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CAR T cells in cancer immunotherapy

A review on the prodigy of modern cancer treatment

Bachelor's Thesis

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Abbreviations

***ALL**, acute lymphoblastic leukaemia*

***APC**, antigen presenting cell*

***CAR**, chimeric antigen receptor*

***CDC/ADCC**, complement- or antibody-dependent cytotoxicity*

***CRS**, cytokine release syndrome*

***CSM,** co-stimulatory molecule*

***DLBCL**, diffuse large B cell lymphoma*

***FITC**, anti-fluorescein isothiocyanate*

***GCV**, ganciclovir*

***HSV-tk**, herpes simplex virus tyrosine kinase*

***iCAR**, inhibitory CAR*

***iCasp9**, inducible Caspase 9*

***IFN-γ**, interferon gamma*

***ITAM**, tyrosine-based activation motifs*

***ITIM**, tyrosine-based inhibitory motif*

***MHC**, major histocompatibility complex*

***MM**, Multiple Myeloma*

***R/R**, relapsed or refractory*

***scFvs**, single chain variable fragments*

***TAA**, tumor associated antigen*

***Tan-CAR**, tandem CAR*

***Tc**, cytotoxic T cell*

***Teff**, effect T cell*

***Th**, T helper cell*

***TIL**, tumor-infiltrating lymphocytes*

***TMPK**, human thymidylate kinase*

***TNF-α**, tumor necrosis factor alpha*

***uCAR**, universal CARs*

Abstract
Over the past decades, significant advances have been made in the field of cancer treatment as novel immunotherapies made their entrance. In particular cell based immunotherapies targeting tumor associated antigens (TAAs) have evolved exceedingly. Strategies in which autologous T cells are genetically engineered *in vitro* to express a TAA recognizing chimeric antigen receptor (CAR) offer a solution for the suppressed anti-tumor responses observed in cancer. CAR T cell therapy has proven to be successful in the treatment of especially haematological malignancies, with currently 4 CAR T cell therapies being FDA approved for the treatment of acute lymphoblastic leukaemia (ALL) or diffuse large B cell lymphoma (DLBCL). Over the years the design of the CAR has been further and further fine-tuned in order to enhance CAR T cell activity and specificity, and to limit cytotoxic side effects like cytokine release syndrome (CRS) and neurological toxicity. Yet, efficacy of CAR T cells against solid tumors is still very limited due to limited tumor tissue penetration and an immune-suppressive tumor microenvironment, which constitutes a challenge in the design of next-generation CARs. In this review, I will discuss the concept of CAR T cell therapy, the various generations of CARs including their mode of action as well as their therapeutic efficacy and side effects.

Keywords: Cancer; Immunotherapy; TAA; CAR; CAR T cell therapy

Introduction

The human body has developed a complex, yet sophisticated, way to protect itself from infectious diseases and malignancies, i.e., the immune system. The internal immune system consists of 2 arms (consisting of multiple components); the innate 'non-specific' immune system and the adaptive 'specific' immune system. The innate immune system provides a first line of defence and is activated upon pathogen penetration, and invasion, through the physical- and chemical barriers of the human body (e.g., skin and acidic stomach). Moreover, the innate immune system provides a general cellular (pathogen recognizing receptors, phagocytes and NK cells)- and humoral (complement system and cytokines) defence response to protect against pathogens (Romo et al., 2016). Due to the limited specificity of the innate immune system it does not always prevail in eliminating pathogens, infections or diseases. When the innate immune system fails to eliminate the pathogen or pathogenic infection it activates the adaptive immune system.

The adaptive immune system comprises B- and T cells. B cells are involved in the humoral adaptive response as they start producing specific antigen recognizing antibodies upon activation. These antibodies are especially directed against extracellular pathogenic antigens. T cells are responsible for the cellular adaptive immune response. This response is exerted as activated naïve T cells differentiate into CD8+ cytotoxic T cells, which are able to recognize and kill cells infected intracellularly by pathogens (Smith-Garvin et al., 2009). Moreover, CD4+ T cells play a critical role in immune response regulation. Activated CD4+ T cells can either differentiate into T helper cells or into regulatory T cells. T helper cells function to enhance the immune response by activating both B lymphocytes and cytotoxic T cells, whereas regulatory T cells serve to control the immune response by suppressing immune cells. Regulatory T cells also are essential to prevent auto-immune disease (Luckheeram et al., 2012).

Activation of the adaptive immune system depends on the activity of antigen presenting cells (APCs) of the innate immune system. APCs are able to process and present pathogenic- and tumorigenic antigens on their surface in the context of major histocompatibility complexes (MHCs) (Gaudino & Kumar, 2019). There are 2 MHC classes; MHC class 1 and MHC class 2, which present antigen to CD8+ T cells and CD4+ T cells respectively (Wieczorek et al., 2017). Upon binding of the T-cell receptor (TCR) of naïve T cells to their cognate antigen presented by APCs in the context of MHC, T cell proliferation and differentiation occurs. T cells can either develop into effector T cells (Teff), i.e., CD4+ helper T cells (Th) and CD8+ cytotoxic T cells (Tc), or into memory T cells. Th cells function to stimulate the activation of both B cells and Tc cells, whereas Tc cells are capable of directly binding and killing of infected or tumor cells. In fact, Tc cells are the most forceful component of the human immune system against cancer (Raskov et al., 2021). Additionally, B cells can be directly activated by the binding of their B cell receptor to either membrane bound or soluble pathogenic antigens (Treanor, 2012).

In order to maintain cellular homeostasis and to inhibit carcinogenesis a cancer immune surveillance system is incorporated in the immune system. The concept behind this surveillance system is based on the recognition of tumor cells as non-self, resulting in the initiation of a destructive immune response towards these malignant cells (R. Kim et al., 2007).

Unfortunately, the immune system is not always able to detect and destroy neoplastic cells, which could result in the persistence of malignant cells and eventually tumor formation. The inability to detect and destroy tumors is predominantly due to general innate- and adaptive immune evasion mechanisms like; T-cell exclusion from the tumor microenvironment, negative regulation of immune checkpoints (predominantly CTLA-4 and PD-1) (Prestwich et al., 2008, Spranger & Gajewski, 2018) and downregulation of the antigen processing/presentation machinery (Vinay et al., 2015). Via these immune response evading mechanisms tumor cells prevent Teff cell activation, and thus prevent their own destruction (Fig. 1).

