Chapter 9

CARs on the Highway: Chimeric Antigen Receptor Modified T Cells for the Adoptive Cell Therapy of Malignant Diseases

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Additional information is available at the end of the chapter

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Abstract

Adoptive therapy of malignant diseases by chimeric antigen receptor (CAR) redirected T cells takes advantage of the patient's own immune system to recognize and destroy cancer cells. This is impressively demonstrated by the induction of complete and lasting remissions of leukemia with CAR-engineered T cells in early phase trials. Recent developments in optimizing the CAR design, in the recognition of target cells and the production of modified cells for clinical use, have paved the path for a broader application than currently explored. The chapter reviews the differences in CAR design, the success in the treatment of hematologic malignancies, the challenges in treating solid cancer, the treatment-related toxicities, and strategies to improve safety of CAR T cell therapy. Challenges for future applications are discussed.

Keywords: adoptive cell therapy, chimeric antigen receptor, T cell, clinical trial, costimulation, cancer

1. Synopsis

Adoptive cell therapy with redirected T cells has recently shown spectacular success in the treatment of hematologic malignancies supporting the concept that patient's own T cells can control cancer in the long term. A current strategy to specifically redirect patient's immune cells toward cancer is based on the adoptive transfer of cytolytic T cells which are ex vivo engineered with a chimeric antigen receptor (CAR) to provide both targeting specificity and T cell activation upon cancer cell recognition. The CAR is a composite transmembrane receptor molecule with a single-chain fragment of variable region (scFv) antibody binding domain in the extracellular part for recognizing a "tumor-associated antigen" on the surface of the

targeted cancer cell (**Figure 1**). The CAR transmits a T cell activation upon cancer cell recognition. The CAR is a composite transmembrane–activating signal through its intracellular part which is mostly derived from the T cell activation upon cancer cell recognition. The CAR is a composite transmembrane receptor (TCR)/CD3ζ signaling moiety with or without a costimulatory domain. Engagement of the cognate antigen on cancer cells initiates immune cell activation resulting in a lasting anti-tumor cell response [1, 2].

The prototype CAR for redirecting T cell activation has several advantages which are due to the modular design, in particular the combination of the antigen recognition by an antibody with the T cell activating machinery of the TCR. The antibody-mediated CAR recognition is independent of MHC presentation of antigen, which is in contrast to the TCR, and allows recognizing any target for which an antibody is available, including carbohydrates, lipids, or structural variants of an antigen. In contrast to the TCR recognition of antigen presented by the MHC, the CAR recognizes only antigens on the cell surface. However, by using an antibody which recognizes a specific peptide in the context of MHC, CARs can also gain TCR-like specificity; one example is a CAR recognizing a MHC class I presented NY-ESO-1 peptide [3, 4].

Adoptive therapy with CAR-modified T cells takes advantage of the power of cytolytic T cells that actively migrate through vascular endothelia and penetrate tissues, are activated upon antigen recognition, amplify, eliminate cognate target cells and have the capacity for repetitive killing. Once activated CAR T cells can moreover induce a secondary immune response by the release of a variety of pro-inflammatory factors which attract innate immune cells to the targeted tissue. After target elimination and without further re-stimulation, most CAR T cells undergo apoptosis; however, some CAR T cells can persist over years and provide an antigen-specific memory.

The efficiency of CAR-mediated T cell activation depends on various parameters; most of them are empirically defined, including the CAR design, the CAR primary and costimulatory moieties, the binding affinity, the targeted antigen epitope and its accessibility, the density of the cognate antigen on the target cell, and others. Bispecific CARs were engineered to target cancer cells which lost one antigen but retained the other. Several other modifications of the prototype CAR design were explored to increase treatment safety, targeting selectivity, and clinical efficacy. T cells were engineered with two CARs which recognize defined patterns of target antigens and complement in signaling to provide only full T cell activation when both antigens are engaged. Inhibitory CARs (iCARs) provide an inhibitory signal when engaging an antigen on healthy cells, thereby preventing unintended T cell activation against healthy tissues. Switch CARs provide an activating signal while engaging an inhibitory ligand on the target cell, thereby "switching" a suppressor signal to an activating signal for the engineered T cell.

In clinical applications, patient's T cells are ex vivo engineered with the CAR, mostly by lenti- or retroviral gene transfer, amplified to relevant numbers and re-administered to the patient who, prior T cell therapy, received a non-myeloablative lymphodepleting treatment to provide favorite conditions for the transferred CAR T cells. While the genetic modification of T cells by viral transduction is permanent, T cells can be transiently modified by RNA transfection in order to display the CAR for short term on the cell surface. In the majority of trials, the entire population of T cells is genetically modified; apart thereof, T cell subsets such as $CD8^+$ T cells, $CD4^+$ T cells, γδ T cells, cytokine activated killer (CIK) cells, or NK cells are also used. CAR-engineered regulatory T cells (Treg cells) are explored in experimental models to treat autoimmune diseases.

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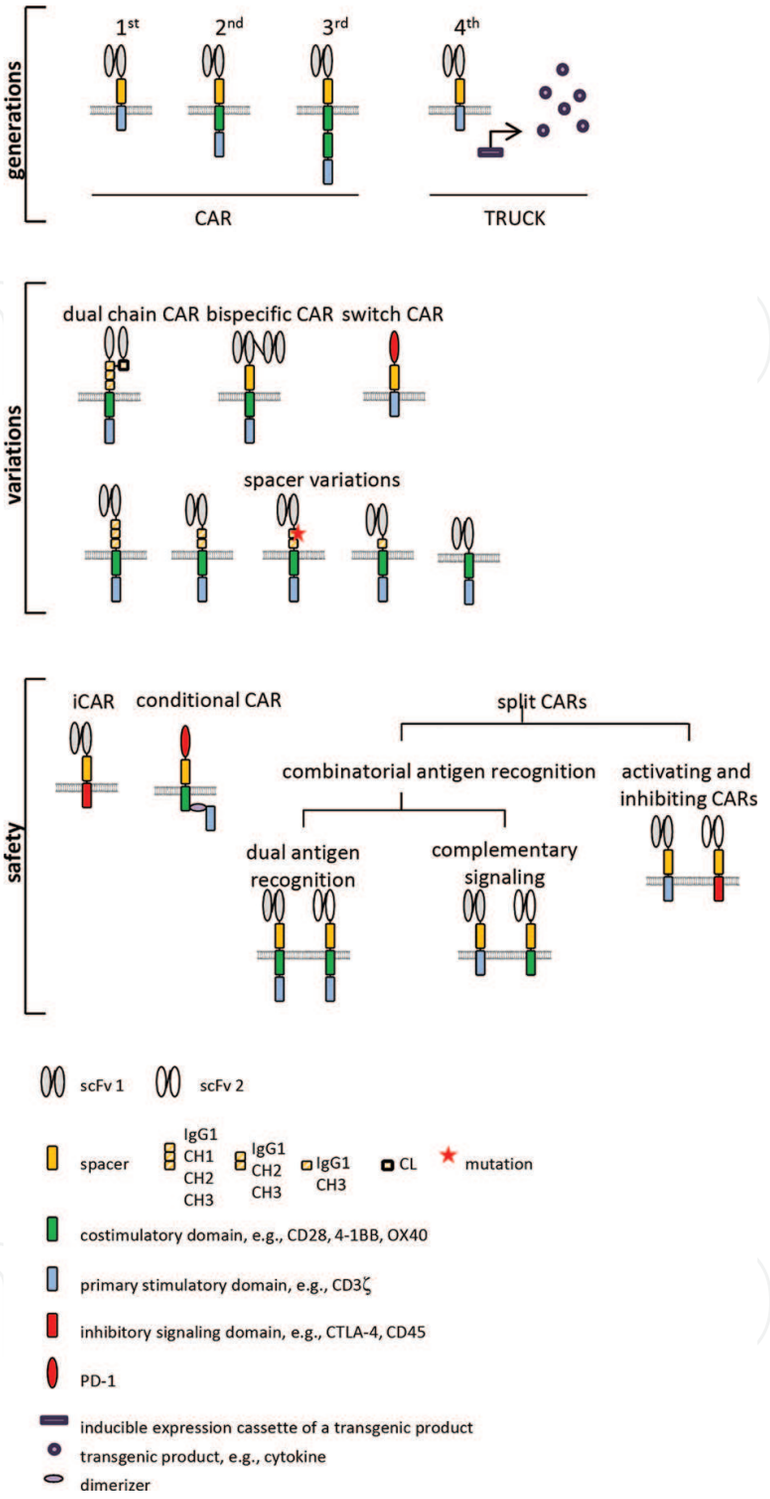

