Treatment with Living Drugs: Pharmaceutical Aspects of CAR T Cells

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Abstract

Background: Adoptive therapy with genetically modified T cells achieves spectacular remissions in advanced hematologic malignancies. In contrast to conventional drugs, this kind of therapy applies viable autologous T cells that are ex vivo genetically engineered with a chimeric antigen receptor (CAR) and are classified as advanced therapy medicinal products. Summary: As “living drugs,” CAR T cells differ from classical pharmaceutical drugs as they provide a panel of cellular capacities upon CAR signaling, including the release of effector molecules and cytokines, redirected cytotoxicity, CAR T cell amplification, active migration, and long-term persistence and immunological memory. Here, we discuss pharmaceutical aspects, the regulatory requirements for CAR T cell manufacturing, and how CAR T cell pharmacokinetics are connected with the clinical outcome. Key Messages: From the pharmacological perspective, the development of CAR T cells with high translational potential needs to address pharmacodynamic markers to balance safety and efficacy of CAR T cells and to address pharmacokinetics with respect to trafficking, homing, infiltration, and persistence of CAR T cells.

Introduction: CAR T Cells Are “Living Drugs”

Adoptive therapy with chimeric antigen receptor (CAR)-engineered T cells aims at redirecting the immune effector cells toward pre-defined tissues. A CAR is a modularly composed, recombinant one-polypeptide chain transmembrane receptor molecule that mediates target recognition by its extracellular part and cellular activation by the intracellular part. Target recognition is provided by an antibody-derived binding domain, mostly a single-chain fragment of a variable region (scFv) antibody. The binding domain is linked by a spacer to the transmembrane domain that anchors the receptor in the cell membrane. The CAR intracellular signaling domain is mostly derived from the T cell receptor (TCR) CD3ζ chain; the Fc epsilon receptor-I signaling chain as well as downstream TCR kinases are also used as CAR signaling...
domains. Thereby the CAR uses the TCR downstream signaling machinery in order to drive T cell activation upon engagement of cognate antigen. The first generation of CARs harbors the primary signal (signal-1), while CARs of the second generation combine signal-1 with a costimulatory signaling domain (signal-2), like CD28, 4-1BB, OX40, CD27, or ICOS. Both signals are required for complete and long-lasting T cell activation [1–4]. Third-generation CARs combine two costimulatory domains and show superior to T cells in terminal maturation stages [5]. CAR T cells that are engineered with an additional transgenic “payload” are called “T cells re-directed for antigen-unrestricted cytokine-initiated killing” (TRUCKs) or the fourth generation of CARs [6, 7]. Multiple variants of each CAR prototype were reported and showed beneficial in specific applications; for details, we refer to recent comprehensive reviews [8, 9].

Due to antibody-mediated binding, the CAR recognizes the respective antigen in a major histocompatibility antigen-independent fashion, which is advantageous in targeting tumor cells that are defective in peptide processing or major histocompatibility antigen presentation. Nearly any antigen can basically be targeted, also nonclassical T cell antigens like carbohydrates or lipids, as far as they are expressed on the T cell surface and a specific recognition molecule is available. Targets for CAR T cells are ideally tumor-selective however in most cases tumor-associated since they are also expressed by healthy cells, albeit at lower levels. For instance, targeting epidermal growth factor receptor variant-3 [10, 11] utilizes a tumor-specific mutation, ideally representing a tumor-selective antigen. In contrast, a CAR recognizing human epidermal growth factor receptor-2 (HER2) targets cancer cells with high HER2 levels as well as healthy tissues with lower levels [12]. The situation of physiologically expressed target antigens is basically not an exclusion argument when the CAR-mediated elimination of healthy cells is clinically manageable, like targeting CD19 on leukemia/lymphoma cells and the CAR-mediated elimination of healthy B cells [13]. With the help of TCR-like CARs that recognize presented peptides in the context of human leukocyte antigen, cytoplasmic proteins like cancer-testis antigens such as NY-ESO-1 [14, 15] and viral oncoproteins such as HPV-16 E6 [16] can also serve as targets, furthermore expanding the number of potential CAR targets.

For achieving and extending CAR triggered activation, the intracellular signaling domains, in particular the costimulatory domains, are crucial. CD28 and 4-1BB co-stimulation differ in their impact on T cell function and persistence due to addressing different downstream regulatory and metabolism pathways [17]. In particular, CD28 activates the PI3K/Akt/mTOR pathway which stimulates the glycolytic metabolism and triggers the immediate response effector cell phenotype [18, 19]. In contrast, 4-1BB stimulates the Wnt/β-catenin pathway which induces the oxidative metabolism resulting in a central memory phenotype and long-term survival of T cells [17, 20].

While the prototype CAR confers a defined specificity, so-called universal CARs were designed to target an epitope linked to a tumor-targeting antibody; adding the tagged antibody confers CAR specificity. Various CAR-antibody combinations were so far explored, including biotin-binding immune receptor-recognizing biotinated antigen recognition molecules [21] or FITC-specific CARs binding FITC-labeled antibodies [22]. A CAR with a CD16V-binding domain recognizes the Fc part of a tumor-targeting antibody [23] to initiate antibody-dependent cell cytotoxicity. The universal CAR approach allows adaptable specificity in a time- and dose-dependent manner allowing to target tumors with heterogenous antigen expression. All these strategies have the advantage that in case of unexpected toxicity, the antibody concentration can be reduced or discontinued or competed by irrelevant antibodies without depleting the CAR T cells. From the pharmacological point of view, such CAR T cell systems are composites of two drugs administered to the patient, the CAR T cell and the targeting antibody. While the CAR T cell is expected to persist for months, the administered antibodies exhibit a short half-live in serum as long as they are not captured by the CAR T cell.

