CAR T Cells: A Snapshot on the Growing Options to Design a CAR

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Abstract

Adoptive cell therapy of malignant diseases with chimeric antigen receptor (CAR) modified T cells rapidly advanced from pre-clinical models to commercial approvals within 2 decades. CARs redirect patient's T cells towards cancer cells and activate the engineered cells for a cytolytic attack resulting in the destruction of the cognate target cell. CAR T cells have demonstrated their powerful capacities in inducing complete and lasting remissions of leukemia/lymphoma in an increasing number of trials worldwide. Since the early 90's, the design of CARs went through various steps of optimization until the very recent developments which include CARs with logic gating in the recognition of antigen patterns on target cells and TRUCKs with a target recognition induced delivery of immune modulating agents. Here we review the generations in CAR design, the impact of specific modifications, the strategies to improve the safety of CAR T cell therapy, and the challenges to adapt the CAR design for broader applications.

Introduction

Adoptive therapy with tumor-isolated and ex vivo amplified T cells is showing spectacular success in the treatment of malignant diseases supporting the overall concept that the patient's immune system can control cancer in the long-term. In particular, tumor infiltrating lymphocytes (TILs), isolated and expanded from melanoma lesions, are capable in inducing tumor regressions and long-term remissions in a substantial number of patients.[1] The antigen specificity of most TILs is frequently not known, however, assumed to be redirected towards the respective tumor from which the cells were isolated. The assumption is supported by the recent report that the T cell receptor (TCR), isolated from TILs from a mammary tumor lesion and engineered on peripheral blood T cells, was capable to induce tumor regression.[2]

However, the number of available TCRs with known specificity for tumors is still limited and cancer cells frequently lose the capacity to present antigen, either by deficient antigen processing or by suppressed expression of the major histocompatibility complex (MHC). In this situation Zelig Eshhar and colleagues (Weizmann Institute of Science) designed a chimeric antigen receptor (CAR), previously called "immunoreceptor" or nick-named "T-body", which consists in the extracellular moiety of an antigen binding and in the intracellular moiety of a signaling domain capable to initiate T cell activation upon antigen engagement.[3] The CAR is a composite receptor which for binding frequently uses a single chain fragment of variable region (scFv) antibody; the T cell activating signal is mostly transmitted through the TCR CD3ζ signaling chain in the intracellular part with or without a linked costimulatory moiety (Fig. 1). Engagement of cognate antigen on the surface of cancer cells by the CAR engineered T cell initiates a cascade of signaling events resulting in T cell activation and an antigen-specific response towards the cognate target cells.[3,4]

Adoptive therapy with CAR modified T cells takes advantage of the power of T cells and other cytolytic immune cells that actively migrate through vascular endothelia and penetrate tissues, become activated and amplify upon antigen engagement, and eliminate cognate target cells by granzyme/perforin mediated killing. As a result, tumor antigens are massively released at the site of tumor recognition which are captured and cross-presented by tumor infiltrating DCs, monocytes, and tumor-associated macrophages which recruit and initiate a second wave of immune response.[5]

By using an antibody for target recognition, CAR T cells can recognize target cells independently of MHC presentation of antigen. Consequently, CAR T cells can recognize target cells with downregulated MHC or deficiency in the antigen processing machinery and, moreover, can recognize other targets than proteins, like carbohydrates and lipids, which broadens the panel of potential targets compared with the TCR mediated T cell recognition. CAR T cells are repetitively re-stimulated as long as the antigen is present; withdrawal from antigen turns the CAR T cell to energy or to entry into apoptosis; however, some CAR T cells can persist in the long-term and provide an antigen-specific memory as suggested by experimental models.[6]

After application to the cancer patient, CAR T cells amplify upon antigen engagement giving rise to increasing CAR T cell

Figure 1. The family of Chimeric Antigen Receptors (CARs). The CAR is a recombinant composite receptor that specifically binds a target and provides host cell activation in a well-defined and predictable fashion. On the intracellular side, the CD3ζ activating signaling domain or alternatively the Fc ε receptor-I (FcεRI) γ-chain is used to provide the primary signal; the linked costimulatory domain provides the secondary activating signal required for full and lasting T cell activation. The extracellular CAR binding domain, the spacer, transmembrane and the intracellular signaling domains can be swapped with other domains making up the growing family of CARs. (A) The first, second, and third generation of CARs are defined by their signaling domains: the CAR with only the primary signaling domain (1st generation), with an additional costimulatory domain (2nd generation) or with combined costimulatory domains (3rd generation). CARs of 4th generation, so-called TRUCKs, in addition release a transgenic protein of interest (POI) upon CAR signaling, for instance a cytokine like IL-12 or IL-18. (B) Two co-expressed CARs can integrate the antigen recognition in a specific and logic fashion. T cells with 2 co-expressed, fully signaling CARs are activated upon engagement of either antigen 1 or antigen 2 (Boolian "OR" computation) while T cells with a primary CAR and a costimulatory CAR are only fully activated upon simultaneous engagement of both antigen 1 and antigen 2 (Boolian "AND" computation). T cells with a second generation activating CAR recognizing antigen 1 and an inhibitory CAR recognizing antigen 2 are only activated if no signaling by the inhibitory CAR occurs ("antigen 1 but no antigen 2"); in case of engaging both antigens the T cell is

