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REVIEW ARTICLE

Immunogenicity of Chimeric Antigen Receptor T-Cell Therapeutics

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Abstract
Chimeric antigen receptor (CAR) T-cell immunotherapy has gained significant attention in the past decade due to its considerable potential in the treatment of various types of malignancies, particularly hematological. While success has been achieved in a number of studies, and two CAR-T-cell products were recently approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (YESCARTA®, KYMRIAH®), this treatment modality continues to present challenges for clinical development. One major potential side effect is the ability of CAR-T products to induce host immune responses. Immunogenicity induction risk factors have been shown to be associated with the presence of non-human or partially human sequences in the CAR construct, suicide domain, or other components of the CAR-T, and also with the presence of residual viral proteins or other non-human origin proteins utilized as part of the gene editing step of CAR-T production. Both humoral (antibody-based) and cellular-type responses have been described, leading to various degrees of impact on CAR-T expansion and persistence, and therefore the overall safety and clinically meaningful response of the treatment. In this article we discuss various types of immune responses specific to CAR-T therapy, their impact on treatment outcome, and methodologies used to detect them.

Key Points

CAR-T therapeutics carry a risk of immune response including humoral and cellular-type responses.

Several risk factors have been identified with varying possibilities for induction of response, primarily associated with the non-human or partially human nature of various components of the CAR-T construct as well as the complexity of the CAR-T production process requiring use of viral or other types of gene transfer procedures.

Immune response against CAR-T therapeutics demonstrated an ability to impact the persistence and efficacy of the treatment.

The negative effect of immune response against CAR-T therapeutics can be reduced to a variable degree depending on the modality and extent of immunosuppressive pretreatment.

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1 Introduction

Chimeric antigen receptors (CARs) are engineered proteins expressed on the surface of modified T cells in order to direct the binding of resulting CAR-T cells to targets expressed on malignant cells [1–3]. CAR proteins are designed for a specific binding with tumor-specific antigens in an MHC-independent manner. CARs may be designed as a single-chain variable fragment (scFv). Other formats (e.g., natural ligands) are possible [4]. The actual scFv may be derived from a parental murine or humanized antibody specific to the tumor antigen or may be generated as a result of phage display library screening [5]. CAR proteins allow for a wide variety of binding specificities and affinities, which affect the CAR-T-cell potency. The most common target for a CAR protein is a surface antigen specifically expressed on a tumor cell. For example, an anti-CD19 CAR that can bind an epitope not commonly recognized by immune cells has been developed to treat chronic lymphocytic leukemia and acute lymphoblastic leukemia [1, 6–8].

In addition to the tumor-cell-targeting scFv, CAR proteins also contain one or more intracellular T-cell signaling domains [1, 2, 9–11]. These are intended to enhance CAR-T ability to expand after injection while circulating in vivo and to exert anti-tumor-cell cytotoxicity. Several generations of CAR design have been developed in recent years. While initial CAR proteins included only the CD3z

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intracellular signaling domain, later generations contain various costimulatory domains, including CD27, CD28, CD134, CD137, 4-1BB, or others [1, 12]. One or more costimulatory domains may be included within the intracellular fraction of the CAR construct. The intracellular domain is believed to be able to influence the degree of anti-tumor activity of the modified T cell. Other details of the CAR construct have been viewed as important, including design of the hinge or transmembrane domains, which may contribute to the specificity of antigen binding and efficiency of immunologic synapse [9, 13–15]. The transmembrane domain serves as a membrane anchor and also connects the extra and intracellular domains together. Additional structural adjustments to the CAR-T cells such as removal of the CD52 and T-cell receptor α chain domains have been described [16]. Generalized representation of CAR-T cell construct is shown in Fig. 1.

Methodologies applied to achieve permanent expression of CAR construct on the T cells vary and include lentiviral or onco-retroviral systems as well as non-viral gene transfer procedures [1, 9, 17–20]. Resulting products may vary in safety-related risks due to insertional mutagenesis, level of CAR expression, and complexity of production.

While different T-cell types have been engineered into CAR-T modality, the $CD8^+$ and $CD4^+$ cells are most commonly utilized due to their high anti-cancer cytotoxic properties. Other DNA editing techniques are frequently used to edit or remove domains typically expressed on the surface of T cells, including bacterial-derived gene editing tools such as CRISPR (clustered regularly interspaced short palindromic repeats) and TALEN (transcription activator-like effector nuclease) methodologies [16, 21].