Figure 1. Modularity of the Chimeric Antigen Receptor (CAR) design. The CAR is an artificial composite receptor in order to bind a target in a specific fashion and to provide host cell activation in a predictable manner. The extracellular CAR binding domain, the spacer, the transmembrane and the intracellular signaling domains can be swapped with diverse other domains. On the extracellular side, various recognition domains were used, mostly single chain fragment of variable region (scFv) antibodies, or receptor derived binding modules. On the intracellular side, a panel of signaling domains can be used; the primary activating signaling domain is mostly derived from the TCR CD3ζ, Fc ε receptor-I (FcεRI) or downstream kinases. The costimulatory domain providing the secondary activating signal is linked to the primary signaling domain. Alternatively, inhibitory signals can be used to block T cell activation.

Currently, an overwhelming number of clinical trials has been initiated, and most of them are extraordinary successful in the treatment of hematologic malignancies [5]. As a treatment with "living drugs", the CAR T cell therapy provides a significant advancement toward a specific and individualized cell therapy of cancer which is going to establish in clinical practice. However, the CAR T cell treatment of solid tumors is still challenging demanding further developments of the basic strategy. CAR T cells with the inducible release of a transgenic payload, so-called TRUCKs, are envisaged to overcome some of the current hurdles [6]. In the following, recent developments are reviewed in the context of clinical applications, safety concerns, and challenges for future applications.

2. The prototype CAR

A CAR of the classical design consists of an antibody-derived binding domain, a spacer, a transmembrane domain, and one or more signaling domains; the attributes for an optimal CAR function are so far empirically defined and multiple variations of the prototype CAR design were described during the last two decades (**Figure 1**). Most CARs use an antibody-derived binding domain in a single-chain format, the so-called single-chain fragment of variable region (scFv) antibody. The scFv is engineered by joining the heavy and light immunoglobulin (Ig) variable regions by a flexible peptide linker, for example, $(Gly_4Ser)_3$, resulting in a continuous polypeptide chain in the order V_H-linker-V_L or V_L-linker-V_H. Such an antibody format facilitates the combination with the transmembrane polypeptide chain for membrane anchoring and the intracellular signaling chain. Since the conversion of a natural antibody into a scFv format does not always conserve the binding affinity and specificity of the antibody, a CAR format was recently reported which uses the entire Ig heavy and light chains with their constant domains to form a natural antibody on the surface of the engineered T cell; the Ig heavy chain is at the N terminus linked to the transmembrane and intracellular CAR domain [7]. The two CAR chains form a stable heterodimer, a so-called dual chain CAR (dcCAR), and bind with high affinity and in a specific fashion to their cognate antigen. The dual chain CAR format seems to be universally applicable and broadens the CAR T cell therapy toward a variety of antigens for which a scFv antibody is not available. Instead of an antibody, a naturally occurring receptor or ligand can also be used for binding. For instance, a CAR with a mutated IL-13 extracellular domain was designed to selectively bind to IL-13 receptor-α2 which is over-expressed by a broad variety of solid tumors but less by healthy tissues [8–10].

The CAR extracellular domain additionally incorporates a spacer of various lengths and a hinge domain to provide some flexibility. Various spacers were explored in the context of various antigens since the design and length of the spacer domain can be decisive for optimal CAR expression and function (**Figure 1**). This became obvious through discrepancies in the potency of CARs to activate T cells; some CARs are expressed and are active when the scFv is directly fused onto the signaling domain, and others are only active with a spacer [11]. Targeting of some antigens requires a spacer region between the antigen recognition and signaling domains implying some impact of the antigen itself. The spacer is typically

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derived from the IgG1 or IgG4 constant domain; other spacers such as CD4 or CD8 are also used [2, 12]. The spacer length can be varied by using different moieties, for example, IgG1 CH1-CH2-CH3 vs CH2-CH3 vs CH3; for each antigen, there seems to be an optimal CAR design providing the best suitable distance between the interacting CAR T cell and target cell [13]. The optimal distance moreover depends on the position of the targeted epitope on the antigen; higher order structural requirements and multimerization driven by the extracellular spacer domain may additionally apply demanding a more thorough exploration in the context of a particular antigen.

Apart thereof, the commonly used IgG1 constant domain in the extracellular CAR moiety can bind to Fc γ receptors (FcγR) (CD64) on myeloid cells, thereby initiating an unintended "off-target" activation of both T cells and innate cells. It is therefore essential to abrogate binding to Fc receptors by either deleting the IgG1 CH2 domain or by replacing through the IgG4 domain. Alternatively, the FcγR binding motif within IgG1 CH2 was modified by deleting the Asn_{297} glycosylation site [12, 14].

CARs typically have a membrane spanning region consisting of 20–23 hydrophobic amino acids, rich in leucines, isoleucines, and valines; a variety of membrane spanning receptor domains have been used in CAR design, including those of CD3ζ, CD4, CD8, CD28, or OX40 [2]. The choice for a transmembrane region is so far empiric, and some evidences imply that CARs with CD3ζ transmembrane domain incorporate into the endogenous TCR/CD3 complex and may be more robust in expression and signaling than others [15].

"First generation" CARs used the TCR-derived intracellular CD3ζ or the Fc ε receptor-I (FcεRI) derived γ chain for signaling; CD3ζ has become the most widely used signaling component [16, 17]. The CD3ζ harbors three immunoreceptor tyrosine activation motifs (ITAMs), the γ chain one ITAM. Upon CAR engagement of antigen, the ITAMs become phosphorylated and serve as specific adaptors for a panel of signaling proteins, thereby utilizing the endogenous TCR downstream signaling machinery for initiating the cascade of activation events. In this context, downstream kinases like lck or fyn can also be used as CAR activation domains.