numbers of CAR T cells in the peripheral blood within the first weeks; anti-CD19 CAR T cells and concomitant B cell aplasia can persist for years.[7] There is increasing evidence that CAR T cells need to persist in a substantial number and with active effector functions providing long-term control of the disease; relapse of the disease was frequently observed when the number of CAR T cells in the patient after initial amplification dropped to undetectable levels. In some cases of CD19 CAR T cell loss, relapse was heralded by recovering healthy B cells. In this situation, monitoring healthy B cell counts may identify patients with a high risk of relapse.[8] In the treatment of neuroblastoma, duration of CAR T cell persistence in the peripheral blood beyond 6 weeks was associated with superior clinical outcome; long-term persistence was concordant with the percentage of $CD4^+$ cells and central memory cells in the manufactured cell product[9] implying that those cells promote the extended low-level persistence in patients. Experimental models indicate that in the presence of constant antigenic stimulation CAR T cells can provide long-term, antigen-specific tumor rejection,[6] moreover indicating that there is a persistent, functional population of CAR T cells capable to re-expand and eradicate secondary tumor challenge. The endogenous CD19 antigen seems to be required to promote persistence of $CD4^+$ CAR T cells, and to some extend of $CD8^+$ T cells, implying repetitive antigen stimulation as driving force for CAR T cell persistence in the long-term.[10] CAR T cell persistence in the long-term is thereby not equivalent to canonical T cell memory in the absence of antigen. Classical T cell memory depends on appropriate costimulation; costimulation through ICOS increased the number of $CD4^+$ CAR T cells which in turn promote persistence of $CD8^+$ CAR T cells redirected by a CD28 or 4–1BB signaling CAR.[11] As a consequence, a third generation CAR providing ICOS and 4-1BB costimulation may be superior in promoting long-term CAR T cell persistence and anti-tumor activities. However, some patients stayed in remission although CAR T cells dropped below detection limit in the peripheral blood[12] which indicates that apart from the specific CAR T cells, a recruited secondary immune response may also substantially contribute to control the disease.

Treatment of B cell malignancies with CD19 specific CAR T cells induces clinical and molecular remissions with high frequencies which can last over years.[13] However, CD19 specific CAR T cells deplete also the healthy $CD19^+$ B cell compartment resulting in hypogammaglobulinemia within the first weeks of CAR T cell infusion; most plasma cells are spared by anti-CD19 CAR T cells due to lack of CD19.[14] The symptoms due to B cell aplasia are clinically manageable by immunoglobulin replacement and in some cases by prophylactic antibiotic therapy.[15,16] B cell depletion rapidly reverses after CAR T cell ablation[17] which, however, not necessarily indicates a pending relapse of the disease; complete lymphoma remissions can continue after the disappearance of an effective anti-CD19 T cell response.[12] Recovering non-malignant B cells in peripheral blood of treated

patients were $CD19^+$ and $CD20^+$, were polyclonal, and of naive phenotype indicated by IgD and lack of CD27.

Up to today, more than 1000 patients were treated with anti-CD19 CAR T cells in the US alone. Currently, 439 clinical trials are listed, more than 270 trials are actively exploring CAR T cells in the treatment of hematologic and solid cancer around the world with clear hotspots in frequencies in the US and in China; a minority of trials are performed in Europe (https://www.the-scientist.com/infographics/cell-and-gene-therapy-tracker-64450). Based on the considerable remission rate in patients with B cell lymphoma, the anti-CD19 CAR T cell product tisagenlecleucel (Kymriah™, Novartis) was approved on August 30th, 2017, by the U.S. Food and Drug Administration (FDA) and subsequently by the European Medicines Agency (EMA) for the treatment of relapsed and/or refractory B cell acute lymphoblastic leukemia. Shortly thereafter, the second anti-CD19 CAR T cell product axicabtagene ciloleucel (Yescarta™, Gilead/Kite) was approved for the treatment of adult patients with relapsed and/or refractory large B cell lymphoma (https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm574154.htm). The anti-CD19 CAR T cell product lisocabtagene maraleucel (liso-cel; JCAR017, Celgene/Juno) received an FDA breakthrough therapy designation for the treatment of DLBCL.

The mentioned anti-CD19 CARs differ in their design; the Kymriah™ CAR signals through 4-1BB-CD3ζ as does the JCAR017 while the Yescarta™ CAR uses the CD28-ζ chain. While CD28 costimulation induces IL-2 release along with IFN-γ secretion and rapid expansion of CAR T cells, costimulation through 4-1BB does not induce IL-2, but IFN-γ secretion and a more prolonged T cell activation and amplification period. All 3 CARs use the murine FMC63 scFv for binding while the extracellular hinge and transmembrane domains differ: Kymriah uses the CD8, Yescarta the CD28 and JCAR017 use the IgG4 hinge and transmembrane domains. The Kymriah and JCAR17 CAR are transduced into T cells by lentiviruses while the Yescarta CAR by retroviruses. Given the CAR design, the functional capacities of engineered T cells and the patient eligibility differences caution needs to be taken when comparing data across studies.

