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Adeno-Associated Viral Vectors for Homology-Directed Generation of CAR-T Cells

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Immunotherapy with T cells expressing chimeric antigen receptors (CAR) is an emerging and promising treatment against refractory cancers. However, the currently adopted methods of modification of T cells pose a risk of insertional oncogenesis because lentiviral and retroviral vectors integrate the CAR transgene in a semi-random fashion. In addition, this therapy is only available using autologous cells, which create problems in production and limit the access for patients who have their T cells depleted. One modification method that shows the ability to overcome both drawbacks is the knock-in of the CAR simultaneously knocking-out genes that prevent allogeneic therapy, such as the endogenous T cell receptor. In this mini-review, the authors present recent efforts to develop safer universal CAR-T cells. More specifically, the combined application of target-directed nucleases, which create a double-strand break at a specific genome locus, and the delivery of CAR DNA via adeno-associated viral vectors for subsequent integration via homologous recombination and silencing of the targeted gene is focused on.

1. Introduction

Chimeric antigen receptor (CAR) T cell therapy is an emerging cancer treatment. In this therapy, a patient's T cells are collected through leukapheresis and transduced using viral vectors to express a CAR.[1,2] This CAR is a fusion protein of a single-chain variable fragment from a specific monoclonal antibody that targets a tumor's antigen, and one or more T cell receptor (TCR) intracellular signaling domains.[3] Upon re-infusion of the transformed T cells, the chimeric antigen receptors direct the lymphocytes to attack cancer cells. Anti-CD19 CAR-T cells targeting B-cell neoplasms have been especially promising, inducing remission in recent clinical trials involving children and young adults with both acute[4–7] and chronic lymphoid leukemia.[8,9] Another promising antigen is the B-cell maturation antigen (BCMA), which have been successfully targeted against multiple myeloma.[10–12] In 2017, the Food and Drug Administration approved the first therapy based on anti-CD19 CAR-T cell modified with lentiviral vectors, tisagenlecleucel (Kymriah) (approval letter available at https://www.fda.gov/media/106989/download). This therapy was approved for the treatment of young patients with acute lymphoid leukemia (ALL) and, in 2018, for the treatment of adults with diffuse large B-cell lymphoma (DLBCL) (approval letter available at https://www.fda.gov/media/112803/download). Also in 2017, a second product was approved for the treatment of DLBCL, axicabtagene ciloleucel (Yescarta), which is based on modification with retroviral vectors (approval letter available at https://www.fda.gov/media/108458/download).

However, CAR-T cell therapy still requires significant improvements, as the product is currently highly variable. Several gene transfer platforms have been developed and are available to introduce the CAR transgene into primary T cells. In most of the cases, transgene insertion is performed by lentivirus or γ-retrovirus-mediated transduction. Nevertheless, these vectors integrate into diverse sites of the T cell's genome. The lentivirus favors the integration into active genes, while γ-retrovirus tends to integrate next to regulatory elements.[13,14] This variable integration can result in negative side effects, such as insertional oncogenesis.[15–17] In addition, available therapy is currently autologous. Endogenous TCR present on donor-derived CAR-T cells may identify mismatched human leukocyte antigen I (HLA-I) on the recipient's cells, which could generate graft-versus-host disease.[18,19] Reversely, the patient's endogenous T cells can also recognize allogeneic CAR-T cell's HLA-I, leading to CAR-T cell rejection.[20] However, the need to use autologous T cells creates a 3-week delay in CAR-T cell therapy due to manufacturing, increases the cost of treatment, and denies the therapy for patients whose T cells have been depleted due to cancer or its treatment.[21,22]

Therefore, there have been recent attempts to use vehicles other than retroviruses and lentiviruses and genetic editing techniques to create a more consistent and robust product. This review will outline current efforts to use targeted nuclease-mediated gene editing and adeno-associated viral vectors for the generation of CAR-T cells.

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2. Site-Specific Nucleases

Five classes of nuclease proteins can be used for targeted genome modification by generating a double-strand break (DSB) in