Mechanisms responsible for humoral and cellular immune responses to CAR-T cells are discussed herein. The extent of specific risk may be altered due to patient pre-conditioning treatments, such as chemotherapy, known to influence the immune system, which is also briefly discussed.

Other factors such as activation-induced cell death of CAR-T cells observed after repeated antigen stimulation may limit CAR-T-cell persistence. This and similar mechanisms impacting CAR-T exposure will remain outside the scope of the current review [22].

2 Key Immunogenicity Risks of CAR-T Therapies

The potential immunogenicity of CAR-T therapies has been now broadly recognized and noted as possibly impactful on both efficacy and safety outcomes of the treatment. Several immunogenicity risk factors have been identified and are listed below.

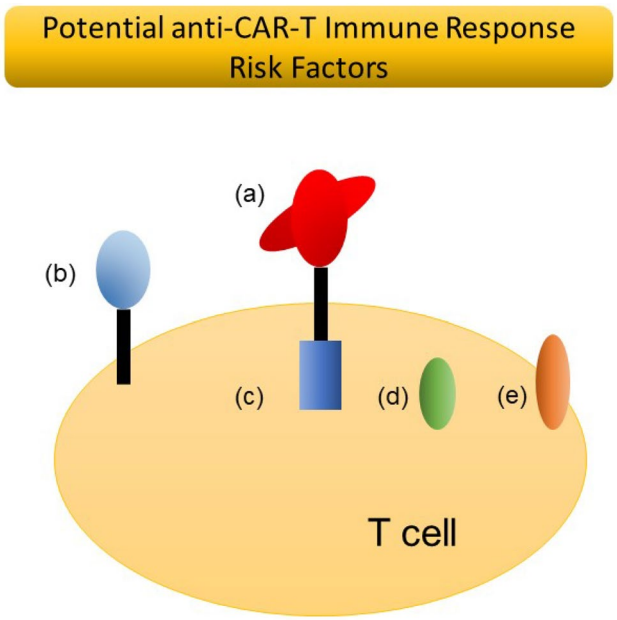

Fig. 1 Schematic representation of CAR-T construct immune response risk factors: (a) CAR antigen-binding moiety, frequently designed as scFv; (b) safety or suicide domain, (c) intracellular signaling stimulatory domain, (d) and (e) residual viral and other non-human proteins associated with gene editing steps of the CAR-T manufacturing process. *CAR* chimeric antigen receptor, *scFv* single-chain variable fragment

- Non-human or partially human nature of the extracellular domain of the CAR construct. Similar to protein-based biotherapeutic modalities, the presence of a non-human sequence was shown to significantly increase immunogenicity of therapeutics. This includes use of an scFv domain of murine parental anti-target antibody as well as a non-germline sequence found in the idiotype of a humanized or fully human antibody protein, which does not exclude immune response to human sequences.
- Fusion nature of the CAR construct where scFv is typically presented as a domain of a larger protein that includes trans-membrane and one or more intracellular signaling domains.
- Presence of non-human germline sequence in linker domain connecting various domains of the CAR construct.
- Presence of non-human germline sequence in other domains introduced on the CAR-T construct, including suicide domain.
- Presentation of CAR domain and other engineered sequences in a cell surface context, which may lead to enhancement of the antigenic potential of non-human and human sequences.
- Potential presence of residual viral proteins.

Table 1 Main CAR-T therapeutic modality immunogenicity risk factors and proposed associated mitigation strategies

Risk factor	Probability of immune response induction	Examples of response specificity	Potential response type	Proposed monitoring strategy	Proposed mitigation strategy
Non-human or partially human nature of the CAR-T construct components including CAR, suicide domain or other domains of the construct	High	Anti-extracellular CAR domain, anti-suicide domain response	Antibody and cellular	Pre- and post-dose monitoring of antibody and cellular response	Reduction or complete elimination of non-human sequence in the extracellular domain of CAR; use of a suicide domain of human origin
Presence of residual viral proteins, Presence of other residual non-human origin proteins, e.g. TALEN Gene editing-associated deletions and mis-expressions	Medium, depends on the level of residual material present	Anti-viral protein response, anti-TALEN protein response	Antibody and cellular	Risk-based approach based on the level of residual proteins present in final product	Control for non-human protein presence

CAR chimeric antigen receptor, *TALEN* transcription activator-like effector nuclease

- Potential presence of other residual non-human origin proteins, e.g. TALEN construct-related proteins.
- Gene-editing-associated deletions and mis-expressions.

