Applications and explorations of CRISPR/Cas9 in CAR T-cell therapy

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Abstract

Chimeric antigen receptor (CAR) T-cell therapy has shown remarkable effects and promising prospects in patients with refractory or relapsed malignancies, pending further progress in the next-generation CAR T cells with more optimized structure, enhanced efficacy and reduced toxicities. The clustered regulatory interspaced short palindromic repeat (CRISPR/Cas9) technology holds immense promise for advancing the field owing to its flexibility, simplicity, high efficiency and multiplexing in precise genome editing. Herein, we review the applications and explorations of CRISPR/Cas9 technology in constructing allogenic universal CAR T cells, disrupting inhibitory signaling to enhance potency and exploration of safer and more controllable novel CAR T cells.

Key words: CAR T cells; CRISPR; Cas9; cancer immunotherapy; genome editing

Introduction

Cancer immunotherapy is the fourth mainstream treatment after surgery, chemotherapy and radiotherapy. Adoptive T-cell immunotherapy, particularly chimeric antigen receptor (CAR) T cell therapy, has revolutionized cancer therapy especially after the FDA approval of Kymriah and Yescarta (CD19-directed CAR T cells in B-cell leukemia and lymphoma) [1–3]. CARs are synthetic receptors typically containing an antibody-derived target-binding extracellular domain, a hinge region, a transmembrane domain and an intracellular signaling moiety capable of activating T cells [4,5]. T cells programmed with CARs can specifically recognize and kill antigen-expressing cells without the restriction of major histocompatibility complex (MHC). Clinical data has demonstrated that CAR T-cell therapy can induce durable complete remissions (CRs) in patients with a variety of hematologic and solid cancers, especially in relapsed/refractory acute lymphoblastic leukemia (ALL) and multiple myeloma with striking response rates of 80–100% [6–8]. Despite of promising efficacy of CAR T-cell therapy, there are several challenges awaiting for solutions, such as insufficient quantity and poor quality of autologous T cells, CAR T cell exhaustion and tumor suppressive microenvironments, potential self-killing and uncontrollable proliferation.

Optimization of the CAR T designs is supposed as one of the main tracks to tackle these limitations. The first generation of CAR T cells with only CD3 zeta intracellular chain was found to have modest proliferative and cytotoxic capacity [9–12]. The second generation of CARs contains a single costimulatory domain (CD28 or 4-1BB), proven to attain an improved efficacy and in vivo survival, whereas the third generation has two or more costimulatory domains (CD28, 4-1BB, ICOS or OX40), not superior to the second generation [13–15]. More functional elements are considered to be added to the next generation of CARs, like interleukins genes to increase potency, chemokine receptors genes to improve T-cell trafficking and on–off switches or suicide genes to enhance safety and controllability [16–18]. The structures and features of every generation of CAR-T are shown in Table 1.

The development of genomic editing technologies opens a window to accelerate the fourth generation of CAR T cells.
There are currently three major genomic editing technologies, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regulatory inter-spaced short palindromic repeat (CRISPR-associated) protein 9 (CRISPR/Cas9) [19–21]. Although ZFNs and TALENs have been applied to engineer T cells in clinical trials, the recognition of targetable DNA sequences is based on complicated protein conformation, a pair of Zn-finger binding domains or a pair of TALE DNA binding domains, accompanying with complex designs and relatively low gene-editing efficiencies [22,23]. CRISPR/Cas9, directed by a small guide RNA (sgRNA) to the target site, has become the most popular and developed of these tools due to its simplicity, flexibility, high efficiency and multiplexable genome editing capabilities [24–26]. A sgRNA-guided Cas9 nuclease induces a DNA double-stranded break at targeted genomic locations, subsequently repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ, an error prone repair pathway, can result in insertions or deletions of small nucleotide sequences and HDR can knock-in relatively large gene segments in the presence of a homology repair template at the site of interest [27–29]. Therefore, the combination with CRISPR/Cas9 technology will further expand the landscape of T-cell engineering. Besides knock-in of functional genes, such as interleukins and suicide genes, to product next-generation CAR T cells, other strategies comprises knock-out of endogenous genes, such as TCRs and MHCs, to develop ‘off-the-shelf’ universal CAR T cells [30], disruption of inhibitory receptors (such as PD-1 and TGF beta receptor) to ameliorate suppressive microenvironments [31,32], integration of the CAR cassette into the specific gene locus (such as TRAC and TET2) to improve efficiency and safety [33,34], deletions of target genes to avoid self-killing of CAR T cells [35]. CRISPR/Cas9 technology is unveiling a new era for CAR T-cell therapy. All gene-edited CAR T cells discussed here are shown in Table 2.

