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The making and function of CAR cells

Maja Zabel, **Peter A. Tauber**, **Winfried F. Pickl***
Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

Abstract

Genetically engineered T cells expressing chimeric antigen receptors (CAR) present a new treatment option for patients with cancer. Recent clinical trials of B cell leukemia have demonstrated a response rate of up to 90%. However, CAR cell therapy is frequently accompanied by severe side effects such as cytokine release syndrome and the development of target cell resistance. Consequently, further optimization of CARs to obtain greater long-term efficacy and increased safety is urgently needed. Here we high-light the various efforts of adjusting the intracellular signaling domains of CARs to these major requirements to eventually obtain high-level target cell cytotoxicity paralleled by the establishment of longevity of the CAR expressing cell types to guarantee for extended tumor surveillance over prolonged periods of time. We are convinced that it will be crucial to identify the molecular pathways and signaling requirements utilized by such 'efficient CARs' in order to provide a rational basis for their further hypothesis-based improvement. Furthermore, we here discuss timely attempts of how to: i) control 'on-tumor off-target' effects; ii) introduce Signal 3 (cytokine responsiveness of CAR cells) as an important building-block into the CAR concept; iii) most efficiently eliminate CAR cells once full remission has been obtained. We also argue that universal systems for the variable and pharmacokinetically-controlled attachment of extracellular ligand recognition domains of choice along with the establishment of 'off-the-shelf' cell preparations with suitability for all patients in need of a highly-potent cellular therapy may become future mainstays of CAR cell therapy. Such therapies would have the attraction to work independent of the patients' histo-compatibility make-up and the availability of functionally intact patient's cells. Finally, we summarize the evidence that CAR cells may obtain a prominent place in the treatment of non-malignant and auto-reactive T and B lymphocyte expansions in the near future, *e.g.*, for the alleviation of autoimmune diseases and allergies. After the introduction of red blood cell transfusions, which were made possible by the landmark discoveries of the ABO blood groups by *Karl Landsteiner*, and the establishment of bone marrow transplantation by *E. Donnall Thomas* to exchange the entire hematopoietic system of a patient suffering from leukemia, the introduction of patient-tailored cytotoxic cellular populations to eradicate malignant cell populations *in vivo* pioneered by *Carl H. June*, represents the third major and broadly applicable milestone in the development of human cellular therapies within the rapidly developing field of applied biomedical research of the last one hundred years.

*Corresponding author at: Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Lazarettgasse 19, 1090 Vienna, Austria. winfried.pickl@meduniwien.ac.at (W.F. Pickl).

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1 Introduction

The basic concept of chimeric antigen receptor (CAR) therapy aims at redirecting cytotoxic lymphocytes against antigens expressed on tumor cells, eventually leading to tumor elimination. To provide immune cells with the ability to target new antigens, the cells are engineered to express CARs that basically link two functional domains: an extracellular antigen-recognition domain and an intracellular stimulatory domain. The extracellular antigen-recognition domain most commonly consists of a single-chain variable fragment (scFv) derived from a monoclonal antibody and, unlike *bona fide* T cell receptors (TCR), allows for antigen recognition in an MHC-unrestricted manner [1]. The extracellular domain is linked *via* a transmembrane region to the intracellular stimulatory domain, most frequently the CD3-ζ chain of the T cell receptor (TCR). To eliminate tumor cells, CARs become introduced into immune cells with cytolytic capacity which upon encounter should attack and lyse the tumor cells. Upon binding of the CAR to the (tumor-) antigen, the intracellular ITAM domains become phosphorylated and downstream signaling proteins activated. This activates the CAR cell, which leads to the release of cytokines, the proliferation of CAR-cells and antigen-specific lysis of tumor cells [2].

Various immune cell types have been evaluated as CAR recipients in the past. The first studies focused on $CD8^+$ T lymphocytes (CTLs) due to their inherently strong cytotoxic activity, but it was soon recognized that the sole use of CTLs does not guarantee durable results and that combination with T helper (Th) lymphocytes could be beneficial [3–5]. In addition to T lymphocytes, natural killer (NK) cells are also known to be highly cytotoxic. Indeed, several recent studies have proven their efficacy in tumor elimination and first clinical studies using NK cells as carriers of CARs are currently being performed [6]. Despite the extensive research on both T and NK cells, a more holistic approach combining different immune cell types might be appropriate. To the best of our knowledge, only one study combining T and NK cells was conducted so far, showing better tumor elimination when both cell types were used concomitantly [7].

In the recent past, CAR T cells have demonstrated great success in the treatment of B cell malignancies revealing up to 90% response rates in clinical trials [8]. Since 2017, two CAR cell therapies approved by the FDA – Kymirah (tisagenlecleucel) and Yescarta (axicabtagene ciloleucel) – are available for clinical use, both targeting the B cell differentiation antigen CD19 [9]. While clinical studies look very promising with remissions observed in more than 70% of patients treated, severe therapy associated side effects such as cytokine release syndrome (CRS) and neurologic events, including encephalopathy and aphasia, were frequently observed [10,11]. Moreover, the encouraging results observed during the treatment of hematological malignancies have yet to be translated to solid tumor treatment [12,13]. To overcome these obstacles, various groups have aimed at improving the design

and composition of chimeric receptors. In this review, we first aim to summarize the 'evolution' of the design of CAR signaling domains, followed by the description of attempts of how to optimize and potentially universalize ligand recognition domains. In the second part of this article we want to emphasize the importance of the recipient cell type used for CAR expression.

2 CAR signaling domains

Chimeric antigen receptors (CARs) proven to exert cellular activation and displaying target cell specificity constitute an extended list. Indeed, already in 1993 a paper published by *Eshhar* et al. demonstrated that the CAR T cells which were directed against 2,4,6-trinitrophenyl (TNP) exhibited potent TNP-specific cell lysis [14]. This 1st-generation CAR contained exclusively CD3-ζ as activation domain, comprising all three ITAMs, and although it generated a cytotoxic effect, it only had limited anti-tumor efficacy due to early T cell exhaustion (Fig. 1a and Table 1) [15]. To circumvent this problem, and to take advantage of other potent T cell activation domains, signaling domains 'borrowed' from different costimulatory molecules such as 4-1BB (CD137), CD28, DAP10, OX40 (CD134), or ICOS (CD278) were subsequently fused to the intracellular ζ-chain containing signaling domain in 2nd-generation CARs (Fig. 1b). Multiple studies have documented that these combinations of co-stimulatory and activation domains made 2nd-generation CARs superior to their predecessors, however, optimal combinations with regards to cellular yield and longevity still remain to be determined [15–17]. The most frequently used costimulatory domains are derived from CD28 and 4-1BB and have been compared in several studies, however, results remained controversial as of today. *Zhao* et al. showed that the introduction of the CD28 signaling domain resulted in faster tumor elimination when compared to 4-1BB CAR T cells, with lower numbers of CAR T cells needed for successful tumor elimination [16]. In contrast, *Long* et al. demonstrated that 4-1BB CAR T cells not only have superior activity but also persist for longer periods of time in the host [15]. The latter is an important factor in the treatment of cancer patients since longer T cell persistence relates to a superior clinical outcome [5,18,19].

Although 2nd-generation CARs have shown robust efficacy in the treatment of B-cell leukemias, results of CAR therapy of solid tumors are less promising [12,18]. The tumor microenvironment possesses many immuno-inhibitory elements such as inhibitory cytokines, inhibitory immune cells and a tendency to deplete nutrients essential for immune cell function [19]. To overcome these obstacles, further optimization of CARs was necessary, which led to the development of 3rd-generation CARs, which contain intracellular signaling domains of two different costimulatory molecules paired with constituents of the ζ-chain signaling domain (Fig. 1c). T cells equipped with 3rd generation CARs showed higher proliferation rates and longer *in vivo* persistence with only modest toxic side effects, however, anti-tumor effects were not greatly enhanced [20,21]. An elegant demonstration of how to identify new and powerful combinations of intracellular signaling domains has been conducted by *Duong* et al. who generated new and improved CAR constructs by randomly combining intracellular signaling domains from a collection of intracellular signaling domains. They discovered that the construct consisting of the DAP10, CD3-ζ and CD27 signaling domains exhibited the most potent cytolytic activity [22]. Whether such alignment

of intracellular domains truly exhibits higher antitumor efficacy in *in vivo* models remains yet to be determined.

It is well known that T cells require three signals to elicit a productive response: (i) antigen presented by the peptide/MHC complex (ii) a potent costimulus and (iii) growth and differentiation factors in the form of soluble cytokines [23]. CARs of the 2nd and 3rd generation provide the first two signals but fall short with regards to providing the third, cytokine dependent, signal. To actively deliver also the third signal to CAR expressing lymphocytes, *Kagoya* et al. have linked the cytoplasmic domain of the interleukin (IL)-2 receptor β-chain and a STAT3-binding tyrosine-X-X-glutamine (YXXQ) motif with the CD3-ζ and CD28 signaling domains (Fig. 1d). Notably, the inclusion of the third signaling module resulted in superior *in vivo* persistence of modified T cells, which delivered a more potent anti-tumor effect [24]. To the best of our knowledge, this is the only study so far combining all three signaling modules for T cell activation in a single CAR construct.

