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REVIEW

Toxicity and management in CAR T-cell therapy

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T cells can be genetically modified to target tumors through the expression of a chimeric antigen receptor (CAR). Most notably, CAR T cells have demonstrated clinical efficacy in hematologic malignancies with more modest responses when targeting solid tumors. However, CAR T cells also have the capacity to elicit expected and unexpected toxicities including: cytokine release syndrome, neurologic toxicity, "on target/off tumor" recognition, and anaphylaxis. Theoretical toxicities including clonal expansion secondary to insertional oncogenesis, graft versus host disease, and off-target antigen recognition have not been clinically evident. Abrogating toxicity has become a critical step in the successful application of this emerging technology. To this end, we review the reported and theoretical toxicities of CAR T cells and their management.

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INTRODUCTION

Adoptive cellular therapy (ACT) targeting malignant cells was first described decades ago in a xenogeneic murine model.[1] Translation of this finding to human malignancies has long been a goal for cancer immunotherapy.[2–5] Administration of allogeneic T cells as a component of hematopoietic stem cell transplantation (HSCT) or through donor lymphocyte infusion has been utilized to generate graft versus leukemia response secondary to human leukocyte antigen disparity in leukemia patients.[6] Limiting this therapy is the propensity to develop graft versus host disease (GVHD) when utilizing unselected donor T cells. In solid tumors, the isolation and infusion of autologous tumor-infiltrating lymphocytes has shown remarkable clinical results in patients with melanoma, demonstrating the potential of ACT without the risk of GVHD.[7] The process by which tumor-infiltrating lymphocytes are isolated and expanded is technically difficult, labor intensive, and time consuming, necessitating a method to more rapidly generate a pool of tumor-specific effectors. In response, advances in *ex-vivo* growth and genetic engineering of T cells have enabled rapid generation of effector cells with selectivity for tumor-associated antigens, thereby broadening applicability for cancer immunotherapy.[8–14] The genetic modification of T cells to confer tumor antigen recognition is typically through transgenic expression of a high-affinity T-cell receptor or a chimeric antigen receptor (CAR).[15]

The most common and reliable method of genetic delivery is via lentiviral and γ-retroviral-based transduction methods.[16–19] These allow for stable integration with prolonged expression of the desired transgene. Alternative technologies with a goal of less durable integration and expression include electroporation as well as transposon/transposase delivery systems. T cells that are genetically modified to express a high-affinity T-cell receptor rely upon human leukocyte antigen-matched antigen presentation, which limits applicability to a diverse patient population. Additionally, given their recognition of small peptide epitopes, there exists the potential of cross-reactivity with an array of normal antigens. Alternatively, antigen recognition by CARs can be independent of human leukocyte antigen and typically occurs by engagement with larger epitopes imparting less risk of cross reactivity.[20] For these reasons, CAR modification of T cells may ultimately be more advantageous.

The precision with which engineered immune cells recognize targets has the potential to decrease the general toxicity traditionally seen with conventional chemotherapeutic agents. However, severe immune-mediated adverse events following CAR T-cell infusion have been appreciated. Unique to cellular therapies is the extraordinary long-term persistence of up to 10 years with ACT in human trials.[21,22] This persistence extends the timeline of potential toxicities far beyond that of conventional small-molecule pharmaceuticals. Adverse events following T cell-based therapies may be immediate, delayed, mild, severe, and/or persist for the duration of the genetically modified T-cell lifespan. The focus of this review will be on the reported toxicities following CAR T-cell infusion. In an effort to provide the reader a comprehensive description of toxicities, theoretical and potential sequelae of CAR T cells will also be included (Figure 1). Preventing or managing unwanted toxicity has therefore emerged as a key component in the successful clinical application of this novel technology. Ideally, prediction of per-patient severity and onset would allow consideration of prophylactic therapy to guard against toxicity with resultant improved management.

TOXICITIES OF CAR T-CELL THERAPY

Cytokine release syndrome (CRS)

To date, the most prevalent adverse effect following infusion of CAR T cells is the onset of immune activation, known as CRS.[23] CRS has

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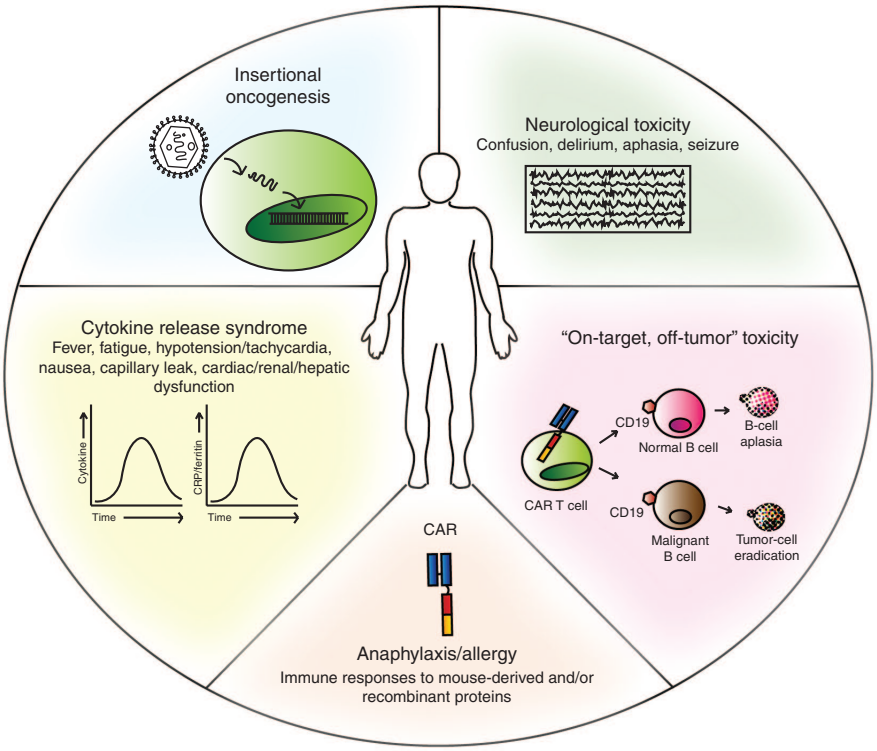

