CAR-NK cells [70].

Another innovative approach to restore specific anti-tumour reactivity is the genetic manipulation of primary NK cells by generation of DNAX-activation protein (DAP)-12 based CARs, also referred to as anti-prostate stem cell antigen (PSCA)-DAP12, for retargeting primary NK cells towards PSCA-positive bladder tumours [72]. PSCA shows low-to-moderate expression levels on normal prostate tissue, but is highly expressed on primary prostate tumours and prostate cancer metastases [73, 74]. Unlike several other CAR-specialised research groups working with common activation domains (e.g., CD3ζ-signalling domain), this preclinical study was focused on a novel strategy for the stimulation of signal transduction pathways to trigger NK cell-mediated cytotoxicity by using DAP12 as a cell type-specific intracellular signalling domain [72, 75]. The key signal transduction receptor DAP12 is usually found in NK cells and induces surface expression of the associated activating receptor NKG2C (NK group 2, member C) [76] and the natural cytotoxicity receptor NKp44 [77, 78]. PSCA-DAP12-engineered NK cells showed retargeted killing activities after antigen binding via intracellular ζ-chain-associated protein kinase 70 phosphorylation and interferon-γ (IFN-γ) secretion against PSCA-positive tumour cells, which was markedly improved by killer cell immunoglobulin-like receptor (KIR)-HLA mismatch [72]. These data underline the hypothesis that NK cell-specific activation domains (e.g., DAP12) are essential for a powerful, redirected CAR-NK cell cytotoxicity and, thus, for an improved immunotherapy against solid tumours. Besides NKG2C activation, which is responsible for immunosurveillance of cytomegalovirus infections in humans and mice [79–81], it is also advisable to recruit additional activating receptors such as NKG2D. NKG2D recognises multiple ligands, such as major histocompatibility complex class I chain-related peptides A/B (MICA, MICB), and several UL-16-binding proteins (ULBP), which are preferentially expressed after cellular stress on malignant and trans-

formed tumour cells [82, 83], thus resulting in an NK-G2D-mediated anti-tumour activity [84–87]. The NK-G2D target-associated antigen (TAA)-crosslink leads to activating signals via phosphorylation of NK-typical DAP10, inducing NK cell-dependent cytotoxicity against tumour cells [88, 89]. As NKG2D – already expressed by NK cells – recognises tumour antigens of the NKG2D ligand families induced after DNA damage, for example, it is attractive to fuse this natural cancer-targeting molecule to signal enhancers. Those activation cascades have been used successfully in a preclinical study to generate a retroviral construct encoding a chimeric receptor containing NKG2D, DAP10, and CD3ζ [90]. Activated NK cells were transduced with the NKG2D-DAP10-CD3ζ expression vector and tested for their anti-tumour activity in vitro and in vivo [90]. The cells showed increased NK-G2D surface expression, enhanced tumour necrosis factor-α and IFN-γ release, and improved, redirected anti-tumour response against several tumour entities (prostate, colon, gastric, lung squamous cell, hepatocellular and breast carcinomas), and, in particular, to osteosarcoma, rhabdomyosarcoma, and neuroblastoma cells without mediating adverse effects against non-malignant cells, such as mesenchymal cells [90]. Since NKG2D-DAP10-CD3ζ-modified NK cells showed improved anti-tumour activity and off-target safety in vitro, they may be an interesting format for further approaches in the treatment of cancer and as a therapeutic option for infectious diseases [90]. Regarding their nomenclature, NKG2D-CARs are not "classical" CARs since NKG2D is not an scFv, but are referred to as "CARs" in the literature [90].

Recently, a novel, single-centre pilot study by mRNA-electroporated CAR-NK cells with transiently increased specificity and cytotoxicity against NKG2D-ligand was initiated in cancer patients diagnosed with metastatic NKG2D ligand-positive solid tumours. The goal is to evaluate and assess the safety and feasibility aspects of those autologous or allogeneic CAR-transfected NK cells (NCT03415100; https://clinicaltrials.gov; Table 1).