Recent efforts have broadened the potential of CAR T cell therapies beyond oncology towards the treatment of auto-immune diseases. In particular, regulatory T cells (Tregs) were engineered with and activated by CARs[18]; in a pre-clinical model, FoxP3 induced Treg cells were redirected by an engineered CAR towards myelin oligodendrocyte glycoprotein (MOG) in order to repress murine experimental auto-immune encephalomyelitis.[19] T cells were engineered with CAR redirected specificity for the auto-antigen Dsg3 to eliminate auto-immune B cells in order to treat auto-immune diseases like pemphigus vulgaris.[20]

CAR T cell therapy is frequently associated with toxic side effects, mostly due to on-target effects depending on the antigen specificity of the CAR and the degree and kinetics of redirected T

blocked by the inhibitory CAR. A bispecific CAR (TanCAR) transmits the activating signal upon engagement of either antigen 1 or antigen 2 or both. (C) To switch-on the conditional CAR, a synthetic dimerizer molecule is administered that links the primary signal to the costimulatory CAR; upon antigen engagement and in the presence of the dimerizer, the CAR provides the signal for a lasting T cell stimulation. Withdrawal from dimerizer results in dissociation of the signaling domains and abrogates T cell activation despite antigen engagement. The synNotch system uses a receptor molecule to switch on the CAR expression; upon antigen 1 binding the receptor releases a transcription factor that induces the expression of the CAR that provides full activation upon recognition of antigen 2. (D) The inhibitory CAR (iCAR) provides a blocking signal upon antigen engagement. On the other hand, a CAR that engages an inhibitory ligand (like PD-L1) can provide an activating signal to the T cell thereby converting a physiologically blocking signal into an activating signal. (E) The scFv antibody is commonly used for binding; various other binding domains can also be used for antigen recognition. In case no scFv is available, the physiological immunoglobulin-G (IgG) light and heavy chains can be coexpressed and used for binding; the IgG heavy chain is anchored to the cell membrane and linked to the signaling chains; the IgG light chain associates with the Ig heavy chain to build up the antigen binding domain.

cell activation. Most toxic effects are reversible or disappear upon CAR T cell elimination or termination of engraftment. This is in contrast to cytotoxic chemotherapy that can cause permanent genetic alterations in healthy tissues with substantial risk of secondary malignancies in the long-term. For further information on clinical aspects, we refer to a recent review by June and Sadelain.[13]

We here discuss some major aspects in the CAR design and review recent developments towards a safer CAR which intend to make T cell therapy applicable to a broad variety of diseases and to a growing number of patients.

The modular composition of the CAR

The prototype CAR comprises in the extracellular domain a single chain fragment of variable region (scFv) antibody for binding and a spacer of various lengths linked to a transmembrane domain. The intracellular signaling moiety is mostly derived from the CD3ζ intracellular chain with or without a linked costimulatory domain (Fig. 1A). The scFv is genetically engineered by joining the coding regions of the heavy chain (V_H) and light chain (V_L) variable regions of an antibody in the order V_H-linker-V_L or V_L-linker-V_H while the linker is frequently a short glycine-serine peptide sequence. The activating domain is mostly the CD3ζ intracellular chain of the TCR; the Fcϵ receptor-I (FcϵRI) signaling γ-chain is used in some constructs. CARs of the "first generation" contain only the primary signal (signal-1) like CD3ζ or FcϵRI γ while "second generation" CARs in addition harbor a costimulatory moiety (signal-2), like CD28, 4-1BB, OX40, ICOS, or CD27, mostly at the membrane proximal position followed by CD3ζ in the distal position. Both the primary signal-1 and the costimulatory signal-2 are required for inducing full T cell activation and for protecting from activation-induced cell death.[21-23] Consequently and in contrast to the first-generation CARs, T cells redirected by the second-generation CAR show durable in cytokine release, amplification and anti-tumor activity making them suitable for clinical applications. "Third-generation" CARs contain a combination of costimulatory domains along with the primary signal and provide some benefit in sustaining survival of more matured T cells.[24]

The overall modular design of the prototype CAR has several advantages with respect to both the exchange of binding and signaling moieties on the engineering level and the combination of the antigen recognition with the T cell activating machinery on the functional level.

(a) The scFv antibody provides targeting specificity to the CAR; exchange of scFv facilitates redirecting T cells towards a variety of targets. Antibody-mediated recognition by the CAR is independent of antigen presentation by the MHC; this is of advantage in case of recognizing cancer cells, which frequently lose MHC expression or are deficient in antigen processing. The CAR can potentially recognize any targetable epitope for which an antibody is available, including carbohydrates, lipids, or variants of an antigen.