Figure 1 Toxicities of chimeric antigen receptor (CAR) T-cell therapy. Depiction of reported/potential toxicities following the use of CAR T cells: insertional oncogenesis (theoretical); neurological toxicity; "on-target, off-tumor" toxicity (engagement of target antigen on nonpathogenic tissues); anaphylaxis/allergy (host reaction to foreign antigen expressed by the CAR T cell); cytokine release syndrome (systemic inflammatory response following activation of CAR T cells). CRP, C-reactive protein.

also been seen following the infusion of therapeutic monoclonal antibodies (mAbs), systemic interleukin-2 (IL-2), and the bispecific CD19-CD3 T-cell engaging antibody blinatumomab.[24–29] In the setting of early CAR T-cell trials utilizing "first-generation" constructs (without costimulatory signaling elements), insufficient T-cell proliferation/cytokine production and lack of antitumor response were noted.[30] The addition of costimulatory signaling in second-generation CAR design (CD28 or 41BB) translated to improved T-cell activation/expansion, cytokine production, and most notably dramatic antitumor responses in patients with hematologic malignancies.[3,31,32] However, the "double-edged sword" of CAR T cells is demonstrated in the similarly impressive and potentially life-threatening CRS following CAR T-cell administration.[3,31–34]

The hallmark of CRS is immune activation resulting in elevated inflammatory cytokines. Clinical and laboratory measures range from mild CRS (constitutional symptoms and/or grade-2 organ toxicity) to severe CRS (sCRS; grade ≥3 organ toxicity, aggressive clinical intervention, and/or potentially life threatening).[23,35] Clinical features include: high fever, malaise, fatigue, myalgia, nausea, anorexia, tachycardia/hypotension, capillary leak, cardiac dysfunction, renal impairment, hepatic failure, and disseminated intravascular coagulation.[23] Dramatic elevations of cytokines including interferon-gamma, granulocyte macrophage colony-stimulating factor, IL-10, and IL-6 have been shown following CAR T-cell infusion.[3,31,32,36] The cost and technical difficulties inherent to "real-time" monitoring of serum cytokines have precluded the clinical application of this methodology to identify evolving CRS. Currently, under investigation is the use of C-reactive protein, which is made by hepatocytes in response to IL-6, as a laboratory marker of CRS onset and severity.[31] The presence of CRS generally correlates with expansion and progressive immune activation of adoptively transferred cells. It has been demonstrated that the degree of CRS severity is dictated by disease burden at the time of infusion as patients with high tumor burden experience a more sCRS.[3,31,32] In reports of patients treated with CD19-specific CAR T cells for relapsed/refractory B-cell acute lymphoblastic leukemia, the incidence of sCRS has ranged from 19 to 43%, with variability likely due to differences in clinical identification of the syndrome, chimeric receptor designs, and infused cellular phenotypes.[3,23,31,32,36] Clinical outcome is not predicated on the development of sCRS as patients may exhibit an antitumor response in the absence of this toxicity. However, in the context of hematologic malignancies the majority of patients who respond exhibit at least mild CRS (fever) following CAR T-cell infusion.

Following diagnosis of CRS, a challenge has been choosing appropriate therapy to mitigate the physiological symptoms of uncontrolled inflammation without dampening the antitumor efficacy of the engineered cells. Systemic corticosteroid has been shown to rapidly reverse symptoms of sCRS without compromising initial antitumor response.[31,32] However, prolonged use (*e.g.*, >14 days) of high-dose corticosteroids has also resulted in ablation of the adoptively transferred CAR T-cell population potentially limiting their long-term antileukemia effect.[31] As an effective alternative IL-6 receptor (IL-6R) blockade with the Food and Drug Administration-approved mAb, tocilizumab has demonstrated near-immediate reversal of CRS.[3,35] Investigators are now determining the effect of IL-6R blockade on CAR T-cell proliferation, persistence, and most importantly, antitumor effect. Despite this unknown, the use of IL-6R blockade has generally been accepted as front-line treatment for sCRS following CAR T-cell infusion.[23,31,36] It is also unknown whether blocking other cytokine/receptor partners would effectively treat CRS and maintain antitumor efficacy. Future studies are warranted and ongoing.

Neurological toxicity

The development of neurologic toxicities including confusion, delirium, expressive aphasia, obtundation, myoclonus, and seizure has been reported in patients receiving CD19-specific CAR T cells.[3,31,32] The causative pathophysiology of these neurologic side effects is unknown, though given similar events reported with blinatumomab administration,[37,38] it is plausible that elevated cytokine levels are partly responsible for the neurologic sequelae. Conversely, direct CAR T-cell toxicity on the central nervous system is possible but has not been demonstrated. Patient correlates have not been informative as reports have conflicted on the correlation between the number of engineered or nonengineered T cells in spinal fluid and status of central nervous system leukemia with neurological complications.[3,31,32] Similarly, EEG traces have not reliably identified seizure activity despite clinical symptoms demonstrating such activity. To date, the neurologic toxicity has been reversible in a majority of cases and it is unclear if this toxicity is restricted to CD19-specific CAR T cells or will be exhibited by the targeting of other tumor-associated antigens.[3,31,32]

