**Introduction**

Despite the variety of traditional methods available to treat cancer, such as surgical resection, chemotherapy, and radiation therapy, immunotherapy has emerged as an attractive alternative for cancer patients. Adoptive cell transfer (ACT) is a rapidly emerging immunotherapy approach, and a subcategory, genetically engineered T cells, which includes chimeric antigen receptor (CAR) T cells and T cell receptor (TCR)-modified T cells, has shown promising clinical benefits in treating malignant tumors [1]. CAR-T cells are modified with a chimeric receptor molecule, composed of an extracellular antigen-binding domain, a hinge, a transmembrane domain, and intracellular domain(s), to recognize antigens on cell surfaces independent of the major histocompatibility complex (MHC) [2]. The extracellular domain of most CARs is derived from the single-chain variable fragment (scFv) of antibodies to target tumor-specific antigens. After withdrawing blood from patients, T cells are genetically engineered with CARs and infused back into patients after ex vivo expansion. CAR-T cell therapy is highly effective in relapsed or refractory (R/R) B cell hematological malignancies, especially in targeting CD19/CD20 expressing B cell leukemia and lymphoma. However, the efficacy of CAR-T cells seen in blood malignancies does not carry over to solid tumors, partly due to the lack of specific surface tumor antigens and inefficient trafficking and infiltration [2].

In contrast to CAR-T cell therapy, TCR-T cell therapy has made greater progress in treating solid tumors. TCRs recognize and interact with the peptide-MHC complex (pMHC), where peptides are cleaved from intracellular antigens and presented by MHC molecules [3]. A T-cell receptor is composed of an α chain and a β chain to form a heterodimer, and each consists of a constant region to anchor the chain inside the T cell membrane and a variable region to recognize the MHC-presented antigen. Since the majority of protein antigens are only expressed intracellularly [4], thousands of pMHC molecules are broadly available to be targeted as antigens for TCR-T cells compared to CAR-T cells. Furthermore, TCR-T cell therapy is found to be associated with a lower risk of cytokine release syndrome (CRS) and neurotoxicity than CAR-T cell therapy, due to a lower amount of cytokine secretion [5].
TCR-T cell therapy is particularly more efficacious than CAR-T cell therapy in treating solid tumors, possibly due to its ability to trigger a T cell response despite a relatively low copy number of peptide antigens present on the surface of tumors.

However, both CAR-T and TCR-T cell therapy have limitations for their applications in clinics. They have difficulties in eradicating metastatic tumors due to the immunosuppressive microenvironment of tumors, which leads to the vulnerability and low persistence of CAR-T and TCR-T cells [6]. The expression of inhibitory checkpoint molecules is often increased in tumor cells, such as programmed death-ligand 1 (PD-L1), which inhibits cell proliferation and cytokine secretion of CAR-T or TCR-T cells. In addition, constitutive expression of CAR or TCR in T cells mainly depends on the delivery by gamma retroviral or lentiviral vectors. The random integration mediated by retroviral or lentiviral vectors remains a safety issue, as it may result in transcriptional silencing, potential oncogenesis, and variated transgene expression [7].

Applications of Precise Gene Knock-in in Engineered T cell Therapies

There are three potential purposes for the precise integration of exogenous DNA in a specific locus: Silencing the target gene, correcting a mutated functional gene, and introducing an exogenous gene. Using Cas9 RNP and an exogenous single-stranded DNA template, Schumann et al. successfully replaced 12 nucleotides in CXCR4 and PDCD1 with a HindIII restriction enzyme cleavage site, which disrupted CXCR4 and PDCD1 genes by homology directed repair (HDR)-mediated genetic knock-in [11]. The optimization of the CRISPR-Cas9 genome-targeting system enables the precise knock-in of large DNA fragments in the genomes of the human primary T cell, thus allowing for more therapeutic gene modifications. IL2RA deficiency is an immunodeficiency disorder associated with mutations in the interleukin 2 receptor alpha gene of T cells [12]. Two heterozygous mutations in IL2RA were previously characterized. One mutation is c.530A>G, which creates a premature stop codon. The other mutation is c.800delA, which disrupts CXCR4 and PDCD1 genes by homology directed repair (HDR)-mediated genetic knock-in [11].

Specific insertion of exogenous CARs or TCRs into the T cell genome not only minimizes the risks caused by random integration, but also provides additional benefits by transcriptional silencing of the targeted gene locus. During preparation of “off-the-shelf” universal CAR-T cells [14], the endogenous TCR is disrupted by knocking out the TCR α or β constant gene (TRAC or TRBC) to eliminate the potential reaction of the engrafted T cells with allogeneic antigens, which may elicit graft-versus-host diseases. Daniel T. MacLeod first reported the gene-editing approach to insert the anti-CD19 CAR gene while simultaneously disrupting the endogenous TRAC gene [15]. The process was based on the I-CreI homing endonuclease and AAV6-delivering HDR template. Using the more widely used gene-editing tool, CRISPR/Cas9, Justin further demonstrated that targeting the coding sequence of CAR to the TRAC locus, and putting it under the control of endogenous transcriptional regulatory elements, enhances the anti-tumor ability of engineered T cells by preventing accelerated T cell differentiation and exhaustion [16]. In addition to targeting the endogenous TCR locus, inhibitory immune checkpoint molecules like PDCD1 are also suitable candidates for gene insertion. Dai et al. successfully integrated the genes of anti-CD19 and anti-CD22 CAR into TRAC and PDCD1 locus, and generated CD19 and CD22 bispecific CAR-T cells at a bulk efficiency of 21.7%. The bi-specific CAR showed superior potency in killing the leukemia cell line NALM 6 (CD19- CD22+) [17].