TRUCKs: “Living Drugs” Turn into “Living Factories”

CAR T cells that encode and deliver a transgenic “payload” into the targeted tissue upon CAR signaling are classified as the 4th-generation CAR T cells, also nicknamed TRUCKs [24]. T cells are engineered with a CAR and additionally equipped with a constitutive or inducible expression cassette for the release of a transgenic protein as “payload” upon CAR engagement of target; abrogated CAR activation leads to withdrawal of transgenic protein expression and release. Technically, an “all-in-one” vector allows one-step genetic modification of T cells, facilitating genetic engineering and good manufacturing practice (GMP)-compliant manufacturing [25, 26]. Examples for “payloads” are transgenic cytokines in order to modulate the tumor immune environment and to attract other immune cells; antibodies to mediate antibody-dependent cell cytotoxicity; or immune checkpoint...
inhibitors to modulate the suppressive environment. A major advantage of the strategy is the local deposition of the protein in high concentrations while avoiding systemic toxicity. This is the case for IL-12 that is highly toxic upon systemic application; however, local production and depositing seems to be associated with tolerated toxicities while being efficacious against tumors in experimental models [27]. Locally deposited IL-12 moreover recruits and activates macrophages capable to control antigen-negative tumors [7]. TRUCKs releasing IL-18 were designed to improve the cytolytic T cell activity by orchestrating the levels of Tbet and FoxO1 transcription factors [28]. CAR T cells secreting a PD-1 blocking [29] or PD-L1 blocking antibody [30] counteract T cell suppression in a locally restricted fashion while avoiding impact on systemic immunity.

From the pharmacological point of view, TRUCKs are genetically engineered CAR T cell products with constitutive or inducible production and release of a transgenic protein. Thereby, CAR T cells as “living drugs” turn into “living factories,” producing a therapeutic protein on demand and as long as the CAR T cell is appropriately stimulated in the targeted tissue. The transgenic production capacity of TRUCKs can be ex vivo recorded by respective “potency assays” under standardized conditions. However, the dose of the produced protein within the targeted tissue is not predictable and depends on a number of physiologic variables including the number of T cells triggered by the CAR, the degree of T cell activation in situ, the protein half-life, consumption by target cells, and entry into circulation.

First CAR T Cell Products Are Approved by the FDA and EMA

To date, more than 500 CAR T cell trials have been initiated, mainly for the treatment of hematologic malignancies and most of them conducted in Eastern Asia, followed by the USA and Europe. More than half of the studies target CD19, others target alternative markers for the treatment of B cell leukemia/lymphoma; a growing number of CAR T cell trials is addressing solid tumors [31]. Currently, there are five CAR T cell products approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) to treat B cell malignancies: Kymriah™ for the treatment of pediatric and young adult patients with relapsed and refractory (r/r) B cell acute lymphoblastic leukemia (B-ALL) and r/r diffuse large B cell lymphoma [32]; Yescarta™ for the treatment
of adult patients with aggressive r/r B cell non-Hodgkin’s lymphoma (NHL) including diffuse large B cell lymphoma [33]; Tecartus™ for the treatment of r/r mantle cell lymphoma [34]; the recently approved Breyanzi™ targets CD19 for the treatment of patients with r/r large B cell lymphoma [35]; Abecma™ targets BCMA for the treatment of r/r multiple myeloma (Table 1). The approved CAR T cell products showed impressive therapeutic efficacy achieving remission rates of about 70–90% among children and adults with relapsed B-ALL [36]. Another anti-CD19 CAR T cell product, ARI-0001, was approved by the Spanish Agency of Medicines and Medical Devices (AEMPS) under the hospital exemption approval pathway foreseen by the European Regulation [37]. The treatment of patients with CLL, including Richter’s transformation, showed a response rate of 87.5% [38]; however, CD19-negative relapses occurred in 2 patients. In contrast to a centralized marketing authorization pathway which allows access to all member states, an advanced therapy medicinal product (ATMP) under hospital exemption approval is intended to be placed on the European market as a custom-made product used only in the member state where it was developed [39].

As a caveat, direct comparison of trial data is difficult due to a number of differences in trial parameters like CAR design, type of genetic modification, technical differences in the production process, in vivo expansion of CAR T cells after administration, patient preconditioning, disease entity and disease burden, administered CAR T cell dose, and dosing scheme, among others; each variation substantially impacts the trial outcome. Apart of these differences, meta-analysis of CD19 CAR T cell trials revealed that lymphodepletion and the CAR T cell dose as well as tumor burden are key factors for clinical efficacy [40, 41].

**Strategies to Improve CAR T Cell Therapy**

CAR T cells are capable to fight cancer and to induce lasting remissions in the treatment of hematologic malignancies, albeit there are still pivotal challenges. One cause of relapsed or failed CAR T cell therapy can be the loss or downregulation of the targeted antigen [42] or mutation of the targeted epitope [43], resulting in tumor relapse. Alternative strategies addressing this situation include:

1. Targeting alternative antigens expressed by the same malignant cell [44];
2. Targeting multiple antigens by using (i) bispecific CARs targeting two co-expressed antigens [45], (ii) co-expressed CARs on T cells, each CAR targeting a distinct antigen, or (iii) a pooled CAR T cell mixture of different monospecific CAR T cells;
3. Combining CAR T cell therapy with (i) immune checkpoint inhibitors such as atezolizumab, nivolumab, or pembrolizumab [46], (ii) immunomodulatory agents like the tyrosine kinase inhibitor ibrutinib [47, 48], or (iii) oncolytic viruses to synergize their lytic effects with the CAR T cell attack [49, 50].

Another approach to enhance CAR T cell efficacy is to equip CAR T cells with an additional targeting receptor. These so-called armored CARs carry additional transgenic receptors as “weapons” to execute their designer function in a more effective fashion. Armored CAR T cells as a pharmacological drug are engineered T cells with two co-expressed transgenic receptors, where one is the CAR and the other the auxiliary receptor for improving CAR T cell function. Examples are co-expression of the chemokine receptor CCR4 to improve lymphoma infiltration and overall antitumor activity [51], a co-expressed dominant-negative TGF-β receptor acting as decoy for TGF-β in the targeted tumor tissue [52], and a so-called switch receptor that converts binding of a suppressive factor into a positive stimulatory signal [53]. Armored CAR T cells can also express ligands for costimulatory molecules like CD40L improving activation [54] or 4-1BBL enhancing persistence of CAR T cells in preclinical models [55]. Currently, armored CAR T cells are explored in a clinical trial for the treatment of NHL and CLL (NCT03085173) [56].