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the DNA: zinc-finger nucleases (ZFNs); transcription activator like effector nucleases (TALENs); homing endonucleases (HEs); meganucleases; and clustered, regularly interspaced, short palindromic repeat (CRISPR) RNA-guided nucleases, such as Cas9. ZFNs are synthetic proteins derived from the restriction enzyme *Fok*I fused to an array of zinc finger DNA-binding domain that specifically binds to DNA. Similarly, TALENs are fusions of the *Fok*I nuclease to the transcription activator-like effector (TALE) that can be created to recognize a specific DNA sequence.[23] Homing endonucleases are proteins that have been found in numerous organisms, including bacteria, yeast, fungi, and algae and are able to bind to sequences of 20 to 30 base pairs, generating DSB.[24] In addition, synthetic, engineered nucleases can be derived from HEs, such as I-*Cre*I,[25] where mutations alter the recognition pattern of the HE. Meganucleases exploit the nuclease activity of HEs by the tethering of the protein to another DNA-binding domain, such as megaTAL, in which the HE is fused to the TALE domain.[26] Instead of relying on DNA recognition by protein-binding domains, the CRISPR system recognition is based on Watson–Crick base pairing. The CRISPR-associated nuclease proteins are directed by a guide RNA (gRNA) to a protospacer-adjacent motif sequence, where the active sites of protein cause a double-strand break 3 bp upstream the motif.[27] This DSB is then repaired through one of the two pathways: either non-homologous end-joining, which can generate insertions or deletions that can disrupt the targeted locus, or homology-directed repair (HDR), where a donor template with homology to the targeted locus is inserted.[28–31] The insertion of a CAR transgene into a defined location of the T cell genome (**Table 1**) may result in a safer and more efficient product by reducing the chances of insertional oncogenesis and allowing for the disruption of specific proteins that may negatively affect the therapeutic function.

3. Adeno-Associated Viruses

Adeno-associated viruses (AAVs) are 5 kb long non-pathogenic replication-defective non-enveloped single-stranded DNA viruses [32] of the family *Parvoviridae*.[33] Their genome contains the genes *Rep*, which is required for DNA replication, and *Cap*, which is required for capsid formation, all flanked by inverted terminal repeats (ITRs),[34] which recombine within transduced cells to form a circular extra genomic episome.[35] As their name suggests, AAVs are dependoviruses for replication.[32] Recombinant AAVs (rAAVs) containing a gene of choice can be produced, independently of adenoviral co-infection, by expressing *Rep*, *Cap,* and adenoviral helper genes in *trans* while inserting the gene of choice between the ITRs.[36] Once inside the cell, the genetic material primarily remains in an episomal conformation. In the event of integration, the vector integrates into safe, non-tumorigenic genomic locations (mitochondrial DNA or a specific locus on chromosome 19, designated AAVS1).[37,38] No case of vector-induced malignancies has been reported in humans following the use of non-integrating vectors like AAVs.[39] Furthermore, AAVs are stable for long-term storage at −80 °C,[40] making them an interesting commercial product.

The single-stranded nature of AAVs makes them an optimal vehicle for the delivery of a DNA template for HDR (reviewed in ref. [41]). This template contains the gene expression cassette, flanked by regions of homology with the locus target. Homology arms of 150 and 500 bp on the 5′ and 3′ ends, respectively, are necessary for efficient targeting.[42] AAVs have been shown to stimulate HDR more effectively than any other plasmid donor systems or viral vectors and, therefore, provides an appropriate platform for the delivery of CAR-coding sequence as a donor template.[43–45] In addition, after a double-strand break, AAV-mediated HDR is enhanced by >100-fold.[46] Several serotypes and more than 100 variants of AAVs have been isolated, with each having a different tropism and ability to transduce different cell types.[47] In the context of gene therapy, this provides flex-

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Table 1. Generation of CAR-T cells through AAV6-mediated HDR.

Nuclease	Delivery method	Target gene	CAR target	HDR rate	Remarks	Reference
megaTAL	mRNA electroporation	*CCR5*	HIV	14%	Cell activation following target antigen stimulation	[53]
			CD19	9%	Engagement and direct killing of CD19+ cells	
megaTAL	mRNA electroporation	*TRAC*	CD19	40%	Functional responses similar to randomly integrated CAR-T cells	[54]
			BCMA	40%		
Homing Endonuclease	Electroporation	*TRAC*	CD19	48.9%	Potent functional responses in vitro and in vivo	[25]
Cas9	RNP electroporation	*TRAC*	CD19	>40%	Delayed differentiation and exhaustion compared to the retrovirus-mediated CAR-T cells	[48]
Cpf1	mRNA electroporation	*TRAC*	CD22	45.5%	Simultaneous *TRAC*/*PDCD1* knockout	[56]
		TRAC and *PDCD1*	CD22 and CD19	21.7%	Robust in vitro functionality.	

ibility and specificity for targeting a range of different tissues. AAV Serotype 6 (AAV6) is considered the most suitable serotype for the transduction of T cells. Wang et al.[45] have identified that AAV6 has a high tropism for T cells, both CD4+ and CD8+, resulting in more than 75% of transduced cells. In addition, AAV6 was evaluated as a homology donor to zinc-finger-mediated DSB in the *CCR5* locus, to yield integration rates of >45% in CD8+ cells and >40% of CD4+ cells.[45]