### Production of allogeneic universal CAR T cells

Although currently widespread-used autologous CAR T cells have shown promising results in cancer therapy, limitations exist. Almost 10–15% of enrolled patients were unable to receive infusions of CAR T cells because of poor quality and insufficient quantities of autologous T cells unavailable for manufacturing or rapid disease progression and even death before successful production of certain amount of CAR T cells [1–3]. A UPenn team recently reported a patient relapsing after infusion of anti-CD19 CAR T cells with CD19-negative leukemia that aberrantly expressed the anti-CD19 CAR because the CAR gene was unintentionally introduced into a single leukemic B cell during T-cell manufacturing [36]. The development of universal ‘off-the-shelf’ CAR T cells from healthy donors can circumvent the constraints and potentially be the mainstream direction in the future. The major barriers of such universal CAR T cell products are graft-versus-host disease (GVHD) and rejection of the infused allogeneic T cells. Endogenous αβ T cell receptors (TCRs) on adaptively transferred donor lymphocytes can recognize alloantigens in human leukocyte antigen (HLA) mismatched recipients resulting in GVHD; conversely, recognition of foreign HLA molecules on donor T cells may lead to rejection.

ZFNs and TALENs were successfully used to knock-out TCRα constant (TRAC) and TCRβ constant (TRBC) to generate TCR-negative CAR T cells to prevent GVHD without compromising CAR-mediated cytotoxicity [37,38]. Previous researches demonstrated that genetic knock-out of either TRAC or TRBC loci was sufficient to eliminate expression of α/β TCR on the T cell surface [39]. The Collectix firstly reported the generation of TALEN-edited allogeneic universal anti-CD19 CAR T(UCART19) cells in which TRAC and CD52 genes were knocked out [40]. CD52 disruption in the CAR T cells allowed effective targeted depletion of patients’ autologous T cells using an anti-CD52 antibody (alemtuzumab). The first-in-man application of the products was two infants with high-risk CD19-positive ALL who achieved molecular remission after receiving the infusion of UCART19 cells and attained successful bridge-to-transplantation [41,42]. The remarkable results led to two clinical trials of UCART19 cells: CALM trial in adults and PALL trial in pediatric patients (NCT02746952 and NCT02808442). Pooled data of 20 patients showed acceptable and manageable safety with 15% (3/20) of

### Table 1. Structure and features of every generation of CAR-T

| Structure of CAR | First-generation CAR | Second-generation CAR | Third-generation CAR | Next-generation CAR | Universal CAR |
|-----------------|----------------------|-----------------------|----------------------|---------------------|--------------|
| **Similarities** |                      |                       |                      |                     |              |
| An extracellular antigen-recognition region consisting of an scFv | A flexible hinge region derived from a CD8 molecule or CD28 or Fc region of an antibody | A transmembrane derived from CD8 or CD28 |                       |                     |              |
| **Differences** |                      |                       |                      |                     |              |
| Intracellular domain | only CD3ζ | CD3ζ; One costimulatory molecule: CD28 or 4-1BB | CD3ζ; ≥ 2 Costimulatory molecules: CD28, 4-1BB, ICOS or OX40 | CD3ζ; One costimulatory molecule: CD28 or 4-1BB | CD3ζ; One costimulatory molecule: CD28 or 4-1BB |
| In vivo Persistence | Low (days to 2 months) | Improved (3 months to years) | Improved (ORR depending on the tumor type) | Not superior to 2nd-generation | Exploration |
| Antitumor Effects | Low (ORR 0–40%) | Improved (ORR to years) | Not superior to second-generation | Exploration | Exploration |

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and genetically modified human T cells used in clinical trials to nently and completely eliminate HLA-I expression in primary also enhanced T-cell potency and delayed T-cell exhaustion [33].

The CRISPR/Cas9 technology not only minimized the risks of insertional oncogenesis and TCR-inducedGVHD, but also enhanced T-cell potency and delayed T-cell exhaustion [33]. A MSKCC group showed that directing a CD19-specific CAR to the TRAC locus using CRISPR/Cas9 technology not only minimized the risks of insertional oncogenesis and TCR-inducedGVHD, but also enhanced T-cell potency and delayed T-cell exhaustion [33].