Based on the "two signal hypothesis" that sustained T cell activation requires both the primary TCR-derived signal and a costimulatory signal, researchers added a costimulatory moiety to the primary signaling moiety to improve T cell activation in the long term [18–20]. Such "second generation CARs" harbor combined primary and costimulatory signaling moieties within the same polypeptide chain (**Figure 1**). CD28 was initially incorporated as a costimulatory domain, and alternative costimulatory molecules are also used including 4-1BB (CD137) and OX40 (CD134). The specific order of signaling domains within the CAR appears to be important for optimal activity; the CD28 domain is located in the membrane proximal and the CD3ζ domain in the distal position; the same applies for 4-1BB while OX40 is also active in a membrane distal position. Due to the modular composition of signaling moieties, two costimulatory domains can also be combined within the same CAR providing a more complex signaling signature to the engineered cell. Such "third generation" CARs incorporating CD28 and OX40, for instance, may be of benefit for T cells during late stages of maturation [21].

The individual costimulatory signals drive T cell activation in a different fashion resulting in a fine tuning of the T cell response with respect to T cell amplification, secretion of pro-inflammatory cytokines, and cytolysis of antigen-positive target cells [22]. CAR T cells with 4-1BB costimulation persisted for more than 6 months in the blood of most patients, whereas CD28 CAR T cells were mostly undetectable beyond 3 months [23]. CD28 CAR T cells show a reprogramming toward $CD45RO^{+}$ $CCR7^{-}$ effector memory maturation while 4-1BB CAR T cells predominantly show a $CD45RO^{+}$ $CCR7^{+}$ central memory cell differentiation [24]. Costimulation moreover orchestrates the metabolism of the CAR T cells; CD28 signals through the PI3K/Akt pathway increase glucose uptake through the Glut1 transporter and PDK1 which inhibits the pyruvate decarboxylation, all resulting in an increased ATP generation. In contrast, T cells with the 4-1BB CAR show an enhanced catabolic activity and oxidative metabolism together with an enhanced mitochondrial respiratory capacity. With that respect, the 4-1BB CAR initiates a long-lasting central memory and the CD28 CAR a more short-lived effector cell response. Both CD28 and 4-1BB CAR T cells in high doses eradicates large established tumors in preclinical models; at lower doses, CD28 CAR T cells show larger degree of exhaustion than the 4-1BB CAR T cells and treatment with 4-1BB CAR T cells more efficiently eradicated tumors [25]. T cells with 4-1BB CAR are still sensitive to tumor-mediated inhibition, however, show less exhaustion and decline in cytolytic capacities and cytokine secretion upon repeated antigen encounter than CD28 CAR T cells. Therefore, the criteria for selecting a CAR design depend on multiple parameters including T cell persistence, resistance to repression, the pattern of costimulatory and co-inhibitory ligands on targeted tumor cells and CAR T cells, the CAR density on the modified T cell, and require variations in CAR design such as affinity, the spacer, and transmembrane domains among others.

3. TRUCK: a CAR T cell releasing a transgenic product

CAR T cells of the "fourth generation," so-called TRUCKs, are T cells engineered with a CAR and an additional "payload", that is, a transgenic product (**Figure 1**) [6]. TRUCKs are aimed at depositing a protein in the CAR-targeted tissue in order to locally achieve therapeutic effective concentrations of the transgenic protein while avoiding systemic toxicity. The transgenic product may by a cytokine or any other protein which is produced and released upon CAR signaling. Technically, the CAR T cells are engineered with an additional expression construct for the transgenic protein directed by a constitutive or a CAR responsive promoter. Such TRUCKs deposit the transgenic product at the place of activation as long as the CAR T cell remains activated [26, 27]. In a specific development, TRUCKs were engineered to release a transgenic immune modifier upon CAR signaling to shape the targeted tumor environment in a specific fashion without causing systemic toxicity. In an example, IL-12 TRUCKs were shown to release IL-12 upon CAR activation into the targeted tumor tissue where the accumulated IL-12 recruited innate immune cells, such as NK cells and macrophages, which in turn drive a secondary immune response [28]. The strategy is of particular interest with respect to the fact that cancer cells which have down-regulated the cognate target are invisible to CAR T cells and may give rise to tumor relapse despite the presence of CAR T cells. Tumor relapse through antigen loss cancer cell variants is becoming an increasing obstacle when solid cancer

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lesions with a substantial genetic or phenotypic heterogeneity are treated by mono-specific CAR T cells. Combining the CAR T cell attack with the local deposition of an immune modifier represents a strategy to initiate a broader immune response. Beyond the treatment of cancer, TRUCKs may be envisioned for the therapy of virus infections, autoimmune diseases, or metabolic disorders delivering a therapeutic protein into the diseased tissue.

So-called armored CARs include the 4-1BB ligand in addition to the CAR in order to provide increased costimulation through the 4-1BB pathway [29]. Recently described examples are TRUCKs that co-express catalase to protect T cells from oxidative stress-mediated repression [30] and heparanase to improve T cell penetration through tumor stroma [31]. T cells engineered to secrete Toll-like receptor (TLR) ligands including TLR5 ligand can stimulate the TLR on T cells and on antigen-presenting cells which then activate a broad panel of tumor-reactive T cells [32, 33].

4. CAR targetable antigens: neo-antigen, tumor-associated antigen, and activation-induced antigen

The CAR T cell treatment of tumors demands both specificity in antigen recognition and selectivity in cancer cell targeting to avoid destruction of healthy tissues. Ideally, the targeted antigen is required for cancer cell survival and harbors mutations that are large enough to produce new epitopes, so-called neo-antigens, which then can be specifically recognized by the CAR [34]. Although mutations are thought to occur frequently enough in cancer cells to provide multiple new targetable epitopes, the identification of neo-antigens requires deep sequencing of the tumor material. Only a subset of such neo-antigens may be presented by the MHC to be recognized by the TCR; neo-antigens on the cell surface to be recognized by the CAR occur less frequently. Currently, such neo-antigens are rarely identified, asking whether the technical ability to predict relevant neo-antigens are sufficiently advanced and whether antibody-based binders for the use in a CAR can be engineered in due time. The situation is even more complex since the tumor lesion is genetically extremely heterogeneous which requires targeting more than one antigen in order to deplete the majority of cancer cells and to minimize the risk for a tumor relapse by remaining cancer cells.

Since the peptide processing is frequently impaired in cancer cells, T cell epitopes associated with impaired peptide processing and derived from broadly expressed self-antigens are potential targets for cell therapy since they are not restricted by central tolerance [35]. Since such antigens do not require the cellular peptide transporter, tumors defective in TAP transporter remain targetable by CAR T cells despite downregulation of the antigen presentation machinery.

Basically, subpopulations of tumor cells with “stem cell–like” properties, so-called cancer stem cells (CSCs), and the expression of stem cell–associated antigens may be good targets since CSCs are thought to trigger disease progression and tumor relapse and to be responsible for the resistance to conventional therapy. CAR T cells are capable to eliminate those CSCs in experimental models including melanoma; one trial is going to explore the clinical efficacy [36–40]. CAR T cell targeting of activation antigens expressed by stem cells may be superior to targeting

lineage-associated antigens since activation associated antigens are transiently expressed during maturation. For instance, CAR T cells targeting CD30 spare $CD30^+$ hematopoietic stem and progenitor cells while eliminating $CD30^+$ lymphoma cells in an experimental model [41]. In contrast, lineage-associated antigens increase in expression during cell maturation, like folate receptor-β and CD123; targeting those antigens increases the risk to destruct tissue stem cells [42].