**CAR T Cells as “Living Drugs” Can Cause Severe Toxicities**

Adoptive therapy with CAR T cells as “living drugs” display specific properties due to their active migration, amplification, and particular cellular functions triggered by the CAR. These specific properties may cause a panel of toxicities, including cytokine release syndrome (CRS), neurotoxicity, hemophagocytic lymphohistiocytosis/macrophage activation syndrome, and others.

CRS is due to a primary systemic inflammatory reaction with supraphysiological serum levels of inflammatory cytokines, particularly IL-6, following extensive amplification of CAR T cells early after administration to the patient [57]. CRS is characterized by flu-like symptoms like fever, fatigue, headache, rash, arthralgia, and myalgia and is mostly self-limiting but can be life-threatening with capillary leak and multi-organ failure, requiring immedi-
ate intensive care intervention [58]. Grading systems and clinical management profiles for CRS are established [59]; first-line treatment is the FDA-approved drug tocilizumab, an anti-IL-6 receptor antagonist, to rapidly abrogate IL-6 signaling [60]. Prophylactic treatment with tocilizumab prior infusion of CD19 CAR T cells reduces CRS incidence and severity [61].

Neurotoxicity in CAR T cell therapy is defined as “immune effector cell-associated neurotoxicity syndrome” (ICANS) that often correlates with CRS [62]. ICANS usually appears 1–3 weeks after infusion and is thought to arise due to activated CAR T cells overcoming the blood-brain barrier. Parker et al. [63] identified CD19 expression in brain mural cells that are critical for blood-brain barrier integrity, suggesting that CD19 targeting may lead to toxicity. Symptoms of ICANS are aphasia, tremor, dysgraphia, and lethargy, among others. Standard treatments to toxicity. Symptoms of ICANS are aphasia, tremor, dysgraphia, and lethargy, among others. Standard treatments for ICANS include corticosteroids. Neurethic in CAR T cell therapy is defined as “immune effector cell-associated neurotoxicity syndrome” (ICANS) that often correlates with CRS [62]. ICANS usually appears 1–3 weeks after infusion and is thought to arise due to activated CAR T cells overcoming the blood-brain barrier. Parker et al. [63] identified CD19 expression in brain mural cells that are critical for blood-brain barrier integrity, suggesting that CD19 targeting may lead to toxicity. Symptoms of ICANS are aphasia, tremor, dysgraphia, and lethargy, among others. Standard treatments for ICANS include corticosteroids. Neurotoxicity in CAR T cell therapy is defined as “immune effector cell-associated neurotoxicity syndrome” (ICANS) that often correlates with CRS [62]. ICANS usually appears 1–3 weeks after infusion and is thought to arise due to activated CAR T cells overcoming the blood-brain barrier. Parker et al. [63] identified CD19 expression in brain mural cells that are critical for blood-brain barrier integrity, suggesting that CD19 targeting may lead to toxicity. Symptoms of ICANS are aphasia, tremor, dysgraphia, and lethargy, among others. Standard treatments for ICANS include corticosteroids. Neurotoxicity in CAR T cell therapy is defined as “immune effector cell-associated neurotoxicity syndrome” (ICANS) that often correlates with CRS [62]. ICANS usually appears 1–3 weeks after infusion and is thought to arise due to activated CAR T cells overcoming the blood-brain barrier. Parker et al. [63] identified CD19 expression in brain mural cells that are critical for blood-brain barrier integrity, suggesting that CD19 targeting may lead to toxicity. Symptoms of ICANS are aphasia, tremor, dysgraphia, and lethargy, among others. Standard treatments for ICANS include corticosteroids.

In most of the clinical trials with second-generation CAR T cells, CD28 or 4-1BB were used as costimulatory domains, differing in their toxicity profile while showing quite similar clinical efficacy [71]. CAR T cells with a CD28-derived costimulatory domain seem to have an earlier onset of CRS and higher rates of neurological toxicities than 4-1BB CAR T cells [34, 35, 72, 73], albeit comparison between trials is difficult due to additional variables beside the costimulatory domain, like different scFvs, transmembrane, and/or hinge domains and grading systems. A distinct toxicity profile was mostly observed in the treatment of B cell lymphoma patients but less in B-ALL. One trial comparing CD28 and 4-1BB in CAR T cell treatment revealed differences in the response pattern, i.e., peak reaction time and cytokine secretion [74]. Also, the costimulatory domain can affect/affects the pharmacodynamics of the CAR T cell as 4-1BB CAR T cells showed longer persistence than CD28 CAR T cells [36, 72, 75].

Manufacturing the Therapeutic Drug: CAR T Cells Are ATMPs

CAR T cells are classified as ATMPs that are defined as a class of innovative, research-driven biopharmaceuticals including gene therapy medicinal products (GT-MPs), somatic cell therapy medicinal products, tissue-engineered products, and combined products [76]. The legal and regulatory framework for ATMPs in the European Union was established by the EU Commission in 2007 [39]. Together with the Directive 2009/120/EC amending Directive 2001/83/EC, the documents define specific requirements and a centralized procedure for marketing and authorization [77, 78]. The quality, safety, and efficacy of ATMPs are reviewed and classified by the Committee for Advanced Therapies (CAT) at the EMA [79]. Within the ATMP category, CART cells are subclassified as a GTMP that has to meet the requirements for GMP during the manufacturing process [80]. The official standards published in the European Pharmacopeia (Ph. Eur.) provide the legal and scientific basis for the quality control of medicinal products [81].

