Fasten the seat belt: Increasing safety of CAR T-cell therapy

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Abstract
After the recent success and approvals of chimeric antigen receptor (CAR) T cells in haematological malignancies, its efficacy is currently evaluated in a broad spectrum of tumor entities including melanoma. However, severe and potentially life-threatening side effects like cytokine release syndrome, neurologic toxicities, and the competing risk of morbidity and mortality from the treatment itself are still a major limiting factor in the current CAR T-cell landscape. In addition, especially in solid tumors, the lack of ideal target antigens to avoid on-target/off-tumor toxicities also restricts its use. While various groups are working on strategies to boost CAR T-cell efficacy, mechanisms to increase engineered T-cell safety should not move out of focus. Thus, the aim of this article is to summarize and to discuss current and potential future strategies and mechanisms to increase CAR T-cell safety in order to enable the wide use of this promising approach in melanoma and other tumor entities.

KEYWORDS
adoptive T-cell therapy, cancer, chimeric antigen receptor, cytokine release syndrome, neurotoxicity

1 INTRODUCTION

While the first engineered T cells in adoptive cell therapy (ACT) were used over a decade ago with a tumor antigen specific T-cell receptor (TCR) targeting a human leucocyte antigen (HLA)-A2-restricted peptide from a melanocytic differentiation antigen in melanoma patients and indeed showed great potential,[1-3] major breakthrough was reached with the introduction of a chimeric antigen receptor (CAR) into T cells.[4-8] Especially for therapy-refractory B-cell acute lymphoblastic leukaemia (B-ALL) and diffuse large B-cell lymphoma (DLBCL), CD19-directed CAR T-cell therapy revealed impressive response rates in clinical trials.[9-12] This has led to the approval of two CD19 CAR T-cell constructs in the United States and other parts of the world including Europe.[5] As the CAR construct comprises a single chain variable fragment (scFv) derived from a monoclonal antibody, whole cell surface antigens on cancer cells are recognized independent of antigen processing and major histocompatibility complex (MHC) presentation, which represents an advantage over TCR T cells.[5,13,14] The intracellular structures of a CAR typically include the zeta subunit of the CD3 complex as a signalling domain derived from the TCR and one or more co-stimulatory motifs, for example CD28, 4-1BB or OX40.[5,14]

After the recent success and approvals of CAR T cells in haematological malignancies, its efficacy is currently evaluated in a broad spectrum of tumor entities.[15-19] Especially the application of CAR T cells in solid tumors including melanoma, however, still lacks a major breakthrough.[15-19] Reasons for a lower impact comprise hampered T-cell trafficking into cancer site,[19-22] as well as the immunosuppressive tumor microenvironment (TME) due to, eg immunosuppressive cytokines, regulatory T cells (Tregs) and upregulation of inhibitory receptors on T cells.[23-26] Other obstacles include tumor escape mechanisms like antigen downregulation or antigen loss which can be developed by
tumor cells in order to by-pass immune recognition.\textsuperscript{[26-28]} One of the biggest challenges in solid tumors, however, is represented by the identification of an optimal target antigen, which should not be expressed on healthy tissues to prevent on-target/off-tumor toxicities.\textsuperscript{[26,29,30]}

While various groups focus on the optimization of CAR T cells to increase their efficacy towards the use in solid tumors, safety concerns emerge as fatal side effects like cytokine release syndrome (CRS) or neurotoxicity are observed.\textsuperscript{[4,5,31,32]} The full clinical manifestation of CRS, which is mainly caused due to increased interleukin-6 (IL-6) levels, are highly variable from fevers and myalgias to life-threatening multi-organ dysfunction.\textsuperscript{[31,32]} Neurologic toxicities include hallucinations, encephalopathy, seizures, aphasia, headaches and rapidly progressive cerebral oedema which can be refractory to IL-6 directed therapy and lead to fatal herniation.\textsuperscript{[31,32]} A number of studies have indeed detected CAR T cells in the central nervous system (CNS) of patients who experienced neurotoxicity.\textsuperscript{[31,32]} Therapeutically, the use of anti-IL-6 antibodies tocilizumab and siltuximab, as well as Janus kinase (JAK) inhibitors and corticosteroids, has proven to be effective. Severe side effects refractory to these therapies, however, are unfortunately still frequently observed.\textsuperscript{[32]}

In this context, while focusing on mechanisms to increase CAR T-cell efficacy for its use in a broad spectrum of tumor entities, mechanisms to increase CAR T-cell safety should not move out of focus. Thus, the aim of this article is to describe and to discuss current and potential future strategies and mechanisms to increase engineered T-cell safety.