Precise comparison of CAR signaling with *bona fide* T cell receptor (TCR) signaling might reveal important underlying differences that could form the basis for further optimization steps during future CAR construct development. While low-level tonic TCR signaling enhances T cell responsiveness to foreign antigens, tonic CAR signaling may lead to cellular exhaustion. This could be avoided by using different intracellular signaling domains [25]. CAR signaling also needs to be adapted to the cell type used since it was shown that NK cells exhibit grater cytotoxicity when expressing a CAR with a DAP12 intracellular domain in comparison to T cells expressing CD3-ζ containing CAR [26]. A better understanding of the effects of each individual CAR domain on cell activation will help to further improve CAR cell function. While CD28 augments CAR T cell persistence, its lck-binding domain promotes production of IL-2, which stimulate functional programs in Tregs that in turn may dampen the initially induced anti-tumor effects. Indeed, the deletion of the lck-binding domain of CD28 resulted in enhanced proliferation and better *in vivo* tumor control [27].

Despite the great importance of the intracellular domains of CARs for the function and longevity of lymphocytes expressing them, the contributions of other elements to CAR function, such as the transmembrane and linker domains connecting the scFv ligand binding domains and the respective transmembrane regions, should not be underestimated. Since some antigens are localized closer to the plasma membrane of the tumor cell surface, a longer linker might be needed for their efficient recognition [28]. While a longer linker might enhance the chances for antigen recognition, it also increases the chances for generating an immunogenic molecule, which might become recognized by other immune cells, potentially leading to the deletion of the respective CAR T cells *in vivo* [29]. CARs are powerful receptors to target cell types of choice. Nevertheless, it is noteworthy that as of today no optimized design for the composition of the signaling modules has been agreed upon on. Although 1st generation CAR constructs, exclusively consisting of a ζ-chain intracellular domain, have ‘evolved’ into constructs consisting of various domains derived from different signaling molecules, such as the recently described CAR construct endowed for instance with a JAK-STAT signaling domain, only little knowledge exists when it comes to deciding which type of combination would be optimal for a given purpose. Studies exploring the T cell activating potential of different chimeric constructs may guide us

towards the creation of improved signaling modules. For example, simple clustering of chimeric constructs harboring the Syk kinase domain led to increased Ca^{2+} flux and cytolysis [30]. Interestingly, clustering of ZAP70 chimeric constructs failed to induce similar effector programs while co-clustering of ZAP70 with the Scr-kinases Lck or Fyn could overcome this impairment [30]. Thus, it may be reasonable to establish constructs containing signaling modules consisting of an active Src kinase or Syk domain together with the critical ζ-chain or ZAP70 phosphorylation sites. Such constructs could trans-phosphorylate each other upon antigen encounter and dimerization may enhance signal propagation and thus, more robust T cell effector responses. Moreover, signaling domains of other, non-cytotoxic immune cell types, *e.g.,* B cells, could be adopted to enhance T/NK cell function. Our increasing understanding of the physiologically triggered TCR and associated co-stimulatory and cytokine-mediated signaling pathways will improve and certainly also expand the scope of application of CAR technology.

3 Targeting

Effective CAR cell activation not only depends on the appropriate function of the intracellular activation domains but also on proper recognition of the targeted antigen. For CAR cell activation to efficiently occur, a sufficient quantity of the antigen needs to be present on target cells [25]. While the paucity of CAR-specific antigens might reduce the outcome of CAR treatment, ‘on-target, off-tumor’ effects, in which CAR cells recognize normal, non-malignant cells and tissues that may express the targeted antigen, might occur with higher frequency [31,32]. The selection of appropriately targeted antigens is therefore of crucial importance.

3.1 Extracellular ligand recognition

This chapter will summarize our current knowledge on ligand recognition domains that have been used to generate CARs. The following four classes will be discussed: (i) single-chain variable fragments (scFvs) derived from antibodies, (ii) fragments antigen-binding (Fab), (iii) natural ligands that engage their cognate receptor independently of MHC, and (iv) *bona fide* TCR ligands (Fig. 2).

3.1.1 Single chain variable antibody fragments—ScFvs consist of the antibody variable light-chain (VL) tethered to the variable heavy-chain (VH) of the same antibody by virtue of a peptide linker (Fig. 2a). They comprise the predominant type of extracellular ligand recognition domains (ELRD) used for the creation of CARs [33,34]. ScFv fused to T cell receptor subunits were first described by *Eshhar* et al. in 1993 and many different scFvs for CAR T-cell therapy have been developed since then [14]. While several CARs have been evaluated in preclinical studies, some have entered clinical trials such as CD20 (NCT03576807) and a few have already been FDA/EMA approved such as those targeting CD19 used in Kymriah and Yescarta [35,36]. Other targets of scFvs currently being investigated clinically or pre-clinically are mesothelin, an antigen expressed on many different malignant tumors such as ovarian and pancreatic cancers, the prostate specific membrane antigen (PSMA) as well as epidermal growth factor receptor (EGFR) vIII, representing a mutated form of EGFRIII present in glioblastoma which is commonly

expressed also on other tumors [37–39]. B cell maturation antigen (BCMA, CD269) represents another important differentiation molecule targeted by a CAR. BCMA is a cell surface receptor of the TNF-receptor superfamily 17 (TNFRSF17), which is highly expressed on malignant myeloma cells and specifically binds B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) [40–42]. Ligand-induced signaling *via* BCMA promotes the growth of myeloma cells [43]. An scFv-based CAR directed against BCMA (CART-BCMA) has been tested in a Phase I clinical trial with patients suffering from relapsed multiple myeloma recently where it showed a toxicity profile similar to that of CD19-specific CAR T cells with excellent clinical response rates ranging from 20 to 64% [44].

One interesting general question in that context is the selection of scFvs with optimal affinity for the target antigen. Does high affinity binding of the scFv necessarily translate into better CAR cell efficacy? For example, choosing an scFv with too high a affinity might result in profound T cell activation, however, at the expense of reduced mobility of the CAR T cell as it might get stuck to the cell expressing the target antigen and thus preventing serial killing [45,46]. On the other, too low affinity might increase the number of non-engaged CARs and thus lead to inappropriate cellular activation.

3.1.2 Fabs—Although scFvs are the most frequently used extracellular ligand recognition domains nowadays, the first chimeric receptors targeting antigen and inducing antigen-specific signaling were antibody variable regions fused to T cell receptor constant regions (Fig. 2b) as described by *Kuwana* et al. as early as in the year 1987 [47]. However, this concept has by far not been as intensively pursued as the more widely used scFvs. Nevertheless, an adaptation of this Fab concept has experienced a recent revival in a study describing the fusion of CD19-specific antibody Fab fragments to TCR γ and δ constant regions. This design allows for the assembly of so-called ‘antibody TCRs’ which efficiently associate with the CD3 signaling complex of molecules endowing them with several advantageous features over classical CARs, like reduced cytokine release associated with less T cell exhaustion [48]. Moreover, compared to the classical concept applying Fab fragments fused to αβTCR chains this new concept avoids mispairings with the endogenously expressed αβTCR chains because usually these two chain types do not mispair [49]. The refinement of TCR constant domain-based CARs might prove to be successful, since this CAR generation relies on the interplay of the individual components of the primary TCR signaling machinery, instead of a non-physiologic, chimeric intracellular domain, possibly permitting improvement of cellular activation.

3.1.3 Natural ligands, natural receptors and B cell receptor ligands—CAR cells expressing the cytokine IL-13 (Fig. 2c) and intended to target tumor cell-associated IL13 receptor(R)α2 have been designed and underwent clinical testing in patients with glioblastoma in which the IL-13Rα2 is overexpressed on the malignant but not in the normal brain tissue of the majority of patients [50]. An elegant example of natural receptors used for targeting virus infected cells with CARs is Ly49H, the activating natural killer cell receptor, which has been shown in one study in mice to protect from lethal CMV infection [51]. Notably, $CD8^+$ T cells expressing the chimeric antigen receptor comprised of Ly49H as the

ligand recognition domain and fused to the ζ-chain intracytoplasmic signaling domain effectively protected mice by clearing cells, which expressed viral antigen (Fig. 2c) [51].

In principle, the majority of CARs known as of today have been designed for the treatment of malignant diseases in which, ideally, the targeted antigen is densely expressed on the surface of malignant but not normal cells. However, recently the exploitation of CARs for the treatment of auto-immune diseases was increasingly moving into the scientific focus. Herein, the selective targeting of subsets of autoantigen-specific immune cells becomes the primary goal to alleviate or even cure disease. In initial studies targeting autoantibody-producing B cells, *Nguyen* et al. equipped CAR T cells with hen egg lysozyme linked to intracellular signaling domains (Fig. 2d). Such T cells showed cytolytic potential *in vitro* and *in vivo* against antigen-specific B cells even in the presence of secreted, CAR-specific antibodies. However, when transferred into mice, these CAR T cells were found to be immunogenic because they induced an antibody response against the extracellular ligand recognition domain, *i.e.,* HEL, showing that despite their ability to selectively kill antigen specific B cells, some B cells may escape elimination resulting in persistent antibody responses [52]. In another study relying on similar technology and conducted by *Ellebrecht* et al. the target antigen desmoglein-3 was used as ligand recognition domain of a CAR (Fig. 2d) to efficiently and specifically kill anti-desmoglein-3 antibody producing hybridoma cells *in vivo* [53]. Desmoglein-3 is one of the key target antigens in *pemphigus vulgaris,* an auto-immune disease characterized by the aberrant production of auto-antibodies against this important adhesion protein expressed by keratinocytes, who's functional blockage results in the loosening of the interconnections between the lower layers of keratinocytes within the dermis resulting in the formation of skin blisters and detachment of the epidermis [54]. A further elegant approach established along those lines was the use of blood clotting factor VIII CAR expressing cytotoxic T cells, to kill anti-blood clotting factor VIII producing hybridomas *in vitro* and *in vivo* recently [55]. Moreover, blood clotting factor VIII -/- mice treated with blood clotting factor VIII CAR expressing cytotoxic T cells in the same study were able to protect from a factor VIII immunization-related increase in anti-factor VIII antibodies *in vivo* [55]. Hemophilia patients requiring substitution with recombinant blood clotting factor VIII to avoid bleeding, frequently mount neutralizing antibody responses against factor VIII, which represents a severe impairment for long-term treatment. In a very recent approach, one group aimed at targeting IgE-producing B cells with a CAR consisting of the high affinity FcεRI on its extracellular ligand recognition domain [56]. These CARs were able to target IgE producing cells but not effector cells (basophils) binding free IgE *via* their FcεRs [56]. The major disadvantage of this approach is that high levels of IgE would inhibit such CAR T cells by interfering with their binding to IgE^+ B cells. However, mutated forms of the CARs' extracellular FcεRI domain were generated which are supposed to circumvent this problem of interception by free IgE due to reduced affinity [56]. The last examples underscore, how elegantly and precisely wellknown target antigens in auto-immune and auto-inflammatory diseases can be adopted to specifically target antigen-specific B cells/plasma cells, which may allow to ameliorate or even cure a variety of autoimmune/allergic diseases with almost no off-target effects in the future. Especially in the field of allergies this concept could be highly interesting, since many allergens are nowadays characterized on the molecular level and humanized mouse models are available [57–59].