Compared to monotherapies with CAR-NK cells, combinations of second-generation designed epidermal growth factor receptor-CAR-NK cells and oncolytic *Herpes simplex* virus led to higher killing rates against breast cancer cell lines and enhanced inhibition of the tumour progression in a breast cancer mouse model [91]. This seems to be an extensible application for CAR-NK cells in combination with oncolytic viruses to effectively eliminate resistant solid tumours [91]. Moreover, it is urgent to include predominantly overexpressed TAAs from resistant cancer identities for the generation of target-oriented CAR constructs to induce redirected NK cell responses. CAR-driven NK cell cytotoxicity depends on stable and moderate surface expression levels of the retargeted antigen. If the antigen expression is too low, tumour cells can escape the monitoring of CAR-engineered effector cells. However, the enhanced optimisation of CAR-TAA-mediated molecule affinity to recognise and cross-link very low antigen surface levels on target cells would lead to undesirable side effects against healthy tissue and non-transformed cells, resulting in on-target/off-tumour interactions. Therefore, in case of resistant tumour cells, a solution to known limitations could be the development of dual-specific CAR-NK cells for recognition and cross-linking of both corresponding TAAs in order to minimise the observed adverse side effects against normal tissue and healthy cells.

CAR-Expressing NK-92 Cells for Retargeting of Solid Tumours

In the past and present, it has often been shown that the NK-92 cell line can be effectively transduced with several different CARs against several malignancies for testing in preclinical approaches and currently in first clinical studies. CAR-NK-92 cells were quite successful in overcoming the tumour barrier and retargeted anti-tumour cytotoxicity against several resistant solid tumours, including epithelial cancers by targeting of human epidermal growth factor receptors (HER1 [ErbB1], HER2 [ErbB2]), neuroectodermal tumours by GD$_2$, brain tumours by HER1 and HER2, and ovarian carcinomas also by HER2 [4, 6, 92, 93]. However, there are some limitations to using this cell line. Since the transformed NK-92 cell line originated from undifferentiated NK-cell precursors [11–13], these NK cells lack ADCC-inducing CD16 receptors, which is also the case in other NK cell lines [94]. Consequently, these effector cells are unable to recognise tumour-targeted antigens by ADCC mechanisms. To overcome these cytotoxic limitations, NK-92 cells were genetically manipulated to express the high-affinity V158 variant of the Fc-gamma receptor (FcγRIIIa/CD16a, termed haNK™) and to produce endogenous, intracellularly retained IL-2 [95, 96]. In an ongoing phase I trial it will be evaluated whether infused haNK™ cells are safe and potent in the treatment of patients with histologically confirmed, non-resectable, and locally advanced or metastatic solid tumours (NCT03027128; https://clinicaltrials.gov; Table 1).