As cancer is the cause of approximately 10 million deaths annually and thereby the second leading cause of death globally (WHO, 2021), new improved and specified treatments are demanded. Whereas the traditional cancer treatments like surgery, radiation and chemotherapy, are rather non-specific, newly developed immunotherapies show to be very specific and offer an opportunity for precision medicine in cancer treatment (Liu & Guo, 2018). As of today, the traditional treatment options are still the standard of care, yet immunotherapeutic approaches are increasing, and hold promise because of their potential to be tailored to specific forms of cancer. Current cancer immunotherapies include cancer vaccines, immune checkpoint inhibitors and adoptive T-cell transfer therapy.

The two main therapies base on T-cell transfer are tumor-infiltrating lymphocytes (TIL) therapy and chimeric antigen receptor (CAR) T-cell therapy (National Cancer Institute, 2020). In TIL therapy lymphocytes are isolated from the patient's blood and tested for their tumor recognizing ability. The most potent lymphocytes are then selected and in the next step expanded *ex vivo*. At last, the expanded number of TILs is reinfused into the patient to expectantly target the tumor. TIL therapy was originally developed for the treatment of advanced melanoma patients (Eberlein, 2012) and is nowadays also used in several medical centres for the treatment of metastatic melanoma with observed overall response rates of approximately 50% (van den Berg et al., 2020). Like in TIL therapy lymphocytes are isolated from the patient's blood in CAR T-cell therapy. After the isolation of the patient's T cells a genetic modification (e.g., via a viral vector, electroporation, nanoparticles or transposon transfection) of these cells is exerted, resulting in the expression of CARs on the surface of the T cells. These CAR constructs are antigen specific and do not require presentation by MHC molecules. After the T cells have been successfully modified, these cells are expanded in the laboratory. Finally, the CAR T cells are reinfused into the patient to attack the cancer cells (Fig. 2).

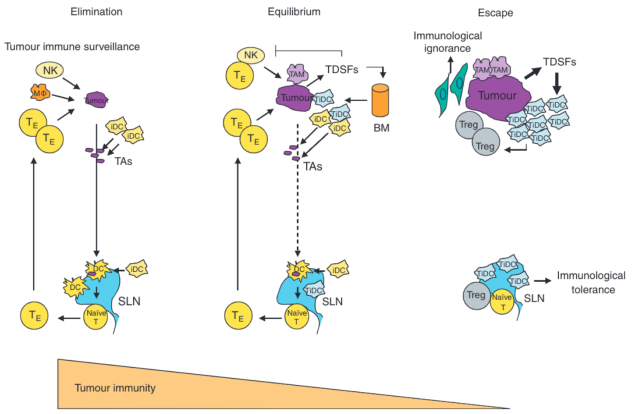

Figure 1. Three essential phases of immune system – tumor interactions towards cancer immune evasion (R. Kim et al., 2007). Initially, the tumor is detected and destroyed as a consequence of cancer immunosurveillance (phase 1 - elimination). During tumor growth, tumor remodelling occurs resulting in a decrease in tumor immunogenicity and an increase in tumor immune resistance (e.g.,, via CTLA-4 and PD-1), impairing the tumor eliminating function of the immune system (phase 2 – equilibrium). Finally, tumor progression results in the secretion of soluble factors further impairing tumor recognition and complete immunological ignorance is obtained (phase 3 – escape). TA= tumor antigen; NK = natural killer cell; MΦ= macrophage; T_E= effector T cell; iDC= immature dendritic cell; DC= dendritic cell; TiDC= tumor-associated DC; SLN= sentinel lymph node; TAM= tumor-associated macrophage; TDSFs= tumor-derived soluble factors; Tregs= regulatory T cells; BM= bone marrow.

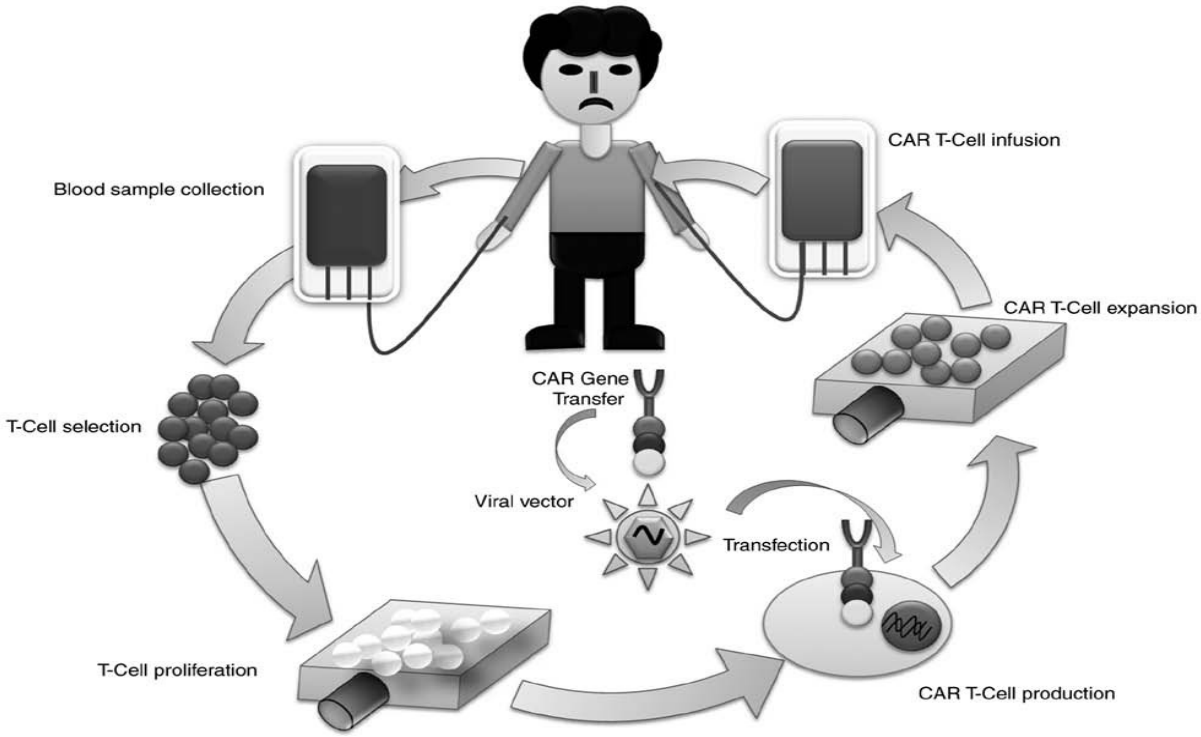

Figure 2. CAR T cell therapy (Mohanty et al., 2019). A graphical overview of the several procedures in CAR T cell therapy in chronological order; blood sample collection, T cell selection, T cell proliferation, the transfection of the T cell with a viral vector encoding for the CAR, CAR T cell expansion and CAR T cell infusion.