The risk-factor-specific degree of impact on the final immune response will greatly depend on a variety of therapy-specific parameters including whether the patients undergo a pre-conditioning treatment known to weaken the immune system. The main immunogenicity risk factors and proposed monitoring and mitigation strategies are summarized in Table 1.

Both antibody (humoral) and cellular immune responses have been described in the literature and are reviewed in the following sections of the manuscript. A combination of both types of responses may be expected, which should be viewed as a distinctive feature of the immune potential of CAR-T therapies. In at least one example, cellular response was detected prior to the antibody (human anti-chimeric antibody, HACA) response [23]. As both antibody and cellular responses may be impacting CAR-T-cell survival and proliferation in vivo, a parallel assessment of responses may be needed.

3 Humoral Immune Response to CAR-T Therapeutics

Similar to a broader class of protein-based biotherapeutics, and specifically monoclonal antibody (mAb)-based biologics, induction of humoral immunity can be anticipated in response to administration of CAR-Ts carrying non-human origin extracellular CAR domain, such as scFv originating from a mouse monoclonal antibody protein. Anti-idiotypic responses to the scFv may also be induced, similarly to anti-idiotypic and neutralizing anti-drug antibody responses reported for humanized or even fully human mAb-based biologics [24]. Humoral anti-idiotypic and neutralizing response was reported for an autologous T-cell therapeutic genetically modified to express CAR specific to the carbonic anhydrase IX (CAIX) antigen found on the surface of renal cell carcinoma (RCC) cells [23]. The non-human chimeric nature of the extracellular domain of the CAR (based on a murine monoclonal antibody cG250) resulted in an HACA antibody response that was monitored by an ELISA protocol. An anti-idiotypic HACA response was detected as early as day 37 after the second T-cell treatment. Anti-CAR HACA response interfered with the flow cytometry protocol based on the use of anti-G250 antibody reagent and was designed to detect circulating CAIX-CAR-T cells while detection was still possible by a qPCR-based method. In addition, HACA response inhibited specific cytotoxic activity of the CAIX-CAR-T cells. Although based on a low patient number, a high incidence of HACA response was reported (6/7 or 85.7%), which contrasted with previously reported anti-cG250 antibody response incidence in patients treated with the cG250 protein alone (30% or less) [25–27]. This is consistent with the view that immunogenic determinants presented in the context of the cell surface may enhance stronger antibody response in comparison with the same antigen in a soluble form.

Induction of anti-CAR-T antibody response was described for the CART72 construct targeting tumor-associated glycoprotein TAG-72 based on a humanized TAG-72-specific antibody (CC49) for a treatment of metastatic colorectal cancer (CRC) [3]. Tumor biomarkers carcinoembryonic antigen (CEA) and TAG-72 known to be shed by proliferating CRC cells were assessed to determine residual tumor burden. TAG-72 was detected using the radio immunoassays (RIA) method using CC49 as a capture reagent. A significant reduction in concentration of the TAG-72 protein was reported in several patients as soon as 5 weeks after initial CAR-T infusion while the CEA levels remained largely unchanged. The apparent reduction in the TAG-72 concentrations was linked to development of anti-CAR construct (anti-CC49)-specific antibodies. Assessment of patient serum samples for the ability to bind to a murine version of the CC49 in an ELISA method demonstrated induction of idiotypic anti-CC49 antibodies targeting the TAG72 binding domain of the CAR construct.

Although persistent levels of CART72 were observed after the first treatment, in some cases reaching up to 0.1% of peripheral blood mononuclear cells (PBMC), an increased clearance of CAR-T cells was detected after the second and third infusions in several patients. The reduction in the CAR-T-cell levels aligned with the development of anti-CAR-specific antibodies. In the case of CART72, the initial TAG-72 biomarker assay in essence served as an anti-CAR antibody response test. Anti-CAR antibody ability to bind to the murine version of the CC49 antibody suggested immunogenic potential of the residual non-human germline sequences in the humanized CC49 domain of the CAR construct. As may be expected, the original murine CC49 was shown to be highly immunogenic when administered as a single agent with a significant number of treated patients developing anti-idiotypic or human anti-mouse antibody (HAMA) immune response [28].