Another unfavourable aspect is the absence of some KIRs, with the exception of KIR2DL4 (CD158d) on the surface of NK-92, which may contribute to a possible stimulation of graft-versus-host disease [12, 97–99]. Thus, it should be noted that activated CAR-modified NK-92 cells must be irradiated with at least 10 Gy before infusion in tumour patients, resulting in a lower cell persistence and a loss of effector-mediated anti-tumour functions [99]. Despite these disadvantages, preclinical results were described for CAR-expressing NK-92 cells
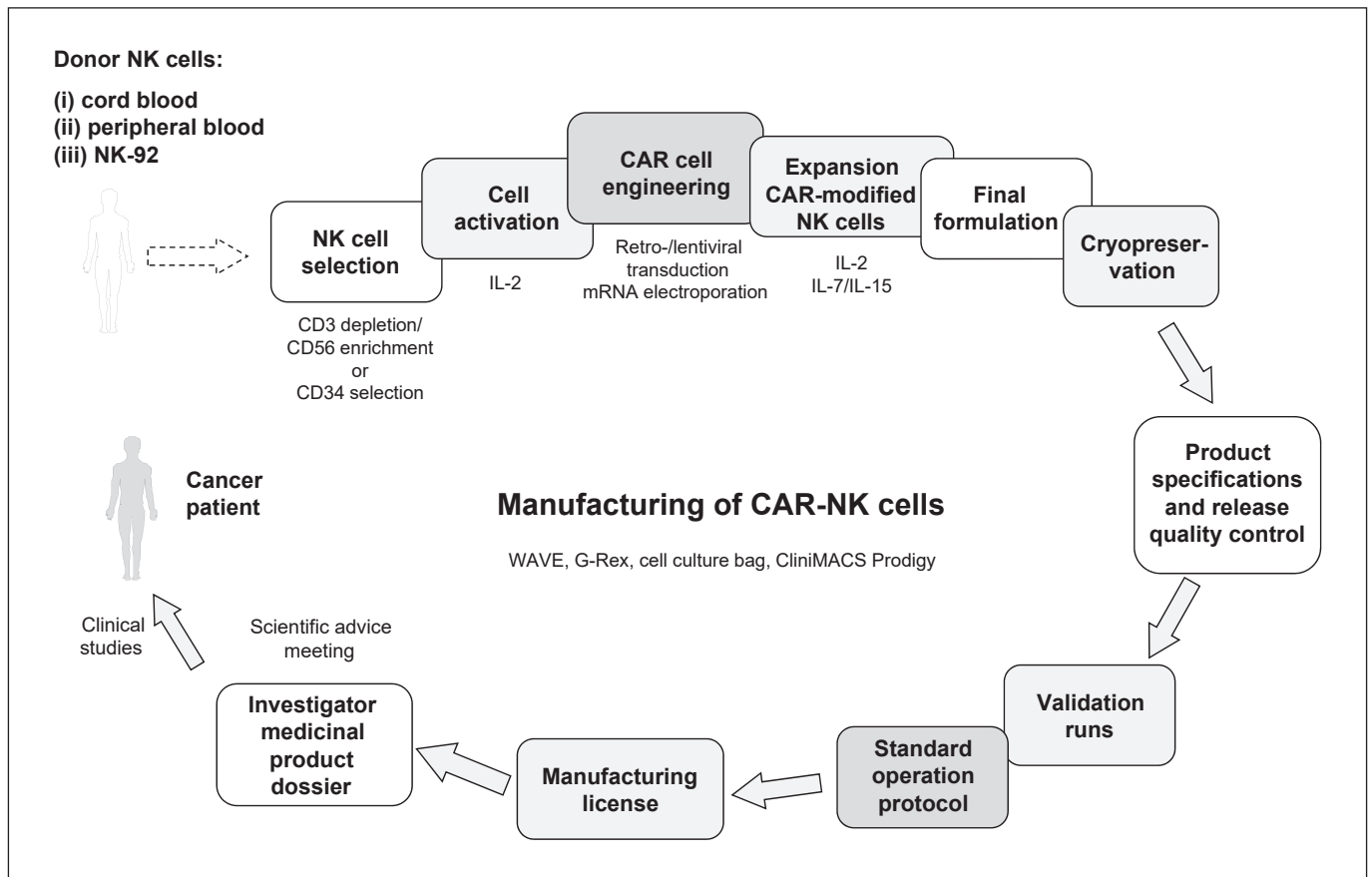
Fig. 1. GMP-compliant manufacturing of CAR-T cells.

targeting a wide range of tumour antigens [100, 101]. To date, only a few clinical trials using CAR-modified NK cells against haematological malignancies and especially against solid tumours have been initiated (Table 1). Recently, a phase I/II trial aimed to investigate the safety and efficacy of CAR-NK cells in patients with overexpressed MUC1-positive relapsed or refractory solid tumours, especially carcinomas (hepatocellular/pancreatic/breast/colorectal/gastric), non-small cell lung cancer, and glioblastoma (NCT02839954; https://clinicaltrials.gov; Table 1) [reviewed in 92].

Conclusion and Outlook

Both CB- and PB-derived primary human CAR-NK cells as well as CAR-NK-92 cells are complex medicinal products combining important features: cell products that are genetically modified and applicable as cellular immunotherapy. The entire manufacturing process following GMP requires between 10 days and several weeks using bags or more harmonised automation platforms like the CliniMACS Prodigy® (Miltenyi Biotec GmbH). These different strategies allow NK cell activation, trans-

duction, amplification, and final harvesting of CAR-NK cells with high transduction frequencies and mostly efficient cell numbers (Fig. 1). In contrast to CAR-T cells, CAR-NK cells have the advantage of "off-the-shelf" manufacturing, but still face several challenges. This includes the improvement in cell numbers, making the intracellular signalling more specific for NK cells, as reviewed by Oberschmidt et al. [9], and finally, finding the best source of NK cells. Also, the question of whether long-living immature or short-living mature CAR-NK cells are more suitable as therapeutic products has not been answered yet.

Other concepts are aiming to combine the advantages of T and NK cells, leading to the development of the so-called T-cell receptor (TCR)-CARs, which, like NKG2D-CARs, do not contain scFv antibody domains [102]. The stable expression of a functional peptide-specific TCR-CAR in NK-92 cells was proven feasible [102]. Moreover, TCR-CAR NK-92 were highly cytotoxic against formerly almost NK-insensitive cancer cell lines, whereas the recognition of unloaded major histocompatibility complex resulted in killing only at high effector:target ratios [102]. Notably, TCR-CAR NK-92 cells, like T cells, redirected other killer cells [102].

Disclosure Statement

The authors declare no conflicts of interest.

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