In the absence of truly tumor-specific neo-antigens, so-called tumor-associated antigens are preferred; some of those antigens are expressed in a polarized fashion by healthy cells and topologically sequestered from redirected T cells, while uniformly distributed on cancer cells. An example of such antigen is carcinoembryonic antigen (CEA) which is expressed on the luminal surface of gastrointestinal and lung epithelia but homogenously by cancer cells where it can be recognized by CAR T cells.

For the treatment of B cell malignancies, CD19 and CD20 are most frequently targeted by CAR T cells with the consequence that healthy $CD19^+$ B cells are also depleted. Although B cell deficiency is clinically manageable, researchers are looking for alternative targets to avoid CAR T cell–induced immune deficiency in the long term. In the case of a malignant B cell clone with an immunoglobulin κ (Igκ) light chain, CAR T cells targeting Igκ may be of benefit since the Igλ B cells and plasma cells remain untouched [43]. Moreover, Igκ B cell deficiency does not increase the risk of infection making Igκ a good candidate target for treatment. An alternative target is the receptor tyrosine kinase-like orphan receptor-1 (ROR1) which is expressed by cells of chronic lymphocytic leukemia (CLL), mantle cell lymphoma, B-ALL, and numerous types of solid tumors [44–47], however, also by many healthy tissues.

5. CAR T cells recognizing multiple antigens

Cancer cells may lose the expression of particular antigens due to various mechanisms during tumor progression making them invisible to specific CAR T cells demanding targeting of multiple antigens on the cancer cells. Instead of engineering a panel of CARs with different specificities and applying a panel of T cells with different CARs, a CAR with multiple specificities can be engineered by linking the scFvs to each other (**Figure 1**). A so-called TanCAR is a bispecific CAR which harbors two linked scFvs of different specificities in the same CAR polypeptide chain and is aimed at targeting two antigens in order to control tumors with a growing number of antigen loss cancer cell variants [48]. Such TanCAR induces a T cell response upon engagement of either antigen; both antigens are not needed to be simultaneously present on the same cell to initiate CAR T cell activation. With this respect, CD19-CD20 bispecific CARs were engineered in order to mitigate a B cell leukemia relapse through cells which lost either antigen [49]. In particular, leukemia relapses occurred upon therapy with CD19 CAR T cells, and the relapse is predominantly driven by $CD19^-$ $CD20^+$ leukemic cells which are likely recognized by CD19- and CD20-specific TanCAR T cells. Simultaneous engagement of two antigens by a bispecific CAR has moreover the advantage to increase the avidity and the interaction of the CAR T cell with the respective target cell which stabilizes the formation of the synapse and improves T cell activation toward target cells with low antigen levels. Apart from tandem scFvs, diabodies, two-in-one antibodies, and dual variable domain antibodies may also be used as bispecific binding domains in the context of a CAR.

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Strategies were developed to increase selectivity in cancer cell targeting. The sensing of an antigen pattern is more selective in cancer cell recognition as long as the targeted antigens are co-expressed by the cancer cells and less by healthy cells. The antigen pattern is recognized by a pair of cooperating CARs; each CAR specific for a different antigen and providing different signals, one CAR the primary activating, the other CAR the costimulatory signal; both signals need to complement in order to induce a T cell response (**Figure 1**). CAR T cell targeting of an antigen pattern is thought to minimize off-tumor toxicities toward healthy tissues. The design is in contrast to a second generation CAR which provides both the primary and costimulatory signal through the linked signaling moieties upon engaging the one cognate antigen.

Some examples of combinatorial antigen recognition were reported, for instance, targeting ErbB2 by the CD3ζ CAR and Muc1 by the CD28 CAR [50], or targeting mesothelin by the CD3ζ CAR and folate receptor-α by the CD28 CAR [51]. T cell activation by combinatorial antigen recognition requires a subthreshold primary signaling to ensure a dependence on costimulation for a productive T cell response. Such fine-tuning of signaling strength is required to avoid unintended activation against cells with one antigen only and to achieve a complete T cell response against cells with both antigens. In this situation, de-tuning of the primary activation signal can be achieved by using binding domains of lower affinities [52].

6. CARs with exchangeable antigen recognition

In the current situation, each CAR has specificity for a defined antigen; changing specificity requires engineering T cells with a new CAR. To obtain some exchangeability in targeting specificity once the CAR T cells are applied to the patient, strategies were developed which use the CAR for binding a "linker" molecule which targets the cancer cell. In particular, the CAR binds, for example, via CD16, to the immunoglobulin Fc region of an antibody which binds to the cancer cell; the CAR will bind the antibody and in turn gains specificity for the targeted antigen [53]. The strategy allows using an universal CAR which is grafted with tumor specificity by an applied antibody. In an alternative approach, the CAR binds to a protein epitope, which is not encoded by the human genome and which is linked to cancer-targeting antibodies [54]. In another example, folate receptor-positive cancer cells were marked by a fluorescein isothiocyanate (FITC)-conjugated folate which binds to the cancer cells and is recognized by a FITC-specific CAR [55, 56]. By using more than one FITC-labeled molecule which mark the cancer cells, the same CAR T cell can target multiple types of cells within the tumor lesion. However, the strategy requires sufficient concentrations of the marking molecule and adequate numbers of CAR T cells in the tumor lesion for productive T cell activation.

7. Conditional CARs to control toxicity

In order to combine primary and costimulatory signal only "on demand", a conditional CAR was designed which consists of two chains: one chain providing the extracellular and transmembrane moiety together with the primary signaling moiety and the second chain providing the costimulatory signaling moiety (**Figure 1**). Both chains keep "switched off" and are

only "switched on" upon adding a small dimerizer molecule which enables the formation of a functional CAR heterodimer synapse and the delivery of both signals to the T cell in a temporally limited fashion [57]. Withdrawal of the dimerizer terminates switch-on of the CAR. The strategy is thought to be safe as in the absence of a dimerizer no signaling occurs. Moreover, the T cell activity may be fine-tuned by titrating the dose of the dimerizer.

An alternative strategy is based on synthetic Notch (synNotch) receptors which enable the conditional expression of a targeting receptor upon engagement with a tissue-specific ligand [58, 59]. The strategy is based on Notch, which is composed of an extracellular receptor, a transmembrane domain, and an intracellular transcription regulator, and upon activation mediates proteolysis of the internal domain which is releasing the intracellular transcription regulator. The synNotch receptor activity is cell contact dependent and controls cellular responses in a spatially defined fashion [58]. These properties can be used in a CAR-like synNotch receptor which controls the transcription of an authentic CAR and thereby uses combinatorial antigen recognition to spatially control CAR T cell function [59]. The CD19-specific synNotch receptor was tested in a model recognizing CD19 and releasing a transcription regulator to induce the expression of a CAR against mesothelin. The described synNotch receptor is composed of a CD19-specific scFv and of the transcriptional effector domains Gal4-VP64 or TetR-VP64 required to induce CAR expression; the engineered T cell is only activated when both the synNotch ligand and the CAR ligand were engaged on the target cell. There is a dose-response relationship between ligand concentration and CAR engagement. The kinetics of the "on-switch" determines the selectivity in targeting cancer cells while protecting healthy tissues; the induction of CAR expression by synNotch ligand binding needs to be fast enough to engage the tumor while the decayed CAR expression needs to be timely enough to spare healthy tissues expressing the cognate antigen.