The regulatory landscape for CAR T cells differs between Europe and the USA. The FDA is the only regulatory body in the USA, while in the EU, the EMA works closely together with the national authorities of each member state as well as with the local-state authorities [77]. In the USA, the subclassification compromises two major groups of products, i.e., gene therapy and cellular therapy products as defined by the “Guidance for Human Somatic Cell Therapy and Gene Therapy” [82]. The criteria for the classification as a GTMP in the USA is a biological product that contains “genetic material,” whereas it is termed as a biological product containing “recombinant nucleic acid(s) of biological origin” in the EU [78]. In the EU, a product aimed at the prophylaxis or treatment of infectious diseases is classified as vaccines, therefore excluding them from being classified as a gene therapy product [78]. In the USA, vaccines for infectious diseases are not specifically excluded but have their own guidance for development [78]. A GMP-compliant manufacturing process of CAR T cells is certified by a qualified person in EU countries while it is assessed by paper review in the USA [77].
The CAR T cell manufacturing process requires 7–22 days, usually 12 days, and starts, in short, with the isolation of T cells from the leukapheresis product of a patient, followed by activation and genetic modification of the cells in order to express the respective CAR (shown in Fig. 1). These cells are expanded, finally formulated, and reinfused to the pre-treated patient. The manufacturer has to show that the product is consistently manufactured in a pre-defined quality and that the product is safe and efficacious in patients [79]. There are numerous variables that impact the quality of the final CAR T cell product, like the efficiency in genetic modification, the level of CAR expression, the transgene copy number per cell, the phenotype, and maturation stage of CAR T cells, among others; all having impact on the safety, performance, and efficacy in their therapeutic use [80]. The manufacturing process starts with bulk T cell populations obtained from leukapheresis; T cell subsets are more frequently used, for instance purified CD4+ and CD8+ T cells [83], naive cells [84], central memory cells [85], or memory stem cells [86]. After isolation, T cells are stimulated for transduction with replication-defective retroviral or lentiviral vectors; the viral vector stock can be produced in large quantities and stored at −80°C for at least 4 years [87]. Other gene transfer procedures using mRNA transfection [64] or transposon systems are also applied [88]. After genetic modification, cells are amplified to clinically relevant numbers in the presence of stimulatory cytokines. The manufacturing process is accompanied by a panel of quality control tests and release testings for cell identity, process-related impurities, mycoplasma, endotoxin, bacterial, and fungal contaminations as well as testing for replication-competent retroviruses/lentiviruses [77].
The final cell product consists of amplified "living cells" with engineered capacities to recognize target cells and to respond with a defined program of effector functions dependent on the maturation stage of the engineered T cell. To record CAR T cell capacities, quantification of the CAR expression and binding to the target is determined by flow cytometry as an indirect potency assay in early phase trials. However, the assay does not predict the CAR T cell performance and efficacy in the individual patient. In later phase trials, validated functional assays will be mandatory, such as cytotoxicity assays and secretion of cytokines upon target recognition to test for functional capacities of the applied CAR T cells. To identify adverse effects in the treated patients in the long-term, the FDA recommends an observation period for 15 years posttreatment [89].

As an autologous cell product, the CAR T cells are individually manufactured for each patient. Due to the resulting overwhelming labor load, great efforts are made to transform the hands-on manufacturing procedure into a fully automated manufacturing process that allows reproducible and supervised CAR T cell production and ensures appropriate in-process control and tracking of the used products. One example for an automated process is the Cocoon Bioreactor™ (Octane Biotec) [90], another example is the CliniMACS Prodigy™ (Miltenyi Biotec) [91]. With such a device, decentralized and standardized manufacturing of patients’ cells at the point of care (PoC) in the hospital becomes possible.

The device-based manufacturing of 1 patient product at a time mitigates the risk of cross-contaminations, is adaptable to an individual program, and will decrease the costs and risks to the product due to transportation, extended delivery time, and freezing for shipping [91]. On the other hand, decentralized PoC manufacturing requires continuously trained, highly qualified GMP personnel. The overall costs for running a GMP facility and for the consumables are high and challenging for small academic groups or hospitals. As a solution in this situation, a strong academic network is mandatory to harmonize the production protocols in the production facilities and to collect data and experience in a comparative fashion. At the end, two manufacturing lines are needed: PoC manufacturing to show safety and efficacy enabling fast transfer of new products from academia to clinical application and centralized manufacturing to establish high-technology platforms enabling production upscaling and cost efficiency.

Even if manufactured and amplified by the same process, there is still a substantial heterogeneity between the T cell products due to different donors, cellular composition and functional fitness, and cellular senescence [92]. Such an individualized manufacturing of patient’s cells substantially differs to a centralized pharmaceutical production line of a conventional drug where the same product is processed in high numbers along the same line.

Genome-Edited CAR T Cells

Large-scale clinical application of CAR T cells is currently limited due to the individualized, expensive, and time-consuming process in manufacturing the cell product. The process may additionally be limited by insufficient leukapheresis due to patient’s lymphopenia. In this situation, allogeneic CAR T cells from healthy donors may be an alternative option. Deletion of the TCR abolishes the capability of third-party cells to recognize allogeneic antigens, thus abolishing the risk of graft-versus-host disease (GvHD). To make such third-party CAR T cells less visible to the host immune system, the human leukocyte antigen class I loci of these cells can additionally be disrupted or deleted by genome-editing technologies involving clustered regularly interspaced short palindromic repeats-associated nucleases (CRISPR-Cas9) [93], transcription activator-like effector nuclease [94], or zinc finger nuclease [95]. However, mismatches of minor histocompatibility antigens may still cause GvHD. Clinical trials are currently evaluating safety and efficacy of CRISPR/Cas9-engineered allogeneic CD19 CAR T cells (NCT03166878, NCT03229876) [96, 97]. Treatment of two children with relapsed, highly refractory CD19+ B-ALL with transcription activator-like effector nuclease gene-edited TCR-deficient universal CAR19 (UCART19) T cells achieved molecular remissions [98], demonstrating the feasibility of the approach.

While currently CAR T cell products are manufactured starting from peripheral blood T cells, induced pluripotent stem cells (iPSCs) are an alternative source which takes advantage of the unlimited proliferative capacities of iPSCs [99]. A first-of-class hiPSC-derived CAR T cell product (FT819) was generated by reprogramming peripheral blood T cells and targeted insertion of a CD19 CAR into both alleles of the TCR-α (TRAC) locus [100]; FT819 has been translated into clinical exploration [101, 102] with the first patient treated in a phase I study for the treatment of r/r B cell malignancies (NCT04629729).
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CAR-redirection immunotherapy with T cells for the treatment of solid tumors is still challenging due to the limited penetration into the tumor tissue or trafficking through the suppressive tumor microenvironment. Apart from T cells, innate immune cells have the capacity to enter and survive within the tumor tissue, induce a broad immune response, and show unique effector functions. In addition, innate cells show a more favorable toxicity profile due to lack of GvHD in allogeneic setting and reduced risk for CRS and neurotoxicity [103].