On-target/off-tumor recognition

The ideal target antigen is restricted to the tumor cell and provides a critical survival signal for the malignant clone. Unfortunately, most targets of CAR T cells have shared expression on normal tissues and some degree of "on-target/off-tumor" toxicity occurs through engagement of target antigen on nonpathogenic tissues.[30] The severity of reported events has ranged from manageable lineage depletion (B-cell aplasia) to severe toxicity (death). "On-target/off-tumor" recognition is predictably seen in a variety of organ systems, including gastrointestinal, hematologic, and pulmonary. One of the earliest trials utilizing a carboxyanhydrase-IX-specific CAR T cell for renal cell carcinoma resulted in the development of cholestasis due to expression of carboxyanhydrase-IX on bile duct epithelium.[39,40] Targeting of carcinoembryonic antigen by CAR T cells in patients with colon cancer resulted in severe, albeit transient, colitis due to antigen recognition of normal colonic tissue.[41] In the setting of CD19-specific CAR T cells, the targeting of normal B cells results in B-cell aplasia which may require intermittent infusion of pooled immunoglobulin as prophylaxis from infectious complications.[33,42] Finally, in a fatal example of "on-target/off-tumor" recognition, a patient treated with CAR T cells specific for the cancer-associated antigen HER-2/neu developed rapid respiratory failure, multi-organ dysfunction, and subsequent death attributed to reactivity against pulmonary tissue expression of HER-2/neu.[43] However, this unforeseen toxicity was potentially provoked by the substantial dose of infused CAR T cells (1×10^{10} CAR T cells) as subsequent studies utilizing a different HER2/neu-specific CAR (without prior conditioning chemotherapy) have proven safe at significantly lower CAR T-cell doses.[14]

Anaphylaxis

The majority of genetically modified T cells utilized in clinical trials contain antigen-recognition domains derived from murine mAb.[30] Therefore, it comes as little surprise that both cellular and humoral rejection of CAR T cells have been demonstrated due to the immunogenicity of foreign protein.[44–46] Efforts are ongoing to humanize the components of expressed proteins with a goal of improving persistence and potentially, efficacy.[47] A more immediate toxicity is host recognition of infused foreign components resulting in acute anaphylaxis, as seen with one patient treated with mesothelin-specific CAR T cells.[48] In this report, one of four patients treated with multiple infusions of mesothelin-specific CAR T cells developed cardiorespiratory failure at the conclusion of the third infusion.[48] The design of this study employed multiple infusions of T cells expressing a transient CAR (mRNA vector) in an effort to reduce "on-target/off tumor" toxicity. Detailed investigation following the cardiorespiratory event confirmed the presence of human anti-mouse antibodies and elevated trypsin in the patient's serum supporting the finding of an IgE-mediated anaphylactic event.[48] Diligent surveillance, prompt recognition, and immediate treatment of this life-threatening side effect are critical for patients receiving genetically modified T cells.

Insertional oncogenesis

The risk of insertional oncogenesis in human cells has been established in the context of gene therapy of hematopoietic stem cells for X-linked severe combined immunodeficiency and chronic granulomatous disease.[49–53] In the majority of cases retroviral vector insertion near the LMO-2 oncogene has been implicated.[53] Insertion of a transgene into differentiated T cells also carries the risk of induced malignant transformation. However, to date, no cases of transformation have been reported following infusion of genetically modified T cells. Notably, the LMO-2 oncogene is silent in T cells making this site an unlikely locus of retroviral integration. In practice, the use of genetically modified T cells has a decade-long safety profile without evidence of vector-induced immortalization, clonal expansion, or enrichment for integration sites near genes implicated in growth control or transformation.[22] Taken together, the risk of insertional oncogenesis following gene transfer into T cells is seemingly low; however, investigators must remain vigilant and adhere to strict monitoring set forth in current clinical trial design.

Graft versus host disease

ACT with autologous/patient-derived tumor-specific CAR T cells has demonstrated clinical benefit for patients with cancer. In the context of CD19-specific CAR T cells, a number of patients have been treated following allo-HSCT with confirmation that infused CAR T cells were donor in origin. Despite the risk of alloreactivity, CD19-specific CAR T cells collected post allo-HSCT have not demonstrated the propensity to induce GVHD.[3,31,32] Even more impressive is the lack of reported GVHD in a study utilizing CD19-specific CAR T cells generated from allogeneic donors in adult patients with relapsed/refractory B-cell malignancies following allo-HSCT.[54] In this study, patients were required to tolerate donor lymphocyte infusion without evidence of GVHD prior to donor-derived CAR T-cell infusion.[54] It is unclear, if this selection resulted in a population of patients/donors with mitigated risk for alloreactivity. Despite this proven safety, the generation of a "personalized" product (patient or allo-HSCT donor derived) on a patient-by-patient basis is time consuming and expensive. Establishment of an "off-the-shelf" or "third-party" cell bank is an attractive solution with the possibility of reducing time to treatment and cost. Two methodologies are currently at the forefront of these efforts: CAR-transduced viral-specific cells and endogenous T-cell receptor silencing.[13,55–57] Studies utilizing these technologies are in process and may ultimately broaden the applicability of engineered cell technology.