Anti-CAR-T antibody response was reported in a study with T cells engineered to express folate receptor (FR)-specific CAR domain based on a murine anti-FR antibody. The FR-specific CAR-T cells were developed for treatment of metastatic ovarian cancer [29]. They were readily detected shortly after the initial infusion, accounting for up to 1% of circulating lymphocytes, but cleared within 1 month in the majority of treated patients. Post-infusion patient sera were shown to effectively inhibit CAR-T cytotoxic activity against FR-expressing tumor cells, while no inhibitory activity was detected in the pre-treatment samples. Such an immune response against CAR-T cells in this study is not too surprising considering the clinical protocol that required patients' intact immune system as evidenced by a positive reaction to Candida albicans skin test, mumps skin test, or tetanus toxoid skin test on a standard anergy panel. Anti-CAR-T inhibitory activity in serum was reduced by extracting immunoglobulins by Protein G treatment, confirming the antibody nature of the immune response.

In the study reported by Till et al. [30], patients with relapsed or refractory indolent B-cell lymphoma or mantle cell lymphoma were treated with autologous anti-CD20-specific CAR-T therapy. T cells were modified to express anti-human CD20-specific scFv domain of the murine antibody (Leu-16). Specific CAR-T cells were observed in circulation in vivo up to 9 weeks. Humoral anti-CAR-T response was evaluated using an enzyme-linked immunosorbent assay (ELISA) method designed to detect anti-murine Leu-16 antibody in patients' sera because the Leu-16 murine sequence was expected to be the dominant immunogen within the CAR construct. While antibody or cellular immune responses were not initially detected, some patients did develop HAMA responses several months after the CAR-T-cell infusion.

Information related to antibody responses against two approved CAR-T-cell products has been made available. During clinical trials, it was observed that YESCARTA® (the anti-CD19 CAR-T cell) has the potential to induce anti-product antibodies that were detected by an ELISA with specificity against the FMC63 protein, the originating antibody of the anti-CD19 CAR construct. It was shown that three patients tested positive for the presence of anti-FMC63 antibodies at baseline as well as at the month 1, 3 and 6 time-points. Overall, the time course of YESCARTA® CAR-T expansion as well as persistence of the cells post-dose were not apparently impacted by the presence of pre- or post-dose anti-CAR-T antibodies [31].

Similarly, immunogenicity against KYMRIAH® (the anti-CD19 CAR-T cell) was evaluated by detection of anti-murine CAR19 antibody response. Assessment was conducted at baseline (pre-dose) and after administration of the therapeutic (post-dose). A significant prevalence of pre-dose anti-murine CAR19 antibody was detected in the study patients (86% and 91.4% in two separate studies). Limited (5%) induction of anti-murine CAR19 antibodies was reported post-dose, although it is not clear whether and how the presence of pre-dose antibodies impacted this conclusion. Overall, the time course of KYMRIAH® CAR-T expansion, persistence of the cells post-dose as well as the clinical responses, including safety and efficacy, were not apparently impacted by the presence of pre- or post-dose anti-CAR-T antibodies [32].

Both registered CD19 CAR-T-cell products are generally administered as a single dose for infusion and preceded by a lymphodepleting chemotherapy treatment, which is intended to foster CAR-T-cell expansion and persistence, but which also seems to have the beneficial effect of reducing clinically meaningful anti-CAR-T immune responses. For example, Turtle et al. report that the addition of fludarabine to

lymphodepleting cyclophosphamide chemotherapy reduced the incidence of detectable anti-CAR immunoreactivity [33]. Therefore, the persistence of CAR-T cells, duration of remission, and disease-free survival were improved by additional chemotherapy with fludarabine. In such a clinical setting, neither of these products showed any adverse effect of anti-CAR-T immunoreactivity on CD19 CAR-T-cell expansion or antitumor efficacy.

It remains unclear why a high prevalence of pre-dose immunity and the non-human nature of the CAR construct did not significantly impact overall outcome of the treatment. Both the single-infusion nature of the therapy and/or pre-treatment with immunomodulatory compounds may be the cause, although it is likely that prior lymphodepletion treatment plays a bigger role. As CAR-T cells expand and circulate in vivo, they are expected to be exposed to the host immune system for a prolonged period, despite the fact that initially a single infusion is given.