ZFNs were also used to target the HLA-A locus to permanently and completely eliminate HLA-1 expression in primary and genetically modified human T cells used in clinical trials to evade rejection [44]. In addition, elimination of HLA heavy chains or beta-2-microglobulin (B2M), the non-polymorphic subunit of HLA-I complex, would prevent rapid rejection of allogeneic cells [45]. However, ideal universal CAR T cells should be silenced both TCR and HLA to avoidGVHD and rejection without reducing persistence and cytotoxicity in vivo. CRISPR/Cas9 has an obvious advantage in simultaneously multiplex and highly efficient genomic editing compared with ZFNs and TALENs. CRISPR/Cas9 was readily applicable to generate double-knock-out (B2M and TRAC, DKO) UCART19 cells with as similar safety and efficacy as wild-type anti-CD19 CAR T cells in preclinical studies [46]. One-shot CRISPR protocol for multiplex genome editing by incorporating multiple gRNA cassettes into a single CAR vector) (incorporating multiple gRNA cassettes in a single CAR vector) was 71.3 ± 6.7% [42].

| Target of CAR | Delivery of CAR | Target locus | Gene-editing method | Delivery | Editing efficiency | Reference |
|---------------|-----------------|--------------|--------------------|----------|-------------------|-----------|
| CD19          | SB electroporation | TRAC and TRBC | ZFNs | mRNA electroporation | 15–37% | [33] |
| CD19          | Lentivirus       | TRAC and CD52 | TALEN | mRNA electroporation | 10-60% | [36] |
| CD19          | AAV vector       | TRAC (insert CAR to TRAC) | CRISPR/Cas9 | Electroporation | ~70% | [29] |
| CD19          | SB electroporation | HLA-A | ZFNs | Nucleofection | 40.70% | [40] |
| CD19          | Lentivirus       | B2M and TRAC | CRISPR/Cas9 | RNA electroporation | 52.55-65.21% | [26] |
| CD19          | Lentivirus       | TRAC, B2M and PD-1 | CRISPR/Cas9 | Lenti viral vector | 37.05-50.97% | |
| CD19          | Lentivirus       | B2M and TRAC | CRISPR/Cas9 | mRNA electroporation | 71.3 ± 6.7% | [42] |
| CD19          | Lentivirus       | TRAC, B2M and Fas | CRISPR/Cas9 | Electroporation | 55.10% | |
| PSMA          | Lentivirus       | dntTGF-βRII | / | Lenti viral vector | 55.20% | [55] |
| CD19          | Retrovirus       | IL-15 and an suicide gene | Inducible caspase-9 | / | 65% | [57] |
| CD19          | Retrovirus       | Safety switch | Inducible caspase-9 | Retroviral vector | 61% ± 5% | [58] |
| CD19          | Lentivirus       | GM-CSF | CRISPR/Cas9 | Lenti viral vector | 82.20% | [61] |
| CD33          | Lentivirus       | CD33 in HSCs | CRISPR/Cas9 | Electroporation | 40–90% | [31, 64, 65] |
| CD7           | Gammaretrovirus  | CD7 in CAR T cells | CRISPR/Cas9 | Electroporation | >80% | [66] |

Disruption of inhibitory signaling molecules

The function of T cells was proven to play a significantly important role in the therapeutic effect of CAR T cells [49]. However, T cells are exposed to persistent antigen in patients with malignant tumors, resulting in T-cell exhaustion [50]. Exhausted T cells lose robust effector functions and express multiple inhibitory receptors, such as programmed cell death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), domain-containing protein-3 (TIM-3) and lymphocyte-activated gene-3 (LAG-3), which inhibit T-cell proliferation and cytokine production leading to immune escape [51]. The inhibitory pathways also contribute to suppressive tumor microenvironment, a major barrier of CAR T-cell therapy in solid tumors. Immune checkpoint inhibitors, anti-PD-1/PD-L1 and anti-CTLA-4 antibodies, have shown promising clinical results and been approved by the FDA [52]. Thus, disruption of multiple inhibitory factors is expected to improve the potency of CAR T cells. Recent studies suggested that anti-CD19 CAR T cells with CRISPR-mediated triple-knockout of the TRAC/TRBC, B2M and PD-1 genes displayed stronger
antitumor functions in contrast to DKO UCART19 cells in vitro and in animal models [30, 47]. High-fidelity Cas9s with the one-antitumor functions in contrast to DKO UCART19 cells by simultaneous disruption of quadruple genes [46]. CTI-4 dual inhibitory pathway-resistant DKO UCART19 cells shot platform showed the feasibility of generating PD-1 and [53]. Researches demonstrated that CAR T cell activity was (TNF-α (dnTGF-βRII) pathway could be blocked by using a dominant-negative TGFBRII suppressive milieu. Previous studies demonstrated that TGF-β of solid tumors, has the potential to improve the immuno-

differentiation into regulatory T cells (Tregs) [56]. Thus, inhibiting cell death (AICD) [54]. Ren et al. utilized CRISPR/Cas9 technology to generate Fas-resistant universal CAR T cells that observed elevation of AICD resistance and prolonged survival.