4. AAV Delivery of CAR

The combination of site-specific nucleases and DNA template delivery via AAV can be used to disrupt endogenous TCR genes and insert the CAR transgene at the same time, generating a universal “off-the-shelf” CAR-T cell[25,48] (**Figure 1**). According to Osborn et al.,[49] the use of megaTAL and CRISPR/Cas9 resulted in the highest levels of TCR disruption, when compared to TALENs. CRISPR/Cas9 and megaTAL also exhibited low levels of toxicity and off-targets.[49] Besides TCR, additional genes can be knocked out by this method, such as the major histocompatibility complex (MHC) genes, whose protein products present the HLA and lead to immunogenic responses from the host.[50,51] Inactivation of MHC class I and MHC class II, through disruption of β2-microglobulin (*B2m*) and class II transactivator (*Ciita*), respectively, and overexpression of CD47 resulted in the loss of immunogenicity in human and mouse induced pluripotent stem cells.[52] The programmed cell death 1 (*PDCD1*) gene and the cytotoxic T-lymphocyte antigen 4 (*Ctla4*) gene are interesting candidates also. A multiplex gene editing protocol knocking out *PDCD1* and *Ctla4* resulted in CAR-T cells resistant to T cell exhaustion and to suppression of activity.[50]

Using a megaTAL targeting the *CCR5* gene and AAV6 as a donor template vector, Sather et al.[53] successfully inserted anti-HIV- and anti-CD19-CAR in 14% and 9% of T cells, respectively. Both *CCR5* gene-edited CAR-T cells showed up-regulation of activation marker CD137 in the presence of their target antigens, and anti-CD19 CAR-T cells were able to engage and specifically kill CD19+ targets.[53] The insertion of a CAR transgene while knocking out the endogenous TCR was described for the first time by Hale et al.,[54] by using a megaTAL targeting the T cell receptor alpha constant (*TRAC*) locus. An average of 40% expression of CAR was achieved, with more than 90% of these CAR+ cells being CD 3-, using either anti-CD19- or anti-BCMA-CAR. No difference regarding the phenotype, activation, or killing response was found in *TRAC*-encoded anti-CD19-CAR-T cells when compared to randomly integrated, lentiviral generated, CAR-T cells.[54] MacLeod et al.[25] used an engineered homing endonuclease from *Chlamydomonas reinhardtii* to target the anti-CD19-CAR encoded in an AAV6 vector to the exon 1 of the *TRAC* locus. Following electroporation of mRNA encoding the nuclease and AAV6 transduction, the CAR transgene integrated into one or both *TRAC* alleles, generating >99% CD3-TCR knockout T cells of which 48.9% expressed CAR. The allogeneic CAR-T cells proliferated in response to antigenic stimulation and showed potent functional activity in vitro and in vivo against target cells.[25]

Eyquem et al.[48] directed CD19-specific CAR to the *TRAC* locus by delivering CRISPR/Cas9 in complex with its gRNA (ribonucleoprotein, RNP) via electroporation and the CAR donor template in an AAV6. The resulting rate of transduction was slightly over 40% at a multiplicity of infection of 10^6, and around 95% of these CAR+ cells have had their *TRAC* locus disrupted. The resulting CAR-T cells showed improved function and delayed differentiation and exhaustion compared to the retrovirus-mediated CAR-T cells in a mouse model of ALL.[48] The recently described CRISPR/Cpf1 system[55] has also been employed for the generation of CAR-T cells. Dai et al.[56] developed a platform for the generation of modular CAR-T cells using Cpf1 and AAV6. T cells were electroporated with Cpf1 mRNA and transduced with AAV6 carrying 2 guide RNAs and one or two CAR DNA templates. Both *TRAC* locus and *PDCD1* were targeted and knocked out by this method, with mutation efficiency of 60.4% and 80.1%, respectively. The researchers also promoted the insertion of anti-CD22 CAR into the *TRAC* locus (45.5% efficiency) via HDR, while knocking out *PDCD1* (59.7% efficiency). A CD19/CD22 bispecific CAR-T cell was also generated by targeting the insertion of anti-CD22 CAR to the *TRAC* locus and of anti-CD19 CAR to *PDCD1* (21.7% of double-positive efficiency). Cytolytic