8. Switch CARs: converting a suppressor into an activator

Solid tumor lesions display a plethora of inhibitory ligands to T cells, thereby actively suppressing the T cell anti-tumor attack; programed cell death ligand-1 (PD-L1) and PD-L2 binding to programed cell death-1 (PD-1) expressed by activated T cells are a major mechanism in this scenario. Kobold et al. [60] and Liu et al. [61] explored the concept to switch the inhibitory signal provided upon PD-1—ligand interaction into a T cell activating signal by a "switch CAR" which consists of the PD-1 extracellular domain for ligand binding and the CD28 intracellular domain for activation (**Figure 1**). The PD-1:CD28 "switch" CAR increased ERK phosphorylation, the release of pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α, the T cell proliferation, and the expression of the cytolytic molecule granzyme B upon PD-L1 binding [62]. Obviously, the switch CAR provided CD28 costimulation overruns the PD-1 mediated suppressive signal in the engineered T cell. The switch receptor moreover competes for available PD-1 ligands on the tumor cells and thereby reduces the number of repressive PD-1 interactions. However, other suppressive mechanisms are still in place in the tumor lesion, for instance through T cell immunoglobulin and mucin-domain containing protein-3 (TIM-3), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), B and T lymphocyte

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attenuator (BTLA), or lymphocyte-activation gene-3 (LAG-3), which all need to be overrun in a sufficient fashion to sustain the T cell anti-tumor attack in the long term.

9. CARs providing inhibitory signals: iCARs

Based on the modular composition of the prototype CAR, the intracellular activating domain can be exchanged by a moiety which delivers inhibitory signals to the T cell, including PD-1, CTLA-4, or CD45 [63]. Such an inhibitory CAR (iCAR) represses T cell activation upon binding to cognate antigen (**Figure 1**). The rationale for iCARs in the context of anti-tumor immunotherapy is to suppress T cell activation when engaging an antigen expressed by healthy cells. T cells which co-express a dominant iCAR along with an activating CAR recognizing a tumor-associated antigen are activated when engaging the cancer cells and repressed upon contact with healthy cells. In this context, the inhibitory CAR signal is aimed at avoiding off-tumor toxicities by overrunning the activating CAR signal when engaging healthy tissues.

10. Universal CAR T cells

The fundamental idea of an "off-the-shelf" CAR T cell is a genetically edited CAR T cell with deleted endogenous $\alpha\beta$ TCR and HLA molecules which can be achieved by the zinc finger nuclease technology [64]. Such CAR T cells are expected to be applied to a number of patients without causing graft-versus-host disease (GvHD) and without being eliminated by the host immune cells while providing CAR-mediated effector functions against cancer cells. Based on the same rationale, T cells were genetically edited by TALEN technology in the TCRα and CD52 locus [65] for the treatment of ALL. In a first in-human application, gene-edited CAR T cells were administered for the treatment of pediatric $CD19^+$ ALL in a patient for whom autologous CAR T cells could not be produced. CAR T cells from an unrelated donor were genetically edited by deleting the endogenous TCR to prevent GvHD and by deleting CD52, present on the patient's malignant B cells, which allowed to eliminate recipient lymphocytes while sparing the infused CD52-negative CAR T cells. Donor-derived, gene-edited allogeneic T cells may have the potential to provide CAR T cell products for numerous recipients; however, the safe treatment with allogeneic CAR T cells still requires additional editing of self-recognition molecules.

11. CAR T cells are successful in first clinical explorations

Almost 100 early phase trials in the adoptive therapy of cancer with second generation CAR T cells have been initiated pushing the field into a new era [66]. During the last years, good manufacturing practice (GMP) procedures have been developed for genetically engineering patient's T cells from autologous leukapheresis products and for amplifying the modified cells by CD3/CD28 bead stimulation during a 10-day period to clinically relevant numbers. In the majority of trials, retro- or lentivirus transductions are used to genetically modify the

T cells; electroporation-mediated DNA or RNA transfer is also applied in some trials. DNA transposons have been used to efficiently insert gene cassettes into the host genomic DNA [67–69]. DNA transposon-based systems, such as the Sleeping Beauty (SB) and the PiggyBac transposon, have been used to engineer CAR T cells for clinical applications [70–72]. Although there is a theoretical risk of insertional oncogenesis, no transforming event in mature T cells after viral or non-viral gene transfer was so far observed.

The ex vivo amplification of CAR T cells is currently performed in the presence of IL-7 and IL-15 or IL-21 [73] which favors a more rapid expansion of less matured T cells which provide a more robust persistence, cytokine release, and cytolytic activity compared with anti-CD3 antibody and IL-2-amplified T cells. While static culture systems have been traditionally used, shaking reactors or bags, and gas-permeable rapid expansion culture-ware (G-Rex) [74] are sustaining T cell expansion to much higher densities. The currently entirely manual process is going to be translated into a closed, fully automated and supervised system. Automated systems will allow the production of multiple batches in parallel which will be required when clinical exploration in trials transforms to standard applications to a huge number of patients.

In a number of trials, CAR T cells targeting CD19 produced significant therapeutic efficacy in the treatment of B cell malignancies, including so far refractory B cell chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), and various other types of B cell malignancies [5, 66]. The success, however, is rather heterogeneous; CAR T cell therapy of pediatric and adult ALL achieved complete remission rates of about 90% and sustained remissions for more than 4 years [75]; in CLL, about 63% overall remissions and 19% complete remissions were achieved [76]. T cells were effective upon one application only and even at low dosage levels of about 1.5×10^5 cells per kg. CAR T cells were capable to expand more than 1000-fold after administration and to persist in the peripheral blood and bone marrow for months and in some patients for years. Clinical trials targeting B cell leukemia/lymphoma are currently entering phase II development by pharmaceutical companies.

The trials have been performed by academic centers and major pharmaceutical companies; some of the CAR T cells provided by Novartis, Juno Therapeutics, and Kite Pharma received "breakthrough designation" by the US Food and Drug Administration in 2014 and 2015. More than 50 trials are currently open to treat B cell malignancies with CD19-specific CAR T cells [66]. While the anti-CD19 CAR used in clinical exploration contains a murine scFv domain, fully human CARs are currently developed to avoid an anti-CAR immune response and finally depletion of CAR T cells through the host immune system. Most currently active trials use CARs with CD28 or 4-1BB costimulatory domain, alternative costimulation by OX40 [77], ICOS [78, 79], CD27 [80], CD40-MyD88 [81], CD2 [82], CD244 [83], and others are currently being studied.

Current efforts are aiming at sustaining engraftment and improving CAR T cell amplification and persistence in vivo. A key factor is thought to be the "preconditioning" of the patient's immune system through non-myeloablative lymphodepletion. A number of trials are exploring modifications of the basic regimens, and only a small minority of them does not perform preconditioning. Patient treatment furthermore includes reducing the bulk of tumor mass prior therapy, CAR T cell administration, systemic cytokine supplementation, and clinical managing of comorbidities and toxicities.