Transforming growth factor-β (TGF-β) represses effector T-cell activities through binding the TGF-β receptors (TGFBRI and TGFBRII) to induce heterodimerization of the respective receptors and phosphorylation of the major TGF-β signal mediators SMAD2 and SMAD3, resulting in reduced cytokine production, cytotoxicity and amplification [55]. TGF-β also drives T-cell differentiation into regulatory T cells (Tregs) [56]. Thus, inhibiting TGF-β signaling, a potent immunosuppressive factor in a variety of solid tumors, has the potential to improve the immunosuppressive milieu. Previous studies demonstrated that TGF-β pathway could be blocked by using a dominant-negative TGFBRII (dnTGF-βRII), which lacked the intracellular domain necessary for downstream signaling [57]. Foster et al. [31] used a clinical grade retrovirus vector to construct dnTGF-βRII-expressing human antigen-specific cytotoxic T lymphocytes (CTLs) and found that TGF-β-resistant CTLs had a functional advantage over unmodified CTLs in the TGF-β-secreting lymphoma [31]. The clinical trial (NCT00368082) showed that TGF-β-resistant CTLs could safely expand and persist in patients with Hodgkin lymphoma without lymphodepleting chemotherapy and induced complete responses [58]. It was testified that adding dnTGF-βRII to PSMA-targeted human CAR T cells promoted T-cell proliferation and augmented prostate cancer eradication [59]. Chang et al. recently described a novel TGF-β CAR containing a scFv based on the sequences of TGF-β-neutralizing antibodies, demonstrating the ability to not only inhibit endogenous TGF-β signaling but also convert TGF-β into a stimulant of T-cell growth [60]. Above results support the potential value of the countermeasure of using CRISPR/Cas9 technology to generate TGF-β-resistant CAR T or UCART cells to improve potency of engineering T cells in solid tumors.

Exploration of safer and more controllable novel CAR T cells

Albeit unprecedented efficacy of CAR T-cell therapy, it is accompanied by serious and even life-threatening toxicities, including CRS, on-target/off-tumor toxicity, neurotoxicity, macrophage activation syndrome/ hemophagocytic lymphohistiocytosis and tumor lysis syndrome, which need to be paid more attention [61]. The most significant and common toxicity of CAR T-cell therapy is CRS, an inflammatory syndrome caused by multiple cytokines, including interferon γ, interleukin (IL)-1, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α and granulocyte/macrophage colony-stimulating factor (GM-CSF), produced by the CAR T cells themselves and by other cells [62]. Tocilizumab, IL-6 receptor blockade, was approved by FDA for treatment of CAR T cell-induced severe or life-threatening CRS [63]. Thus, blocking relevant cytokines signaling is a hopeful strategy to ameliorate the dilemma and CRISPR/Cas9 can effectively knock-out related molecules. Sterner et al. [64] performed a study of multiple tumors to investigate whether CTIs can knock out multiple genes simultaneously.
described CRISPR/Cas9 mediated knock-out of GM-CSF and showed that GM-CSF-negative CAR T cells produced less GM-CSF without weakening antitumor activity in vivo compared to wild-type CAR T cells. Single or combined knock-out of other critical relevant cytokines in CAR T cells using CRISPR/Cas9 are needed to be further explored. Long-lasting B cell aplasia is a classical on-target/off-tumor toxicity of anti-CD19 and anti-CD20 CAR T-cell therapy [65, 66]. The insert of safety switches gene into CAR vector is a feasible method to terminate the effects without jeopardizing clinical responses. Diaconu et al. [67] demonstrated that the iC9 safety switch eliminated CD19-specific CAR T cells in a dose-dependent manner in a humanized mouse model, allowing either a selective containment of CAR T expansion in case of CRS or complete deletion on demand granting normal B-cell reconstitution.

There are two reported cases indicating the risks of unexpected situations in the manufacture of CAR T cells and potential carcinogenicity in vivo. One CD19-negative relapsed patient after CD19-targeted CAR T cell therapy was found that the CAR gene was unintentionally introduced into a single dominantly-proliferative leukemic B cell and its product bound in cis to the CD19 epitope on the surface of leukemic cells, masking it from recognition by CAR T cells [36]. Another case was a 78-year-old man with advanced relapsed/refractory chronic lymphocytic leukemia who obtained CR after the second infusion. Unexpectedly, 94% of CAR T cells at the site of the response originated from a single clone in which lentiviral vector-mediated insertion of the CAR transgene disrupted the methylcytosine dioxygenase TET2 gene [34]. Therefore, there is a need to incorporate inducible safe switches or suicide genes into the CAR T cells, which can provide a means to eliminate the CAR T cells in case of unexpected toxicities. Hoyos et al. [68] generated a novel anti-CD19 CAR construct that incorporates the IL-15 gene and an inducible caspase-9(iC9)-based suicide gene and >95% of transgenic cells could be efficiently eliminated within 24 h upon pharmacologic activation of the suicide gene [68]. Adding inducible safe switches or suicide genes to generate more controllable and safer CAR T cells will be widespread-used by multiplexed CRISPR/Cas9 technology. There are three ongoing clinical trials (NCT02107963, NCT01822652 and NCT02439788) incorporating the iC9 construct into CAR T-cell products to provide a method to eliminate autologous CAR T cells in case of potential off-target toxicity.