Macrophages interact with a variety of cells, present antigen, exhibit high infiltration rate into the tumor tissue, and have the capability to ingest malignant cells [104]. On the tumor site, however, there are several mechanisms to protect themselves from phagocytosis such as the “do not eat me” signal via the CD47/SIRPα axis [105]. Manufacturing of engineered macrophages is still limited [106] since the cells do not sufficiently amplify in vitro and can therefore be applied only in limited numbers to patients [104]. Preclinical models indicated that redirected by a CAR, macrophages traffic to tumor tissues, execute phagocytosis, and reduce tumor load in mouse models [107]. CAR macrophages combine several mechanisms of action as they enter immunologically “cold” tumors and secrete pro-inflammatory cytokines and chemokines to “warm up” the tumor tissue [108]. The FDA has approved an anti-HER2 CAR macrophage (CT-0508, CARISMA Therapeutics) for the treatment of patients with r/r HER2 overexpressing solid tumors which is currently being evaluated in a multicenter clinical trial (NCT04660929).

Natural killer (NK) cells have a broad and antigen-unrestricted killing capacity. The activation of NK cells is regulated by a balanced expression of activating and inhibitory signaling receptors and results in the secretion of pro-inflammatory cytokines and chemokines. NK cells have a reduced risk for alloreactive immune reactions making them potential effectors for CAR-redirected cytotoxicity [103]. The combination of CAR-dependent killing capacity and intrinsic cytotoxic mechanisms enables CAR NK cells to eradicate CAR-targeted as well as antigen-negative tumor cells. CAR-independent killing is initiated through NKG2D and KIRs independently of CAR engagement [109]. Due to the low risk for allogeneic immune reaction, CAR NK cells can be produced in advance for a number of patients as “off-the-shelf” cell product offering an opportunity for patients for whom autologous cells are not available. Results of early phase clinical trials underline that umbilical cord blood-derived CAR NK cells can induce complete remissions without major side effects (NCT03056339) [110]. However, there is a risk of contaminating B and T cells in the final CAR NK cell product that may cause GvHD [111]. Alternative NK cell sources are NK-92 cell line, CD34+ hematopoietic stem cells, and iPSCs (reviewed by Xie et al. [103]). The NK-92 cell line is the only human NK cell line so far that has entered clinical trials due to their high cytotoxic activity against tumor cells [112]. As an established cell line, however, NK-92 cells need to be irradiated before application which substantially reduces their persistence after application. CAR-engineered NK-92 cells were used for intracranial injection in patients with recurrent HER2+ glioblastoma in a phase I clinical trial (NCT03383978) [113]. Currently, worldwide 19 CAR NK trials are ongoing for the treatment of hematologic malignancies as well as solid tumors [114].

From the pharmacological perspective, CAR NK cells and CAR macrophages are different cellular products compared to CAR T cells, although they may have the same CAR in common. In contrast to a classical drug, the different cell products execute different cellular functions and release a different panel of effector cytokines, while triggered by the same CAR, impacting the therapeutic efficacy in a different fashion.

Alternative Engineering Strategies: mRNA Electroporation, SB Transposon System

An alternative strategy to engineer T cells ex vivo with a CAR is the transfection with CAR-encoding mRNA. RNA-modified T cells differ from virally engineered T cells in some pharmacokinetic and pharmaceutical aspects. One of the main differences is the short half-life of the CAR-encoding mRNA and the rapid dilution by each T cell division due to the lack of genomic integration. The half-lives of both the mRNA template and the translated product impact substantially the pharmacokinetics of the cell product, whereas the processing pathways of the mRNA-encoded protein are determinants of its pharmacodynamics [115]. The limited CAR T cell persistence reduces the risk for long-term “on-target off-tumor” toxicities, however may compromise therapeutic efficiency in the long-term.

A phase I trial with RNA-modified T cells expressing a mesothelin-targeting CAR showed migration of CAR T cells to primary and metastatic tumor sites without dose-
limiting toxicities or CRS [116]. The transient expression of the CAR made repeated applications of the CAR T cell product necessary which required a well-defined dosing schedule. A case report describes an anaphylactic reaction as a severe side effect most likely caused by IgE antibodies specific to the CAR [117]. In order to avoid Ig class switch from IgG to IgE, the interval between two infusions may not be longer than 10 days [118].

Another approach for virus-free CAR gene transfer is the Sleeping Beauty (SB) transposon technology mediating stable integration of DNA sequences into the host genome [119]. Shortly, the transposase enzyme is delivered to the target cell together with the transposon DNA by transfection or electroporation leading to the integration of the transposon into the cellular genome. The CAR-AMBA trial is the first-in-human clinical trial using SB-produced SLAMF7-specific CAR T cells for the treatment of multiple myeloma (NCT04499339) [120]. Here, the SB gene transfer system consists of mRNA encoding an optimized hyperactive SB100X transposase and a minicircle vector encoding the SB transposon with the CAR. Taken together, the virus-free approaches are likely reducing the manufacturing costs as GMP-grade production of nucleic acids is less time-consuming and work-intensive as the GMP-grade production of a viral vector.

Alternative Mode of Application: CAR-Encoding Vectors

Ultimately, in vivo production of CAR T cells may further reduce costs and production time. The approach uses T cell-targeted lipid nanoparticles (LNPs) packaged with modified mRNA encoding the CAR [121]. After injection, the LNPs are endocytosed by the targeted cell type, resulting in release of the mRNA into the cytoplasm and finally transient production of CAR T cells. An early proof of concept (POC) is shown with CD5-targeted LNPs for producing FAP CAR T cells to treat cardiac injury in a mouse model [121]. However, the approach needs further optimization with specific focus on LNP composition, targeting to the specific cell type and repetitive, fine-tuned dosing. A significant hurdle currently is the complex pharmacology of the in vitro-transcribed mRNA that may lead to different mRNA dose-protein-effect relationships across patients [115]. In principle, clinical-grade GMP manufacturing of in vitro-transcribed mRNA is cost-effective compared to current ex vivo CAR T cell manufacturing; the product is moreover broadly applicable compared with the individualized production of patient’s CAR T cells. Further approaches are needed to enable broad access of CAR T cells to a large number of patients in a due time between diagnosis and treatment.