One could imagine the use of “Allergen-CARs” in the future where the major allergen towards which the patient reacts could be used for the construction of a chimeric antigen receptor. This would allow for the selective elimination of allergen-specific B cells and specific Ig bearing mast cells and basophils thus also inhibiting the type I hypersensitivity reaction associated with allergen-specific IgE. However, the risk of such a therapy potentially leading to effector cell activation is hard to predict. Nevertheless, future developments in the field of CAR cell therapy could make the therapy safe even for this application. This would be especially interesting for food allergens which are supposed to be one of the most common causes of anaphylaxis [60].

3.1.4 Bona fide TCR ligands Bona fide TCR ligands—

One of the clear benefits of CAR cell therapy is its independence of MHC restriction. However, since auto-reactive T cells commonly recognize peptide antigens only when they are presented by the respective MHC, some groups have resorted to the application of peptide/MHC (pMHC) complexes as antigen-recognition domains of CARs (Fig. 2e). Such pMHC expressing CAR T cells eliminate auto-reactive T cells expressing pMHC-specific T cells which express pMHC-specific TCRs of different clonality [61]. This opens the possibility for the treatment of autoimmune diseases that are preferentially driven by auto-reactive T cells.

For instance, in type I diabetes, CTLs are implicated to be important for the development of the disease. To specifically target diabetogenic $CD8^+$ T cells, a CAR consisting of the insulin peptide InsB15-23 linked to β-2 microglobulin, the monomorphic binding partner of HLA Class I alpha chains, was fused to the intracellular CD3ζ signaling domain [62]. Notably, receptor modified T cells (RMTCs), which expressed this CAR, efficiently eliminated insulin-reactive $CD8^+$ T cells which resulted in a lower incidence and a delayed onset of diabetes in a preclinical model of diabetes [62].

In experimental autoimmune encephalomyelitis (EAE), which is used as a preclinical model for multiple sclerosis in humans (MS), $CD4^+$ lymphocytes play an important role for disease initiation and propagation. In fact, they represent the major cell type found in the inflammatory infiltrates in the brain [63]. Because these cells recognize their cognate peptide presented by MHC class II molecules, *Mekala* et al. have used a combination of myelin basic protein (MBP) and MHC class II molecules to build the extracellular antigen-recognition domain of that 1^{st}-generation CAR [64,65]. *In vivo* cytolysis of target cells by such RMTCs was found to be efficient, with CAR cells undergoing vigorous proliferation. Moreover, the prevention of EAE by such CARs was significant but more importantly it was also able to alleviate established disease, showing lower disease severity and increased survival of animals already suffering from EAE [64,65]. CAR systems relying on HLA molecules as extracellular ligand recognition domain could also be beneficial to remove unwanted clonal T cell populations as observed in allergic diseases and their potency could be easily evaluated in preclinical humanised allergy models [57,66].

3.2 Prevention of antigen escape

Despite the high efficacy of CAR treatment of B-cell malignancies, antigen escape remains an important cause of relapse. In CD19 CAR trials, relapse rates varied between 29% and

57%, with up to 75% of relapsed patients presenting with $CD19^-$ B cell blasts [31]. While such leukemia cells lack the initially targeted CAR antigen, they continue to express other B cell specific differentiation antigens. Therefore, initial targeting of multiple antigens by CAR cells might help to prevent the selection of antigen escape variants.

CAR T cells expressing two different receptors (Fig. 1e), each recognizing a B cell-specific antigen, *e.g.,* CD19 and CD20, have shown to efficiently prevent relapse due to antigen escape [22,67,68]. Along those lines, *Hegde* et al. demonstrated that lymphocytes concomitantly transduced with both CAR constructs showed greater efficacy than co-administration of two CAR T cell lines expressing the respective CARs on two separate cellular entities [68]. *Bielamowicz* et al. have developed this concept even further by transducing T cells with a tri-cistronic expression vector coding for three chimeric receptors recognizing three distinct tumor antigens (Fig. 1f) and treatment with such CAR cells was shown to be efficient in an *in vivo* mouse model of glioblastoma [69]. Increasing the number of targeted antigens may be useful for the treatment of tumors, which express various degrees of tumour antigens, however, this maneuver potentially also increases the frequencies of 'on-target, off-tumor' effects, which must be taken into consideration.

Instead of expressing two (or three) separate chimeric receptors, *Hegde* et al. have created 'tandem CARs' (tanCAR) that consecutively connect two antigen-binding domains – HER2-binding scFv and IL13R-α2 – in a single CAR (Fig. 1g). Such 'OR-gate' CARs are activated upon binding of either of the two antigens and it was shown that this prevents antigen escape and improves survival in animal tumor models [70]. A similar concept was put forward by *Zah* et al. targeting both CD19 and CD20 antigens. Besides their importance for the prevention of antigen escape, CARs with dual specificity have also demonstrated that the length of the linkers between two scFv domains and those linking VH and VL chains of an individual CAR greatly affect T cell activation, pointing to the importance of a thoughtful CAR design with well-specified linker lengths [71].

3.3 Prevention of 'on-target, off-tumor' activity

A clinical case of a patient, treated with an ErbB2-specific CAR therapy who experienced a detrimental cytokine storm five days after CAR T cell infusion, because of the preferential trafficking of the CAR T cells to the healthy lungs of the patient, possibly due to the low level expression of ErbB2 in the lungs under physiological conditions, clearly highlights the importance of the specificity of CAR treatment [32].

A possible strategy to increase target cell specificity would be to target two tumor antigens that are simultaneously expressed in tumor but not healthy tissues. *'AND-gate'* CARs are specific for two different antigens but unlike *'OR-gate'* CARs, that are activated in the presence of any of the two antigens, *'AND-gate'* CARs exhibit cytotoxic effects only in the concomitant presence of both antigens (preferably on the same cell). This technology is used by the *'synNotch'* receptor, which consists of an antigen receptor linked to the intracellular transcription factor TetR-VP64 or Gal4.VP64. Upon ligation of the synNotch receptor the transcription factor becomes cleaved from the receptor and subsequently binds to its respective tetracycline-controlled trans-activator (tTA), which in turn directs the expression of the CAR that recognizes a second tumor antigen (Fig. 1h). It was shown that such

receptors successfully eliminated GFP^+CD19^+ tumors while sparing those expressing only a single antigen. In theory, also '*AND-gate*' CARs could become primed at one site in the body, express the CAR gene and then get translocated to another site where they may cause undesired 'on-target, off-tumor' effects. However, this was not observed in animal models carrying two distantly-located tumors, one expressing both antigens and the other only expressing a single antigen. One of the mechanisms that prevented the translocation of the T cells is possibly brought about by the rapid downregulation of CAR expression upon cessation of the first synNotch-related stimulus [72].

Since for efficient tumor eradication, CAR constructs need to express both an activation domain (usually provided by CD3-ζ) and a costimulatory domain (most often provided by CD28 or 4-1BB), the separation of the two on distinct antigen recognition molecules may increase overall specificity. Indeed, *Kloss* et al. have shown in a prostate cancer specific model that the dissociation of the costimulatory CD28–4-1BB domain expressed along with an PSMA-scFv from a CD3-ζ signaling domain linked to the PSCA-scFv efficiently eradicated prostate tumors expressing both prostate-specific antigens but showed only limited activity against tumors expressing a single tumor antigen (Fig. 1i) [73]. Nevertheless, when selecting CD19 and PSMA as a model for two unrelated targeting antigens, CAR T cells still showed strong residual cytotoxic activity against cells exclusively expressing the CD19 antigen, pointing to the importance of appropriate antigen selection [73]. *Lanitis* et al. made similar observations with such CARs, showing preferential targeting of tissues expressing two tumor antigens, while lower numbers of CAR T cells accumulated in tumors expressing either one of the tumor antigens [74]. Whether diminished but not abolished activity against tissues expressing only one targeted tumor antigen would be sufficient to avoid serious side effects against normal tissues remains unanswered so far.