4 Cellular Immune Response to CAR-T Therapeutics

In addition to humoral immunity, there is also strong evidence of cellular anti-CAR-T-cell immune response induction. Autologous CD20- and CD19-specific CAR-T therapies were developed to treat recurrent or refractory follicular lymphoma, respectively [34]. The CAR constructs were transferred into the T cells by plasmid vector electrotransfer protocol. The CD19 CAR-T construct additionally expressed fusion hygromycin resistance and HSV-1 thymidine kinase selection-suicide domain (HyTK), while the CD20 CAR-T construct expressed neomycin phosphotransferase domain (NeoR) enabling neomycin-mediated selection of CAR-Ts during the ex vivo expansion phase. By applying the qPCR method, it was determined that the levels of circulating CAR-T cells were significantly reduced after a short period of time, typically between 24 h and 7 days after infusion. To investigate the reason for a quick drop in the CAR-T levels, patients' PBMCs were evaluated for the presence of cellular immune response before and after treatment. The cytotoxic anti-CAR-T-specific activity was evaluated using Chromium 51 (51Cr) release assay, which demonstrated presence of functional effector cells in blood collected from treated patients. A control T-cell construct carrying the NeoR domain but lacking the anti-CD20-specific CAR was used to demonstrate anti-NeoR domain specificity of the anti-CAR-T-specific cytotoxic T cells. Separately, clear evidence of a cellular response against CD19-specific CAR-T expressing HyTK was detected in the absence of humoral anti-CD19 CAR receptor domain immunity. Pre- and post-treatment samples were shown to be negative for the presence of antibodies specific to the CD19 CAR receptor domain as measured by a flow cytometry assay. It should be pointed out that these patients were subjected to the immunosuppressive rituximab therapy prior to the infusion of therapeutic $CD8^+$ cytotoxic T lymphocytes (CTL) expressing a CD20-specific CAR. It was suggested that at least in some cases, the lack of CAR-T-cell persistence was due to immune rejection responses mounted by the patients' endogenous T cells, despite rituximab pretreatment [34].

Patients treated with anti-CAIX-CAR-T therapy [23, 35] were assessed for the anti-CAR-T cellular response using PBMC preparations. Anti-CAIX-CAR-T cytotoxic cellular reactivity was detected in the post-infusion samples after several cycles of co-stimulation of the PBMC preparation with irradiated CAR-T cells. No anti-CAR-T reactivity was observed in pre-dose samples even after similar prolonged co-culture treatment. The response was undetectable when fresh unstimulated PBMCs were evaluated, suggesting a relatively low percentage of anti-CAIX-CAR-T-specific T cells in circulation. A persistent cellular response was observed starting as early as day 36 after the second infusion cycle. Specificity of the anti-CAR-T response was evaluated by assessment of various CAR constructs and by utilizing an alternative non-viral nucleofection method to introduce the CAR construct into the T cells. While the degree of cellular response was patient dependent, specific anti-CAR-T activity was detected in PBMCs collected from several patients. Overlapping 15-mer peptides derived from the CAR construct were used to stimulate T cells found in patients' PBMC preparations pre-stimulated with the CAR-T cells in vitro. A single peptide (epitope) per patient was identified in five patients evaluated. The peptides that belonged to the sequence of the complementarity determining region (CDR) were found on the VH (three patients) or the VK (two patients) framework domains of the CAR protein. Some patients developed a cellular response not related to the CAR protein, as was demonstrated by the lysis of transduced T cells expressing irrelevant control protein (human CD24). This was attributed to the anti-retroviral epitope response and was confirmed by comparing anti-CAR-T activity detected in the PBMCs against CAR-T cells generated by viral transduction versus nucleofection protocol. It was concluded that virally derived immunogenic epitopes can be expressed on the retrovirus transduced CAR-Ts resulting in a cellular immune response in the treated patients. A mixed response can therefore be expected where cellular immunity can be induced to either the CAR and/or anti-retroviral epitopes.