CAR T-cell therapy is a very novel strategy in the treatment of cancer with only 4 FDA approved CAR T-cells; 1. Tisagenlecleucel for acute lymphoblastic leukaemia (ALL) (2017), 2. Axicabtagene ciloleucel for diffuse large B cell lymphoma (DLBCL) (2017), 3. Brexucabtagene autoleucel for mantle cell lymphoma (2020) and 4. Lisocabtagene maraleucel for relapsed or refractory (R/R) DLBCL (2021) (Nature, 2021). CAR T-cells are thus currently only applicable and successful in the treatment of hematopoietic malignancies, whereas their effectiveness against other forms of cancer is still under investigation (Mohanty et al., 2019).

In this essay, I will review the concept of CAR T cell therapy for cancer treatment focusing on the various generations of CARs that have been designed, their mode of action as well as their therapeutic efficacy and side effects. In addition, I will highlight current challenges and new developments in the design and applicability of CAR T cell therapies.

General concept and design of a CAR

The idea behind CAR T cell therapy is to counteract the inability of autologous TCRs to recognize tumor associated antigens (TAAs). To this end, patients' T cells are transduced with genetic constructs instructing the cells to synthesise a specific immunoreceptor, the CAR.

The construct of this CAR consists of four major elements; an extracellular binding region, a hinge (spacer) region, a transmembrane domain and an intracellular (cytoplasmic) signalling domain (Dotti et al., 2014; Sadelain et al., 2013) (Fig. 3).

The specificity and efficacy of the CAR is determined by its extracellular binding region, which recognizes TAAs and thus directs the T cells (Gacerez et al., 2016). The predominant strategy in specifically directing T cells is the use of single chain variable fragments (scFvs) as extracellular binding region. This scFv is derived from antibodies directed against TAAs. Variable regions of the light and heavy chains of this specific antibody are fused using a short linker peptide (Feinberg et al., 2019). The variable regions used to form the scFv can be considered as partial replacements of the autologous α- and β TCR chains. As mentioned earlier, this generic extracellular binding region of the CAR is able to directly recognize and bind TAAs, whereas autologous non-modified TCRs require MHC antigen presentation. Moreover, other strategies besides the use of a scFv as extracellular binding region are currently being investigated.

The effectiveness of alternative CAR extracellular binding regions, like ligand-based receptors and natural receptors (e.g., second generation modified IL13Rα2-specific CARs and second generation modified T1 E28z CARs), has already been investigated in multiple studies towards haematological and solid malignancies and both preclinical and clinical results look promising (Murad et al., 2018).

The hinge determines the flexibility and stability of the extracellular binding region of the CAR. Furthermore, by extending the length of the extracellular part of the CAR, receptor dimerization can occur. Via these characteristics of the hinge region the CAR T cell-target cell interaction is affected, increasing the strength of the activation signal. The hinge region in most CARs is composed of CD8-α and CD28 spacer regions, or of constant heavy chain domains of immunoglobulins (Lipowska-Bhalla et al., 2012).

The transmembrane domain is formed by a hydrophobic α-helix derived from either immunoglobulins, CD8, or CD28 and mainly functions to anchor the extracellular part of the CAR to the T cell membrane (Feinberg et al., 2019). Moreover, CAR stability and expression levels are substantially affected by the transmembrane domain. More specifically, recent research indicated that the transmembrane domain is responsible for the regulation of the magnitude of CAR signalling via the control of the CAR expression level, whereas the hinge region is responsible for the regulation of the CAR signalling threshold (Fujiwara et al., 2020).

Lastly, the intracellular or cytoplasmic signalling domain of the CAR is of importance for the final downstream signalling resulting in CAR T cell activation and the cytotoxic response directing against the malignant cell(s) (Gacerez et al., 2016). Multiple intracellular signalling domains have been investigated over the past years resulting in the development of currently 5 generations of CAR T cells. The 5 different generations of CAR T cells and their specifications will be discussed in the next paragraphs.

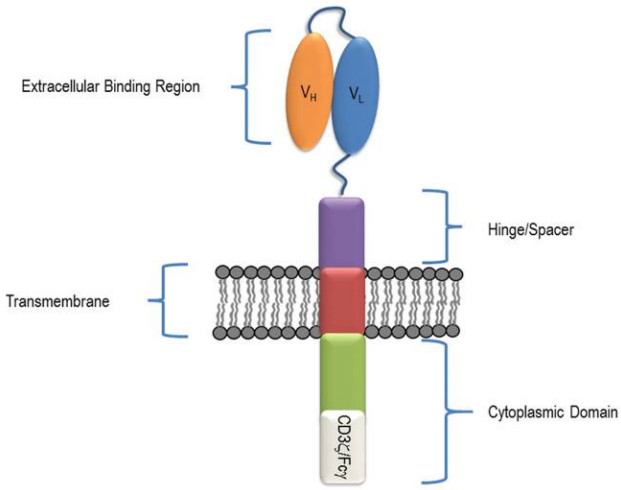

Figure 3. The general construct of the chimeric antigen receptor (Gacerez et al., 2016). The 4 major elements of the CAR are indicated; the extracellular binding region, the hinge (spacer) region, the transmembrane domain (e.g., CD8- α or CD28) and the intracellular (cytoplasmic) signalling domain. The extracellular binding region is mostly formed by a scFv, which consist of variable light and heavy chain (Vh and Vl) regions of an antibody. The generally used intracellular signalling domains in CARs, CD3ζ and Fcγ, is depicted here and is in later generations paired with a costimulatory component (green).

First generation CARs. The construct of the first generation CAR T cells consists of a TAA binding scFv forming the extracellular binding region, a hinge or spacer protein, a transmembrane protein and the intracellular signaling domain CD3ζ (Fig. 4). This CD3ζ – chain functions via its tyrosine-based activation motifs (ITAM), which enables CAR T cell activation in a non-MHC restricted way. Important to notice is that activation of first generation CAR T cells only occurs through the downstream signaling via this CD3ζ domain and no further co-stimulatory molecules are involved in CAR T cell activation. Initial preclinical studies, in for example *in vitro* target-cell specific killing- and murine tumor models (Almåsbak et al., 2016), proved the efficacy of first generation CAR T cells, and subsequently first generation CARs entered into phase 1 trials for cancer patients with B cell malignancies, ovarian cancer, renal cell carcinoma and neuroblastoma (Ramos et al., 2014).