How to Test Pharmacology of CAR T Cells in Clinical Trials

To test conventional drug candidates, three pharmacodynamic endpoints are usually evaluated in a clinical trial, including target engagement for proof of mechanism (POM), phenotypic change for proof of principle (POP), and clinical outcome for POC [122, 123]. Classically, the phase 0 trial aims to evaluate the pharmacodynamics and pharmacokinetics of a candidate drug through micro-dosing and/or to validate the POM and POP through biomarkers [122, 123]. Phase I trials with 20–100 healthy volunteers evaluate the safety and define the maximum tolerated dose of a drug candidate; toxicity, pharmacokinetic, and pharmacodynamic data are also recorded. Phase II trials with 20–300 patients evaluate efficacy, while phase III trials with 300–3,000 patients record clinical outcomes and evaluate the overall risk/benefit ratio. Finally, phase IV studies are performed post-marketing and usually record safety and explore additional drug uses [124]. The basic concept of clinical trial evaluation applies for CAR T cell evaluation as well; however, it needs some specific adaptations.

As phase 0 trials are not implemented in CAR T cell studies, phase I trials evaluate safety, dose-finding, and feasibility of CAR T cell treatment and prove POM, POP, and POC; further validation of safety and efficacy is conducted in phase II trials. POM is evaluated by recording biomarkers in serum that are released by activated CAR T cells upon target engagement, for instance, elevated serum levels of cytokines, in particular IL-6, IL-8, IFN-γ, and chemokines at different times after CAR T cell application [125]. In addition, CAR T cell expansion in peripheral blood is recorded by flow cytometry or quantitative polymerase chain reaction (qPCR). POP recording for CAR T cells aims at recording the reduction of healthy and malignant cells in peripheral blood or tissues like bone marrow. POC aims at evaluating the clinical benefit for the patients after treatment, including tumor burden reduction and event-free survival.

To determine cellular kinetics in phase I trials, CAR T cells are specifically recorded in serum, bone marrow, or cerebrospinal fluid by flow cytometry or qPCR which allows calculation of the area under the plasma concentra-
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The function of persisting CAR T cells can ideally be evaluated by isolating these cells from blood or other specimens and by inducing IFN-γ release or CD107a as marker for degranulation upon engagement of target cells [125].

Immunogenicity of CAR T cells and finally immune elimination of CAR T cells is a general concern as the CAR is an artificial protein containing foreign domains like the scFv and junctions elements which may elicit a humoral and/or cellular immune response [83, 127]. To date, immunogenicity does not seem to be a limiting issue, at least in a number of CD19 CAR T cell trials analyzed in this respect [36, 125, 126, 128, 129]. Patients treated with mRNA-engineered CAR T cells may develop a human anti-mouse antibody response due to repeated application of the product. Therefore, the infusion schedule has to be carefully adapted to reduce the window between two infusions and the overall infusion period [117].

**CAR T Cells Do Not Follow Classical Pharmaceutical Drugs**

CAR T cells are complex pharmaceutical products and differ from conventional pharmaceutical drugs in multiple aspects [77, 130]. The CAR is designed as one gene product to recognize a specific marker and acts as the active pharmaceutical ingredient. In contrast to conventional drug formulation, the active pharmaceutical ingredient is not combined with inert ingredients but transduced into patient’s T cells. Engineered CAR T cells execute a variety of cellular effector functions upon target recognition and together with non-transduced T cells define the final product.

During the manufacturing process, the CAR-encoding DNA is permanently integrated into the genome of the patient’s T cell; the latter migrates after infusion to the diseased tissue, becomes activated, amplifies, executes a panel of cellular effector functions, and persists in the long-term or enters apoptosis after some rounds of activation. In comparison, conventional pharmaceutical formulations harbor in addition also a panel of pharmacological inert substances to improve drug absorption, distribution, metabolism, and elimination which finally sustain the therapeutic drug efficacy and minimize the adverse effects. In comparison, CAR T cell products use the physiological behavior and effector functions of the patient’s T cell as a “living drug” to initiate the execution of their cellular therapeutic capacities while the CAR per se acts as a targeting and activation reagent.

From the regulatory view, the CAR T cell is a drug that fulfills the criteria of an ATMP. The CAR T cell product has its intrinsic properties like the CAR design but however is also influenced by extrinsic factors like patient preconditioning and tumor burden making every CAR T cell product unique even if the CAR is the same. The classical pharmacokinetic considerations absorption, distribution, metabolism, elimination as well as pharmacodynamic considerations are only in parts applicable to the CAR T cell product. Absorption is not an issue as CAR T cells are usually infused intravenously followed by initial accumulation in the lung and redistribution to the spleen and bone marrow within hours [131]. Alternatively, CAR T cells are locally applied within or near the tumor lesion. Biodistribution is influenced by chemokine-driven CAR T cell infiltration into tissues and persistence in the long-term which is finally determined by the maturation stage of the applied T cell. The elimination from circulation depends on the induction of exhaustion, lack of survival factors, and/or activation-induced cell death [132].

While being a “living drug,” CAR T cells exhibit a number of differences to classical drugs (Table 2):

1. A classical drug is chemically defined; CAR T cells are a highly complex mixture of thousands of proteins, lipids, nucleic acids, and organic compounds [133]. The cell product “CAR T cell” is defined by being a T cell genetically engineered with a CAR. Thereby, the definition covers criteria for T cells like CD3, CD45, CD62L expression along with the expression of the CAR. The latter can be detected by using an antibody directed against the scFv-binding domain of the respective CAR, i.e., an anti-idiotypic antibody, or using the protein L that binds sequence-independently to the scFv or using a cognate antigen that binds to the CAR. For instance, the clinically used anti-CD19 CAR with the FMC63-binding domain is detected by the anti-idiotypic antibody [134]. Alternatively, the CAR is detected by recording the extracellular spacer domain, mostly the IgG1 Fc region, or an integrated tag.