Off-target antigen recognition

The majority of CAR T cells recognize antigen through single-chain variable fragments derived from mAbs. For some, the corresponding

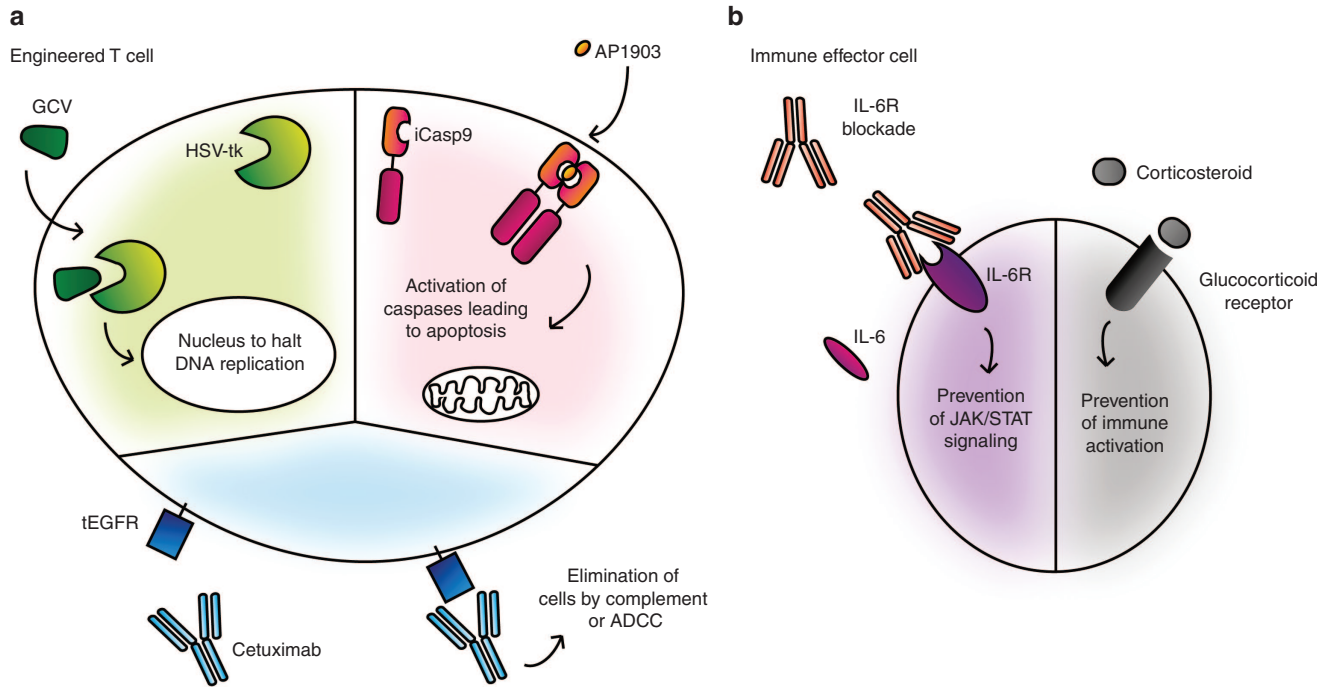

Figure 2 Management of chimeric antigen receptor (CAR) T-cell toxicity. (**a**) CAR T cells can be further engineered to express "suicide genes" or "elimination genes" such as herpes simplex virus thymidine kinase (HSV-tk) which converts the prodrug ganciclovir (GCV) into GCV-triphosphate resulting in cell death by incorporation into replicating DNA; inducible caspase 9 (iCasp9) a chimeric protein that binds the small molecule AP1903, leading to caspase 9 dimerization and ultimate apoptosis; truncated endothelial growth factor receptor (tEGFR) which is a targetable antigen which allows for elimination of modified cells following infusion of associated anti-EGFR MAb (cetuximab). (**b**) Pharmacological immunosuppression will ameliorate toxicity from CAR T including blockade of IL-6R and/or treatment with systemic corticosteroid. ADCC, antibody-dependent cellular cytotoxicity.

mAb has established a proven safety record in clinical use. For others, the toxicity profile is uncertain. Confounding this is a discrepancy when comparing mAb to CAR T-cell binding, as illustrated in the case of trastuzumab (anti-HER2/neu). The toxicity profile of the mAb (cardiotoxicity) is not mirrored by the toxicity profile of HER-2/neu-specific CAR T cells.[43] In addition to target-antigen binding, there is a distinct possibility of off-target antigen recognition by genetically modified tumor-specific T cells. In cases where "first-in-human" antigen recognition motifs are used, prediction of off-target antigen recognition and subsequent toxicities is exquisitely difficult. To date, off-target recognition of cross-reactive antigens has not been evident in CAR T-cell trials. However, fatal cardiac toxicity due to off-target reactivity has been seen in 2/2 patients infused with autologous T cells engineered to express an enhanced affinity T-cell receptor directed against the cancer testis antigen MAGE-A3.[58,59] Cross reactivity occurred against titin, a protein for which expression is only evident during contraction and expansion of cardiac tissue.[60] This finding illustrates the difficulty in predicting off-target antigen toxicity especially in the case of complex antigen presentation at different stages of cellular differentiation and activity. To this end, diligent surveillance and prompt recognition of any potential toxicity is a requisite when CAR T cells target novel tumor-associated antigens.

TOXICITY MANAGEMENT

In hematologic malignancies, significant clinical benefit in targeting of CD19 antigen on malignant B cells has been demonstrated. However, the intense immune activation has resulted in unwanted side effects despite optimism that direct targeting of tumor cells would avoid systemic toxicity. Management of these toxicities has become an integral step in the successful clinical application of CAR T cells. Several methods have been proposed to ameliorate toxicity, including nonspecific immune suppression or selective depletion of modified cells through "suicide" or "elimination" genes (Figure 2). An obvious caveat to any suppression or elimination of the tumor-specific cell population would be the concurrent abrogation of any persistent antitumor surveillance.