Riddell et al. conducted a study to evaluate potential use of $CD8^+$ human immunodeficiency virus (HIV)-specific cytotoxic autologous T cells in treatment of individuals seropositive for HIV [36]. Using a retroviral transduction technique, T cells were modified to express the HyTK domain, allowing for a positive/negative selection. A cellular

immune response was reported in five of six treated patients resulting in complete rejection of the introduced modified T cells. Modified T cells were detected for up to 4 weeks after infusion and then quickly cleared from circulation. A cellular immune response to the HyTK protein was suspected, which was corroborated by the evidence demonstrating cytolytic activity against modified T cells detected in patients' PBMCs. This finding demonstrates that genetically modified T cells can induce cellular immune responses in HIV-infected individuals who may be immunocompromised but who may also have a dysregulated immune system [37, 38].

The following examples, although not directly related to CAR-T cell products, illustrate mechanisms that can lead to cellular immune responses against modified T cells. The use of an inducible suicide gene has been proposed as a mitigation measure to address potential vector-induced iatrogenic tumors [39]. Examples of suicide genes include herpes simplex virus thymidine kinase (small molecule induction, HSV-TK), inducible caspase 9 gene (small molecule induction [40]), CD34/CD20 combination domain (anti-CD20 induction [16, 41]), EGFR polypeptide (anti-EGFR antibody induction [42]), and other. Additional details can be found in the review published by Jones et al. [43].

In the case of the HSV-TK, introduction of anti-herpes virus drugs, acyclovir or ganciclovir, will effectively result in killing of the modified cells. Allogeneic bone marrow transplantation (BMT) of donor lymphocytes has been applied in treatment of hematological malignancies [44, 45] where the infused donor T cells are able to exert graft versus leukemia (GVL) activity but can also result in a graft-versus-host-disease (GVHD) condition. HSV-TK or similar control switch domains may enable effective control over the risk of the GVHD concern. Acute cases of GVHD in patients injected with HSV-TK expressing donor cells were addressed by the ganciclovir treatment with or without glucocorticoids [44, 45]. Due to the foreign nature of the HSV-TK protein, an anti-HSV-TK immune response may be expected, although it may not preclude an effective use of the GVL treatment [46]. In another example, hematologic malignancy patients treated with HSV-TK expressing allogeneic donor lymphocytes developed a cellular immune $CD8^+$ response to the TK domain [47]. The potential impact due to previous exposure to the herpes virus was suspected although it was concluded that a newly generated anti-TK immune response is not directly connected to the history of allogeneic donor exposure to the virus. Immunocompetent status of treated patients was noted as a critical factor in order to generate an anti-HSV-TK response [47].

Immune response to HSV-TK-modified donor T cells in hematopoietic cell transplantation recipient patients was evaluated to determine distinct immunogenic epitopes [48]. While no pre-existing cytolytic anti-HSV-TK T cells were found in the patients' PBMCs collected prior to the infusion, HSV-TK-specific $CD8^+$ T-cell response was readily detected as soon as 2 weeks after the initial infusion of HSV-TK modified T cells. The response was further enhanced after an additional infusion of the HSV-TK-carrying T cells. It was concluded that even a single administration of HSV-TK modified cells was sufficient to induce a $CD8^+$ cytolytic T-cell response resulting in efficient clearance of the infused cells [48]. Stimulation of patient T cells with lymphoblastoid cell lines transduced with various domains of the HyTK construct demonstrated induction of cellular response against several distinct epitopes of the HyTK including immune response to either bacterial origin hygromycin B or viral origin HSV-TK domains [36]. Evidence of robust durable memory T-cell response in treated patients was shown up to 7 years after the initial infusion of HSV-TK modified cells.

5 Impact of Humoral and Cellular Immunity

It has been generally accepted that success of CAR-T treatment is highly dependent on the persistence of CAR-T cells in circulation. Very long exposures have been achieved; for example, in the case of the CAR-Ts modified to express CD4 linked to the CD3z signaling domain (CD4z) [49]. The CD4z CAR-Ts were generated using a retroviral gene transfer protocol. The CD4z CR-T cells with retained function were found in patient blood 11 years after the initial infusion. The decay half-life was estimated to exceed 16 years. These results are in stark contrast with other reports where CAR-T exposures were observed for a much shorter period of time. Frequently, quick clearance of the CAR-Ts from circulation was associated with development of anti-CAR-T-specific humoral or cellular immunity [23, 50]. A robust immune response was reported to reduce CAR-Ts below detectable levels within 18–34 days after repeat infusion [23].