(b) CAR targets need to be cell surface antigens that are accessible to the CAR binding domain; intracellular antigens are not visible to CAR T cells. However, by using an antibody recognizing a specific peptide/MHC complex, CARs can provide TCR-like specificity to the host cell as shown for a CAR with specificity for HLA-A2/NY-ESO-1 peptide.[25,26]

(c) The use of a scFv single chain antibody for CAR targeting moreover allows the design of a 1-polypeptide-chain receptor molecule. However, by the conversion from a native antibody, a number of scFvs lose their specificity and affinity. To circumvent the situation, we proposed an alternative CAR format that is composed of two, non-covalently linked polypeptide chains, that is, 1 chain is composed of the Ig heavy chain with the variable and constant region linked to the transmembrane and signaling moieties; the second chain is composed of the Ig light chain without a linked transmembrane domain (Fig. 1E). When co-expressed both chains spontaneously associate forming a fully functional antibody anchored to the T cell membrane by the linked intracellular signaling domain to provide T cell activation upon antigen engagement.[27]

(d) Instead of antibodies, any other binding domains or ligands of natural receptors can be alternatively used for targeting. As an example, mutated IL-13 was linked to the extracellular CAR moiety for targeting the IL-13 receptor-α2 which is over-expressed by a broad variety of solid tumors but less by healthy tissues, thereby providing improved cancer selectivity.[28-30] Adnectin, a protein fragment derived from fibronectin, was used for targeting the epithelial growth factor receptor (EGFR).[31] Another example of an alternative binding domain is a designed ankyrin repeat protein (DARPin) that is composed of ankyrin repeats of 33 amino acids in length and forms a β-turn followed by 2 anti-parallel α-helices and a loop reaching the β-turn of the next repeat.[32]

(e) The CAR-mediated T cell activation requires a distance of about 15 nm between the T cell and the target cell[33] as it is the case for the physiological TCR-MHC interaction. To allow optimal interactions a spacer is inserted into the extracellular moiety between the antigen binding and the transmembrane domain; targeting a membrane distal epitope on the antigen requires a short spacer, targeting a proximal epitope requires a long spacer within the CAR. By using spacers of various lengths, the CAR-redirected T cell activation can be optimized. For empiric adjusting the distance of the binding domain to the membrane, the constant region of IgG1 is practical since it allows creating spacers of various lengths, that is, by using the CH1-CH2-CH3 or CH2-CH3 or CH3 moiety.[34] Fine tuning the spacer length adjusts the distance between the T cell and the target cell and thereby improves the CAR T cell activation and finally efficacy in eliminating the target cells.[35] Higher order structural requirements and CAR dimerization driven by the extracellular spacer domain may additionally impact T cell activation demanding a more thorough exploration in the context of a particular target antigen. The currently used anti-CD19 CARs harbor different spacers, that is, the Kymriah[TM] CAR harbors the CD8 hinge domain, Yescarta[TM] the CD28 spacer domain and the JCAR017 the IgG4 hinge domain. While there are additional differences between the individual CARs, the different spacers may create different lengths of the extracellular CAR domain and thereby different distances between the CAR T cell and the CD19$^+$ B cell to initiate a cytolytic attack. The differences in CAR design demand a more detailed analysis to understand the clinical performance of the various anti-CD19 CAR T cells.

The commonly used IgG1 constant domain as a spacer in the extracellular CAR moiety can bind to Fcγ receptor (FcγR) (CD64) with the risk to initiate an unintended "off-target" activation of both T cells and myeloid cells. To avoid the situation, the FcγR binding motif within IgG1 CH2 was modified by deleting the Asn297 glycosylation site[36,37] or by deleting the IgG1 CH2 domain to abrogate binding to Fc

receptors.[35] CARs with the CH2CH3 spacer moreover show a pronounced tonic signaling which accelerates T cell senescence which can be mitigated by removing the CH2 region.[35] These specific IgG1 properties provide the rationale to use alternative spacers like the constant domain of IgG4 or the extracellular domains of CD4 and CD8.[4,36]

(f) CARs of the prototype design are also functional in NK cells based on the capacity of the TCR derived signaling domain to efficiently recruit and associate with kinases to initiate a productive signaling cascade in NK cells[38–44]; NK cell-derived signaling chains, like DAP10 or DAP12, are also applied.[45–47]