Other applications

CAR T-cells therapy has an obvious barrier in acute myeloid leukemia (AML) because myeloid-directed immunotherapy will eradicate normal as well as malignant cells, leading to bone marrow failure, as has been shown in several preclinical studies of CD33 or CD123 directed CAR T cell therapy [69, 70]. Several groups developed a novel approach to circumvent the problem with potent anti-CD33 CAR T cells followed by infusions of CRISPR/Cas9-modified CD33-knockout normal hematopoietic stem cells (HSCs), thus allowing persistent antigen-specific cytokotoxicity along with reconstitution of effective hematopoiesis [35,71,72]. Extending the success of CAR T cells to T-cell malignancies is also problematic because most target antigens are expressed on both normal and malignant cells, resulting in CAR T-cell fratricide. CD7 is a transmembrane protein highly expressed in T-cell acute leukemia (T-ALL) and largely confined to T cells and natural killer cells. Studies showed that CD7-specific CAR T cell impaired expansion due to self-killing of the CAR T cells. Diogo et al. [73] explored that targeted disruption of the CD7 gene using CRISPR/Cas9 prior to CAR expression minimized fratricide in T cells and allowed the expansion of the CD7-knock-out anti-CD7 CAR T cells with robust antitumor activity for preclinical and potential clinical application. Hence, the CRISPR/Cas9 system can be applied to disrupt the targeted antigens to avoid self-killing of the CAR T cells and broaden the therapeutic index.

Conclusion and outlooks

The unprecedented responses of CAR T cells in advanced malignancies promote the rapid growth of the therapeutic approach and the development of the smarter and commercialized CAR T cells is an inevitable mainstream trend, such as a split, universal and programmable CAR system to prevent relapse, mitigate overactivation and enhance specificity [74]. CRISPR/Cas9 genomic editing technology holds promising explorations and applications to create the next-generation CAR T-cell products, including universal CAR T cells by disrupting endogenous TCR and HLA, more potent CAR T cells by ablating inhibitory modulators, more controllable CAR T cells by adding inducible safe switches or suicide genes and novel CAR T cells by knock-out of the targeted antigens to avoid self-killing.

However, the gene-editing specificity and efficiency of CRISPR/Cas9 technology are of significant importance in therapeutic application. The first concern of CRISPR/Cas9 gene editing is off-target effects, which introduce random mutations, hence activating oncogenes or impacting tumor-suppressor genes to unintentional deleterious consequences [75]. Multiple strategies, such as careful selection of the target site, optimized sgRNA design and Cas9 activity, prior off-target detection assays, have been attempted to minimize the safe risks of off-target effects [76–78]. Attempts to increase HR frequencies using HR enhancers or NHEJ inhibitors are currently ongoing and may further promote precise gene engineering [79, 80]. Another challenge for therapeutic gene editing is efficient and nontoxic delivery into CAR T cells. There are three main methods to deliver CRISPR/Cas9 system, including a DNA plasmid-based system, an all-RNA-based system and a Cas9 ribonucleoprotein complex as delivery [81]. Viral vectors with high efficiency and potential hazards, such as mutagenesis, immunogenicity and off-target effects, are widely applied for donor DNA delivery and electroporation has emerged as new method to deliver CRISPR/Cas9 elements with safety, simplicity and flexibility [82]. Viral and non-viral vectors have specific merits and beneficial combinations of different delivery means are being explored to ensure efficiency and safety [33]. As technical progresses to reduce off-target effects and improve delivery efficiency, CRISPR/Cas9 technology provides an extraordinary potential to construct novel CAR T cells and streamlines the burgeoning realm of immunotherapy.

Key Points

- CAR T-cell therapy has shown promising responses in both hematologic and solid cancers. However, there are some limitations awaiting for solutions, such as insufficient quantity and poor quality of autologous T cells, CAR T cell exhaustion and tumor suppressive microenvironments, potential self-killing and uncontrollable proliferation.
Genomic editing technologies, especially CRISPR/Cas9 with flexibility, simplicity, high efficiency and multiplexing open a window to develop next-generation CAR T cells. CRISPR/Cas9 genomic editing technology holds promising explorations and applications to create next-generation CAR T cell products, including universal CAR T cells by disrupting endogenous TCR or HLA, more potent CAR T cells by ablating of inhibitory modulators and more controllable CAR T cells by adding inducible safe switches or suicide genes. CRISPR/Cas9 technology is unveiling a new era for CAR T cell therapy.

Conflict of interest
The authors declare that they have no conflict of interests.