An alternative approach was followed by *Fedorov* and colleagues, who established inhibitory CARs (iCARs) (Fig. 1j). Inhibitory CARs accommodate inhibitory intracellular domains instead of activating ones and thus inhibit cellular activation upon recognition of the respective antigen. Co-expression of the activating CD19::CD28::ζ CARs with an inhibitory PSMA::CTLA-4 or PSMA::PD-1 CAR resulted in strong CAR T cell inhibition upon recognition of both antigens. However, such inhibition could be overruled in cases in which the primary tumor antigen was recognized exclusively [75]. While the co-introduction into CAR T cells of inhibitory receptors proved to be efficient in preventing 'on-target, off-tumor' effects, the identification of appropriate antigens for the activation of such inhibitory receptors might prove to be difficult due to the variability of antigens expressed on the many non-tumor tissues.

To circumvent the need for the selection of two different antigens, 'masked CARs' have been developed which take advantage of the presence of proteases in the tumor microenvironment for their unmasking (Fig. 1k). These CARs are based on pro-bodies, and consist of a scFv domain protected by a linker that can be cleaved-off by proteases which are typically secreted by the tumors of interest. *Han* et al. showed that the masking linkers were efficiently cleaved off once CAR T cells entered into the tumor and their resulting effects on tumor elimination *in vivo* were comparable to the respective conventional CAR constructs.

However, it was also observed that some tumors tended to secrete lower amounts of proteases resulting in incomplete activation of CAR T cells in such environments [12].

3.4 Elimination of CAR T cells

While the prevention of 'on-target, off-tumor' activity avoids undesired targeting effects, tumor targeting may still evoke severe side effects such as neurotoxicity and cytokine release syndrome (CRS). Although CRS can be effectively treated with the anti-IL-6 monoclonal antibody tocilizumab, additional immunosuppressive treatment with corticosteroids may be needed [76]. Of note, long-term persistence of CAR T cells may lead to the permanent elimination of cells carrying the targeted antigen which can have undesired side effects, such as those observed in patients undergoing CD19 CAR therapy and presenting with B-cell aplasia [76].

To alleviate such side effects, various groups have investigated the possibility to eliminate CAR cells by the activation of suicide genes once full remission has been achieved. Inducible caspase 9 (iCasp9) represents such a suicide gene that connects the intracellular domain of the pro-apoptotic protein caspase 9 to the extracellular FK506 binding protein (FKBP). Upon administration of the chemical inducer for dimerization (CID) iCasp9 dimerizes and leads to programmed cell death [77]. Several studies have demonstrated that this mechanism can be efficiently applied *in vivo* and several clinical studies introduced iCasp9 into their model as a safety switch (*e.g.,* NCT03016377, NCT03696784) [78–80].

Co-expression on CAR lymphocytes of neo-antigens that can be deliberately targeted with antibodies and allow for their elimination *via* antibody-dependent cellular cytotoxicity (ADCC) represent further possibilities for the targeted elimination of CAR cells. Accordingly, a truncated version of human epidermal growth factor receptor (huEGFRt) that can be targeted by the anti-EGFR monoclonal antibody cetuximab was shown to efficiently eliminate CAR T cells *in vivo*. Additionally, huEGFRt was shown to be useful for the purification of CAR T cells during the production process as well as for tracking purposes (*e.g.,* NCT03085173, NCT03618381) [81].

3.5 Universal CAR systems

Commonly, CAR T cells target their specific antigens with the help of pre-defined cell-surface expressed scFvs, which are linked to potent intracellular T cell signaling domains, endowing the transduced lymphocytes with a monospecific reaction potential. Thus, the creation of CAR systems that could switch their target antigen specificity would represent certain advantages over conventional CAR expression systems. For instance, UniCARs express an extracellular "anti-Tag" scFv that specifically binds to the tagging sequence, which had been attached to the respective targeting-antibodies and which become co-applied with the CAR T cells to opsonize the tumor cells of choice (Fig. 1l). The tagging sequence is specifically recognized by the UniCAR "anti-Tag" scFv and such recognition leads to specific lysis of target cells to which the tagged antibody has bound [82].

An even more straightforward system was proposed by *Urbanska* et al. who used a biotin-binding immune receptor (BBIR) containing a dimeric form of avidin that binds to biotinylated tumor targeting antibodies with high affinity (Fig. 1m) [83,84]. When such

CAR cells are incubated with two different antibodies this allows for the targeting of two different antigens, although sequential targeting is also possible by sequential administration of antibodies. Although biotin is physiologically present in all bodily fluids, even concentrations 20-times higher than the physiological levels were found to be unable to block the recognition of the biotinylated antibodies by the avidin expressing CARs [85].

The *Split Universal Programmable (SUPRA)* CAR system allows for even more sophisticated fine-tuning of the targeting and killing process. It consists of an RR zipCAR that contains a leucine zipper instead of an antigen-recognizing scFv and recognizes EE zipFv-tagged antibodies (Fig. 1n) [86]. Upon binding of RR zipCar to the EE zipFv tag the CAR cells become activated, which leads to the elimination of the target cells. Additionally, this system allows to fine-tune antibody affinity *via* the modification of the leucine zipper, which helps to regulate killing efficiency. However, the addition of the competitive RR zipFv leads to the binding of EE zipFv to RR zipFv which results in neutralization of the EE zipFv tag and thus works as an “OFF switch”. Since zipCARs can be linked to different intracellular signaling domains, distinct signaling pathways may be activated upon binding of receptors to different antigens, including the possibility of specific transduction of different cell types. Indeed, such systems offer a multitude of possibilities for variation and optimization, from “OFF switch” to affinity modulation and targeting of antigen combinations, however, its efficacy and applicability in the human system still needs to be thoroughly evaluated.

Another interesting concept was described by *Clémenceau* et al. in 2006 which made use of a fusion construct consisting of the FcγRIIIa extracellular domain and FcεRγ1 intracellular part and allowed T cells to kill an autologous B-lymphoblastoid cell line upon coating with anti-CD20 mAb [87]. The principal idea of this concept was to combine such transduced T cells with a monoclonal antibody for targeting malignant cells which had been opsonized with a monoclonal antibody. Although interesting, we believe this concept will face many disadvantages in the real-life clinical setting such as the potential binding of autoantibodies to the T cell expressed FcR eventually generating self-reactive T cells. Moreover, occupation of the T cell-expressed FcR by other Ig present in the patient serum might lower the efficiency of the binding of the specific antibody even when administered in excess.

With the discovery of the new disease-related antigens the applicability of CAR therapy is broadening and could be further extended and clinically implemented to non-neoplastic diseases such as autoimmune and infectious diseases, or allergies. To be able to target the pathognomonic cell types in such diseases the appropriate antigens need to be identified. That gave rise to various antigen-finding platforms, such as MCR-TCR chimeric receptors, that could help further broaden the applicability of CAR therapy [88]. Although the identification of novel antigens is important, the affinity to these antigens might also play an important part since a too high affinity is known to potentially lower the killing efficiency due to interference with serial killing [45,46]. Universal CAR systems that enable the modification of the CAR affinity could help determine optimal affinities in that respect.

Concepts such as the UniCAR systems promise certain fascinating advantages such as flexibility and adaptability. Moreover, since the activity of the CAR T cell is supposed to be

dependent on the presence of the respective tagged antibody/scFv from here on referred to as "module" also the pharmacological properties of the CAR T cell activity get linked to this module. This linking might also allow for a better control and monitoring of the therapy. We believe that such platforms offer certain highly advantageous features since they are more flexible as the target antigen can be switched easily. One major disadvantage of the UniCAR concept compared to the traditional CAR concept could be, that in the case of malignant diseases the UniCAR would lose its immune-surveillance function in the absence of regular application of the respective module. On the other hand, UniCAR cells can easily be switched off which, in the case of using CD19 as the targeting structure, would allow for reconstitution of B cells in the further presence of UniCAR cells. Nevertheless, similar effects could be achieved by applying the classical CAR concept together with a kill-switch, *e.g.,* dimerizable caspases. The implementation of such a safety switch which even allows dose dependent CAR T cells reduction has already been described in preclinical studies [80].

4 Enhancing the activity of CAR cells

Lately the focus of CAR cell improvement has expanded to the coexpression of cytokines and/or additional stimulatory receptors in CAR cells. In this chapter we focus first on the co-expression of stimulatory ligands or cytokines and their receptors. While the cell activity can be enhanced by direct stimulation (*e.g.*, with cytokines (Fig. 3b)), the interference with inhibitory signals may have a similar effect. Upon CAR ligation, such interference can be achieved by the expression of inhibitory antibodies or chimeric antigen receptors that convert an inhibitory into a stimulatory signal (Fig. 3d and e). In addition to the direct anti-tumor effect, such modulated CAR cells may have a beneficial impact also on the tumor microenvironment and other immune cells for instance by stimulating the innate immune cells and directing them towards cancer cells [89].

4.1 Co-expression of stimulatory ligands

Co-stimulatory proteins have proven to be effective for tumor elimination. One such protein is the CD40 ligand (CD154) which becomes expressed on activated T cells. When it binds to its receptor CD40, expressed on tumor cells, direct tumor cell apoptosis is induced, while engagement of the same receptor on dendritic cells increases the recruitment of T cells with anti-tumor activity [90]. Antibodies targeting the CD40L/CD40 axis have shown promising clinical results indicating that the co-expression of CD40L along with a chimeric TCR receptor on cytotoxic lymphocytes may lead to augmented anti-tumor activity [91]. Indeed, *Curran* et al. have shown enhanced cytotoxicity of CD19-CAR T cells against $CD40^+$ cancer cells leading to prolonged survival of tumor-bearing mice [92].