Pharmacological immunosuppression

As noted, the most common toxicity following CD19-specific CAR T-cell infusion has been uncontrolled immune activation in the form of CRS. The use of tocilizumab to provide IL-6R blockade has demonstrated near-immediate reversal of CRS symptomatology including fever and hypotension. The impact of IL-6R blockade on the neurologic sequela following CD19-specific CAR T cells is unknown. Immunosuppression with systemic corticosteroid can also improve the symptoms of CRS, with dexamethasone as a logical first choice agent due to its superior central nervous system penetration. Not surprisingly, the use of prolonged systemic corticosteroids has been shown to diminish the persistence and potentially, the efficacy of CAR T cells.[31] Alternative immunosuppression with cell-specific mAbs or lymphodepleting chemotherapy (cyclophosphamide) is theoretically possible but as of yet, untested or reported.

Suicide genes

Given the wide range of expected and unexpected toxicities, integration of a "suicide gene" to allow for selective depletion of CAR T cells may be an essential component in the evolution of this technology. Several methodologies have been described

and successfully reported in preclinical or clinical testing.[61–70] The first suicide gene evaluated in human trials was the herpes simplex virus thymidine kinase whose expression renders modified cells susceptible to treatment with the acyclic nucleoside analog ganciclovir.[61–63] Once expressed, herpes simplex virus thymidine kinase catalyzes phosphorylation of ganciclovir resulting in competitive inhibition of guanosine incorporation with subsequent disruption of DNA polymerization and synthesis. While effective, this approach is limited by the immunogenicity of herpes simplex virus thymidine kinase expression with resultant rejection of modified cells. Secondly, reliance on inhibition of DNA replication as a method of cell death may delay clinical benefit. Furthermore, the widely used antiviral ganciclovir is precluded from therapeutic use as administration would result in toxicity to modified cells. Another approach utilizes the transgenic introduction of a mutated tymidylate kinase responsible for phosphorylation of the HIV prodrug 3′-azido-3′-deoxythymidine with subsequent DNA chain termination and cell death in modified cells.[71] However, this system was proven to have suboptimal efficacy when compared with herpes simplex virus thymidine kinase.[69]

Selective depletion of genetically modified cells can also occur through the dimerizable death molecules inducible Fas or caspase 9 (ICasp9).[64,66] These dimerizable chimeric elements are engineered to contain components of an FK506-binding protein with cross-linking triggered by exposure to an otherwise inert bivalent small molecule. In the case of both inducible Fas and ICasp9, dimerization leads to activation of downstream caspases with induction of the apoptotic pathway. As a proof-of-principal, ICasp9-modified donor T cells were evaluated in haploidentical HSCT recipients.[68,70] A single dose of a small-molecule dimerizing agent (AP1903) eliminated more than 90% of ICasp9-modified T cells within 30 minutes of administration.[68,70] This rapid onset of action without adverse event resulted in reversal of GVHD without recurrence. Despite these dramatic results, when ICasp9 is introduced into other engineered T-cell platforms, the minority cell population unaffected by suicide gene activation may perpetuate toxicity. Conversely, there is a possibility of tonic ICasp9 dimerization in the absence of the small-molecule dimerizing agent, seen in transduced cell lines *in vitro*.[72] The resultant increase in basal apoptosis potentially limits widespread utility.

Elimination genes

Another method to induce selective depletion of genetically modified cells is the expression of a targetable moiety. Modified cells can be programmed to express a known cell-surface antigen, such as CD20 or EGFR, with subsequent cell death triggered via infusion of the associated mAb (rituximab for CD20 and cetuximab for EGFR).[65,67,73] This is an attractive strategy given the familiarity of clinicians with the use and safety profile of several Food and Drug Administration-approved mAbs. Expression of known antigens on the surface of CAR T cells also enables for the selection (*ex vivo*) and tracking (*in vivo*) of genetically modified cell populations. Factors limiting this methodology are any on-target side effects inherent to mAb binding to normal tissue as well as dependence on antibody-dependent cellular cytotoxicity for clearance of the cell population. Heavily pretreated cancer patients with dysfunctional immune systems may also have limited ability to remove unwanted populations of genetically modified cells, especially in the setting of toxicity. In a robust preclinical comparison of various safety switch technologies, the IC9 system and the combination of CD20 and rituximab were the methods most likely to have translatable clinical utility based on both rapid onset of action and superior efficacy.[69]

Targeted activation

Functional control of the intensity or toxicity of T-cell activation is possible through the inclusion of an "on-switch" in CAR design. T-cell response can be controlled through combinatorial antigen targeting with separation of T-cell activation signals.[74,75] This requires the identification of two target antigens with coexpression on malignant tissue for T-cell activation. Dual-antigen binding is then necessary for complete T-cell activation.[74] In contrast, normal tissue (expressing one target antigen) provides incomplete activation thereby limiting "on-target/off tumor" toxicity.[74] Alternatively, if presentation of dual antigens is exclusive to normal tissue, inclusion of inhibitory signaling in CAR design allows for selective targeting of malignant/pathogenic tissue (expressing one antigen) while normal tissue is spared.[76] Finally, separation of the antigen recognition moiety of a CAR from the intracellular signaling domain is a strategy that promises exquisite control by incorporating heterodimerizable elements responsive to small-molecule binding.[77] This approach has the potential to control T cell activation and toxicity through titration of the small-molecule "on-switch".[77]

CONCLUSION

The utility of CAR T cells as ACT for the treatment of malignancy will depend both on the ability to easily manufacture the cellular product as well as the feasibility of safe administration. Given the dramatic responses seen in hematological malignancies, we should attempt to diminish the barriers to widespread access and perfect the response seen in solid tumors. Toxicity management should necessarily become a focus of implementation to allow for administration beyond specialized centers. Improved understanding of the immunological response following CAR T-cell infusion will improve clinical management and enhance our investigation into activation or elimination of CAR T cells thereby reducing hazards following infusion. It is our hope that toxicities will be anticipated and manageable, allowing for improved quality and universal benefit.