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References
1. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. N Engl J Med 2017;377(26):2531–44.
2. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N Engl J Med 2018;378(5):439–48.
3. Schuster SJ, Bishop MR, Tam CS, et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. N Engl J Med 2019;380(1):45–56.
4. Guedan S, Calderon H, Posey AD, Jr, et al. Engineering and design of chimeric antigen receptors. Mol Ther Methods Clin Dev 2019;12:145–56.
5. Kulemzin SV, Kuznetsova VV, Mamonkin M, et al. Engineering chimeric antigen receptors. Acta Nat 2017;9(1):6–14.
6. Fry TJ, Shah NN, Orentas RJ, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. Nat Med 2018;24(1):20.
7. Adaniya SPS, Cohen AD, Garfall AL. Chimeric antigen receptor T cell immunotherapy for multiple myeloma: a review of current data and potential clinical applications. Am J Hematol 2019;94:528–33.
8. Raje N, Berdeja J, Lin Y, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. N Engl J Med 2019;380(18):1726–37.
9. Sadelain M, Brentjens R, Riviere I. The promise and potential pitfalls of chimeric antigen receptors. Curr Opin Immunol 2009;21(2):215–23.
10. Brocker T, Karjalainen K. Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes. J Exp Med 1995;181(5):1653–9.
11. Kershaw MH, Westwood JA, Parker LL, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res 2006;12(20):6106–15.
12. Till BG, Jensen MC, Wang JJ, et al. Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. Blood 2008;112(6):2261–71.
13. Salters AI, Ivey RG, Kennedy JJ, et al. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. Sci Signal 2018;11(544). doi: 10.1126/scisignal.aat5753.
14. Till BG, Jensen MC, Wang JJ, et al. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. Blood 2012;119(17):3940–50.
15. Ramello MC, Benzaid I, Kuenzi BM, et al. An immunoproteomic approach to characterize the CAR interactome and signalosome. Sci Signal 2019;12(568). doi: 10.1126/scisignal.aag9777.
16. Chmielewski M, Abken H. TRUCKs: the fourth generation of CARs. Expert Opin Biol Ther 2015;15(8):1145–54.
17. Moon EK, Carpenito C, Sun J, et al. Expression of a functional CCR2 receptor enhances tumor localization and functional eradication by retargeted human T cells expressing a mesothelin-specific chimeric antibody receptor. Clin Cancer Res 2011;17(14):4719–30.
18. Guedan S, Calderon H, Posey AD, Jr, et al. Engineering and design of chimeric antigen receptors. Mol Therapy-Methods Clin Dev 2019;12:145–56.
19. Urmov FD, Rebar EJ, Holmes MC, et al. Genome editing with engineered zinc finger nucleases. Nat Rev Genet 2010;11(9):636–46.
20. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating, and targeting genomes. Nat Biotechnol 2014;32(4):347–55.
21. Miller JC, Tan S, Qiao G, et al. A TALE nuclease architecture for efficient genome editing. Nat Biotechnol 2011;29(2):143–8.
22. Carroll D. Progress and prospects: zinc-finger nucleases as gene therapy agents. Gene Ther 2008;15(22):1463–8.
23. Moscou MJ, Bogdanove AJA. Simple cipher governs DNA strand cleavage. Science 2009;326(5959):1501–1.
24. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339(6121):819–23.
25. Haurwitz RE, Jinek M, Wiedenheft B. CRISPR-Cas systems: the fourth generation of genome editing technologies. Nature 2013;493(7422):58–63.
26. Mali P, Yang LH, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. Science 2013;339(6121):823–6.
27. Ehrke-Schulz E, Schiwon M, Hagedorn C, et al. PAM-guided homing of CRISPR-Cas9 systems for gene editing. Cell 2013;154(5):1139–51.
28. Zhang JP, Li XL, Li GH, et al. Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage. Genome Biol 2017;18(1):35.
29. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, et al. CRISPR-Cas9-mediated gene tagging. Nat Methods 2018;15(2):165–76.
30. Raje N, Berdeja J, Lin Y, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. N Engl J Med 2019;380(18):1726–37.
31. Adaniya SPS, Cohen AD, Garfall AL. Chimeric antigen receptor T cell immunotherapy for multiple myeloma: a review of current data and potential clinical applications. Am J Hematol 2019;94:528–33.
32. Raje N, Berdeja J, Lin Y, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. N Engl J Med 2019;380(18):1726–37.
33. Adaniya SPS, Cohen AD, Garfall AL. Chimeric antigen receptor T cell immunotherapy for multiple myeloma: a review of current data and potential clinical applications. Am J Hematol 2019;94:528–33.
34. Raje N, Berdeja J, Lin Y, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. N Engl J Med 2019;380(18):1726–37.
35. Adaniya SPS, Cohen AD, Garfall AL. Chimeric antigen receptor T cell immunotherapy for multiple myeloma: a review of current data and potential clinical applications. Am J Hematol 2019;94:528–33.
36. Raje N, Berdeja J, Lin Y, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. N Engl J Med 2019;380(18):1726–37.
37. Adaniya SPS, Cohen AD, Garfall AL. Chimeric antigen receptor T cell immunotherapy for multiple myeloma: a review of current data and potential clinical applications. Am J Hematol 2019;94:528–33.
38. Raje N, Berdeja J, Lin Y, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. N Engl J Med 2019;380(18):1726–37.
39. Adaniya SPS, Cohen AD, Garfall AL. Chimeric antigen receptor T cell immunotherapy for multiple myeloma: a review of current data and potential clinical applications. Am J Hematol 2019;94:528–33.
33. Eyquem J, Mansilla-Soto J, Giavridis T, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 2017;543(7643):113–7.