The growing family of CARs

TRUCK: a CAR T cell producing and releasing a transgenic protein product

CAR T cells target the defined tissues and are activated upon antigen engagement; the mechanism can be used to release a transgenic polypeptide product "on demand" upon CAR signaling. CAR T cells are additionally engineered with an expression cassette for the transgenic protein in order to deliver the protein in a therapeutic concentration in the targeted tissue while the protein concentrations in the periphery remain low. "T cells redirected for universal cytokine-mediated killing", so-called TRUCKs or the "fourth generation" of CAR cells, combine the redirected CAR T cell attack with the locally restricted release of a biologically active protein while avoiding its systemic toxicity (Fig. 1A).[48] The protein for release can be produced in a constitutive or in an inducible fashion upon CAR signaling; the expression of the inducible protein is under control of the $NFAT_6$-IL-2 minimal promoter that induces protein synthesis upon CAR signaling and remains silent as long as no T cell activation occurs. The TRUCK concept is universal with respect to the delivered protein; nearly every protein can be produced and released in this fashion converting CAR T cells into "living factories". The delivery of IL-12 and IL-18 to the targeted tissue was reported to induce different biological effects[49–55]; CAR IL-12 T cells (IL-12 TRUCKs) recruited and activated an innate immune response in the targeted tumors,[51] resisted suppression by Treg cells,[52] and showed an increased cytokine release and expansion.[56] IL-18 TRUCKs converted the CAR T cells into Tbet^{high} FoxO^{low} killer cells with the reduction of Treg cells and the increase in Th17 cells in the targeted tumor tissue[54]; TRUCKs delivering both IL-12 and IL-18 were equally potent in this respect as IL-18 TRUCKs. The transgenic release of IL-15 improved T cell amplification and anti-tumor activity.[57] Since IL-15 is potentially leukemogenic,[58] IL-15 TRUCKs need to be controlled by a suicide gene in case of uncontrolled T cell amplification.[59] CAR T cells can be protected from oxidative stress through the release of catalase,[60] and tumor penetration can be sustained by targeting the tumor vasculature by delivering the soluble HVEM ectodomain[61]. A variety of other examples can be envisaged, some are currently evaluated in pre-clinical models. A clinical trial (NCT02498912) is currently testing CAR T cells targeting Muc16 and secreting IL-12,[62] other combinations will follow in the near future.

bispecific CAR T cells

Treatment with antigen-redirected therapies is associated with the risk of antigen/epitope loss and subsequent risk of relapse of the disease. This is the case in a substantial number of acute B cell

lymphoma/leukemia patients treated with CD19 CAR T cells.[63] To reduce the risk of relapse upon antigen loss, T cells are engineered with a CAR with 2 linked scFvs of 2 specificities (tandem CAR, "TanCAR"); binding to either antigen is sufficient to induce CAR T cell activation (Fig. 1B).[64] TanCAR T cells with specificity for CD19 and CD20 target even those leukemic cells which lost CD19 upon a primary CAR T cell attack.[65] Pediatric acute B cell lymphocytic leukemia with heterogeneity in CD19 and CD20 expression can be controlled by bispecific CD20-CD19 CAR T cells in a transplanted mouse model.[66] In the same line, dual antigen targeting is explored for the treatment of B-ALL by targeting CD19 and CD123,[67] CD22,[68] ROR1,[69] and immunoglobulin kappa light chain (Igκ).[70]

logic gating CAR T cells

By combining CARs of different specificities, CAR T cells can recognize an antigen pattern in a Boolean logic computation aiming at reducing toxicities towards healthy tissues. Two CARs of different specificities are co-expressed, 1 CAR providing the primary CD3ζ, the other CAR the costimulatory CD28 signal (Fig. 1B). Consequently, engagement of both antigens and thereby signaling through both signaling chains are simultaneously required to induce full T cell activation; engagement of only 1 antigen is not sufficient. Such Boolean "AND" gating was exemplarily shown for targeting ErbB2 and Muc1,[71] mesothelin and folate receptor-α,[72] and targeting PSCA and PSMA,[73] the latter using combined CD28 and 4-1BB costimulation. The synNotch system can alternatively be used for logic gating; synNotch activation controls the transcription of a CAR (synNotch CAR) for recognizing a second antigen on cancer cells for providing a signal required for final T cell activation (Fig. 1C).[74–76] Thereby a synNotch CAR integrates dual antigens through transcriptional regulation of CAR expression rather than through signaling.[77] In contrast to "AND" integration, a fully signaling CAR or a bispecific CAR provides "OR" computation which initiates T cell activating also upon engagement of either cognate antigen (Fig. 1B). T cells with a second-generation activating CAR recognizing antigen 1 and an inhibitory CAR recognizing antigen 2 are only activated if no signaling by the inhibitory CAR occurs ("antigen 1 but no antigen 2"); in case of engaging both antigens, the T cell is blocked by the inhibitory CAR (Fig. 1B).

CARs with "universal" antigen recognition

Changing the specificity of a prototype CAR requires engineering of a new CAR molecule. To make the strategy more flexible, a CAR with specificity for CD16 was used to capture any antibody through binding the constant domain.[78] By adding specific antibodies, CARs are loaded with defined tumor specificities; different antibodies provide different specificities to the same CAR T cell, for example, CD16 CAR T cells target Her2$^+$ cancer cells in the presence of the anti-Her2 antibody. CARs recognizing epitopes artificially linked to the targeting antibody can likewise be used, like CARs with specificity for fluorescein isothiocyanate (FITC),[79,80] biotin[81] or a protein epitope.[82] The strategy will have substantial potential in simultaneous targeting different antigens on tumors by the same CAR T cell loaded with various targeting antibodies. An additional benefit of the strategy is that the toxicity can be controlled by titrating the amount of targeting antibody; withdrawal from antibody administration is thought to reduce and limit potential side effects.