In general, these phase 1 trials demonstrated that first generation CARs lacked the ability to sufficiently activate the chimeric T cells via their single intracellular signaling domain CD3ζ, and thus failed to generate an adequate antitumor response. Furthermore, first generation CAR T cell therapy trials generally showed limited persistence of these cells over time, limiting anti-tumor responses (Brocker & Karjalainen, 1995). Yet, studies in which B cell lymphoma- and neuroblastoma patients were treated with α-CD20-CD3ζ- and scFv-CD3ζ CAR T cells respectively, lasting therapeutic effects were observed (Duong et al., 2015), emphasizing the potential of CAR T cell therapy.

Second generation CARs. To improve and ensure full T cell activation, a second intracellular signaling domain, besides the original CD3ζ domain, was incorporated into the second generation of CARs, i.e., a co-stimulatory molecule (CSM) (Fig. 4). The CSM domain in second generation CARs is attached to the CD3ζ domain and provides a second signal in the activation of the chimeric T cell via binding of the CSM to CD80 on tumor cells. Moreover, the insertion of this domain between the transmembrane domain and the CD3ζ domain prevents induction of T cell anergy observed in first generation CARs due to the lack of signal amplification and modulation by CSMs (Hay & Turtle, 2017; van der Stegen et al., 2015). CSM domains like CD27, CD28, 4-1BB, inducible T cell co-stimulator (ICOS) and OX40, have been shown to enhance both CAR T cell function and persistence in both in vitro and in vivo studies (Abate-Daga & Davila, 2016). Most spectacular results were observed in phase 1 clinical trials in which patients suffering from relapsed or refractory (R/R) B cell acute lymphoblastic leukemia were treated with CD-19 targeted second generation CAR T cells (CD28 or 4-1BB CMS domain), with complete remission rates of up to 90% (Brentjens et al., 2010).

Third generation CARs. To further improve chimeric T cell activation, proliferation, persistence, cytokine production and thus antitumor functionality, a second CSM domain was inserted into the intracellular signaling domain (Fig. 4). For example, the intracellular signaling domain of a third generation CAR construct consists of CD3ζ-CD28-4-1BB or CD3ζ-CD28-OX40 (Lock et al., 2017). Preclinical trials confirmed the expected improved proliferation, persistence and antitumor activity after which third generation CARs entered phase 1 trials for the treatment of relapsing or refractory B cell malignancies and chronic lymphocyte leukemia. In both phase 1 trials patients were infused with α-CD19-CD3ζ-CD28-4-1BB CAR T cells. In the former phase 1 trial no enhancement in the primary response rate was observed compared to second generation CAR T cell therapy, whereas in the latter trial complete remission rates in over 80% of the patients were reported (Huang et al., 2020; Tang et al., 2016). In conclusion the addition of another CSM domain may be beneficial in complementary co-stimulation and thus CAR T cell efficacy. Yet, the addition of a second CSM domain increases the rate of severe side effects (e.g., cytokine release syndrome) and accelerates CAR T cell aging, which both may be attributed to repeated signal delivery by both CSM domains (Huang et al., 2020).

Fourth generation CARs and fifth generation CARs. Both fourth- and fifth generation CARs are based on the construct of second generation CARs. Additionally, both constitute an additional component within their intracellular signal domain. The additional component in the intracellular signaling domain of fourth generation CARs is a cytokine (e.g., IL-12) expression system, whereas the extra component in fifth generation CARs is a cytokine receptor domain (e.g., IL-2Rβ) (Kim & Cho, 2020) (Fig. 4). Both components are designed to overcome two big challenges encountered with earlier generation CARs, which are the targeting of TAA negative cancer cells and the targeting of solid tumors.

The cytokine expression system in fourth generation CARs induces the expression of innate immune- and T cell activating immune modifiers around the tumor lesion. The attracted innate immune cells (e.g., macrophages and natural killer cells) have the special ability to target and kill TAA negative cancer cells, which are invisible to CAR T cells (Chmielewski et al., 2011).

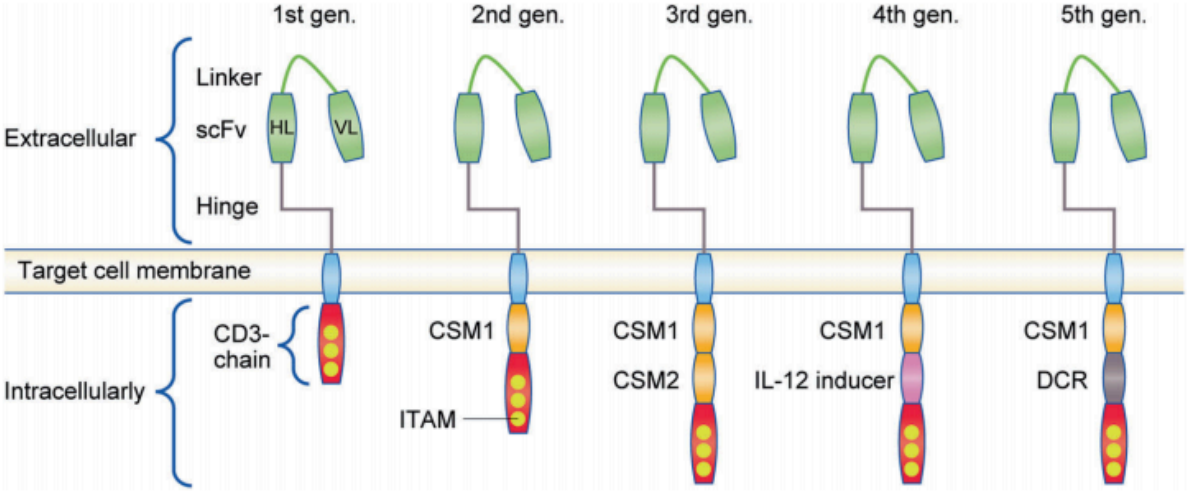

Figure 4. Different generations of chimeric antigen receptors (CARs) (Raskov et al., 2021). The general CAR construct constitutes of an antigen binding scFv, a hinge (spacer) domain, a transmembrane domain and an intracellular (cytoplasmic) CD3ζ signalling domain. The CD3ζ domain signalling functions via its tyrosine-based activation motifs (ITAM). The different generations differ in their intracellular signalling domain; first generation CARs contain only the CD3ζ chain in their intracellular signalling domain, second generation CARs contain the CD3ζ chain + a co-stimulatory molecule (CSM) domain (e.g., CD28 or 4-1BB0), third generation CARs contain the CD3ζ chain + 2 CSM domains, fourth generation CARs contain the CD3ζ chain + a CSM domain + a cytokine (e.g., IL-12) expression system domain and fifth generation CARs contain the CD3ζ chain + a CSM domain + a domain of a cytokine receptor (DCR).