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While a number of centers are performing clinical trials, a direct comparison of therapeutic efficacies is difficult to make due to a number of differences in the CAR design and study protocols. However, a recent meta-analysis of CD19 CAR T cell trials confirmed lymphodepletion and CAR T cell dose as key factors for successful treatment, while IL-2 co-administration is not recommended [23]. Most patients with 4-1BB-CD3ζ CAR T cell therapy did not receive further treatment; patients treated with CD28-CD3ζ CAR T cells frequently underwent subsequent allogeneic stem cell transplantation; the clinical decision is partly based on the observation that 4-1BB-CD3ζ CAR T cells persist over years while CD28-CD3ζ CAR T cells persist only for a few months.

CAR T cell persistence is crucial to obtain lasting remission of the disease; no patients with B-ALL relapsed 1 year after CAR T cell infusion. If the CAR T cells do not persist that long, a consolidation approach such as allogeneic stem cell transplantation may be required. To improve CAR T cell persistence in vivo, virus-specific T cells are applied which engage viral antigens through their TCR in a repetitive fashion and thereby obtain survival signals independently of CAR engagement of tumor target. For instance, Epstein-Barr virus (EBV) specific, autologous CAR T cells persisted longer after infusion than non-virus specific CAR T cells from the same patient [84]. T cells of other specificities toward endogenous virus antigens are also envisaged. In addition, the stage of maturation impacts the T cell persistence. Less differentiated T cells such as naïve, stem cell memory, and central memory T cells seem to provide a more persistent anti-tumor response as compared with effector T cells [85–87]. In particular, $CD4^+$ $CD45RO^+$ $CD62L^+$ memory T cells seem to be superior in the long-term providing the rationale to explore $CD62L^+$-enriched CAR T cells for clinical application.

Apart from CD19, alternative targets for B-cell malignancies such as CD20, CD22, the Igκ light chain, ROR-1 for B-NHL and B-ALL, and CD30 for Hodgkin's lymphoma are being actively studied as CAR T cell targets. The Fcμ receptor seems to be a more selective candidate target for the treatment of CLL in experimental settings in order to spare healthy B cells from a CAR T cell attack [88].

12. CAR T cell therapy of solid cancer is still challenging

The capability of T cells to home to specific targets throughout the body basically allows the elimination of widespread and metastatic tumor lesions. However, the treatment of larger solid cancer lesions is challenging due to multiple reasons.

First, CAR T cells need to traffic to the tumor lesion in the periphery which depends on a number of soluble and cell bound factors, in particular chemokines. Extensively amplified CAR T cells often express an altered panel of chemokine receptors; transgenic co-expression of chemokine receptors can enforce specific trafficking, for example, transgenic CXCR2 (CXCL1 receptor) improves trafficking to melanoma [89] and CCR2b to neuroblastoma [90]. On the other hand, blocking of migration inhibitory receptors like endothelin-B receptor improves T cell infiltration into the tumor lesion [91]. Targeting vascular endothelial growth factor (VEGF) receptor-2, which is over-expressed by tumor endothelial cells, improves vascular evasion of

CAR T cells [92]. Normalization of vasculature by low-dose angiogenesis inhibitors may also be efficacious in the long term [93]. T cells can penetrate the central nervous system (CNS) by migrating through the blood-brain barrier [94]; T cells also infiltrate other immune-privileged sites such as the testes and eyes [95]. The profound migratory capacity of T cells allows the treatment of tumors which are otherwise difficult to access like brain tumors and prostate cancer. However, T cell extravasation and migration are frequently inhibited by various means including the loss of adhesion molecules on vascular endothelial cells [96–98], an altered chemokine milieu [99, 100] and immune suppression by a plethora of inhibitory molecules. Thus, the risk of targeting healthy tissues and the localization of bulky tumor mass demands to decide between local and systemic application of T cells. In some trials, CAR T cells are locally applied by endoscopy or puncture into or in near vicinity of the tumor lesion [101].

Second, tumor tissues execute immune suppression by various means through cells like regulatory T (Treg) cells or myeloid-derived suppressor cells (MDSCs), suppressive cytokines such as IL-10 or TGF-β, or other factors such as IDO, glucose depletion, nutrient deprivation, and acidosis. The inhibitory ligands in tumors are furthermore increased upon an immune attack. On the other hand, CAR T cells express immune repressive receptors upon activation, including programed cell death-1 (PD-1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), or Fas, which upon ligand interaction repress the T cell response. To overcome the situation, a growing number of strategies are explored to make CAR T cells more resistant to repression. For instance, the anti-tumor activity of CAR T cells in the presence of Treg cells is improved by abrogation of IL-2 release through deletion of the lck binding site in the CD28 CAR signaling domain [102]. On the other hand, CAR-mediated CD28 costimulation overcomes TGF-β repression resulting in an improved tumor cell killing [103]. Suppression by TGF-β can moreover be prevented by engineering T cells with a dominant negative mutant of TGF-β [104, 105]. PD-1 upregulation within the tumor suppresses the T cell anti-tumor response; blocking the PD-1/PD-1 ligand pathway through PD-1 antibody checkpoint blockade, cell-intrinsic PD-1 shRNA blockade, or a PD-1 dominant negative receptor, improves CAR T cell activity in a preclinical model [25].

PD-1 expression correlated with exhaustion is moreover triggered by the CAR provided costimulation. In particular, CAR T cells with 4-1BB costimulation are less exhausted upon repetitive re-stimulation and retained their cytotoxic and cytokine secretion functions longer than CD28 CAR T cells. PD-1/PD-L1 blockade may be an effective strategy for improving the potency of CAR T cell therapies. Accordingly, antibody-mediated checkpoint blockade is currently explored in a clinical trial; PD-1 and CTLA-4 blockade are also explored in combination. In addition, antibodies to neutralize immune suppressive cytokines including GM-CSF, IL-6, IL-10, and VEGF may also improve the CAR T cell response. In the end, the best combination of checkpoint blockades to tackle the complex network of immune repression needs to be explored in clinical trials.

Third, some solid tumors have a strong stroma barrier which hampers the penetration of CAR T cells into the lesion. Evidences are increasing that successful treatment of advanced tumors requires breaking the barrier and eliminating the stroma cells; the latter is mediated by IFN-γ accompanied by M1 macrophage infiltration [106]. Costimulation increased IFN-γ release

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into the targeted tumor lesion which then acted on stroma cells in an antigen-independent fashion by both stroma destruction and activating the non-T cell immune compartment.

13. CAR T cell therapy-associated toxicities

CAR redirected T cell targeting is antigen specific, however, mostly not tumor selective as long as antigens are targeted which are also expressed by healthy cells. For instance, targeting CD19 produced a lasting depletion of healthy B cells which is clinically manageable by substitution with immunoglobulins and by antibiotic protection. In addition, antigen-independent toxicities may occur when a huge number of CAR T cells are heavily activated. The following toxicities are frequently observed (**Table 1**).