Conditional CARs

In case of uncontrolled toxicity, CAR T cells need to be selectively and rapidly eliminated, preferentially by a co-expressed suicide gene. CAR T cell apoptosis can be induced by dimerization of the transgenic inducible caspase-9 (iCasp9) upon addition of the dimerizing agent AP1903; as a result, more than 90% of T cells undergo apoptosis within minutes.[83–86] However, spontaneous dimerization of iCasp9 occurs to some extent in such engineered CAR T cells resulting in a certain baseline frequency of apoptotic cells. Alternatively, CAR T cells can be eliminated by antibody-dependent cellular cytotoxicity (ADCC) using antibodies targeting an integrated CAR domain, for instance by application of rituximab targeting a CD20 epitope integrated into the extracellular CAR domain, or a co-expressed truncated receptor molecule like truncated EGFR which is targeted by cetuximab.[87–89] It remains a matter of debate whether in case of toxicity cancer patients with a dysfunctional immune system have sufficient capacities to remove all CAR T cells by ADCC in due time. Another strategy uses the "switch-on/switch-off" key to control the CAR T cell response by splitting the CAR signaling moieties onto 2 polypeptide chains which dimerize in the presence of a small dimerizer molecule ("switch-on") while being dissociated without dimerizer and "switched-off" (Fig. 1C).[90,91] Increase in the concentration of the dimerizer improves CAR-mediated T cell activation and withdrawal switches off CAR activities. The CAR design is based on inducible MyD88/CD40 (iMC) to provide the activation switch upon administration of the drug rimiducid that links the 2 signaling domains required for T cell activation.[92]

In the opposed application, co-expressed inhibitory CARs (iCARs) with inhibitory signals instead of activating signals are used to block CAR T cell activation, for instance, when engaging antigens on healthy cells (Fig. 1D).[93]

CARs targeting inhibitory ligands

T cell activation is frequently blocked by inhibitory signals present in the tumor environment. To prevent T cell repression, CARs were engineered which target the inhibitory ligands by their extracellular domain and provide a stimulatory signal to the T cell by their intracellular moiety in order to overrun the inhibitory signals (Fig. 1D). For instance, a CAR recognizing PD-L1 through its extracellular PD-1 domain provides CD28 costimulation to sustain T cell activation when engaging PD-L1$^+$ target cells.[94–96]

Armored CAR T cells with recombinant cytokine receptors

In order to increase T cell amplification, CAR T cells were equipped with the transgenic IL-7 receptor-α chain with IL-2β signaling in order to stimulate T cell amplification in presence of IL-7.[97–99] Similarly, an IL-4 binding/IL-7 signaling receptor improved anti-tumor activity of T cells with anti-PSCA CAR in pancreatic cancer with increased IL-4 levels.[100] On the other hand, a dominant negative TGF-β receptor on CAR T cells competes with TGF-β in a melanoma model providing CAR T cell resistance in the presence of the suppressive cytokine.[101]

CAR T cell production for clinical application

For adoptive therapy, T cells from each individual patient are ex vivo genetically engineered with the CAR, amplified to clinically relevant numbers and re-infused to the patient after non-myeloablative pre-conditioning. In particular, the procedure includes collecting the cells by leukapheresis, genetic transduction by retro- or lentivirus infection or by RNA electroporation, T cell amplification, and quality control of the final cell product, all processes in accordance to good manufacturing procedure (GMP) guidelines.[102]

The viral transduction procedure has the advantage of a gentle gene transfer to the T cell by infection with low toxicity as long as the T cells are activated and replicate; lentivirus infection requires much lower activation than retroviruses. The viral vector integrates into the host genome in a "semi"-random fashion; episomal DNA vectors are gradually lost in cycling cells with the consequence of loss of transgene expression. While genotoxic and mutagenic effects elicited by vector insertion near proto-oncogenes may occur, to our best knowledge, no malignant transformation of mature T cells upon vector integration occurred in mature T cells during clinical trials so far. While genetic modification of T cells by lenti- and retroviruses is highly efficient, the virus production is extremely labor-, time- and cost-intensive including the generation and characterization of a master cell bank for virus production which makes non-viral transfection systems increasingly attractive.

Efforts are currently made to obtain permanently modified T cells by non-viral vectors; transposon-based vectors like Sleeping Beauty (SB) and PiggyBac as well as CRISPR/Cas9 mediated insertions are currently adopted to clinical CAR T cell applications.[103–105] The sleeping beauty transposon system combines the benefit of an integrating viral vector with long-lasting transgene expression. Naked mini-circle DNA, which can be easily produced under GMP conditions, is transferred into T cells by electroporation; the sleeping beauty integration takes place into "safe harbor" genomic loci with low risk of unintended genetic mutations. Delivering the sleeping beauty transposase by mRNA transfer and delivering a CAR by mini-circle DNA electroporation improves the transposition efficacy and finally the number of genetically modified T cells.[106] Compared with viral vectors, transposon-based vectors can transfer larger genetic elements into the target cell which makes the system convenient for co-expressing 2 or more CARs, for delivering larger transgenic "payloads" by TRUCKs, and for others.