Delivery Strategies for Precise Gene Insertion in Human T cells

To achieve the specific insertion of exogenous genes into the genome of T cells, the DNA endonuclease and HDR templates should be co-delivered into cells by either viral or non-viral systems. The delivery strategies need to meet several criteria, including high delivery and expression efficiency, low toxicity, low immunogenicity, and limited off-target effects [18]. We briefly reviewed several current strategies for gene knock-in in T cells, and discussed other prospective tools that could be applied in the future.

Combination of AAV-delivered HDR template and electroporated endonuclease

AAV-based delivery methods are widely applied in gene therapy because of its high infection efficiency, long-lasting transgene expression, mild immunogenicity, and general safety [19]. However, due to its limited packaging range (approximately 4.7 kb), it is difficult to load larger Cas9 variants [20]. To maximize the transduction
efficiency, AAV are used for delivery of HDR templates containing a gene expression cassette like CAR or TCR, while CRISPR endonuclease is usually electroporated in the form of mRNA or ribonucleoprotein (RNP). Justin Eyquem performed T-cell electroporation of Cas9 mRNA and gRNA followed by recombinant AAV6 infection, and successfully directed anti-CD19 CAR to the TRAC locus. The knock-in efficiency of anti CD19 CAR exceeded 40% [16]. Xiaoyun Dai developed a platform for specific targeting of multiple loci in human primary T cells based on Cpf1, which exhibits higher potential in HDR-mediated precise gene editing. In this platform, Cpf1 crRNA array and HDR template flanked with homologous arms were constructed into one AAV plasmid backbone, and Cpf1 mRNA was electroporated into T cells 2-4 hours before the AAV infection. This “AAV–Cpf1 KIKO” platform allows for multiple precise gene insertions into different loci in one step [17].

Co-delivery of the endonuclease and HDR template by electroporation

Despite the high delivery efficiency of AAV, the manufacturing and quality control of viruses can be complicated and time-consuming. With the development and optimization of electroporating tools, it is feasible to co-electroporate human T cells with Cas9 RNP and long linear dsDNA HDR template with minor toxicity effects, which simplifies the process of T cell engineering. Roth et al. developed the co-electroporation strategy and replaced the endogenous TCR by integrating an approximately 1.5 kbp DNA cassette into the first exon of the TCR-α constant region. The DNA cassette contained a TCR-β and TCR-α pair (1G4) that recognized the NY-ESO-1 tumor antigen, and was expressed efficiently on the T cell surface [13].

Perspective delivery strategies for precise gene-editing system

In addition to the current delivery approaches using AAV and electroporation, emerging technologies like nanocarriers and Microfluidics-based CellSqueeze also exhibit great potential in transporting gene-editing components into T cells for precise gene integration. A novel vehicle named CRISPR-Gold was developed to deliver donor DNA and Cas9 RNP, and induced HDR in vivo [21]. To generate the CRISPR-Gold, HDR template, Cas9 RNP, and the endosomal disruptive polymer poly(N-(N-(2-aminoethyl)2-aminoethyl) aspartamide) (PAsp(DET)) were mixed to form a DNA complex and then conjugated to gold nanoparticles (GNPs). Due to the cationic nature of PAsp(DET), when complexed with CRISPR components, CRISPR-Gold can be internalized by cells via endocytosis. Moreover, local administration of CRISPR-Gold equipped with Cas9 RNP and HDR template efficiently corrected the DNA mutation that causes Duchenne muscular dystrophy in mice. The CellSqueeze technology utilizes a microfluidic approach to deliver cargos, and cells are mechanically deformed as they pass through a constriction 30–80% smaller than the cell diameter. This technology has been demonstrated to deliver a wide range of materials into various cell types such as immune cells and embryonic stem cells [22-24].

Conclusions

Genome editing strategies hold great promise in producing the next-generation of T-cell products. Compared to gene knockout strategies, gene knock-in technology could delete endogenous TCR or inhibitory molecules and express CAR or exogeneous TCR in one single step. CARs or TCRs can be precisely integrated into a specific gene locus through homologous recombination to avoid random integration and subsequently reduce the off-target effects that is seen when lentiviral vectors are utilized. Moreover, integration of CAR or TCR into endogenous TCR loci enables the expression of the genes to be driven naturally instead of artificially overexpressed. This could protect T cells from secreting abundant harmful cytokines to avoid CRS, and prevent them from being exhausted to remain more persistent in vivo. In addition, the frequency of homologous recombination (HR) could be further promoted by using HR enhancers or NHEJ inhibitors. Viral and non-viral delivery methods could be combined to ensure better efficacy and safety. With such genetic modifications for functionality improvement, CAR-T and TCR-T cell therapies will be more effective in fighting tumors.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

Gao Q. and Ding R. wrote the manuscript, and Chao CC. and Gao Q. revised the manuscript.