REFERENCES

1. Mitchison, NA (1955). Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. *J Exp Med* **102**: 157–177.
2. Hinrichs, CS and Rosenberg, SA (2014). Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunol Rev* **257**: 56–71.
3. Maude, SL, Frey, N, Shaw, PA, Aplenc, R, Barrett, DM, Bunin, NJ *et al.* (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* **371**: 1507–1517.
4. Manzo, T, Heslop, HE and Rooney, CM (2015). Antigen-specific T cell therapies for cancer. *Hum Mol Genet* **24**: R67–R73.
5. Themeli, M, Rivière, I and Sadelain, M (2015). New cell sources for T cell engineering and adoptive immunotherapy. *Cell Stem Cell* **16**: 357–366.
6. Kolb, HJ (2008). Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood* **112**: 4371–4383.
7. Rosenberg, SA, Restifo, NP, Yang, JC, Morgan, RA and Dudley, ME (2008). Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* **8**: 299–308.
8. DiGiusto, D and Cooper, L (2007). Preparing clinical grade Ag-specific T cells for adoptive immunotherapy trials. *Cytotherapy* **9**: 613–629.
9. Hollyman, D, Stefanski, J, Przybylowski, M, Bartido, S, Borquez-Ojeda, O, Taylor, C *et al.* (2009). Manufacturing validation of biologically functional T cells targeted to CD19 antigen for autologous adoptive cell therapy. *J Immunother* **32**: 169–180.
10. Vera, JF, Brenner, LJ, Gerdemann, U, Ngo, MC, Sili, U, Liu, H *et al.* (2010). Accelerated production of antigen-specific T cells for preclinical and clinical applications using gas-permeable rapid expansion cultureware (G-Rex). *J Immunother* **33**: 305–315.