Another protein of interest is 4-1BB (CD137), a stimulatory receptor belonging to the TNF surface receptor family that is expressed on T cells and other immune cells. 4-1BB increases survival and induces cytolytic cell activity upon ligation with its ligand, *i.e.,* 4-1BBL that is expressed on antigen presenting cells (APCs) [93]. To benefit from 4-1BBL-driven signals, *Zhao* et al. have expressed full-length 4-1BBL on the surface of T cells together with a CD19-CD28::ζ CAR (Fig. 3a). This combination engaged both the CD28 and 4-1BB signaling pathways in CAR T cells and, in turn, reduced PD-1-, LAG-3- and TIM-3-driven

inhibitory signals. The *in vivo* application of these CAR T cells resulted in higher survival rates, stronger and more rapid T cell proliferation, longer T cell persistence and faster tumour eradication [16].

4.2 Secretion of cytokines

Cytokines are small proteins which are important for the differentiation and function of immune cells. Accordingly, many cytokines (*e.g.,* IL-2, -12, -18 and -21) have been tested in clinical trials for their potential to treat cancer [94]. However, systemic administration of *e.g.,* IL-12 for the treatment of multiple myeloma resulted in serious side effects [95]. To reduce the toxic side effects, intratumoral administration of IL-12 was tested in head and neck squamous cell carcinoma patients, which attracted and activated CTL and NK cells, however, still at the expense of serious toxicity [95,96].

To overcome that problem but still taking advantage of the cytokines' action, *Pegram* et al. have transduced T cells with a bicistronic vector coding for a CD19-CAR and IL-12, resulting in constitutive expression of IL-12 from CAR T cells (Fig. 3b). These CAR T cells were preferentially homing to tumor sites where they secreted IL-12 which contributed to the efficient eradication of the tumor [97]. CD19-IL-12 CAR T cells were also resistant to the inhibitory effects of the regulatory T (Treg) cells, which gets important when T cells move to the tumor and become exposed to its microenvironment since it is known that Treg cells within the tumor dampen the anti-tumor response [97,98]. Additionally, the T cells' persistence at the tumor site was prolonged along with clear signs for the activation of macrophages [99,100]. Nevertheless, even under these circumstances, IL-12 secretion levels needed to be tightly regulated since toxic effects of constitutively expressed IL-12 in CAR T cells were observed when T cells were administered in large numbers. However, exuberant toxicity could be avoided when IL-12 expression was induced exclusively upon antigen stimulation [101]. In addition to the aforementioned benefits of IL-12, *Pegram* et al. also demonstrated that preconditioning with cyclophosphamide was not strictly required to establish CAR T cells upon adoptive transfer, while other studies obtained different results [97,101].

Another cytokine that potently stimulates NK and $CD8^+$ T cells is IL-15. Although administration of IL-15 seems to be less toxic, its activity is limited due to a very short *in vivo* half-life. While mono-therapy with IL-15 showed only limited effects on tumor regression, it revealed great potential when combined with other immunotherapeutic agents [102]. For instance, constitutive expression on one cistron of IL-15 together with a 1^{st} generation CD19-CAR was shown to significantly improve the antitumor activity of T cells *in vivo* leading to greater expansion and longer-lasting persistence of CAR T cells [78].

4.3 Co-expression of cytokine receptors

Another aspect which has to be taken into account is the fact that only cells that carry the appropriate receptor for a respective cytokine will also sense the presence of that very cytokine. IL-2 is known as a potent activation and differentiation factor of T cells, however, the IL-2 receptor (IL2R) is expressed on all T cells in different configurations (high-affinity, low affinity), and it can be especially well sensed by Treg cells due to their expression of the

high-affinity IL-2R CD25 [103]. The secretion of IL-2 by activated T cells was shown to lead to Treg cell accumulation within the tumor and thus lower efficacy of infiltrating CAR T cells [104]. While the IL2R is present on all T cell subsets in different configurations including Treg cells, IL-7 preferentially stimulates naive and memory T cells without having an effect on Treg cells that characteristically down-regulate the respective high-affinity IL-7 receptor-α chain (IL7R-α, CD127) [105,106]. The lack of expression of the IL7R-α on cytotoxic T cells limits its use for the expansion of CAR T cells [107]. To overcome this shortfall, *Perna* et al. have transduced virus-specific CTLs with a GD2-CAR and an expressible IL7R-α chain (Fig. 3c). This resulted in proliferation of these CAR T cells upon administration of IL-7 but had no effects on Treg cells. Of note, when such CAR- and IL-7Rα-expressing T cells were administered together with IL-7 growth of xenografted neuroblastoma cells was controlled even in the presence of Treg cells and supplementation with IL-2 [108].

4.4 Secretion of antibodies – Interference with inhibitory signals

In general, the tumor microenvironment is well-known for its expression of several inhibitory factors [109]. While stimulation of T cells may protect them from the inhibitory cues derived from the inhibitory tumor microenvironment, specific interference with tumor-derived inhibitors could also be actively used for enhancing T cell activity.

Programmed cell death protein 1 (PD-1) is an immunomodulatory receptor expressed on T cells [110]. PD-L1, its immunosuppressive ligand is known to be highly expressed in many tumors, and it is responsible for the inhibition of immune responses within the tumor. PD-1 antibodies inhibit the interaction between PD-1 and PD-L1 resulting in enhanced T cell function [111]. In fact, systemic administration of PD-1 inhibitors has led to a clinically significant antitumor response in various types of tumors [112]. To combine the PD-1/PD-L1-based inhibition with CAR therapy, *Rafiq* et al. have co-expressed a CD19–CAR together with a secreted form of an anti-PD-1-specific scFv able to inhibit PD-1/PD-L1 interaction (Fig. 3d). This resulted in constitutive production and secretion of the PD-1-specific scFv *in vivo* at the site(s) CAR cells re-located to. Secreted PD-1 antibodies bound to PD-1 and protected not only CAR T cells but also bystander T cells present within the tumor, while the cells outside of the tumor were not affected. This resulted in the generation of a more vigorous anti-tumor response and CD19-PD-1-CAR T therapy was shown to be superior to the combination therapy of CAR T cells and PD-1 inhibitors [113].

4.5 Chimeric cytokine receptors

A similar concept was followed using cytokine receptors, combining receptors that express inhibitory, cytokine-specific extracellular domains linked to the intracellular domain of an immunostimulatory cytokine receptor, thus converting any inhibitory stimulus into a stimulatory one (Fig. 3e) [114].

To take advantage of such conversion, *Stegen* et al. have ligated the extracellular domain of the IL4R-α chain to the intracellular, signaling domain of the IL2R-β chain, thereby creating a receptor termed 4αβ. The co-expression of 4αβ with an ErbB-CAR allowed for a selective expansion of CAR T cells upon addition of IL-4 to the cell culture medium [115]. Intra-

tumoral application of such CAR T cells generated with the help of exogenously added IL-4 is now being tested in a clinical study of advanced head and neck cancer (NCT01818323). A similar approach was adopted by *Mohammed* et al. in which they have applied a PSCA-28::ζ CAR with a chimeric receptor linking the extracellular part of the IL-4 receptor to the intracellular IL-7Rα signaling domain. They have shown that addition of IL-4 leads to selective expansion of CAR T cells even in the absence of added IL-2 [116]. This might also help avoiding undesired expansion of Treg cells as mentioned in chapter 4.2. Moreover, it has been shown that the inhibitory PD-1 signal can be directly converted into an activation signal, by fusion of the extracellular domain of PD-1 to the intracellular domain of CD28 and co-expressed together with a 2nd generation CAR using a 4-1BB::ζ intracellular domain. This prevented the tumor-induced inhibition of T cell function and mice treated with the combination of PD1::CD28 CAR T cells had a longer survival, which was associated with significantly increased tumor regression [117].

The advances in the field of the CAR cell activity enhancement are shifting the focus from the mere optimization of the CAR construct to more holistic approaches in which the overall cellular activation of the CAR cells is being addressed. The introduction of additional cytokine stimuli puts the need for costimulatory domains within the CAR construct in question. Additionally, such enhanced cell therapies could lower the need for multiple drug application (*e.g.*, co-application of PD-1 antibodies along with CAR cells) or chemotherapeutic pre-conditioning.

5 Cells used in CAR therapy

Immune cells represent the pivotal backbone of CAR therapy and the therapeutic power of the treatment not only depends on CAR design but also on the phenotype, further differentiation and longevity of the cells that become equipped with CARs and are transferred back into the patients [118]. For the production of CAR cells, usually peripheral blood mononuclear cells (PBMCs) are collected from a patient which then become expanded in the presence of different stimuli (*e.g.,* anti-CD3 antibodies, interleukins and/or feeder cells) (Table 2). CAR fusion genes are then introduced into their genome either by electroporation or viral transduction (Table 3) and cells become further expanded. Once adequate numbers of cells are obtained the cells are re-infused into the patient [119]. Although research initially centered on the use of autologous cytotoxic T cells, other cell types such as NK cells but also NK cell lines have gained increased attention recently. This chapter focuses on T cells and NK cells as cellular backbones for the expression of therapeutic CARs as well as genetically modified cell lines designed for universal application.

5.1 T cells and their subtypes

T lymphocytes are well-known for their central role in cell-mediated immunity [34]. They consist of several subsets, each one able to execute very different effector functions [120].