Currently the use of fourth generation CAR T cells in the treatment of advanced breast cancer is under investigation. Early preclinical studies using fourth generation CAR T cells targeting FRα, a TAA highly expressed on breast cancer cells, show promising results demonstrated by prominent cytotoxicity towards breast cancer cell lines in *in vitro* experiments. (Luangwattananun et al., 2021). However, results in preclinical trials look promising it is also observed that severe side effects may occur when the expressed immune modifiers cause high systemic levels of cytokines, i.e., cytokine release syndrome (CRS) (Chmielewski & Abken, 2015).

The cytokine receptor domain in fifth generation CARs contains a binding site for the transcription factor STAT3. Upon antigen-dependent activation of the CAR STAT3 binding enables the activation of the JAK-STAT pathway which is a key pathway in cytokine signaling. Via synergistic signaling through the CD3ζ domain, the co-stimulatory domain and the cytokine receptor domain, T cell activation and proliferation is enhanced in fifth generation CAR T cells, theoretically increasing their efficacy (Kagoya et al., 2018). The efficacy and safety of these CARs is still to be determined as research into this latest receptor design is still in its early stages and statements concerning future clinical applications cannot be made yet.

Toxic side effect of CAR T cell therapies and approaches to prevent these

As mentioned earlier, current FDA approved CAR T cell therapies and novel CAR T cell clinical trials show the effectiveness and potential of the use of genetically modified T cells in the treatment of cancer, and especially in the treatment of patients suffering from R/R hematologic malignancies. Unfortunately, clinical trials have also indicated that CAR T cell therapies are specifically related to a number of severe toxic side effects that may cause significant morbidity and even mortality (Neelapu et al., 2017). Toxic side effects of CAR T cell therapy include CRS, neurological toxicities (e.g., encephalopathy syndrome), off-tumor effects, and anaphylaxis (Boroojerdi et al., 2020). CRS is the most common prevalent toxicity observed in CAR T cell therapy and is caused by excessive release of cytokines by the infused CAR T cells (Brudno & Kochenderfer, 2016).

To reduce these side effects, it is of great importance to extend knowledge on possible CAR T cell induced immune responses and systemic effects. Moreover, to ensure patient wellbeing it is crucial to incorporate safety mechanisms into the CAR T cells to prevent unwanted scenarios like off tumor activity.

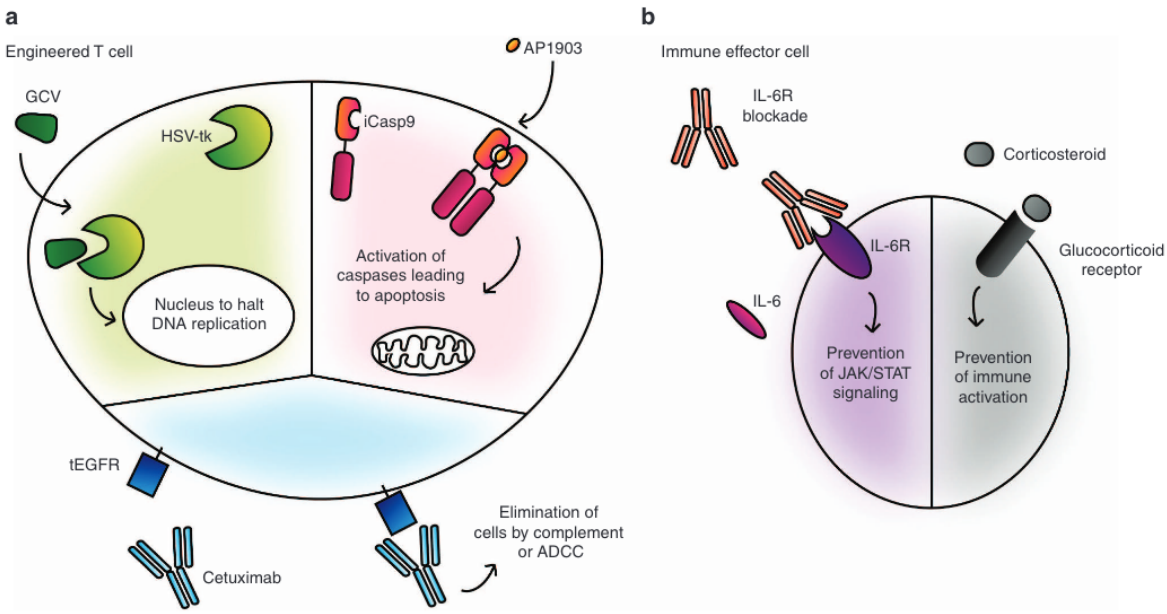

Figure 5. Overview of toxicity management strategies in CAR T cell Therapy (Bonifant et al., 2016). a) CAR T cells can be incorporated with suicide genes like herpes simplex virus thymidine kinase (HSV-tk) and inducible caspase 9 (iCasp9) to ensure selective induction of CAR T cell depletion with regard to toxicity management. HSV-tk converts the administered prodrug ganciclovir (GCV) into GCV-triphosphate, which incorporates into replicating DNA, resulting in cell death. iCasp9 binds a specific administered small molecule (AP1903) and dimerizes, activating the caspase 3 apoptotic pathway. Furthermore, elimination genes such as truncated endothelial growth factor receptor (tEGFR) can be incorporated in CARs. tEGFR is a surface marker, which can be targeted with infused anti-tEGFR antibodies (cetuximab), resulting in CDC/ADCC towards the CARs. **b)** Pharmacological immunosuppressants are used to manage CAR T cell toxicity including antibodies (e.g., anti-IL-6R) and corticosteroids.

As of today, CAR T cells can be modified in 2 ways to enhance and control their safety being the inclusion of so-called suicide genes or elimination genes. A brief explanation on these individual modifications and their mode of action will be given in the following paragraphs. Furthermore, in cases of exaggerated and/or uncontrolled immune responses (e.g., CRS) pharmacological immunosuppressants like corticosteroids and monoclonal antibodies are used to diminish the persistence and toxic effects of CAR T cells (Fig. 5) (Davila et al., 2014).