Limitations & challenges	Potential solutions
CAR design	Identify best suitable combination of CAR domains for targeting, T cell activation and counter-acting suppression in the specific tumor tissue by evaluating a panel of results effective variables, including the targeted epitope, the distance of the epitope from the membrane, the affinity of the targeting scFv, the spacer and transmembrane domains, the primary and co-stimulatory signaling, the number of CARs on T cell surface
T cell suppression	Make CAR T cells more resistant toward repression, for instance by secreting inhibitors of immunosuppressive cytokines such as IL-6, IL-10, and TGF-β; by secreting inhibitors of factors involved in the induction of MDSCs or Treg cells; by modified CD28 signaling deficient in IL-2 induction
PD-1/PD-L1 upregulation within the tumor tissue	Interfere PD-1/PD-L1 pathway through antibody checkpoint blockade; PD-1 suppression by shRNA; coexpression of a PD-1 dominant negative receptor
Impaired T cell migration and trafficking	Engineer CAR T cells to express chemokine receptors such as CXCR2 or CCR2b
Cytokine release syndrome (CRS)	Apply fractionated T cell doses; neutralize IL-6 function by application of tocilizumab
Vascular leakage syndrome (VLS)	Substitute peripheral blood volume; deplete serum from cytokines by plasmapheresis
Tumor lysis syndrome (TLS)	Reduce CAR T cell dose; split CAR T cell dosing; de-bulk tumor mass before CAR T cell therapy
Macrophage activation syndrome (MAS)	Neutralize IL-6 function by application of tocilizumab
Neurotoxicity	No specific treatment so far available; toxicity is transient and fully reversible
Anti-CD19 CAR T cell induced B cell aplasia	Replace immunoglobulins; provide antibiotic prophylaxis; co-express an inhibitory CAR to protect normal B cells; target an alternative, more selective antigen

Limitations & challenges	Potential solutions
"on-target off-tumor" toxicities	Identify more tumor-selective antigens, e.g., tumor-specific antigens, neo-antigens, antigens expressed only by non-essential healthy tissues, antigens physiologically expressed on apical surfaces, activation associated antigens; block target antigen with high dose of a specific antibody; co-express iCARS to prevent activation against healthy tissues; use combinatorial antigen recognition by two complementing CARs; use CARs with optimized recognition of cancer cell associated antigens; express the CAR transiently by RNA transfer; activate the CAR conditionally by a dimerizer; administer CAR T cells intratumorally
CAR T cell elimination	Co-express suicide genes, e.g., HSV-TK, iCasp9; deplete epitope marked CAR T cells by antibodies, e.g., targeting truncated CD34, EGFR, CD20; use a conditional CAR which is inactive in the absence of a dimerizer
GvHD after allogeneic T cell therapy	Engineer T cells with genetically edited endogenous TCR and HLA molecules
Commercialization	Provide "off-the-shelf" CAR T cell products with genetically edited allogeneic T cells
Tumor relapse by antigen escape cancer cells	Target more than one antigen by applying a CAR T cell mixture or T cells engineered with bispecific CARs
Poor in vivo expansion	Improve patient's pre-conditioning and/or cytokine supplementation

Table 1. Limitations, challenges, and potential solutions of CAR T cell therapy.

(i) "On-target on-tumor" toxicity describes a tumor lysis syndrome which is mediated through the rapid destruction of a large tumor mass in response to therapy. The release of tumor cell components into the circulation causes electrolyte and metabolic disturbances which can induce multi-organ failure.

(ii) "On-target off-tumor" toxicities occur when CAR T cells engage their cognate antigen on healthy tissue. Such autoimmune toxicity can be life-threatening, in particular, when targeting lung, heart, liver, or other essential organs. In the case of anti-CD19 CAR T cell therapy of B-cell malignancies, "on-target off-tumor" toxicity consistently causes lasting B cell aplasia and hypo-gammaglobulinemia which are clinically manageable and considered as biomarkers for the anti-CD19 CAR T cell function. "On-target off-tumor" toxicity is more serious when targeting ErbB2 expressed by a broad variety of epithelia resulting in fatal cardio-pulmonary failure [107]. The strength of T cell activation clearly impacts the severity of "on-target off-tumor" symptoms; reducing CAR signaling and a more cautious dose-escalation regimen lowers the risk of toxicities [108].

(iii) "Off-target off-tumor" toxicity can be induced by CAR T cells independently of cognate target recognition. For instance, the extracellular IgG1 Fc spacer in the CAR can activate cells of the innate immune system such as NK cells and macrophages through binding to the IgG Fc receptor (FcγR) resulting in a systemic inflammatory reaction. Modification of the CAR IgG1 Fc domain [14] or the use of the IgG4 domain reduces the risk of this type of side effects.

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(iv) Activation of a huge number of T cells results in the release of extensive amounts of pro-inflammatory cytokines, in particular IFN-γ and TNF-α, and the release of IL-6 upon activation of monocytes or macrophages, causing a cytokine release syndrome (CRS) with the risk of multiple organ failures. CRS is clinically characterized by fever, nausea, and supra-physiological serum levels of pro-inflammatory cytokines and is closely associated with the systemic macrophage activation syndrome, resembling hematophagocytic lymphohistiocytosis. CRS may also occur together with the vascular leakage syndrome (VLS). The occurrence of CRS is associated with clinical efficacy, a high tumor burden and the dose and potency of applied CAR T cells. However, CRS constitutes a major limitation of CAR T cell therapy, and the clinical management of cytokine-related toxicities is still challenging. The clinical symptoms can be reduced without diminishing the therapeutic efficacy by applying the anti-IL-6 receptor antibody tocilizumab which blocks the IL-6 receptor without eliminating the CAR T cells [109]. An algorithm of treatment has recently been proposed based on tumor burden, age, comorbidities, and other factors to standardize grading of CRS and to develop clinical guidelines for treatment [110, 111].

(v) Apart from CRS, neurotoxicity with aphasia, hallucinations, and delirium, is observed in about 40% of treated patients after CAR T cell application [112]. Neurotoxicity was mostly reversible and may be due to a diffuse encephalopathy caused by IL-6 released by brain infiltrating CAR T cells.

14. Strategies to improve safety of the CAR T cell therapy

Strategies were developed to improve safety while maintaining efficacy against tumors; these include target recognition, CAR design and expression as well as CAR T-cell elimination.

(i) Combinatorial antigen recognition

While truly tumor-specific antigens are rare, a pattern of antigens may be more indicative for cancer cells than the expression of a single marker. Redirecting CAR T cells specifically toward such an antigen signature is thought to provide more selectivity for cancer cells while sparing healthy cells. Therefore, two CARs recognizing two different antigens on cancer cells are co-expressed as follows: one CAR providing the primary activating signal and the other CAR providing the costimulatory signal [50–52]. Since both signals are required, only simultaneous engagement of both antigens initiates a productive T cell anti-tumor response while binding to one antigen is not sufficient.

(ii) Inhibitory CARs

A co-expressed inhibitory CAR (iCAR) is aimed at avoiding T cell activation when engaging healthy cells. The iCAR inhibitory signaling moiety is derived from the intracellular PD-1 or CTLA-4 signaling domain, provides a suppressor signal to the T cell upon recognition of an antigen present on healthy, but not on tumor cells, and is dominant over the activating signal provided by tumor-specific CAR, thereby preventing T cell activation against healthy tissues

[63]. The inhibitory effect by iCARs is present as long as the iCAR engages its cognate target; without iCAR signaling the co-expressed activating CAR triggers the T cell response toward the cognate cancer cell.

(iii) Optimized antigen recognition domain

To make a CAR more tumor selective the antigen binding domain was mutated with respect to improve binding to a variant antigen which is expressed by the cancer cells and less by healthy cells. For instance, a CAR was optimized in binding to the IL-13 receptor-α2 of cancer cells but less to the IL-13 receptor-α1 on healthy tissues [8, 9].