5.1.1 CD4$^+$ and CD8$^+$ T cells—Due to their cytotoxic potential, CD8$^+$ T cells were the cell type of choice in the first CAR study [14]. Although cytotoxic effects were observed, it was recognized only later that the sole infusion of CD8$^+$ T cells does not produce long-

lasting effects in patients. Instead, the co-application of $CD4^+$ lymphocytes was found to increase T cell longevity [121]. Because T cells are usually obtained from the peripheral blood of patients, the inter-individual cellular composition may vary considerably. Even more so in cancer patients who may have received one or more cycles of chemotherapy, resulting in skewed composition of peripheral T cell subsets [122]. To avoid such inter-individual differences, *Moeller* et al. have used a predefined one-to-one ratio between $CD4^+$ and $CD8^+$ T cells as the basis for the generation of CARs, which upon transfusion induced complete tumor eradication in preclinical models, which was confirmed by others [16,123,124]. Of importance, clinical trials applying standardized ratios of $CD4^+$ to $CD8^+$ CAR T cells have shown high complete response rates in lymphoma patients pointing to the superiority of such cellular compositions [125].

$CD4^+$ and $CD8^+$ T cells can be further subdivided into functionally different subtypes, some accounting for increased treatment efficacy. Along those lines, many studies have recognized the importance of the $CD8^+$ central memory T (TCM) cell subset, which guarantees for enhanced engraftment and thus prolonged persistence, while effector memory $CD8^+$ T cells were found to be clearly inferior in that respect [5,126]. Although TCM cells engraft well, their combination with $CD4^+$ T helper cells is still of utmost importance to ensure their long-term persistence, which is not only crucial for complete tumor cell elimination but also for continuous tumor immune surveillance [122].

Some T cell subsets, such as central memory T cells, have a low concentration in peripheral blood which renders their isolation and use for the generation of CARs difficult. Nonetheless, optimized cell culture conditions might improve their enrichment. For instance, the use of IL-7 and IL-15 instead of IL-2 for the expansion of T cells to be transduced with CARs was shown to yield larger amounts of $CD8^+$ T memory stem cells, which, upon transfusion, resulted in increased *in vivo* persistence and higher anti-tumor activity [127]. Thus, improved production protocols may greatly influence the efficacy of the final product.

5.1.2 Virus-specific cytotoxic T cells—Because tumors lack many of the costimulatory factors required to fully activate T cells, CAR T cell might not undergo sustained activation upon antigen recognition. In contrast, virus-specific CTL continuously receive stimulation from viral antigens and costimulatory ligands expressed on antigen presenting cells (APCs). To benefit from such a natural stimulatory environment, virus-specific CTL were used for the expression of CARs in some studies. While such cells could lyse both virus infected and tumor targets they did not show a superior long-term survival in the host [5,128].

5.1.3 γδ T cells—Infusion of genetically engineered T cells may be associated with serious side effects and toxicities and may cause graft-versus-host disease (GVHD) in the case of application of allogenic CAR T cells due to HLA mismatching [129]. Since γδ T cells are usually unable to recognize allogeneic HLA molecules but are still endowed with strong cytotoxic capability, such cells may be obtained from healthy allogeneic donors and would present an ideal option for ‘off-the-shelf’ cellular therapies. In addition, a meta-analysis of expression signatures from ~18.000 human tumors across 39 malignancies has shown that the intra-tumoral γδ T cell signature is among the most favorable prognostic

parameters for survival [130]. This makes γδ T cells a very attractive cellular backbone for CAR therapy and, in fact, various studies have already shown that γδ CAR T cells are perfectly capable of conducting target cell-specific tumor lysis [131,132].

5.1.4 Th17 T cells—Th17 cells are a subset of T helper cells defined by their production of the pro-inflammatory cytokine IL-17 [133]. When appropriately targeted, they can induce significant tumor regression and are superior in tumor eradication when compared to Th1 or Th0 T cells [134]. *Guedan* et al. have used a CAR containing the inducible co-stimulator (ICOS) intracellular domain to generate tumour-specific IL-17-producing effector T cells. Although this study showed an increased secretion of Th17 signature cytokines and enhanced T cell persistence in mouse tumor models, more severe forms of GVHD were observed as a side effect [135].

5.1.5 T regulatory cells—Treg cells have a negative impact on cancer therapy due to their multiple inhibitory effects on T effector cells [136]. Although inhibition of immune responses is undesired during cancer therapy, such inhibition may be useful when it comes to the treatment of autoimmune diseases or allergies. Notably, previous studies have shown that Treg represent an interesting agent for the treatment of autoimmune diseases, GVHD and allergies [137] (ref. Ward2018). In fact, *MacDonald* et al. have used a CD28::ζ CAR targeting HLA-A2 and have introduced it into Treg cells. These cells maintained their suppressive function and were superior at preventing GVHD when compared to Treg cells expressing an irrelevant CAR [138]. CAR Treg cells may impact also on bystander T cells by changing their cytokine profile from IFN-γ producing Th1 cells towards IL-10 producing Tr1 cells, thereby creating a more immunotolerant environment [139].

5.2 Natural killer cells, NKT cells and NK-92 cell lines

NK cells represent another interesting backbone for CAR expression. NK cells are able to target cancer cells in various ways, *via* (i) CAR-dependent antigen-specific mechanisms, (ii) spontaneous cytotoxic activity or (iii) indirectly by activation of adaptive and innate immune cells [140]. Another advantage is the possibility to use different sources for obtaining NK cells, such as umbilical cord blood, human embryonic stem cells and induced pluripotent stem cells or even allogenic NK cells or cell lines since they do not require HLA matching [6].

5.2.1 NK cells—CAR expressing NK cells produce larger amounts of cytokines upon target recognition, which leads to tumor cell lysis with various *in vivo* tumor models confirming their efficacy [141–144]. Although NK cells exhibit strong cytotoxic activity their short lifespan limits their successful application. This drawback can be overcome by the use of IL-12, IL-15, and IL-18, respectively, all cytokines which differentiate NK cells towards a memory-like cell phenotype with improved *in vivo* persistence [145]. Two clinical studies (NCT0095137, NCT01974479) evaluating the effects of CD19-CAR transduced NK cells are currently being performed.

5.2.2 NK-92 based cell lines—The NK-92 cell line was established from a male patient suffering from rapidly progressive non-Hodgkin lymphoma in 1994 [146]. Because

of the need for a standardized ‘off-the-shelf’ CAR backbone, the NK-92 cell line represents an interesting cell type. The transfusion of high doses of irradiated NK-92 cells were well-tolerated in clinical studies and resulted in only mild toxic side effects, while irradiation of the cells did not change their efficacy [147–150]. Additionally, transduction of NK-92 cells with CARs greatly enhanced their cytolytic activity and concomitantly reduced the tumor burden in pre-clinical models [150–152]. Currently, the first patients are being treated in a first clinical study, evaluating anti-HER2 CAR expressing NK-92 cells applied intracranially into glioblastoma tumors (NCT02892695).

5.2.3 NKT cells—NK T cells are a subset of T cells that join the properties of both T lymphocytes and NK cells [153]. Higher levels of tumor-infiltrating NKT cells have been related to a favorable outcome in cancer and the infusion of NKT cells was shown to be well-tolerated in clinical studies [154–158]. Low numbers of these cells in peripheral blood makes them difficult to isolate in sufficient numbers and purity, however, their restriction to monomorphic rather than polymorphic HLA molecules makes the application to the patients of allogeneic donor cells possible [159]. Upon application of CAR expressing NKT cells they produced large amounts of Th1-type cytokines, which resulted in potent antitumor activity [160,161]. Currently, one clinical study evaluating NKT GD2-CAR transduced cells for the treatment of neuroblastoma in pediatric patients is being performed with results still undisclosed (NCT03294954).

5.3 Combination of different cell types

While the majority of studies focusses only on one cell type, the combined treatment with different cell types might provide better results. *Du* et al. have developed a protocol for expansion of T lymphocytes, γδ T cells and NKT cells. Using zoledronic acid, interferon-gamma (IFN-γ), interleukin 2 (IL-2), anti-CD3 antibody and engineered K562 feeder cells PBMCs were greatly expanded with γδ T cells constituting over 20% of the population. The infusion of such cellular combination exhibited superior tumor cell lysis when compared to more ‘conventional’ CAR T cells [7].

5.4 Genetically modified cells for universal application

Due to the great potential of CAR therapy, the interest for ‘off-the-shelf’ CAR cell products is increasing constantly, but the need for the use of autologous lymphocytes considerably limits the large scale application of CAR therapies. To prevent the development of GVHD when using an allogenic cell source, the disruption of the endogenous TCR and HLA class loci would be necessary. A first advancement towards that direction was made with the establishment of the FT819 cell line. This CD19-CAR expressing T cell line was created from an induced pluripotent stem cell (iPSC) line by bi-allelic disruption of the TRAC locus [162,163]. A step further was made by *Ren* et al. who combined lentiviral CD19 CAR transduction with the use of the CRISPR/Cas9 system for the simultaneous disruption of three genes, TCR (TRAC and TRBC), β-2 microglobulin and PD1 genes. This maneuver (TCR and β2-m disruption) resulted in reduced alloreactivity and enhanced *in vivo* activity [164]. Currently, two clinical studies (NCT03166878, NCT03229876) are evaluating the use of such universal CAR cells.