The starting material for the manufacturing of the therapeutic T cell product is usually a leukapheresis product obtained from the patient. T cells are ex vivo isolated, stimulated, transduced by a retro- or lentivirus vector and amplified to obtain a final T cell product containing 10% to 50% CAR-modified cells. The manufacturing process from leukapheresis to the release of the final T cell product requires about 12 to 14 days.[107,108] Rigorous quality control management accompanies the entire process and governs the starting material, the genetic engineering and cell amplification process and the final cell product after harvesting. Most trials are using bulk CD3$^+$ T cell preparations engineered with the respective CAR; isolated CD4$^+$ and CD8$^+$ T cell subsets,[109] naïve T cells,[110] central memory[111] or memory stem cells[112] are also explored in some trials. The most suitable T cell population for CAR therapy is thought to be cells in a naïve or young central memory stage of maturation due to their prolonged persistence. In particular, CD45RO$^+$ CD62L$^+$ memory CAR T cells provide a more durable anti-tumor response than effector T cells.[112–114] Therefore T cells are ex vivo amplified in the presence of IL-7, IL-15 or IL-21 that improve clonotypic T cell persistence and survival and induce a robust cytokine release.[115–117]

The manufacturing process is currently an entirely hands-on manufacturing procedure; process controlled automatic manufacturing is increasingly applied for a variety of T cell products. For a detailed description of the manufacturing processes, we refer to Köhl et al.[102]

Obtained from patients with leukemia, the leukapheresis product and thereby the final CAR T cell product may be contaminated with leukemic cells, although the risk is virtually low. Recently, a single contaminating leukemic B cell was unintentionally transduced with an anti-CD19 CAR during the manufacturing process which initiated a CAR T cell refractory relapse of B cell leukemia.[118] The anti-CD19 CAR bound in cis to the CD19 epitope on the leukemic B cell masking it from recognition by the anti-CD19 CAR T cells. The event seems to be very rare since this is the only reported case of "epitope masking" upon unintentional transduction of leukemic cells out of currently 369 leukemia patients treated so far.

Perspectives and future developments

The ideal "universal" CAR T cell

For most clinical applications, patient's T cells are engineered and amplified on the individual basis which demands an individualized and cost-intensive manufacturing and delivery process. To overcome the limiting situation tremendous efforts are made to produce ideally "universal" allogeneic T cells that are produced in advance for the "off-the-shelf" administration to a number of patients.[119] In this line, donor T cells were disrupted in the TCR α-chain locus using transcription activator-like effector nucleases (TALENs) to obtain TCR-negative T cells which were finally engineered with the CAR.[119–121] In a first clinical application, TALEN edited CAR T cells were administered to 2 pediatric B-ALL patients for whom autologous T cells could not be produced in sufficient numbers, demonstrating the feasibility of the approach.[120] One patient, who was mismatched in all MHC-I alleles, developed graft-versus-host disease potentially due to the expansion of contaminating, non-edited CAR T cells and/or the elimination of edited, MHC-deficient CAR T cells by NK cells. To reduce the risk of MHC deficiency induced elimination, CAR T cells were engineered with HLA-E and deleted from HLA-A, -B, -C which prevents their recognition by host immune cells.[122] Basically the same TCR-negative T cells can be achieved using virus-transmitted zinc finger nucleases[123] or non-virally transmitted megaTALs.[124] Recent research achieved *TCR* gene editing and engineering with the CAR by one CRISPR/Cas9 mediated process[125]; the CAR expression cassette is targeted into the endogenous TCR α-chain and β2-microglobulin locus in order to control CAR expression by the physiologic TCR promoter and to disrupt the endogenous TCR.[126–128] Thereby the T cell function is aimed at being more sustained through diminished tonic signaling and delayed T cell exhaustion.[128] To the end, a cell bank with "universal" CAR T cells for allogeneic cell transfer would provide much more flexibility in the application, more rapid delivery to the patient and less cost-intensive manufacturing, alltogether making the therapy more applicable to a high number of patients.

The target antigen

Since most targetable tumor-associated antigens are also expressed by healthy cells, their elimination by CAR T cells may become a severe side effect dependent on the targeted antigen and the attacked tissue. In the treatment of B cell malignancies by targeting CD19 or CD20, depletion from healthy mature B cells is clinically manageable; the same is the case for CAR T cell targeting B cell maturation antigen BCMA (CD269) in the treatment of myeloma which results in the elimination of healthy plasma cells with BCMA expression (NCT02546167).[129] Current efforts are aiming at identifying truly tumor-selective antigens suitable for a more selective targeting of tumor lesions while sparing healthy tissues. Such tumor-selective antigens may be tumor-specific mutations of surface proteins, like claudin-6,[130] or glycosylation variants like Muc1 or Muc16.[131]

Expression of the targeted antigen by healthy tissues does not exclude per se the application of CAR-redirected T cells. For instance, CAR T cell targeting carcinoembryonic antigen (CEA) proofed to be safe since CEA is expressed in a strictly luminal fashion by healthy epithelia in the gastrointestinal tract and the lung; in contrast, cancer cells show a depolarized expression pattern on the entire cell surface.[132] Two trials showed that systemic anti-CEA CAR T cell administration is safe without inducing treatment related colitis; the same trials provided some clinical efficacy in the treatment of gastrointestinal adenocarcinoma (NCT01212887, NCT02349724).[133,134] Local administration of anti-CEA CAR T cells by hepatic artery infusion was safe and also declined tumor progression (NCT01373047).[135]