11. Jin, J, Sabatino, M, Somerville, R, Wilson, JR, Dudley, ME, Stroncek, DF *et al.* (2012). Simplified method of the growth of human tumor infiltrating lymphocytes in gas-permeable flasks to numbers needed for patient treatment. *J Immunother* **35**: 283–292.
12. Somerville, RP, Devillier, L, Parkhurst, MR, Rosenberg, SA and Dudley, ME (2012). Clinical scale rapid expansion of lymphocytes for adoptive cell transfer therapy in the WAVE® bioreactor. *J Transl Med* **10**: 69.
13. Sun, J, Huye, LE, Lapteva, N, Mamonkin, M, Hiregange, M, Ballard, B *et al.* (2015). Early transduction produces highly functional chimeric antigen receptor-modified virus-specific T-cells with central memory markers: a Production Assistant for Cell Therapy (PACT) translational application. *J Immunother Cancer* **3**: 5.
14. Ahmed, N, Brawley, VS, Hegde, M, Robertson, C, Ghazi, A, Gerken, C *et al.* (2015). Human epidermal growth factor receptor 2 (HER2)-specific chimeric antigen receptor-modified T cells for the immunotherapy of HER2-positive sarcoma. *J Clin Oncol* **33**: 1688–1696.
15. Eshhar, Z, Waks, T, Gross, G and Schindler, DG (1993). Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci USA* **90**: 720–724.
16. Sadelain, M, Rivière, I and Brentjens, R (2003). Targeting tumours with genetically enhanced T lymphocytes. *Nat Rev Cancer* **3**: 35–45.
17. Thomas, S, Hart, DP, Xue, SA, Cesco-Gaspere, M and Stauss, HJ (2007). T-cell receptor gene therapy for cancer: the progress to date and future objectives. *Expert Opin Biol Ther* **7**: 1207–1218.
18. Zhou, X, Cui, Y, Huang, X, Yu, Z, Thomas, AM, Ye, Z *et al.* (2003). Lentivirus-mediated gene transfer and expression in established human tumor antigen-specific cytotoxic T cells and primary unstimulated T cells. *Hum Gene Ther* **14**: 1089–1105.
19. Cavalieri, S, Cazzaniga, S, Geuna, M, Magnani, Z, Bordignon, C, Naldini, L *et al.* (2003). Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood* **102**: 497–505.
20. Casucci, M, Hawkins, RE, Dotti, G and Bondanza, A (2015). Overcoming the toxicity hurdles of genetically targeted T cells. *Cancer Immunol Immunother* **64**: 123–130.
21. Heslop, HE, Slobod, KS, Pule, MA, Hale, GA, Rousseau, A, Smith, CA *et al.* (2010). Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* **115**: 925–935.
22. Scholler, J, Brady, TL, Binder-Scholl, G, Hwang, WT, Plesa, G, Hege, KM *et al.* (2012). Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells. *Sci Transl Med* **4**: 132ra53.
23. Lee, DW, Gardner, R, Porter, DL, Louis, CU, Ahmed, N, Jensen, M *et al.* (2014). Current concepts in the diagnosis and management of cytokine release syndrome. *Blood* **124**: 188–195.
24. Chatenoud, L, Ferran, C, Legendre, C, Thouard, I, Merite, S, Reuter, A *et al.* (1990). *In vivo* cell activation following OKT3 administration. Systemic cytokine release and modulation by corticosteroids. *Transplantation* **49**: 697–702.
25. Wing, MG, Moreau, T, Greenwood, J, Smith, RM, Hale, G, Isaacs, J *et al.* (1996). Mechanism of first-dose cytokine-release syndrome by CAMPATH 1-H: involvement of CD16 (FcgammaRIII) and CD11a/CD18 (LFA-1) on NK cells. *J Clin Invest* **98**: 2819–2826.
26. Winkler, U, Jensen, M, Manzke, O, Schulz, H, Diehl, V and Engert, A (1999). Cytokine-release syndrome in patients with B-cell chronic lymphocytic leukemia and high lymphocyte counts after treatment with an anti-CD20 monoclonal antibody (rituximab, IDEC-C2B8). *Blood* **94**: 2217–2224.
27. Suntharalingam, G, Perry, MR, Ward, S, Brett, SJ, Castello-Cortes, A, Brunner, MD *et al.* (2006). Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med* **355**: 1018–1028.
28. Panelli, MC, White, R, Foster, M, Martin, B, Wang, E, Smith, K *et al.* (2004). Forecasting the cytokine storm following systemic interleukin (IL)-2 administration. *J Transl Med* **2**: 17.
29. Teachey, DT, Rheingold, SR, Maude, SL, Zugmaier, G, Barrett, DM, Seif, AE *et al.* (2013). Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy. *Blood* **121**: 5154–5157.
30. Curran, KJ, Pegram, HJ and Brentjens, RJ (2012). Chimeric antigen receptors for T cell immunotherapy: current understanding and future directions. *J Gene Med* **14**: 405–415.
31. Davila, ML, Riviere, I, Wang, X, Bartido, S, Park, J, Curran, K *et al.* (2014). Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med* **6**: 224ra25.
32. Lee, DW, Kochenderfer, JN, Stetler-Stevenson, M, Cui, YK, Delbrook, C, Feldman, SA *et al.* (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* **385**: 517–528.
33. Kochenderfer, JN, Dudley, ME, Feldman, SA, Wilson, WH, Spaner, DE, Maric, I *et al.* (2012). B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* **119**: 2709–2720.
34. Brentjens, RJ, Davila, ML, Riviere, I, Park, J, Wang, X, Cowell, LG *et al.* (2013). CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med* **5**: 177ra38.
35. Grupp, SA, Kalos, M, Barrett, D, Aplenc, R, Porter, DL, Rheingold, SR *et al.* (2013). Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* **368**: 1509–1518.
36. Maude, SL, Barrett, D, Teachey, DT and Grupp, SA (2014). Managing cytokine release syndrome associated with novel T cell-engaging therapies. *Cancer J* **20**: 119–122.
37. Buie, LW, Pecoraro, JJ, Horvat, TZ and Daley, RJ (2015). Blinatumomab: a first-in-class bispecific T-cell engager for precursor B-cell acute lymphoblastic leukemia. *Ann Pharmacother* **49**: 1057–1067.
38. Topp, MS, Gökbuget, N, Stein, AS, Zugmaier, G, O'Brien, S, Bargou, RC *et al.* (2015). Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, phase 2 study. *Lancet Oncol* **16**: 57–66.
39. Lamers, CH, Sleijfer, S, Vulto, AG, Kruit, WH, Kliffen, M, Debets, R *et al.* (2006). Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* **24**: e20–e22.
40. Lamers, CH, Sleijfer, S, van Steenbergen, S, van Elzakker, P, van Krimpen, B, Groot, C *et al.* (2013). Treatment of metastatic renal cell carcinoma with CAIX CAR-engineered T cells: clinical evaluation and management of on-target toxicity. *Mol Ther* **21**: 904–912.
41. Parkhurst, MR, Yang, JC, Langan, RC, Dudley, ME, Nathan, DA, Feldman, SA *et al.* (2011). T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther* **19**: 620–626.
42. Kochenderfer, JN, Wilson, WH, Janik, JE, Dudley, ME, Stetler-Stevenson, M, Feldman, SA *et al.* (2010). Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* **116**: 4099–4102.
43. Morgan, RA, Yang, JC, Kitano, M, Dudley, ME, Laurencot, CM and Rosenberg, SA (2010). Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* **18**: 843–851.
44. Kershaw, MH, Westwood, JA, Parker, LL, Wang, G, Eshhar, Z, Mavroukakis, SA *et al.* (2006). A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* **12**: 6106–6115.
45. Jensen, MC, Popplewell, L, Cooper, LJ, DiGiusto, D, Kalos, M, Ostberg, JR *et al.* (2010). Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans. *Biol Blood Marrow Transpl* **16**: 1245–1256.
46. Lamers, CH, Willemsen, R, van Elzakker, P, van Steenbergen-Langeveld, S, Broertjes, M, Oosterwijk-Wakka, J *et al.* (2011). Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. *Blood* **117**: 72–82.
47. Maude, SB, Barrett, DM, Ambrose, DE, Rheingold, SR, Aplenc, R, Teachey, DT *et al.* (2015). Efficacy and safety of humanized chimeric antigen receptor (CAR)-modified T cells targeting CD19 in children with relapsed/refractory ALL. *Blood* **126**: 683.
48. Maus, MV, Haas, AR, Beatty, GL, Albelda, SM, Levine, BL, Liu, X *et al.* (2013). T cells expressing chimeric antigen receptors can cause anaphylaxis in humans. *Cancer Immunol Res* **1**: 26–31.
49. Hacein-Bey-Abina, S, Von Kalle, C, Schmidt, M, McCormack, MP, Wulffraat, N, Leboulch, P *et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–419.
50. Kohn, DB, Sadelain, M and Glorioso, JC (2003). Occurrence of leukaemia following gene therapy of X-linked SCID. *Nat Rev Cancer* **3**: 477–488.
51. Howe, SJ, Mansour, MR, Schwarzwaelder, K, Bartholomae, C, Hubank, M, Kempski, H *et al.* (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* **118**: 3143–3150.
52. Stein, S, Ott, MG, Schultze-Strasser, S, Jauch, A, Burwinkel, B, Kinner, A *et al.* (2010). Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med* **16**: 198–204.
53. Hacein-Bey-Abina, S, Garrigue, A, Wang, GP, Soulier, J, Lim, A, Morillon, E *et al.* (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* **118**: 3132–3142.
54. Kochenderfer, JN, Dudley, ME, Carpenter, RO, Kassim, SH, Rose, JJ, Telford, WG *et al.* (2013). Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. *Blood* **122**: 4129–4139.
55. Cruz, CR, Micklethwaite, KP, Savoldo, B, Ramos, CA, Lam, S, Ku, S *et al.* (2013). Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. *Blood* **122**: 2965–2973.
56. Wang, X, Wong, CW, Urak, R, Mardiros, A, Budde, LE, Chang, WC *et al.* (2015). CMVpp65 vaccine enhances the antitumor efficacy of adoptively transferred CD19-redirected CMV-specific T cells. *Clin Cancer Res* **21**: 2993–3002.
57. Poirot, L, Philip, B, Schiffer-Mannioui, C, Le Clerre, D, Chion-Sotinel, I, Derniame, S *et al.* (2015). Multiplex genome-edited T-cell manufacturing platform for "Off-the-Shelf" adoptive T-cell immunotherapies. *Cancer Res* **75**: 3853–3864.
58. Cameron, BJ, Gerry, AB, Dukes, J, Harper, JV, Kannan, V, Bianchi, FC *et al.* (2013). Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. *Sci Transl Med* **5**: 197ra103.