In addition, CAR activity monitoring and tracing currently relies on the detection of CAR cells in peripheral blood, however, the importance of their activity at the tumor site remains only poorly appreciated. In that respect, *Keu* et al. have developed a reporter gene system for noninvasive detection of CAR cells by positron emission tomography (PET) scanning [165]. Such innovative *in vivo* cell monitoring approaches would greatly improve open questions regarding optimal dosing of CAR cells (quantity, frequency) and help to better monitor the longevity of CAR cells in the body.

6 Summary and outlook

CAR cells have become a very powerful and versatile tool for the targeting of malignant and autoreactive cell types of choice. With prototypic CAR treatments for acute lymphocytic leukemia already available and in clinical use, it is foreseeable that the scope of applications of this technology will continuously grow during the next decade. The further success of CAR cell technology will strongly depend on the (i) development of CAR constructs optimally suited for individual applications, (ii) identification of the most appropriate cell types to express CARs and to execute desired effector programs, and (iii) the possibility to safely and completely remove CAR cells upon achievement of full remission of the respective disease. Once solved, adoptive CAR cell therapy may become an affordable, efficient, highly selective and safe treatment modality able to fight apart from leukemias also solid malignancies but also autoimmune and allergic diseases in which (poly)clonally expanded cell types drive pathology.

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Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
BBIR	biotin-binding immune receptor
BCMA	B cell maturation antigen
CAR	chimeric antigen receptor
Cas9	caspase 9
CD	cluster of differentiation
CID	chemical inducer of dimerization
CMV	cytomegalovirus

CRISPR	clustered regularly interspaced short palindromic repeats
CRS	cytokine release syndrome
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
EAE	experimental autoimmune encephalomyelitis
EGFR	Epidermal growth factor receptor
ELRD	extracellular ligand recognition domain
EMA	European Medicines Agency
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcεRI	Fc epsilon receptor I
FDA	Food and Drug Administration
FKBP	FK506-binding protein
GVHD	graft versus host disease
HEL	hen egg lysozyme
HER2	human epidermal growth factor receptor 2
HLA	human leukocyte antigen
huEGFRTt	human epidermal growth factor receptor truncated
iCAR	inhibitory CAR
iCasp9	inducible caspase 9
ICOS	inducible T-cell costimulator
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
IL13Rα2	IL-13 receptor α 2
IL2R	IL-2 receptor
IL7R	IL-7 receptor
iPSC	induced pluripotent stem cell
ITAM	immunoreceptor tyrosine-based motif

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LAG-3	lymphocyte-activation gene 3
MBP	myelin basic protein
MHC	major histocompatibility complex
NK cell	natural killer cell
NKT cell	natural killer T cell
PBMCs	peripheral blood mononuclear cells
PD-1	programmed cell death protein 1
PD-L1	programmed death ligand
PSCA	prostate stem cell antigen
PSMA	Prostate specific membrane antigen
RMTC	receptor modified T cell
scFV	single-chain variable fragment
STAT3	signal transducer and activator of transcription 3
SUPRA CAR	Split Universal Programmable CAR
tanCAR	tandem CAR
TCM cell	central memory T cell
TCR	T cell receptor
Th cell	T helper cell
TIM-3	T-cell immunoglobulin and mucin-domain containing 3
TNP	2,4,6-trinitrophenyl
TRAC locus	T-cell receptor alpha constant locus
TRBC locus	T-cell receptor beta constant locus
Treg cell	T regulatory cell
VH	variable heavy chain
VL	variable light chain

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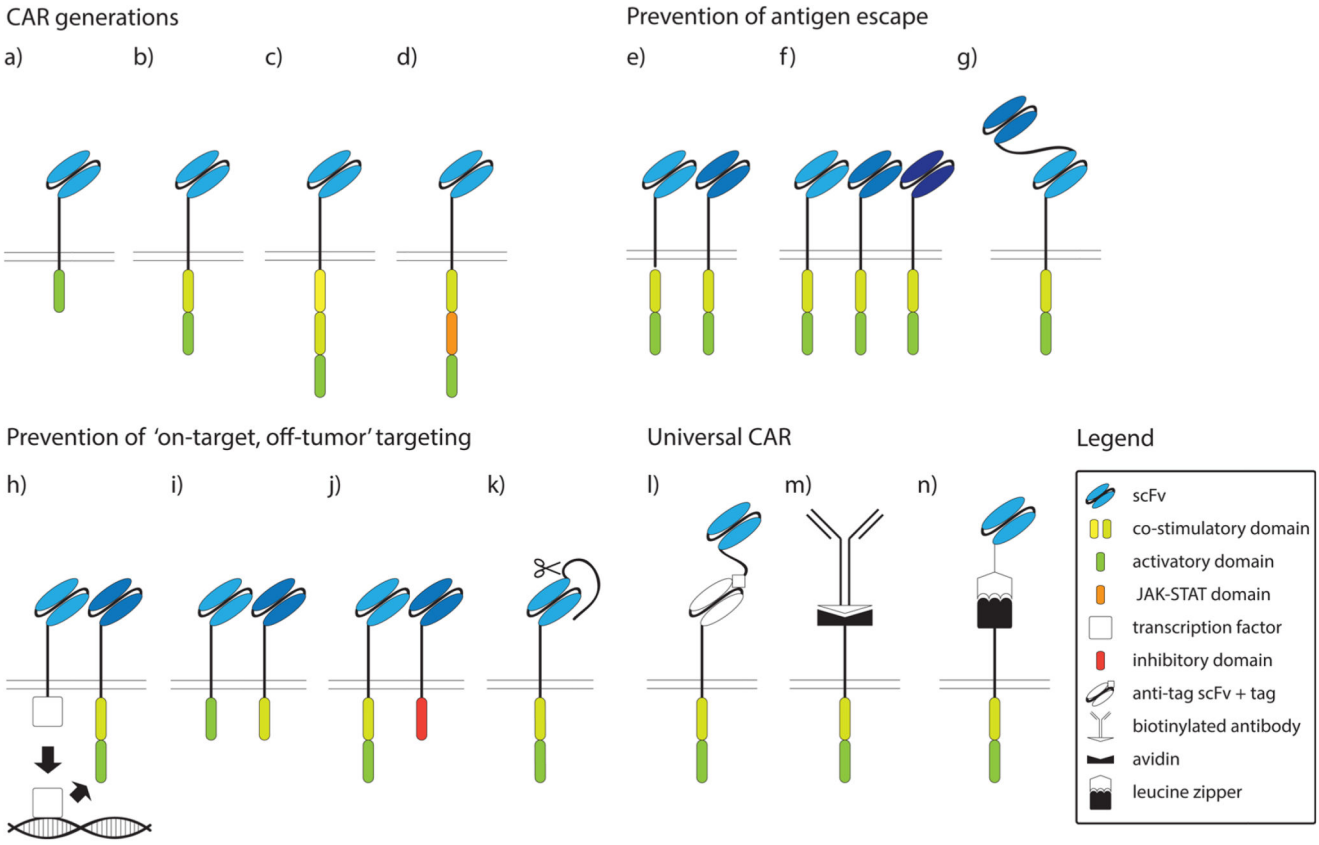

Fig. 1. Intracellular signaling domains and modifications of CARs.

a) First generation CAR, b) second generation CAR, c) third generation CAR, d) JAK-STAT CAR, e) double CAR, f) tripple CAR, g) tandem CAR (tanCAR), h) synNotch CAR, i) CAR with dissociated signaling domains, j) inhibitory CAR (iCAR), k) masked CAR, l) universal CAR (uniCAR), m) biotin-binding immune receptor (BBIR) CAR, n) split universal programmable (SUPRA) CAR. scFv, single chain variable fragment; JAK, Janus kinase; STAT, signal transducers and activators of transcription.

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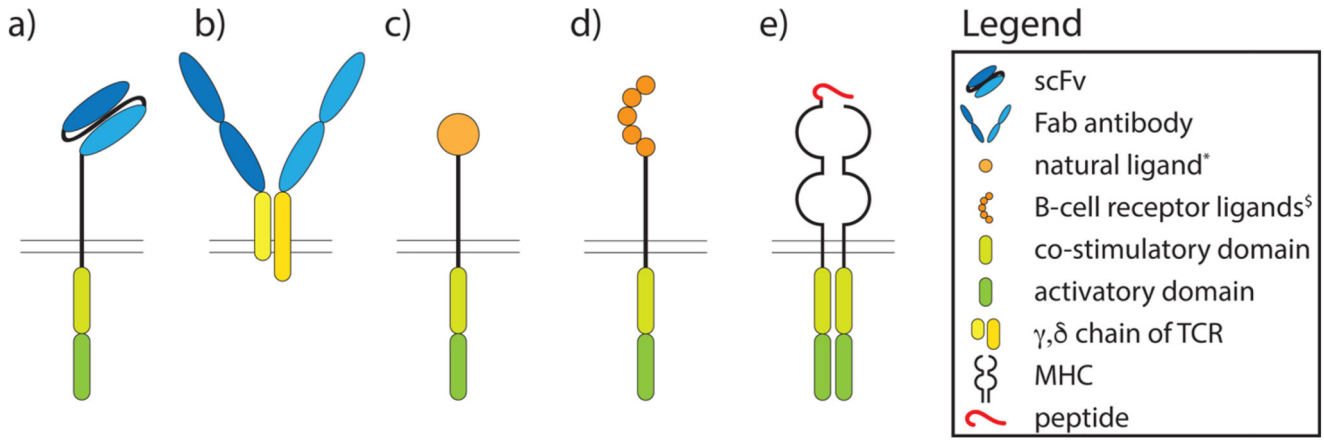

Fig. 2. Extracellular ligand recognition domains of CARs.

a) Single domain variable antibody fragments, b) Fab, c) natural ligands like cytokines and receptors, d) B cell receptor ligands, e) TCR ligands. scFv, single chain variable fragment; Fab, fragment antigen binding; TCR, T cell receptor; MHC, major histocompatibility complex. * IL-13, FcεRI, Ly49H, $ Egg lysozyme, Desmoglein-3, factor VIII.