6 Detection and Characterization of Antibodies Directed Against CAR-T Cells

Currently, methodologies to detect antibody response to biotherapeutic compounds (anti-drug antibody (ADA) assays) have been well established [51–54]. ADA assays are typically viewed as semi-quantitative. Commonly, a titer is reported based on the assay-specific cut-point value defined based on the statistical analysis of the treatment-naïve samples. Most frequently, a ligand binding assay is applied while other platforms remain an option, including RIA, surface plasmon resonance, and more [55]. Similar considerations can be applied towards the assays designed to detect antibodies to the CAR protein or other components of the CAR-T construct. Till et al. [30]

have described a bridge-based ELISA assay for detection of immune response to the anti-scFv component of the CD20 targeting domain of the engineered T cell in treated patients. The scFv domain was derived from the murine antibody (Leu-16) and therefore the assay was constructed using parental murine anti-human CD20 Leu-16 antibody as the capture and detector reagents. An RIA protocol was described for the detection of anti-CAR activity in patients treated with anti-TAG-72-specific CAR-T therapeutics [3].

The cellular nature of the CAR expression offers an opportunity to apply an alternative approach. A flow cytometry protocol was used to evaluate the presence of antibodies specific to the anti-CD20 targeting domain in post-treatment samples [30]. In the assay, patient samples were incubated with Jurkat cells expressing the anti-CD20-specific cTCR, and the bound anti-cTCR antibodies were detected using a secondary fluorescein labeled goat anti-human F(ab')2 reagent. Pre-treatment samples were used as the negative control material. No evidence of humoral immune responses to the cTCR was observed by either the bridge or the flow cytometry approach.

It is common practice to assess anti-drug antibodies for the ability to neutralize specific activity of the drug during the ADA characterization step. Typically, cell or competitive binding (non-cell based) assays are applied [56]. Specific decisions on the assay type and platform are driven by the mechanism of action of the biotherapeutic compound. Similarly, neutralizing anti-CAR antibody activity can be evaluated by constructing assays that assess immunoglobulin ability to neutralize CAR-T binding to its molecular target in a cellular environment or as a recombinant protein. As most relevant to the modality mode of action, an anti-CAR-T neutralizing antibody assay may be designed to assess ADA ability to impact cytotoxic activity of CAR-T against its target tumor cell type.

7 Methods to Detect Cellular Anti-CAR-T Immunity

Chromium 51 (51Cr) release assay is frequently described as a gold standard when detecting activity of CTL. The 51Cr assay is based on utilization of a radioactive material and therefore presents a substantial safety and ease of use challenge. Alternative protocols based on other non-radioactive read-out methods have been recently reported. Examples include protocol based on the detection of luciferase activity expressed as a reporter gene by the target tumor cells [57], lactate dehydrogenase activity released during cell cytolysis [58], fluorescent dye released from pre-labeled target cells [59], and detection of green fluorescent protein (GFP) reporter gene activity in target cells [60, 61]. Anti-CAIX-CAR-T-specific cellular activity was detected in patient PBMCs by measuring interferon-β production. Specific anti-CAIX-CAR-T activity was observed post-treatment and resulted in an accelerated clearance of CAR-Ts [35]. Fluorescent antigen transfected target cell cytotoxic T lymphocyte protocol was developed to detect and quantify presence of antigen-specific cytotoxicity ex vivo [60]. Target cells expressing GFP are co-cultured with effector cells (PBMCs) followed by a flowcytometry-based analysis to determine the fraction of viable GFP expressing cells. The method was successfully applied to assess the presence of anti-influenza and anti-HIV-specific CTLs with an improved versus standard 51Cr release assay sensitivity. A 2D microfluidic xCELLigence system has been applied for detection of real-time cellular proliferation and response to various stimuli and therefore can be applied to detect cytotoxic activity in a sample [62].

8 Mitigation Strategies Aiming to Reduce Immunogenicity Risk of CAR-T Therapeutics

Various strategies aiming to reduce immunogenicity risk of CAR-T treatment have been discussed. These include reduction of the viral protein content, humanization of the CAR construct, and lymphodepletion chemotherapy treatment.