Suicide genes. The concept of the integration of a suicide gene relies on the selective induction of CAR T cell depletion in case of the occurrence of (un)expected toxicities. Several genes have already been investigated for their suicidal abilities in preclinical- and clinical trials, and proven successful (Bonifant et al., 2016). Example suicide genes incorporated in CARs currently are herpes simplex virus tyrosine kinase (HSV-tk), human thymidylate kinase (TMPK) and inducible Caspase 9 (iCasp9) (Brandt et al., 2020). CAR T cells expressing either TMPK or iCasp9 are susceptible for activation of the caspase 3 apoptotic pathway upon administration of an apoptotic pathway inducing small molecule (AP1903), whereas CAR T cells expressing HSV-tk take up and convert the administered prodrug ganciclovir (GCV) into the self-detrimental GCV-triphosphate. The functionality of the iCasp9 gene has especially been validated in patients treated with CAR T cells who experienced onset of graft-versus-host disease effects towards received haploidentical stem-cell transplants, as CAR T cells were quickly depleted upon administration of the detrimental small molecule (Di Stasi et al., 2011).

Elimination genes. Like suicide genes, elimination genes rely on the concept of selective induction of cell death enabling CAR T cell depletion. Yet, the mechanism via which the depletion is induced with elimination genes differs. CAR T cells with an incorporated elimination gene in fact co-express a cell surface elimination marker/antigen (e.g., tEGFR), which is normally not expressed on autologous T cells. These elimination markers can be targeted by infusing clinically approved antibodies, inducing complement- or antibody-dependent cytotoxicity (CDC/ADCC) towards CAR T

cells (Paszkiewicz et al., 2016), resulting in CAR T cell depletion.
A big advantage of this safety mechanism is that it allows positive selection of CAR T cells during the manufacturing process, as cell surface markers are readily detectable. Unfortunately, the efficacy of incorporated elimination genes in patients who received chemotherapy prior to CAR T cell infusion can be compromised as a consequence of their limited CDC/ADCC capacity. Moreover, antibodies might not distribute to poorly vascularized tumors, impairing the depletion of CAR T cells (Brandt et al., 2020). To overcome aforementioned problems a short peptide epitope (E-tag) is built into the extracellular domain of the CAR. This E-tag can be targeted by anti-E-tag CAR T cells which are infused upon toxicity, ensuring the depletion of the initially infused anti-tumor CARs (Koristka et al., 2019).

Challenges in CAR T cell therapies

Despite remarkable results observed in both pre-clinical and clinical trials employing CAR T cells, the risk of specific life-threatening toxicities, as mentioned earlier, still is a major hurdle. Additionally, current CARs are directed and effective against liquid tumors, whereas efficacy in the treatment of solid tumors is still rather poor due to their immunosuppressive tumor-microenvironment (Klebanoff et al., 2016). To overcome these challenges novel CARs are being designed and previously designed constructs are finetuned on specificity and activity. The following section of this review will focus on these so called "next generation CAR T cells" and also highlights recent developments pertaining to the use of natural killer (NK) CARs as an alternative to CAR T cells.

Next generation CAR T cells

Next generation CARs are based on the constructs used for second generation CARs containing a costimulatory- and a CD3ζ component in their intracellular signaling domain. Next generation CARs distinguish themselves from previous generation CARs through their specialized extracellular binding domain. Currently there are 4 next generation CARs based on the construct of second generation CARs; tandem CARs (Tan-CARs), bispecific CARs, physiological CARs and universal CARs (uCARs). Additionally, next generation CARs are designed with an inhibitory intracellular signaling domain, i.e., inhibitory CARs (iCARs), to especially achieve enhanced discrimination between tumorous- and healthy cells (Fig.6).

Tan-CARs. To enhance CAR T stimulation and activation two individual TAA recognizing scFvs are incorporated in the extracellular binding domain on Tan-CARs. The particular scFvs are joined by a linker and placed in tandem on the supplementary part of an individual CAR. Synchronized targeting of two TAAs on a single cancer cell allows CAR T cell activity against both liquid tumors and solid tumors within their microenvironments (Schneider et al., 2017). As CD19 targeting has proven clinical efficacy already, present Tan-CAR studies mostly include the anti-CD19 antigen binding site. The choice of second antigen binding site is based on antigens unique for or aberrantly expressed by tumor cells. The efficacy of Tan-CARs is under investigation for Multiple Myeloma (MM) and B cell malignancies.
MM tumor cells uniquely express a set of surface antigens in relative high numbers (compared to non-tumor cells), which can be utilized as target for a TAA recognizing domain of Tan-CARs. Uniquely highly expressed MM antigens are BCMA, CD38 and TACI (Cronk et al., 2020). Early phase trials have already been performed in which the efficacy and durability of CD19-BCMA CARs, CD38-BCMA CARs and BCMA-TACI CARs was analyzed. Results of these trials were promising with overall response rates of respectively 95%, 87.5% and 43%. All studies reported a few cases of CRS as a consequence of the Tan-CAR therapy, but with a manageable toxicity profile (Zhang et al., 2019; Li et al., 2019; Popat et al., 2019).
Additional uniquely high expressed surface antigens for tumor cells in B cell malignancies are CD20 and CD22. Early phase 1 trials have been performed towards the use of CD19-CD20 Tan-CARs in the treatment of R/R B cell non-Hodgkin lymphomas and the use of CD19-CD22 Tan-CARs in the treatment of B cell ALL. Also, here results were impressive with complete response rates of 55% and 92% respectively. Both trials showed promising toxicity profile with the CD19-CD20 Tan-CAR trial even reporting no dose-limiting toxicities (Shah et al., 2019; Schultz et al., 2019).

Bispecific CARs. Like Tan-CARs bispecific CARs recognize two distinct TAAs. Yet, bispecific CARs express two different CARs on a single surface with each CAR recognizing its own specific TAA. Bispecific CARs have especially been designed to overcome the target antigen loss problem which refers to downregulation of TAAs by the tumor in order to establish immune evasion. Hence, bispecific CARs may offer a solution by simultaneously targeting more than one antigen. This approach is predominantly investigated for the treatment of B cell ALL as earlier CAR T cell therapy trials have indicated the efficacy on CARs against multiple individual TAAs on B cells, like CD19, CD20 and CD22 (Fry et al., 2017). Bispecific CAR T cells conserve their effector capacity in case of one of the TAAs is not being available due to for example cellular hindrance, mutations or antigen loss, and therefore offer a way to circumvent tumor evasion upon occurrence of these events (Majzner & Mackall, 2018). Encouraging results have been reported in one clinical trial utilizing CD19-CD22 CAR T cells for the treatment of R/R B cell ALL with 6/6 patients (adults) achieving a complete response (Dai et al., 2019).