59. Linette, GP, Stadtmauer, EA, Maus, MV, Rapoport, AP, Levine, BL, Emery, L *et al.* (2013). Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood* **122**: 863–871.
60. Kötter, S, Andresen, C and Krüger, M (2014). Titin: central player of hypertrophic signaling and sarcomeric protein quality control. *Biol Chem* **395**: 1341–1352.
61. Bordignon, C, Bonini, C, Verzeletti, S, Nobili, N, Maggioni, D, Traversari, C *et al.* (1995). Transfer of the HSV-tk gene into donor peripheral blood lymphocytes for *in vivo* modulation of donor anti-tumor immunity after allogeneic bone marrow transplantation. *Hum Gene Ther* **6**: 813–819.
62. Tiberghien, P, Cahn, JY, Brion, A, Deconinck, E, Racadot, E, Hervé, P *et al.* (1997). Use of donor T-lymphocytes expressing herpes-simplex thymidine kinase in allogeneic bone marrow transplantation: a phase I-II study. *Hum Gene Ther* **8**: 615–624.
63. Bonini, C, Ferrari, G, Verzeletti, S, Servida, P, Zappone, E, Ruggieri, L *et al.* (1997). HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* **276**: 1719–1724.
64. Thomis, DC, Marktel, S, Bonini, C, Traversari, C, Gilman, M, Bordignon, C *et al.* (2001). A Fas-based suicide switch in human T cells for the treatment of graft-versus-host disease. *Blood* **97**: 1249–1257.
65. Serafini, M, Manganini, M, Borleri, G, Bonamino, M, Imberti, L, Biondi, A *et al.* (2004). Characterization of CD20-transduced T lymphocytes as an alternative suicide gene therapy approach for the treatment of graft-versus-host disease. *Hum Gene Ther* **15**: 63–76.
66. Tey, SK, Dotti, G, Rooney, CM, Heslop, HE and Brenner, MK (2007). Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation. *Biol Blood Marrow Transpl* **13**: 913–924.
67. Wang, X, Chang, WC, Wong, CW, Colcher, D, Sherman, M, Ostberg, JR *et al.* (2011). A transgene-encoded cell surface polypeptide for selection, *in vivo* tracking, and ablation of engineered cells. *Blood* **118**: 1255–1263.
68. Di Stasi, A, Tey, SK, Dotti, G, Fujita, Y, Kennedy-Nasser, A, Martinez, C *et al.* (2011). Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* **365**: 1673–1683.
69. Marin, V, Cribioli, E, Philip, B, Tettamanti, S, Pizzitola, I, Biondi, A *et al.* (2012). Comparison of different suicide-gene strategies for the safety improvement of genetically manipulated T cells. *Hum Gene Ther Methods* **23**: 376–386.
70. Zhou, X, Di Stasi, A, Tey, SK, Krance, RA, Martinez, C, Leung, KS *et al.* (2014). Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. *Blood* **123**: 3895–3905.
71. Sato, T, Neschadim, A, Konrad, M, Fowler, DH, Lavie, A and Medin, JA (2007). Engineered human tmpk/AZT as a novel enzyme/prodrug axis for suicide gene therapy. *Mol Ther* **15**: 962–970.
72. Ando, M, Hoyos, V, Yagyu, S, Tao, W, Ramos, CA, Dotti, G *et al.* (2014). Bortezomib sensitizes non-small cell lung cancer to mesenchymal stromal cell-delivered inducible caspase-9-mediated cytotoxicity. *Cancer Gene Ther* **21**: 472–482.
73. Philip, B, Kokalaki, E, Mekkaoui, L, Thomas, S, Straathof, K, Flutter, B *et al.* (2014). A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. *Blood* **124**: 1277–1287.
74. Kloss, CC, Condomines, M, Cartellieri, M, Bachmann, M and Sadelain, M (2013). Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat Biotechnol* **31**: 71–75.
75. Wilkie, S, van Schalkwyk, MC, Hobbs, S, Davies, DM, van der Stegen, SJ, Pereira, AC *et al.* (2012). Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. *J Clin Immunol* **32**: 1059–1070.
76. Fedorov, VD, Themeli, M and Sadelain, M (2013). PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses. *Sci Transl Med* **5**: 215ra172.
77. Wu, CY, Roybal, KT, Puchner, EM, Onuffer, J and Lim, WA (2015). Remote control of therapeutic T cells through a small molecule-gated chimeric receptor. *Science* **350**: aab4077.