## 2 | THE IDEAL TARGET ANTIGEN

The majority of antigens that have been targeted by CAR T cells are solely protein-based, which poses the risk of non-exclusive cancer-specificity.\textsuperscript{[33]} The fact that these target antigens are expressed in lower amounts on healthy tissue led to hope that no severe side effects will be observed when clinically used.\textsuperscript{[33]} However, to date, several dramatic clinical courses were indeed seen in patients developing on-target/off-tumor toxicities, even resulting in death of some of these patients.\textsuperscript{[32,34-38]} A prominent example is a patient reported by Morgan et al, who suffered from colon cancer metastatic to the lungs and liver and refractory to multiple standard treatments and who was treated with ERBB2/HER2-specific CAR T cells.\textsuperscript{[38]} This patient exhibited severe pulmonary failure, as the ERBB2/HER2 antigen is also expressed in lung epithelial cells leading to destruction of the lung, in addition to a massive cytokine storm.\textsuperscript{[38]}

Looking at the current target antigens in clinical trials running for advanced stage melanoma patients, for instance, we can also observe that these target antigens are not exclusively expressed on melanocytes or melanoma cells (Table 1). Target antigens in these

| Target antigen | Disease | Country | ClinicalTrials.gov identifier | Status |
|----------------|---------|---------|-------------------------------|--------|
| c-Met          | Melanoma, breast cancer | United States | NCT03060356 | Terminated |
| CD20           | Melanoma | Germany | NCT03893019 | Recruiting |
| CD70           | Pancreatic cancer, renal cell cancer, breast cancer, melanoma, ovarian cancer | United States | NCT02830724 | Recruiting |
| GD2            | Sarcoma, osteosarcoma, neuroblastoma, melanoma | United States | NCT02107963 | Completed |
| C7R-GD2        | Sarcoma, breast cancer, neuroblastoma, melanoma | United States | NCT03635632 | Recruiting |
| NY-ESO-1       | Multiple myeloma, oesophagus cancer, lung cancer, melanoma, synovial sarcoma | China | NCT03638206 | Recruiting |
| VEGFR2         | Metastatic cancer, metastatic melanoma, renal cancer | United States | NCT01218867 | Terminated |

Abbreviations: C7R, constitutively activated IL-7 receptor; CD20, cluster of differentiation 20; c-Met, tyrosine-protein kinase Met; GD2, Ganglioside G2; NY-ESO-1, Cancer/testis antigen 1; VEGFR2, vascular endothelial growth factor receptor 2.
clinical trials include, for example the tyrosine-protein kinase c-MET and vascular endothelial growth factor (VEGF). While c-MET is normally expressed by cells of epithelial origin (healthy and malignant tissues), VEGF activity is restricted mainly to cells of the vascular endothelium, although it is known that it also has effects on other cell types, for example monocytes and macrophages. Thus, the results of these clinical studies must first be evaluated in order to be able to decide—apart from the clinical benefit—if the use of these antigens is safe or if it leads to severe on-target/off-tumor toxicities.

Fortunately, it is nowadays possible to use remarkable precision bioinformatics which allows us to access detailed information about tumors, including its antigen expression. This bioinformatics data can be used to fill the gap of lacking appropriate target antigens for cellular therapies. Especially, neoantigens are currently considered particularly promising targets. In this context, Posey et al. demonstrated a promising approach by developing a CAR recognizing a cancer-associated Tn glycoform of cell surface associated Mucin 1 (MUC1), a neoantigen expressed in a variety of cancers. These anti-Tn-MUC1 CAR T cells demonstrated target-specific cytotoxicity and successfully controlled tumor growth in xenograft models of T-cell leukemia and pancreatic cancer. In this preclinical study, the authors demonstrated that specific glycoproteins on tumor-associated antigens can also serve as target antigens for CAR T-cell therapy.

The prevention of CAR T-cell-mediated recognition of antigen-expressing healthy tissue can also be reached through negative differentiation of non-tumor signals. Equipment of T cells with a CAR targeting the killing antigen together with an inhibitory CAR (iCAR) binding to a second antigen co-expressed on cancer and normal bystander cells could override a potentially dangerous T-cell activation. Apart from the extracellular antigen binding domain, the molecular structures of iCARs include programmed cell death protein 1 (PD-1)- or cytotoxic T-lymphocyte–associated protein 4 (CTLA-4)-based inhibitory signalling modules rather than activating TCR related domains. Thus, target cells expressing only the CAR antigen are attacked by CAR/iCAR dual-specific T cells, whereas the additional presence of the iCAR antigen leads to inactivation of the CAR signal and prevents lysis of cells. Fedorov et al. reported that transduction with both a CAR and a PD-1- or CTLA-4-based iCAR resulted in limited CAR-dependent cytokine production, lytic capacity and proliferation ability in a preclinical in vivo study.