34. Fraietta JA, Nobles CL, Sammons MA, et al. Disruption of TET2 promotes the therapeutic efficacy of CD19-targeted T cells. Nature 2018;558(7709):307–12.

35. Kim MY, Kenderian SS, Schreeder D, et al. Genome editing using CRISPR-Cas9 to increase the therapeutic index of antigen-specific immunotherapy in acute myeloid Leukemia. Mol Ther 2016;24(1):S108–8.

36. Ruella M, Xu J, Barrett DM, et al. Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. Nat Med 2018;24(10):1499–503.

37. Torikai H, Reik A, Liu PQ, et al. A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. Blood 2012;119(24):5697–705.

38. Berdien B, Mock U, Atanackovic D, et al. Multiplex genome editing to enable generation of human pluripotent stem cells. Mol Ther 2015;23(6):1128–39.

39. Morgan NV, Goddard S, Cardno TS, et al. Mutation in the TCRalpha subunit constant gene (TRAC) leads to lack of TCRalphaβ+ T cells. J Clin Invest 2011;121(2):695–702.

40. Poiriot L, Philip B, Schiffer-Manniou C, et al. Multiplex genome-edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies. Cancer Res 2015;75(18):3853–64.

41. Qasim W, Zhan H, Samarasinghe S, et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci Transl Med 2017;9(374). doi: 10.1126/scitranslmed.aaj2013.

42. Qasim W, Amrolia PJ, Samarasinghe S, et al. First clinical application of Talen engineered universal CAR19 T cells in B-ALL. Blood 2015;126(23). doi: 10.1182/blood.V126.23.2046.2046.

43. Benjamin R, Graham C, Vallo D, et al. Preliminary data on safety, cellular kinetics and anti-leukemic activity of UCART19, an allogeneic anti-CD19 CAR T-cell product, in a pool of adult and Pediatric patients with high-risk CD19-relapsed/refractory B-cell acute lymphoblastic leukaemia. Blood 2018;132. doi: 10.1182/blood-2018-99-111356.

44. Torikai H, Reik A, Soldner F, et al. Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. Blood 2013;122(8):1341–9.

45. Riolobos L, Hirata RK, Turtle CJ, et al. HLA engineering of human pluripotent stem cells. Mol Ther 2013;21(6):1232–41.

46. Ren J, Zhang X, Liu X, et al. A versatile system for rapid multiplex genome-edited CAR T cell generation. OncoTarget 2017;8(10):17002–11.

47. Ren JT, Liu XJ, Fang CY, et al. Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. Clin Cancer Res 2017;23(9):2255–66.

48. Gornalussen GG, Hirata RK, Funk SE, et al. HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. Nat Biotechnol 2017;35(8):765–72.

49. Ritchie DS, Neeson PJ, Khot A, et al. Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia. Mol Ther 2013;21(11):2122–9.

50. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol 2015;15(8):486–99.

51. Hoos A. Development of immuno-oncology drugs - from CTLA4 to PD1 to the next generations. Nat Rev Drug Discov 2016;15(4):235–47.

52. Hodi FS, O’Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363(8):711–23.

53. Waring P, Mullbacher A. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol Cell Biol 1999;77(4):312–7.

54. Kunkele A, Johnson AJ, Rolczynski LS, et al. Functional tuning of CARs reveals Signaling threshold above which CD8(+) CTL antitumor potency is attenuated due to cell Fas-Fasl-dependent AICD. Cancer Immunol Res 2015;3(4):368–79.

55. Pickup M, Novitskiy S, Moses HL. The roles of TGFbeta in the tumour microenvironment. Nat Rev Cancer 2013;13(11):788–99.

56. Liu VC, Wong LY, Jang T, et al. Tumor evasion of the immune system by converting CD4(+)/CD25(−) T cells into CD4(+) CD25(+) T regulatory cells: role of tumor-derived TGF-beta. J Immunol 2007;178(5):2883–92.