(iv) Transient CAR expression

The transient expression of the CAR by T cells transfected with in vitro transcribed RNA limits the CAR T cell response. The transfected RNA is diluted upon T cell division and degraded with time resulting in a half-life of CARs on the T cell surface in the order of several days. With this rationale, RNA-modified CAR T cells were applied with some anti-tumor efficacy so far [113]; however, repeated doses of CAR T cells produced an anti-CAR response due to xenogenic CAR components [114]. Upon activation of CAR T cells, the time of CAR expression is moreover shortened thereby limiting a potential side effect on healthy tissues [115].

(v) CAR T cell elimination

In case of non-controlled toxicity, CAR T cells can be eliminated by various means including a high-dose steroid treatment as applied in a trial with carboanhydrase IX-specific CAR T cells [116]. Another strategy takes an advantage of marking CAR T cells with a unique cell surface molecule to which an approved therapeutic antibody binds. For instance, the truncated EGFR, co-expressed with the CAR by the same T cell, can be targeted by the antibody cetuximab which efficiently eliminates those marked cells [117]. Efforts are also being undertaken to co-express the targetable epitope within the extracellular part of the CAR, thereby making the CAR itself a target of a depleting antibody [118]. An anti-idiotypic antibody directed against the scFv of the CAR itself may be used for depleting CAR T cells as well [119]. Alternatively, CAR T cells can be eliminated by the action of suicide genes. Basically, two strategies are currently explored, the co-expression of the herpes simplex virus thymidine kinase (HSV-tk) which phosphorylates the guanosine analog gancyclovir into a toxic derivative, or the co-expression of a truncated caspase-9 and a mutated FK506 binding protein which mediates dimerization through a non-toxic synthetic drug, thereby initiating the caspase-9 apoptotic cascade [120].

(vi) Routes of T cell administration

CAR T cell–associated toxicities are mitigated by applying the T cells in tumor burden-adapted doses or in fractionated doses. Usually CAR-modified T cells are administered by i.v. injection upon which the cells accumulate in the lung within 30 min and later on in the liver and spleen [121, 122]. Where possible, more localized routes of CAR T cell administration may avoid off-tumor T cell activation to some extent. For instance, CAR T cells are administered by endoscopic injection into the tumor lesion or liver metastases in some trials [123, 124].

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15. Future perspectives

CAR T cell therapy of hematological malignancies is likely to become established in clinical practice within the next years; CAR T cell therapy for the treatment of solid tumor lesions or for the elimination of residual cancer cells is still in its infancy. The situation is even more complex since the currently accumulating clinical results are difficult to compare due to a number of differences in CAR T cell engineering, clinical protocols, preconditioning of patients, and other relevant factors, demanding a more rigorous standardization of CAR T cell trials in the near future.

(i) Identification of the most suitable target

Tumor-selective antigens are preferred targets, however, are rare demanding the use of tumor-associated antigens as targets for a redirected T cell therapy. An example of a tumor-selective antigen is a glycosylation variant, like Muc1, or a specific mutation of protein identified by deep sequencing the cancer cell transcriptome. By decreasing selectivity, the risk of targeting healthy tissues with life-threatening toxicities increases demanding a thorough preclinical evaluation of potential other target cells and a cautious dose-escalation regimen when entering clinical exploration. Combinatorial antigen recognition, transient CAR expression, and inhibitory CARs are some examples which are expected to increase the safety in CAR T cell targeting, however, still need clinical exploration in a specific tumor context.

(ii) Optimizing the CAR design

There is obviously no universal CAR design which equally fits to each potential antigen; each CAR needs to be optimized with respect to both the particular target and to the T cell subset which is used for the anti-tumor attack. So far, the binding affinity, the targeted antigen epitope, and the extracellular length and transmembrane domain of the CAR as well as the provided costimulation were identified to be crucial for optimal T cell activation in the specific context.

(iii) T cell stage of maturation

CAR T cells with 4-1BB costimulation are primed toward a central memory phenotype, have a more enhanced catabolic activity and oxidative metabolism together with an enhanced mitochondrial respiratory capacity. With that respect, the 4-1BB CAR initiates a long-lasting central memory response while the CD28 CAR mediates a more short-lived effector cell response with enhanced glycolysis [24]. Moreover, the required co-signaling for optimal activation seems to be different for T cells in various stages of maturation; different co-stimuli are needed in different stages of maturation upon repetitive re-stimulation. For instance, T cells in the $CCR7^-$ maturation stage benefit from CD28-OX40 costimulation while CD28 costimulation is sufficient in younger stages of maturation [21].

(iv) Patient preconditioning

Non-myeloablative lymphodepletion of patients prior to T cell therapy seems to be mandatory to allow extensive amplification of CAR T cells after application. There may also be an additional impact of the preconditioning chemotherapy on the tumor milieu by depleting suppressor cells and/or releasing antigens recognized by patient's T cells. With that respect,

the clinical protocols substantially differ and need further standardization to allow conclusions with relevance for future trials.

(v) Clinical exploration and control of side effects

CAR T cell therapy-associated toxicities are life-threatening and need intensive care treatment. With the establishment of a CRS screening and grading protocol [110–112], first steps toward a more standardized clinical management are being made which need further attention in future trials. With the progression in the clinical exploration of CAR T cells, there is a need for novel pharmacokinetic and pharmacodynamic models to sustain the development of optimized mono- and combo-immunotherapies in the near future.

(vi) Hematopoietic stem cell transplantation

A number of patients in clinical remission after CAR T cell therapy were subsequently treated by hematopoietic stem cell transplantation, in particular patients treated with CD28 CAR T cells. While the anti-leukemia efficacy of CAR T cells is clearly documented, the benefit of stem cell transplantation to control the disease in the long term needs to be established.

(vii) SynNotch receptor-mediated immune cell activation

In a broader sense CARs are tools in the growing field of synthetic biology to engineer immune cells with defined specificity and to redirect a cellular response. As such, a modular receptor was designed on the basis of the Notch. At the extracellular side, the receptor binds to targets by an scFv or any other binding domain and at intracellular side effector domains such as transcriptional activators or repressors are released upon proteolytic cleavage which enter the nucleus for function [59, 125, 126]. Such receptors can be used in a broad variety of cells including immune cells to direct the induction of complex cellular responses [59]. The benefit and potential of such receptor types in the adoptive T cell therapy need further exploration.

(viii) CAR T cell–induced anti-tumor immunity

The CAR T cell response against targeted cancer cells can induce a second wave of anti-tumor immune response which is potentially capable of eliminating non-targeted cancer cells. In particular, mice treated with anti-EGFRvIII CAR T cells were shown to resist EGFRvIII negative tumor challenge [127]. The perception rises that a redirected T cell anti-tumor response may additionally have substantial impact on the host immune response itself than rather targeting the cognate cancer cells. Such secondary host response needs to be explored and improved toward a long-term control of cancer.

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Abbreviations

ACT	adoptive cell therapy
CAR	chimeric antigen receptor
CRS	cytokine release syndrome
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
CSC	cancer stem cell
GMP	Good Manufacturing Practice
IFN	interferon
IL	interleukin
ITAM	immunoreceptor tyrosine activation motif
MHC	major histocompatibility complex
PD-1	programed cell death-1
scFv	single-chain fragment of variable region
TCR	T cell receptor

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