Physiological CARs. The scFvs used in the construction of several CARs mainly originate from murine antibodies. Hence, there is a risk that patients develop an anti-CAR T cell immune response which may cause anaphylaxis or limit CAR T cell persistence (Maus et al., 2013). With respect to this problem, researchers have designed physiological CARs to prevent such unwanted immune activation. The construct of physiological CARs consists of the standard intracellular signaling domain CD3ζ and an endogenous (physiological) TAA recognizing receptor/ligand pair forming the extracellular binding domain (e.g., CD27- CD3ζ CAR) (L. Liu et al., 2012; Shi et al., 2014). The use of physiological CARs in trials is still very limited and not much is known about their efficacy. Early results show two major findings; the use of physiological CARs may eliminate immunogenicity concerns, and physiological CARs are able to bind multiple targets (Murad et al., 2018). The latter finding may be advantageous as it could reduce the risk of immune escape due to tumor antigen loss, but may also be a disadvantage as it could increase the risk of off-tumor activity and hence, toxicity.

uCARs. To further fine-tune the activity of CAR T cells and thereby reducing the risks of toxicity, a rapidly switchable chimeric receptor has been designed, which allows for highly controlled and dose dependent CAR T cell activation, i.e., uCAR (Cartellieri et al., 2016). The construction of this uCAR consists of a CD3ζ- and a CSM (e.g., CD28) intracellular signaling domain, a transmembrane domain, and a biotin- or anti-fluorescein isothiocyanate (FITC) labeled TAA recognizing antibody that functions as extracellular binding domain. Additionally, bispecific uCARs are now being designed allowing for direct co-stimulation of the T cell response, which is advantageous over regular bispecific CARs that can only directly engage CD3ζ signaling (Minutolo et al., 2019). As uCARs can be targeted very precisely via their TAA recognizing antibody and for this reason their application for a new highly promising leukemia target, CD123, is studied. Moreover, this TAA is also expressed by certain endothelial-, hematopoietic progenitor and myeloid tumor cells possibly broadening the application of anti-CD123 uCARs. Recent results from early clinical trials indicated the efficacy of CD123 directed uCARs in eliminating CD123-positive leukemic cells. Yet, preclinical trials have indicated that there is a risk of attacking normal cells. On the other hand, the early clinical trials did not report major toxicities so far (Loff et al., 2020). As uCARs are still very novel a very detailed description concerning efficacy and toxicity is still lacking.

iCARs. To overcome the problem of off target CAR T cell activity and to improve discrimination between malignant and healthy cells, iCAR T cells have been designed. iCAR T cell constructs consist of a tandem- or bispecific extracellular binding domain (scFvs), a transmembrane domain and either the CTLA-4- or PD-1 intracellular immune inhibitory signaling domain. The extracellular binding domain is designed to recognize antigen specifically expressed on healthy tissue. Upon binding of the iCAR to its antigen the inhibitory signaling cascade is activated, prohibiting CAR T cell activation.
The immunosuppressive signal of iCAR T cells dominates the CAR T cell anti-tumor effector signal, and via this principle iCAR T cells restrict CAR T cell activity to tumor tissue lacking antigens associated with healthy cells (Fedorov et al., 2013).

Preclinical trials using mouse models have already confirmed the immune regulatory effect of iCARs. Mice infused with iCARs containing the CTLA-4 receptor did not show substantial T cell activation, whereas mice infused with iCARs lacking the receptor showed enhanced T cell activation and proliferation, eventually leading to severe systemic autoimmune disease (Pasquali et al., 2017). Currently iCARs are not used to manage toxicity in approved CAR T cell therapies. Yet, iCARs show the potency to fulfill such a function in the future.

NK CARs

Besides T cells playing a significant role in immunosurveillance and consequently the prevention of tumor formation, also innate NK cells are of great importance for this process. Yet, NK cells do not possess the intracellular signaling domain CD3ζ nor the TAA recognizing TCR via which T cells initiate their anti-tumor response. To distinguish healthy cells from malignant ones NK cells express the tyrosine-based inhibitory motif (ITIM) immunoreceptors on their surface. ITIMs interact with MHC-1, a marker generally expressed by healthy cells, preventing NK cell activation. Importantly, cancer cells almost always lack or only minimally express MHC-I (immune evasion mechanism), making them a target for NK cells as they fail to provide the inhibitory interaction (Chiossone et al., 2018; Guillerey et al., 2016). Thus, NK cells elicit their effector function by directly attacking cells missing MHC-1 markers. (Klingemann, 2014). Upon activation of NK cells, the release of cytotoxic granules containing granzymes and perforin is initiated causing direct lysis of tumor cells. Additionally, NK cells start producing both cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), and chemokines in order to provoke an adaptive immune response.

As NK cells possess essential tumor eliciting- and immunomodulatory abilities the use of these cells in cancer immunotherapies has been investigated over the past decades. Whereas clinical trials towards adoptive T cell immunotherapies indicated the necessity of the use of autologous cells, early clinical trials towards NK cell infusion indicated overall safety of both autologous and allogeneic NK cells (Sakamoto et al., 2015; Rubnitz et al., 2010), allowing for so called “of-the-shelf” NK cell-based immunotherapy (S. Liu et al., 2021).