57. Ebner R, Chen RH, Shum L, et al. Cloning of a type I TGF-beta receptor and its effect on TGF-beta binding to the type II receptor. Science 1993;260(5112):1344–8.

58. Bollard CM, Tripic T, Cruz CR, et al. Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed Hodgkin lymphoma. J Clin Oncol 2018;36(11):1128–39.

59. Kloss CC, Lee J, Zhang A, et al. Dominant-negative TGF-beta receptor enhances PSMA-targeted human CAR T cell proliferation and augments prostate cancer eradication. Mol Ther 2018;26(7):1855–66.

60. Chang ZL, Lorenzini MH, Chen X, et al. Rewiring T-cell responses to soluble factors with chimeric antigen receptors. Nat Chem Biol 2018;14(3):317–24.

61. Wang Z, Guo Y, Han W. Current status and perspectives of chimeric antigen receptor modified T cells for cancer treatment. Protein Cell 2017;8(12):896–925.

62. Brudno JN, Kochenderfer JN. Recent advances in CAR T-cell toxicity: mechanisms, manifestations and management. Blood Rev 2019;34:45–55.

63. Le RQ, Li L, Yuan WS, et al. FDA approval summary: Tocilizumab for treatment of chimeric antigen receptor T cell induced severe or life-threatening cytokine release syndrome. Oncologist 2018;23(8):943–7.

64. Sterner RM, Cox MJ, Sakemura R, et al. Using CRISPR/Cas9 to knock out GM-CSF in CAR-T cells. Jove-J Visual Exp 2019;149(1). doi: 10.3791/59629.

65. Zhang WY, Wang Y, Guo YL, et al. Treatment of CD20-directed chimeric antigen receptor-modified T cells in patients with relapsed or refractory B cell non-Hodgkin lymphoma: an early phase IIa trial report. Signal Transduct Target Ther 2016;1.

66. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 2014;371(16):1507–17.

67. Diaconu I, Ballard B, Zhang M, et al. Inducible Caspase-9 selectively modulates the toxicities of CD19-specific chimeric antigen receptor-modified T cells. Mol Ther 2017;25(3):580–92.

68. Hoyos V, Savoldo B, Quintarelli C, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide
gene to enhance their anti-lymphoma/leukemia effects and safety. Leukemia 2010;24(6):1160–70.

69. Pizzitola I, Anjos-Afonso F, Rouault-Pierre K, et al. Chimeric antigen receptors against CD33/CD123 antigens efficiently target primary acute myeloid leukemia cells in vivo. Leukemia 2014;28(8):1596–605.

70. Mardiros A, Dos Santos C, McDonald T, et al. T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. Blood 2013;122(18):3138–48.

71. Kim MY, Yu KR, Kenderian SS, et al. Genetic inactivation of CD33 in hematopoietic stem cells to enable CAR T cell immunotherapy for acute myeloid Leukemia. Cell 2018;173(6):1439.

72. Borot F, Wang H, Ma Y, et al. Gene-edited stem cells enable CD33-directed immune therapy for myeloid malignancies. Proc Natl Acad Sci U S A 2019;116(24):11978–87.

73. Gomes-Silva D, Srinivasan M, Sharma S, et al. CD7-edited T cells expressing a CD7-specific CAR for the therapy of T-cell malignancies. Blood 2017;130(3):285–96.

74. Cho JH, Collins JJ, Wong WW. Universal chimeric antigen receptors for multiplexed and logical control of T cell responses. Cell 2018;173(6):1426–38 e1411.

75. Peng R, Lin G, Li J. Potential pitfalls of CRISPR/Cas9-mediated genome editing. FEBS J 2016;283(7):1218–31.

76. Liu J, Zhou G, Zhang L, et al. Building potent chimeric antigen receptor T cells with CRISPR genome editing. Front Immunol 2019;10:456.

77. Jiang J, Zhang L, Zhou X, et al. Induction of site-specific chromosomal translocations in embryonic stem cells by CRISPR/Cas9. Sci Rep 2016;6:

78. Torres R, Martin MC, Garcia A, et al. Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. Nat Commun 2014;5:3964.

79. Maruyama T, Dougan SK, Truttmann MC, et al. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol 2015;33(5):538–42.

80. Riesenber S, Maricic T. Targeting repair pathways with small molecules increases precise genome editing in pluripotent stem cells. Nat Commun 2018;9.

81. Jensen TI, Axelgaard E, Bak RO. Therapeutic gene editing in haematological disorders with CRISPR/Cas9. Br J Haematol 2019;185(5):821–35.

82. Xu XJ, Wan T, Xin HH, et al. Delivery of CRISPR/Cas9 for therapeutic genome editing. J Gene Med 2019;21(7). doi: 10.1002/jgm.3107.