Acknowledgments

We sincerely thank the support provided by China National GeneBank and this research was supported by the Guangdong Enterprise Key Laboratory of Human Disease Genomics (2020B1212070028).

References

1. Zhao L, Cao YJ. Engineered T cell therapy for cancer in the clinic. Frontiers in Immunology. 2019;10:2250.
2. Zhang BL, Qin DY, Mo ZM, Li Y, Wei W, Wang YS, et al. Hurdles of CAR-T cell-based cancer immunotherapy
directed against solid tumors. Science China Life Sciences. 2016 Apr 1;59(4):340-8.

3. Hewitt EW. The MHC class I antigen presentation pathway: strategies for viral immune evasion. Immunology. 2003 Oct;110(2):163-9.

4. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. Science. 2015 Jan 23;347(6220):1260419.

5. Harris DT, Hager MV, Smith SN, Cai Q, Stone JD, Kruger P, et al. Comparison of T cell activities mediated by human TCRs and CARs that use the same recognition domains. The Journal of Immunology. 2018 Feb 1;200(3):1088-100.

6. Huang R, Li X, He Y, Zhu W, Gao L, Liu Y, et al. Recent advances in CAR-T cell engineering. Journal of Hematology & Oncology. 2020 Dec;13(1):1-9.

7. Ellis J. Silencing and variegation of gammaretrovirus and lentivirus vectors. Human Gene Therapy. 2005 Nov 1;16(11):1241-6.

8. Gao Q, Dong X, Xu Q, Zhu L, Wang F, Hou Y, et al. Therapeutic potential of CRISPR/Cas9 gene editing in engineered T-cell therapy. Cancer Medicine. 2019 Aug;8(9):4254-64.

9. Zhan T, Rindtorff N, Betge J, Ebert MP, Boutros M. CRISPR/Cas9 for cancer research and therapy. InSeminars in Cancer Biology 2019 Apr 1;55:106-119.

10. Li C, Mei H, Hu Y. Applications and explorations of CRISPR/Cas9 in CAR T-cell therapy. Briefings in Functional Genomics. 2020 May;19(3):175-82.

11. Schumann K, Lin S, Boyer E, Simeonov DR, Subramaniam M, Gate RE, et al. Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. Proceedings of the National Academy of Sciences. 2015 Aug 18;112(33):10437-42.

12. Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the α chain of the interleukin-2 receptor. Proceedings of the National Academy of Sciences. 1997 Apr 1;94(7):3168-71.

13. Roth TL, Puig-Saus C, Yu R, Shifrut E, Carnevale J, Li P, et al. Reprogramming human T cell function and specificity with non-viral genome targeting. Nature. 2018 Jul;559(7741):405-9.

14. Depil S, Duchateau P, Grupp SA, Mufti G, Poirot L. Off-the-shelf allogeneic CAR T cells: development and challenges. Nature Reviews Drug Discovery. 2020 Jan 3;1-5.

15. MacLeod DT, Antony J, Martin AJ, Moser RJ, Hekele A, Wetzel KJ, et al. Integration of a CD19 CAR into the TCR alpha chain locus streamlines production of allogeneic gene-edited CAR T cells. Molecular Therapy. 2017 Apr 5;25(4):949-61.

16. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJ, Hamieh M, Cunanan KM, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017 Mar;543(7643):113-7.

17. Dai X, Park JJ, Du Y, Kim HR, Wang G, Errami Y, et al. One-step generation of modular CAR-T cells with AAV–Cpf1. Nature Methods. 2019 Mar;16(3):247-54.

18. Lino CA, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. Drug Delivery. 2018 Jan 1;25(1):1234-57.

19. MingoZZi F, High KA. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. Nature Reviews Genetics. 2011 May;12(5):341-55.

20. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014 Jun 5;157(6):1262-78.

21. Lee K, Conboy M, Park HM, Jiang F, Kim HJ, Dewitt MA, et al. Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nature Biomedical Engineering. 2017 Nov;1(11):889-901.

22. Adamo A, Sharei A, Adamo L, Lee B, Mao S, Jensen KF. Microfluidics-based assessment of cell deformability. Analytical Chemistry. 2012 Aug 7;84(15):6438-43.

23. Sharei A, Zoldan J, Adamo A, Sim WY, Cho N, Jackson E, et al. A vector-free microfluidic platform for intracellular delivery. Proceedings of the National Academy of Sciences. 2013 Feb 5;110(6):2082-7.

24. Sharei A, PoecevicuTe R, Jackson EL, Cho N, Mao S, Hartoularos GC, et al. Plasma membrane recovery kinetics of a microfluidic intracellular delivery platform. Integrative Biology. 2014 Apr 1;6(4):470-5.