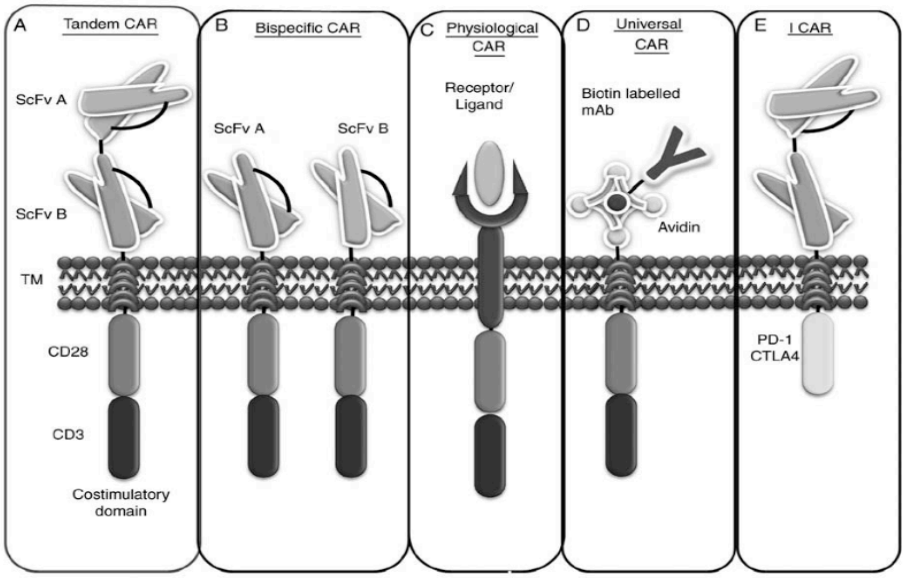

Figure 6. Next generation chimeric antigen receptors (CARs) (Mohanty et al., 2019). A) Tandem CAR; An individual receptor which targets two TAAs via two consecutively linked distinct single-chain variable fragments (scFv A and -B). **B)** Bispecific CAR; Two individual CARs each expressing its own distinct TAA recognizing binding site (scFv A and -B). **C)** Physiological CAR; The extracellular TAA recognizing binding domain comprises a receptor/ligand molecule instead of a scFv. **D)** Universal CAR; The TAA recognizing domain consist of a biotinylated (Avidin) or anti-fluorescein isothiocyanate (FITC) labelled antibody, which is able to recognize nearly all TAAs susceptible for antibodies. **E)** Inhibitory CAR (I CAR/iCAR); CAR with a PD-1 or CTLA4 intracellular signalling domain, which trigger T cell inhibition upon activation of the receptor. iCARs are activated by the binding of specific antigens exclusively expressed on healthy cells.

To specifically target NK cells towards cancer cells, TAA recognizing CAR NK cells have been designed. The construct of CAR NK cells comprises a TAA recognizing scFv extracellular binding domain, a transmembrane domain, and one or two CSMs plus the CD3ζ domain making up the intracellular signaling domain. CSMs used in NK cells are comparable to those used in CAR T cells such as CD28, CD-137, 4-1BB, 2B4, NKG2D and DNAM1, of which the latter four are also naturally expressed by NK cells as activating receptors. The incorporation of these native molecules in the intracellular signaling domain has proven to be more effective in *in vitro* and *in vivo* anti-tumor activity studies compared to incorporation of non-native molecules (e.g., CD28-CD137) (Y. Li et al., 2018).

Besides the cytotoxic effects of NK CARs against cancer cells, recent research suggests a second major function of NK CARs, which is the elimination of immunosuppressive cells in the tumor microenvironment. Finally, as mentioned above, NK CARs secrete cytokines and chemokines, which may be beneficial for the infiltration and functionality of infused CAR T cells. With respect to the characteristics of NK CARs, combined CAR NK – CAR T cell has been proposed for the treatment of solid tumors (Parihar et al., 2019).

Discussion

CAR T cell therapy can be considered as a prodigy in the treatment of cancer with impressive results in preclinical- and clinical trials for a variety of cancer types. Currently 4 types of CAR T cell therapies are FDA approved, with application restricted to last resort treatment of 2 hematological malignancies; ALL and DLBCL. A big advantage of these therapies is the relative short intervention time and only a single infusion of CAR T cells, whereas other cancer therapies require more extensive forms of care with sometimes multiple extensive intervention periods. Additionally, the effect of CAR T cell therapy may persist for decades as the tumor recognizing cells can survive in the host for a longer period without losing their functionality (Perales et al., 2018). Taking into account these characteristics CAR T cell therapy is also referred to as a “living drug” therapy (Zhao et al., 2018).

Yet, treatment of patients with CAR T cells is not without risk. Both preclinical- and clinical trials, as well as FDA approved CAR T cell therapies, report serious therapy associated toxicities, predominantly CRS and neurologic toxicity (Brudno & Kochenderfer, 2019). As these reported toxicities form a great thread to the patient’s health a barrier arises for wider application of CAR T cells. In order to improve toxicity management CAR constructs are still under investigation and further fine-tuned to enhance tumor selective activity, and establish safe and controlled use of CAR T cells. Designs of next generation CARs have aimed to effectuate these goals with specified extracellular binding domains and the inclusion of elimination/suicide genes. Unfortunately, severe toxicities still cannot be ruled out as of today, and immunosuppressive drugs (e.g., corticosteroids and IL-6R antagonist) are necessary at the point of onset of toxicities like CRS. Noted must be that multiple clinical trials have indicated that corticosteroids establish efficacy in treating CRS and thus provide a solution in toxicity management, whereas IL-6R antagonists do not always resolve CRS (Brudno & Kochenderfer, 2019).

Finally, it is important to compare the cost-effectiveness relationship of CAR T cell therapy to the cost-effectiveness relationships of already existing cancer treatments in order to fully understand the potential and feasibility of the therapy. The American Society of Clinical Oncology has estimated the costs of the first two FDA approved anti-CD19 CAR T cell therapies, Axicabtagene ciloleucel and Tisagenlecleucel, at $373.000 (per infusion), with similar costs expected for the other FDA approved- and upcoming CAR T cell therapies (Lin et al., 2019, Fiorenza et al., 2020). The cost-effectiveness is often measured by the price per quality adjusted life year ($/QALY), and depends on the effectiveness- and the long-term outcomes of the treatment. Few cost-effectiveness analysis studies for CAR T cell therapy have been performed yet. One of the few cost-effectiveness analysis studies already performed, was for the treatment of multiply R/R DLBCL patients, receiving either axicabtagene ciloleucel or tisagenlecleucel. As CAR T cell therapy is such a novel treatment cost-effectiveness studies are limited in precise including of QALY factors like quality of life during treatment, post progression care and long-term remission survival. Considering these limitations, the above mentioned study estimated the costs for

both CAR T cell therapies in R/R DLBCL at less than $150.000/QALY (Lin et al., 2019). This threshold is substantially higher compared to current types of cancer treatments and would increase health care costs substantially.
Therefore, for a widespread adoption of CAR T cell therapy cost reduction is a great point of attention, together with improving therapy effectiveness.

Future perspectives

The effectiveness of CAR T cells in the treatment of hematological cancers is proven in clinical trials, resulting in the approval of 4 types of CAR T cells for the treatment of ALL and DLBCL. Yet, CAR T cell therapy still has some obstacles to be overcome before widespread use, such as; the restricted use of autologous T cells, toxicity management, lack of efficacy against solid tumors, and reducing therapy costs. As research continues towards the optimal CAR construct and specific CAR targets, more TAAs will be identified and expectantly simultaneously targeted by the improved CARs. Improved specificity of CARs will result in the decrease of cytotoxic side effects and improved (solid) tumor targeting. Additionally, future research towards the use of genetically modified allogeneic T cells, rather than autologous T cells, could offer a solution in reducing costs. Moreover, the safe use of allogeneic T cells could turn CAR T cell therapy into an “of-the-shelf” immunotherapy (similar to NK CAR cell therapy), and patients can be treated more readily and adequately. All in all, CAR T cell therapy holds great promise in the treatment of cancer and potentially offers a solution for relapsing or refractory patients.

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