2. In contrast to the strictly constant composition of a classical pharmaceutical drug, CAR T cell products vary in their composition. Each CAR T cell product is composed of a mixture of millions of cells with distinct properties leading to a heterogenous cell population. Although the process of genetic engineering and amplification is highly standardized, blood T cells as starting material are highly diverse in maturation and composition. Moreover, random gene insertion during the
genetic engineering process has impact on T cell activity. Consequently, the CAR T cell varies in the final cell product between patients. The amount of transduced CAR T cells in the final cell product is used as manufacturing marker; functional assays have been explored to predict the potency of the CAR T cell product. In order to provide a more standardized final product, some clinical trials are using a defined ratio of CD4+ and CD8+ T cells [83, 135]. Altogether, drug composition in the classical perspective is hard to define; however, normalization of the cellular composition of a CAR T cell product will help to standardize the clinical regimen and evaluate the clinical outcome.

3. Chemical drugs are commonly produced to high purity without significant contaminations by side products; the purity can be defined on a chemical-analytical basis. The definition of CAR T cell purity, in contrast, needs cell-based parameters. Basically, the contamination by non-T cells in the final cell product is recorded by flow cytometry and is commonly below 5%. The homogeneity of the engineered CAR T cell itself is hard to define since during genetic modification multiple events occur at different integration sites in the individual T cell, giving rise to a plethora of genetically diverse CAR T cells. Site-directed insertion of the CAR-encoding transgene, for instance, into the TRAC locus, generates a genetically more homogeneous CAR T cell population with expected more homogeneous functional capacities.

4. The potency of a classical drug is defined as the quantity required to achieve a defined therapeutic effect. In case of applying CAR T cells in tumor therapy, potency will translate to the number of applied CAR T cells capable to reduce tumor burden; the definition however has a number of variables. As a “living drug,” CAR T cells substantially amplify in the peripheral blood after application to the patient; only a minority of them gets in contact to the targeted cancer cells where they execute their antitumor activity. While an in vitro assay for CAR-redirected T cell activation gives some indication of the functional capacity of the CAR T cell product, elimination of established cancer cells in vitro has little correlation with the in vivo potency [136]. Cancer cell elimination may also occur by indirect mechanisms initiated by IFN-γ release or others. On the other hand, it is still unresolved which cells in the CAR T cell product finally mediate the initiating and executing antitumor activity. Evidences indicate that the therapeutic potency is mediated by a minority or particular descendants of cells generated during in vivo expansion. It is therefore difficult to define the potency of a CAR T cell product by in vitro functional
assays as long as the crucial cell or cellular function is not sufficiently defined.

5. Apart from specific CAR T cell effector functions, the pharmacokinetics early after application and in the long-term is essential for the therapeutic efficacy [133]. As the drug “CAR T cell” is a living cell, the term “cellular kinetics” is proposed instead of the term “pharmacokinetics.” Conventional analyses such as maximum plasma drug concentration (Cmax), the AUC, and last measurable plasma concentration can be accordingly applied to the cellular product CAR T cell [126].

Several factors impact on the kinetics of engraftment like manufacturing and amplification conditions, the CAR design and signaling, lymphodepleting chemotherapy prior to T cell application, the stage of disease, applied cell dose, and treatment post-infusion. Early after infusion, the spleen, liver, and lungs are the organs with maximum biodistribution of CAR T cells [137], while there is a highly variable relationship between dose and accumulation at the target site. Pharmacokinetic data indicate a rapid drop in the concentration of anti-CD19 CAR T cells in the peripheral blood within hours upon administration which is likely due to the CAR T cell distribution into tissues [133]. The following CAR T cell amplification occurs in three distinct phases: an initial exponential expansion phase, a short contraction phase, and a sustained persistence phase [138]. Cmax and AUC from CAR T cell administration until day 28 (AUC0–28d) frequently serve as an indicator for CAR T cell engraftment and early CAR T cell expansion [126].

For instance, anti-CD19 CAR T cell tisagenlecleucel (CTL019) levels peaked within the second week after infusion and then declined over time as recorded in ALL and CLL patients by qPCR and flow cytometry [126]. For tisagenlecleucel, the doubling time was calculated to be 0.78 days, the initial decline half-life 4.3 days, and terminal half-life 220 days [138]. In vivo kinetic analyses also revealed that complete responder patients had higher Cmax and AUC during CAR T cell amplification than non-responding patients, implying a correlation between exposure to CAR T cells and clinical response to therapy [126]. Lymphodepletion applied in advance of CAR T cell administration [139] affects early CAR T cell pharmacokinetics as it improves CAR T cell expansion and persistence due to increased levels of cytokines [131, 140]. CAR T cell amplification during the lymphodepleted phase also facilitates CAR T cell persistence in the long-term by overrunning immunological rejection of CAR T cells early after application [128].

6. Pharmacodynamic considerations on CAR T cells as “living drugs” are complex as the type and duration of interactions between CAR T cells and target cells have to be taken into account. Key variables are drug-associated, like the CAR binding affinity and the number of CAR molecules per T cell affecting cellular avidity; are disease-associated, like prevalence of the targeted antigen on cancer cells and in serum; and are treatment-associated like the ratio of effector-to-target cells. Another variable is the engineered effector cell itself as the maturation stage or other cell types such as NK cells or macrophages likely differ in their pharmacodynamic parameters.

7. Recognition of the targeted antigen on healthy tissues may lead to “on-target off-tumor” toxicity by CAR T cells. A prominent example is the induced B cell aplasia following CD19-specific CAR T cell treatment due to recognition of CD19 on healthy B cells [141]. In this specific case, treatment-induced B cell aplasia serves as a pharmacological biomarker indicating persistence of functionally active CAR T cells and predicting some efficacy against leukemia/lymphoma.

**Conclusion and Perspectives**

Our understanding of the pharmacology of CAR T cell products is mostly based on clinical trials using CD19-specific CAR T cells. In these studies, qPCR and flow cytometry are utilized to track the CAR T cells in the patients’ blood upon adoptive transfer [133, 138]. Given the correlation with the clinical outcome, mechanistic insights into the pharmacokinetic processes early after CAR T cell infusion are needed. This becomes obvious due to recent model simulations suggesting the impact of the CAR T cell dose-exposure relationship; the apparent Cmax upon CAR T cell expansion in the blood is more related to the targeted tumor burden than to the initial CAR T cell dose [137]. There is also evidence that the level of CAR T cell persistence correlates with clinical outcome [142]; functional CAR T cells can persist for many years [143, 144]. Research needs to elaborate the molecular mechanisms of persistent functional capacities since the rates are variable and may depend upon a number of potentially connected variables [133, 145]. As CAR T cell expansion correlates not only with efficacy but also with CRS and tumor burden, the therapeutic window in CAR T cell therapy is obviously very narrow asking for...
different modalities in application. As a consequence, upcoming clinical trials need to address the issue from both the therapeutic and pharmacological perspective.