In addition, new molecular strategies were described by different groups in the last years in order to enhance the control of CAR T cells. A prominent idea is the integration of an inducible safety switch in addition to the receptor construct. In this context, an inducible caspase 9 (iCasp9) consisting of the pro-apoptotic protein caspase 9 fused to a modified FK-binding protein was created. Ligation to a synthetic drug activates iCasp9, which rapidly induces apoptosis of cells expressing this construct. The use of iCasp9 was first investigated in allogeneic donor T cells that were infused to patients after haematopoietic stem cell transplantation and led to the termination of graft-versus-host disease (GvHD) through iCasp9-associated elimination of T cells.

Another approach is the use of “ON-switch” CAR constructs that function as small molecule-gated chimeric receptors. With this method, CAR expression can be remotely controlled as the antigen binding and intracellular domains of the CAR are only assembled when a heterodimerizing small molecule, like the rapamycin analog AP21967 or the phytot hormone gibberellin, is present. In vitro examination of the “ON-switch” CAR revealed a strong cytokine production in the presence of a small molecule, comparable to that of conventional single component CAR T cells, and precise control of the timing, location and dosage of CAR T-cell activity.

context, a CAR construct was developed with an integrated synthetic Notch receptor system. After binding to its antigen, instead of T-cell activation in order to eliminate the cancer cell, cleavage of the Notch receptor is induced that releases a transcriptional activator domain. This intracellular factor then enters the nucleus and initiates the assembly of a second CAR with a different antigen specificity leading to the expression of this new receptor on the surface of the cells. By using this strategy, the second CAR that recognizes a second antigen is only expressed when the first CAR encounters its target antigen ensuring a highly specific destruction of tumor cells. Preclinical in vivo analysis revealed that these dual-specific CAR T cells were indeed only equipped and activated in the presence of cancers expressing both antigens rather than single antigen-positive tumors.

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observed with different antigen binding domains, for example targeting CD19 or mesothelin, as well as with the addition of several heterodimerizing small molecules.

An effective approach to eliminate toxic CAR T cells from the host is also represented by the introduction of a tag to engineered cells. The integration of a CAR into T cells can then be combined, for instance, with transfection of a truncated human epidermal growth factor receptor (EGFR) polypeptide (huEGFRt), which was demonstrated by Wand et al. These T cells expressing huEGFRt can easily be eliminated by administration of the anti-EGFR antibody cetuximab. Paszkiewicz et al already demonstrated the feasibility of this approach in an in vivo mouse model as B-cell aplasia caused by CAR T cells expressing huEGFRt was completely reversed by the use of cetuximab. Further clinical studies in this context will most likely follow.

4 | THE IDEAL METHOD OF CELL TRANSFECTION

The most commonly used method to reprogram T cells—including the currently approved CAR constructs—is through stable transduction using DNA-based retroviral or lentiviral vectors. Viral transduction of receptor-encoding DNA results in a permanent transfection of the T cells (Figure 1) and thus, when clinically used, only one single infusion of engineered T cells is needed to get a permanent anti-tumor effect by developing memory T cells. However, possible severe and life-threatening side effects like CRS and neurologic toxicities as well as on-target/off-tumor toxicity could be long-lasting as well.

In this context, an alternative method, especially to increase safety, represents RNA-based receptor transfer, for example RNA electroporation (Figure 1). Due to the absence of chromosomal integration and genetic alteration, RNA-based receptor expression is transient. This represents a safer method as possible side effects, like the unintended reactivity or cross-reactivity of the transgenic receptor, which can result in severe auto-aggression and on-target/off-tumor toxicity, will be transient as well. At the same time, repetitive infusions and a higher number of RNA-transfected T cells will be required when clinically used in patients to obtain a potential anti-tumor effect. This RNA-based method represents an effective strategy to analyze the safety of a CAR when first used in a clinical setting. When no on-target/off-tumor toxicities are observed, DNA-based stable transfection of T cells could be considered in the next step.