The choice of target antigen is also crucial with respect to antigen loss during therapy. The situation became obvious upon CD19 CAR T cell therapy of pediatric B-ALL, which regressed into complete remissions with high frequencies, however, relapsed in about 40% of patients despite persisting CAR T cells.[8,136,137] The dominant cause of relapse seems to be the expression of a CD19 isoform which lacks exon-2 and is not recognized by the CAR with the FMC63 scFv.[63] Loss of CD19 occurred in 28% of patients in a recent trial for the treatment of pediatric and young adult B-ALL making the malignant cells invisible to CD19 CAR T cells[138]; CD19 loss was so far not observed in the treatment of CLL. The risk of relapse due to antigen loss can be reduced by co-targeting a second antigen, for example, CD20 by a bispecific CAR, by T cells with 2 CARs or by the application of 2 CAR T cell products. In the long-term, an antigen-independent anti-tumor response by innate immune cells, which is initiated by a targeted CAR T cell activation, may improve the overall therapeutic efficacy against cancer with profound antigen heterogeneity and antigen loss. TRUCK cells, that is, CAR T cells with the inducible release of transgenic cytokines like IL-12, are capable to induce an innate response against antigen-negative cancer cells in an experimental model[51]; clinical exploration will prove whether the combined activation of a redirected T cell and a cytokine initiated innate response is capable to control cancer with high antigen heterogeneity.

Optimization of the CAR design

While a prototype design of a CAR established during the last decade, each CAR needs optimization with respect to the target antigen and the redirected immune cell. The optimization steps include the targeted epitope of the particular antigen, the binding affinity and the extracellular spacer length to ensure proper accession to the target cell.[139,140] As an example, targeting Her2 by a medium affinity CAR caused no substantial toxicity[141] while a high-affinity CAR targeting a different Her2 epitope caused fatal adverse events.[142] There is an optimal binding affinity by which CAR T cells recognize cancer cells with high antigen load and spare healthy cells with low antigen levels.[143,144]

The T cell subset

The most suitable maturation stage of T cells for adoptive cell therapy is still a matter of debate; naïve or early central memory T cells are preferred due to their enhanced capacity in cytolysis, amplification, and persistence.[110] In previous trials, T cells with a $CD62L^+$ phenotype were used for CAR engineering[145]; other memory T cell subsets may likewise be suitable. Since T cell maturation is modulated by costimulation and/or cytokine signals, efforts are made to shape a T cell response in a specific fashion. Costimulation through 4-1BB in young T cells initiates a central memory T cell response while CD28 mediates an acute effector cell response.[146] In advanced stages of maturation, $CCR7^-$ T cells require combined CD28-OX40 costimulation to be protected from activation-induced cell death.[24] IL-18 differentiates T cells towards $Tbet^{high}$ $FoxO1^{low}$ cytolytic T cells with improved anti-tumor activities; CAR and TCR modified T cells with induced release of transgenic IL-18 showed superior activities against advanced tumors.[54,55] Ibrutinib, a Bruton tyrosine kinase (BTK) inhibitor, reduces PD-1 levels on CAR T cells and finally exhaustion which increases anti-tumor activity of CAR T cells in the long-term.[147]

Manufacturing the CAR T cell product

Currently, each CAR T cell product is manufactured by a manual or semi-automated process by a few specialized centers. The process will come to its limits when thousands of patients in various hospitals require their individualized cell products in due time demanding a de-centralized, in hospital manufacturing process by an automated and entirely controlled procedure with a high degree of standardization. Procedures are going to be set up in this direction; major achievements towards automatization and hospital-based production are expected in the near future.

CARs to manipulate the immune network

Evidences are raising that successful CAR T cell therapy involves the entire host immune system for tumor rejection in the long-term. This is underlined by experimental models that showed resistance to EGFRvIII-negative tumors mediated by host immune cells after transfer of anti-EGFRvIII CAR T cells.[148] A secondary innate cell response can be induced by IL-12 TRUCKs, that is, IL-12 releasing CAR T cells, which activate macrophages in the tumor tissue for eliminating those cancer cells which are invisible to CAR T cells.[51] IL-18 TRUCKs increase the numbers of $CD206^-$ M1 macrophages and $NKG2D^+$ NK cells and reduce Treg cells, suppressive $CD103^+$ dendritic cells and M2 macrophages in the tumor tissue.[54] The strategy can also be used to deliver other immune response modifiers to the tumor tissue in order to shape a broad immune response against cancer. Checkpoints are part of a regulatory network and specific checkpoints like TIM-3 are upregulated upon PD-1 blockade[149]; targeting PD-1/PD-1L by blocking antibodies along with CD19 specific CAR T cell therapy is currently explored in trials (NCT02926833, NCT02650999, NCT02706405).

Acknowledgments

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