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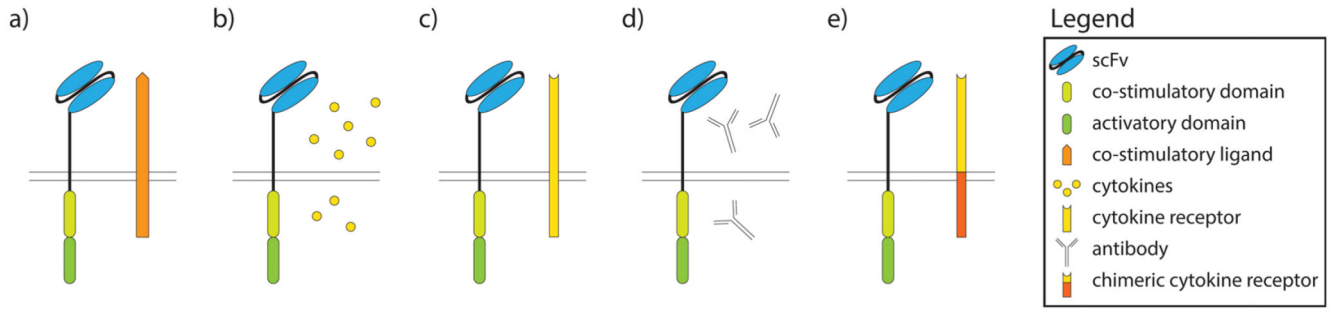

Fig. 3. The strategies to enhance the activity of CAR cells.

a) co-expression of cytokine receptor, b) co-transduction with cytokines, c) expression of co-stimulatory ligands, d) co-transduction with inhibitory antibodies, e) expression of chimeric cytokine receptors.

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Table 1

Composition and variation of CAR intracellular signalling modules, transmembrane and stalk regions.

CAR Domain	Component*	References
Intracellular signalling domain	CD3-zeta§ only	[5,73,74,100,128,142,149,150]
	CD28 only	[74]
	4-1BB only	[73]
	CD28_4-1BB	[74]
	4-1BB_CD3-zeta	[29,67,71,79,117,122,124,129,141,151,152,160,161,166,167,168,169,170,171]
	CD28_CD3-zeta	[16,17,22,68,70,75,78,85,92,99,104,108,113,115,116,117,123,127,131,132,138,143,150,161,170,172,173,174,175,176,177,178,179]
	CD28_4-1BB_CD3-zeta	[2,7,12,86,101,161,180,181]
	4-1BB_CD28_CD3-zeta	[151]
	CD28_OX40_CD3-zeta	[182]
	ICOS_CD3_zeta	[135]
	Dap10	[141]
	Gal 4 DBD v P64$	[73]
	CTLA-4#	[75]
	PD-1#	[75]
	CD28_IL2betaR_CD3-zeta_(YXXQ mutation)	[16]
	IL4Rbetac	[115,178,179]
	Fc epsilon TI gamma	[22]
	2b4	[142]
	2b4_CD3-zeta	[142]
Transmembrane domain	CD28	[2,16,17,22,24,29,68,70,71,73,86,92,99,108,113,115,117,122,123,124,131,132,138,143,150,161,166,169,170,172,173,174,175,176,177,178,179,182]
	CD8	[7,12,67,73,74,75,79,85,97,101,129,141,151,152,160,167,168,169,170,181]
	CD4	[180]
	CD3-zeta	[5,128,142,149,150]
	ICOS	[135]
	2b4	[142]
	4-1BB	[150]

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CAR Domain	Component*	References
	Notch1$	[73]
	IL-4betac	[115,178,179]
	IgG1	[116]
Stalk region	IgG4	[29,71,122,124,131,166,176]
	IgG1	[17,70,104,108,116,127,143,160,161,180,182]
	CD8	[7,12,22,67,73,74,75,79,85,86,101,123,129,141,149,150,151,152,167,168,169,170,172,177,181]
	CD28	[22,24,73,115,173,174,178,179]
	Fc spacer	[132]
	Notch1$	[73]
	G4S	[117]

*Components shown in N- to C-terminal orientation.

$CD3-zeta indicates that all three ITAMs of zeta have been used.

#inhibitory CAR.

$synNotch CAR.

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Table 2

Cell types and corresponding expansion protocols frequently used to express CARs.

Cell type	Culture conditions for expansion of CARs					References
	Antibodies	Interleukins	Feeder cells	Sorting	Others	
PBMC	CD3, CD28	IL-2, IL-15, Il-17, IL-4	NIH-3T3-CD19-CD80 aAPCs, K562-PSCA	Gentamicin resistance, magnetic bead based isolation of CD4+CD25+ T cells	PHA, PMA, Th17 polarizing conditions, anti-ICOS beads	[12,16,17,68,69,70,75,92,100,104,115,116,123,127,129,135,167,169,170,172,173,174,180,182]
Splenocytes, CD3+ splenocytes	CD3, CD28	IL-2, IL-7			Concanavalin A, PHA	[101,113,132,175,181]
CD8+ and CD4+ PBMC	CD3, CD28	IL-2, IL-7, IL-15	CD19+ LCL	Negative or positive selection	With or without defined CD4+/ CD8+ ratio, grown separately or together	[85,86,122,166,1,72,74,79,117,168]
CD8 + TCM and CD4+ PBMC	CD3, CD28	IL-2	CD19+ EBV LCL			[124]
CD8 + TCMs form PBMC	CD3, CD28	IL-2	CD19+ LCL			[29]
CD3+ and CD8+ PBMC		IL2, IL-15		K562, NALM-6, aAPC7mOKT3 expressing OKT3, CD80 and CD83		[24]
CD3+ cells from PBMC	CD3, CD28	IL-2			PMA	[2,78,99]
EBV-specific CTL	CD3	IL-2, IL-7		EBV-LBL		[5,108,128]
Gamma-delta T cells		IL-2, IL-21	Non-specified feeder cells	Negative selection w/ magnetic beads	Zoledronic acid	[97,132]
Treg	CD3	IL-2	Non-specified feeder cells	Sorting for $CD4^{+}$ $CD25RO^{low}$ $CD24RA^{high}$ $CD25^{high}$		[138]

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Cell type	Culture conditions for expansion of CARs					References
	Antibodies	Interleukins	Feeder cells	Sorting	Others	
NK-92, NK-92 MI cell lines		IL-2			Horse sera, human sera, X-VIVO medium	[71,150,151,152,177]
PB NK cells		IL-2		K562-mb15-41BBL		[141,142,143,171]
NK and gamma-delta T cells	CD3	IL-2		K562-mb15-41bbL	Zometa, IFN gamma	[7]

PBMC peripheral blood mononuclear cells, CD cluster of differentiation, PHA phytohemagglutinin, PMA phorbol myristate acetate, CTL cytotoxic T lymphocyte, EBV Epstein-Barr virus, Treg regulatory T cells, NK natural killer, PB peripheral blood, IFN interferon, aAPC artificial antigen presenting cell, ICOS inducible T-cell costimulator, PSCA prostate stem cell antigen, LBL lymphoblastic lymphoma.

Table 3

Expression and packaging vector systems and virus producer cells lines used to deliver CAR constructs to effector cells.

		References
Retroviral vectors	SFG	[2,5,16,17,68,69,70,75,78,79,92,97,99,108,115,116,127,128,142,182]
	pMX	[24]
	pSAMEN	[172]
	pMP71	[12,175]
	pLXSN	[123]
	pMSCV	[68,141,143,171]
	pMSGV	[101,173,174]
Lentiviral vectors	epHIV7	[29,117,122,124,166]
	pLenO	[149]
	pSIN	[89]
	pSIEW	[150,177]
	pELPS	[67,168]
	pELNS	[1,74,85]
	pHR	[72,86]
Packaging constructs	psPAX2	[122,124,166]
	pRDF	[2,69,70,78,141,160,171,182]
	pEQ-PAM3(-E)	[141,171]
	pVSVg	[1,74,85,150,152,168,177]
	pCL	[143,152]
	pMEVSVg	[68]
	pMD2G	[72,86,122,124,149,166]
	pMDLg/p.RRE	[1,74,85,149,168]
	pRSV.REV	[1,74,85,149]
	pTSV.rev	[168]
	pCHGP	[29,117]
	pCMV-g, pCMV-Rev2, pCMVdR8.91	[29,72,117]
	pGALV	[12]
Virus producer cell lines	pG13 cell line	[5,22,115,128,172,173,174]
	GP + e86 cell line	[101,152,181]
	Phoenix Eco cell line	[5,97,113,128,142]
	HEK 293T cell line	[1,2,12,17,29,74,78,79,100,104,108,116,117,124,138,141,143,149,150,160,166,168,171,175]
	GP 293T cell line	[123,175]
	293-Glv9 cell line	[92,99,113]
	Lenti-X 293T	[72]
	NIH 293T cells	[182]
	FLYRD18 cell line	[142]
	H92 cell line	[75,113]