Humanization of the CAR construct is an obvious solution broadly applied for the majority of contemporaneous protein-based therapeutics. Examples include an evaluation of a fully human scFv-based CAR construct specific to human C4 folate receptor alpha that showed an acceptable level of activity in a human ovarian cancer xenogeneic mouse model [11]. In an effort to reduce immunogenicity risk for a CD19-targeting CAR-T construct, Sommermeyer et al. utilized a fully human anti-CD19-specific scFv as well as modified fusion sites between different CAR components [63]. As part of immunogenicity risk assessment, the CAR construct amino acid sequence was evaluated in silico for the ability to recognize MHC-1 alleles and was adjusted accordingly. Resulting human CAR-T constructs showed specific activity against human lymphoma xenografts in an immunodeficient mouse model.

Use of a lymphodepletion chemotherapy treatment with fludarabine and cyclophosphamide has been broadly reported, particularly for the second and third generations of the CAR-T therapies. Locke et al. reported a lack of immune response induction in a study of autologous CD3z/CD28 CAR-T therapy evaluated for the treatment of large B-cell lymphoma where patients were conditioned with cyclophosphamide and fludarabine treatment [64]. Turtle et al. reported that refractory B-cell non-Hodgkin's lymphoma patients infused with an autologous CD19-specific CAR-T

construct and subjected to lymphodepletion chemotherapy by applying cyclophosphamide and fludarabine had a significant improvement in CAR-T expansion and persistence as well as a reduced rate of anti-murine scFv immune responses [33]. Pre-existing immunodeficiency has been associated with a prolonged persistence of adoptively transferred lymphocytes that harbor a foreign gene product [65]. In the study, patients received Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes (EBV-CTL) aiming to prevent or treat EBV-positive lymphoproliferative disease after hematopoietic stem cell transplantation. The neomycin resistance gene-encoding CTL cells were observed in circulation for up to 9 years after initial infusion.

Pre-treatment with an IgG parental to the CAR construct antibody was proposed as an approach to reduce anti-CAR immunity. Patients treated with CAR-T based on the mouse G250 antibody developed anti-CAR-specific antibodies [66] while patients pre-treated with the chimeric G250 antibody developed an immune response at a lower rate [27, 67]. Pre-treatment with the parental antibody protein may result in induction of immune tolerance, although this still needs to be confirmed.

9 Conclusions

CAR-T-cell immunotherapy represents a quickly advancing biotherapeutic modality with great promise in the treatment of various oncological conditions. Many questions remain, including CAR-T potential to induce antibody and/or cellular immune response to various components of the construct. In cases where immune responses were observed, they were associated with a quick reduction in the CAR-T count in vivo and a loss of efficacy. The extent and significance of the immune response are linked with the nature of CAR, the presence of a non-human protein sequence in various domains of the CAR construct and suicide domain, and the presence of residual viral proteins or proteins generated by the DNA editing system used in producing CAR-T (e.g., TALEN). The multi-domain nature of the CAR construct and its cell surface context presentation have been suggested to enhance immunogenicity risk for the CAR-T modality.

Methodologies aiming to reduce immunogenicity risk broadly applied for protein-based biotherapeutics are highly relevant when addressing the non-human nature of the CAR construct [68]. Risk factors related to the presence of viral or DNA machinery editing proteins will require close control during the production phase, which can be paralleled with the control over the residual amount of the host cell proteins in the final drug substance for a typical protein-based biotherapeutic [69]. While some methodologies aiming to evaluate anti-CAR-T immunity in vivo are similar to protocols broadly applied for other biotherapeutics, significant differences exist, particularly when assessing cellular anti-CAR-T response. For example, CD19-CAR-T-cell products effectively utilize lymphodepleting chemotherapy treatment resulting in apparent reduction of clinically meaningful anti-CAR-T immune responses. Additional clinical data will be needed to fully appreciate risks associated with the anti-CAR-T immune responses as well as associated clinical relevance. As CAR-T therapy continues to quickly evolve, more information will be generated by the community of practice allowing for a detailed analysis of modality immunogenicity risk factors.

Compliance with Ethical Standards

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Conflict of interest Boris Gorovits is employed by the Pfizer Inc. which in the past has been involved in the development of CAR-T therapeutics. Eugen Koren is employed by the Precision for Medicine, which supports development of CAR-T therapeutics.

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