In a study performed by our group, these two methods—stable DNA- and transient RNA-based receptor transfer—were combined to transfect the same T cell with two receptors, both specific for different tumor antigens to counteract possible tumor escape mechanisms, which can be developed by tumor cells to bypass immune recognition. Since the antigen glycoprotein 100 (gp100) has been previously used as a target antigen in adoptive T-cell therapy and has proven to be an effective as well as a safe target antigen, we decided to stably integrate a gp100-specific TCR into the T cells via lentiviral transduction. Due to the more distributed expression pattern of the antigen chondroitin sulphate proteoglycan 4 (CSPG4) on malignant as well as healthy tissue, we additionally transiently introduced a CSPG4-specific 2nd generation CAR into the same T cells via electroporation of receptor-encoding RNA. These dual-transfected cells were indeed able to secrete cytokines and showed cytotoxicity after stimulation with one of the targeted antigens or both, while no reciprocal inhibition occurred. With this strategy, the "safer" receptor, which is lentivirally transduced

![Figure 1](image-url)
into the T cells using receptor-encoding DNA, should have a strong and permanent anti-tumor effect, whereas the potentially “more dangerous” receptor, that is transfected into the same T cell using receptor-encoding RNA, should have an additional “boost” effect at the beginning of the therapy. At the same time by combining both methods, potential side effects of dual-transfected T cells are decreased.\cite{71} This could be an effective and safe strategy to massively increase the pressure on the tumor by enhanced induction of direct tumor-cell killing and rapid on-site T-cell expansion and might be an effective and fast way to eradicate large quantities of cancer cells before tumor escape is observed.

Another strategy to transfer receptors in T cells represents the non-viral therapeutic cell engineering with the Sleeping Beauty (SB) transposon system.\cite{79} DNA transposons and retrotransposons are genetic elements with the ability to change their positions within the genome and are mobile units of DNA encoding a gene for a transposase enzyme flanked by inverted terminal repeats (ITRs) that contain the transposase binding sites.\cite{79} These transposons are turned into genetic vectors, one of the two components carrying a DNA sequence of interest between the ITRs, which can then be transferred into T cells in order to integrate into the chromosomal locus.\cite{79} Besides a safe integration profile, this method is especially associated with acceptable cost per treatment and a scalable/exportable vector production to serve large numbers of patients in clinical trials.\cite{79} Whether lower side effects than CRS or neurotoxicity are observed in these patients compared to patients receiving lentivirally transduced CAR T cells has yet to be evaluated in clinical trials.

5 | THE IDEAL CELL TYPE

While usually conventional T cells are used to transfect CARs, efficacy and safety of transfected alternative cell populations, like natural killer cells (NK cells), natural killer T cells (NKT) or γ/δ T cells have also been analyzed in the last years.\cite{80-83}\cite{80} In a recently published clinical phase I/II trial performed by Liu et al, HLA-mismatched anti-CD19 CAR-NK cells derived from cord blood were given to eleven patients with relapsed or refractory CD19-positive cancers (non-Hodgkin’s lymphoma or chronic lymphocytic leukaemia [CLL]).\cite{80} NK cells were transfected with a retroviral vector expressing genes that encode an anti-CD19 CAR, interleukin-15 and inducible caspase 9 as a safety switch.\cite{80} The cells were expanded ex vivo and administered in a single infusion after lymphodepleting chemotherapy.\cite{80} Of the eleven patients who were treated, eight showed a response to this therapy. Of these eight patients, seven (four with lymphoma and three with CLL) had a complete remission.\cite{80} Additionally, the infused CAR-NK cells expanded and persisted for at least 12 months.\cite{80} Most importantly, however, the administration of CAR-NK cells was not associated with the development of CRS, neurotoxicity or GvHD, and there was no increase in the levels of inflammatory cytokines including interleukin-6 over baseline, while the maximum tolerated dose was not reached.\cite{80}

In one of our studies, we introduced a CSPG4-specific CAR into NKT cells and these cells also revealed lower cytokine production compared to conventional CAR-transfected T cells.\cite{81} At the same time, the lytic activity of CAR NKT cells was comparable to that of CAR T cells.\cite{81} Similar effects were observed when CAR-transfected γ/δ T cells were used and compared to α/β CAR T cells in a preclinical setting.\cite{82}

These data indicate that especially in the context of side effects, the use of CAR-NK cells rather than conventional CAR T cells might present a safer but similarly efficient alternative. The use of engineered NKT cells as well as engineered γ/δ T cells needs to be further tested in a preclinical and clinical setting.

6 | CONCLUSION

In summary, the use of CAR T cells already revealed improving success in cancer immunotherapy. Nevertheless, especially to use these engineered cells in a broad spectrum of tumor entities including melanoma, further optimization to increase their safety is required as long as the risk for on-target/off-tumor toxicities is given. Various groups are currently working on innovative and promising CAR-engineering strategies to counteract safety concerns. Genetically encoded tools including new molecules, e.g., sensors and switches to execute sensing-response behaviours may be effective options in this context but need to be further analysed in a clinical setting. While it is essential and necessary to work on improving CAR T-cell efficacy to increase response rates, safety aspects should not move out of focus during these developments in order to fasten the seat belt of CAR T cells.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTION

BS and UU have discussed and wrote the manuscript. Both authors have read and approved the final manuscript.

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