There is also a matter of debate whether randomly integrating vectors encoding the CAR may potentially cause insertional oncogenesis. While after treatment of several 100 patients with γ-retrovirally and lentivirally modified CD19 CAR T cells no single event was reported, insertional mutagenesis with adverse consequences can occur in hematopoietic stem or progenitor cells as used for the treatment of immune deficiency [146, 147]. Also, modifications with a piggyBac transposon was recently reported to have caused transformation in 2 out of 10 cases [148]. Taken together, for virally modified mature T cells, the risk for clonal T cell expansion or insertional oncogenesis is low and far less than for hematopoietic progenitor cells [149]. However, mature murine T cell transformation is in principle possible [150] and targeted CAR cDNA integration into a safe or at least a pre-defined locus like the TRAC locus may alleviate the concerns in this respect [151].

Manufacturing of CAR T cell products as a pharmaceutical drug under GMP conditions and in sufficient amounts is still challenging, in particular, with respect to the infrastructure with clean rooms, quality control, and qualified personnel [91]. Currently, these requirements restrict CAR T cell manufacturing to a limited number of facilities, of runs, and finally, of patients who can be served. Efforts are made to improve the robustness of the manufacturing process, to make decentralized manufacturing feasible, to abrogate the risk of failure, to standardize and simplify each step during the process to enable reproducibility, and to reduce workload and costs. While a fully automated, supervised, and quality-controlled system addresses these issues, a logistic supply chain from the patient’s leukapheresis to CAR T cell manufacturing and finally infusion into the patient need to be established ideally at the patient’s hospital.

There is also a major challenge due to the different requirements among regions and authorities with respect to global manufacturing and exchange of material. Donor screening and testing, traceability and labeling, patient confidentiality, and apheresis requirements are some examples that need to be harmonized in order to allow shipment of donor starting material and final cell product across borders [87]. Although there is a collaboration between the FDA and EMA, it is still challenging to harmonize terminology, classification criteria, recommendations, manufacturing requirements, and others [78].

As a “living drug,” the dose of CAR T cells is currently empirically explored in phase I trials, mostly starting from $1 \times 10^5$/kg and escalating to $1 \times 10^9$/kg CAR T cell product in case of lack of adverse events. Starting from low levels, therapeutic efficacy increases with CAR T cell dose; however, a clear therapeutic dose as for classical pharmaceutical drugs is hard to define due to the various functional capacities of CAR T cells like post-administration amplification, repetitive killing of target cells, active migration, and long-term persistence and memory. Efforts are made to elucidate the dose that is sufficient to mediate efficacy with less toxicity. A recent rate equation-based mathematical model predicts that 1–10% of the currently clinically applied CAR T cell dose can achieve similar efficacy as the full dose as validated by a mouse model [152]. Since a CAR T cell can execute several rounds of target cell killing, increase in infused CAR T cell numbers does not necessarily increase the killing ratio but increases the levels of released pro-inflammatory cytokines like IFN-γ that are required for tumor elimination; the latter however also increases the expression of suppressive ligands by cancer and stroma cells, thereby reducing CAR T cell efficacy. Besides the T cell dose, the tumor mass at the day of treatment seems to be a major determinant of the CAR T cell response. In the near future, more computational simulations will provide us with a more precise prediction model of the therapeutic outcome, making a more rational planning of CAR T cell regimes possible.

The self-replicating and long-term persistence capacities of CAR T cells affect the pharmacodynamic-pharmacokinetic relationship in a complex way [153]; no paradigms are established to predict safe and efficacious dose levels for CAR T cells. Using phase II datasets from tisagenlecleucel, researchers described the CAR T cell kinetic profile in humans and estimated the slopes of distinct kinetic phases; extrapolation to other CAR T cell therapies or doses is limited [138].

Hardiansyah and Ng [154] used tumor dynamics to describe triggered CAR T cell expansion, providing some insight into CAR T cell distribution kinetics and integration of pharmacokinetics and pharmacodynamics. Singh et al. [137] developed a cell-level model to quantitatively describe the activities of CAR T cells, taking into account the CAR affinity, CAR expression by T cells, antigen densities, and T cell-to-tumor cell ratios to determine the rate of saturable tumor cell killing, CAR T cell amplification, and cytokine release. Consequently, adapting CAR T cell dose to disease burden, rather than defining a fixed dose for all patients, is more likely promising to optimize efficacy and safety in each case [155].
Finally, from the pharmacological perspective, the development of CAR T cells with high translational potential in the near future needs to address pharmacodynamic markers to balance safety and efficacy of CAR T cells and to address pharmacokinetics with respect to trafficking, homing, infiltration, and persistence of CAR T cells. To date, the role of tumor stroma to predict antitumor activity is still underestimated. For recording CAR T cells pre- and post-infusion, methods need to be standardized including quantitative and qualitative recording of the manufactured T cell product, like assessment of genetic modifications, cellular homing, persistence, expansion, and efficacy/potency. Also, immune monitoring of patients including the kinetics of reconstitution of host immunity after lymphodepletion and clinical-immunological profiling of the immune response to CAR T cells needs to be harmonized. Standard product profiles need to be envisioned by definitions through the European Pharmacopoeia and through standardized GMP-conform production protocols for CAR T cell ATMPs. Finally, an open and continuous communication should sustain the capability of patients and health care providers to understand and to contribute to the improvement of current and the development of novel CAR T cell products for the future.

Conflict of Interest Statement

Hinrich Abken is the inventor and holds patents in the field of CAR T cells. The authors have no conflicts of interest to declare.

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Author Contributions

Hinrich Abken and Astrid Holzinger designed, wrote and reviewed the manuscript.

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