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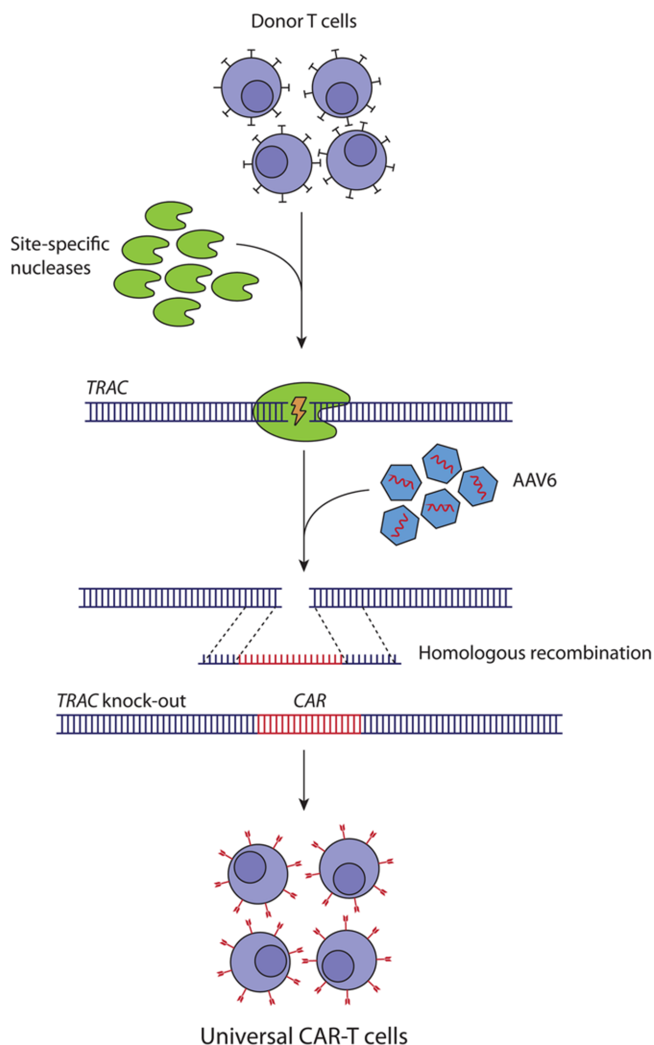

Figure 1. Generation of CAR-T cell through the knockout of the T cell receptor for allogeneic therapy. Donor T cells are modified using site-specific nucleases that target the T cell receptor alpha (*TRAC*) locus and generate a double-strand break. Adeno-associated viral vectors serotype 6 (AAV6) deliver the DNA template containing the gene for the chimeric antigen receptor (CAR). Homologous recombination promotes the simultaneous knockout of the *TRAC* locus and the knock-in of the CAR transgene. Successfully modified cells express only CAR, lacking endogenous TCR.

activity against CD19+CD22+ cell line was robust in both single- and double-knock-in CAR-T cells.[56]

5. Dual-AAV Strategies

One drawback of using recombinant AAV is their limited 4.8 kb packaging capacity.[57] Considering the homology arms, which are necessary for a successful HDR, can take up to 0.8 kb; the actual capacity of an AAV as the donor vector is around 4 kb. To overcome this limitation, a dual-AAV system has been commonly used in the past. To integrate large cassettes in T cells, two AAVs carrying the donor vector were used to trigger two successive homologous recombination events at the same location.[58] The T cells were electroporated with Cas9-RNP complexes and transduced with two AAV6 containing each a segment of the large transgene and homology arms. This resulted in an average of 9% of cells modified with the whole transgene.[58]

In the arena of Cas9 delivery, a dual-AAV was used to deliver a dual-vector system where SpCas9 and the gRNA, packaged in two separate viral vectors, were used to target a single gene, *Mecp2*, in the adult mouse brain in vivo. In this system, >70% of cells were transduced according to immunohistochemistry, illustrating that a dual-AAV system is an efficient vehicle for Cas9 delivery.[59] Chew et al.[60] delivered SpCas9 split between 2 AAVs to myotubes, tail-tip fibroblasts, and spermatogonia and achieved an editing frequency of up to 10.9%, which was similar to the editing levels obtained when CRISPR/Cas9 DNA was electroporated. AAV-CRISPR/Cas9 did not evoke extensive cellular damage and the immune response was mostly evoked by Cas9, illustrating that a split-AAV is a safe delivery vehicle.[60]

6. Conclusion

In conclusion, AAV and targeted nucleases provide adequate technology for gene editing and generation of CAR-T cells. Site-specific nucleases offer the means for the insertion of CAR into safe genomic loci, with the ability to knock out genes of interest to create an allogeneic therapy or more potent responses, while AAV provides a safe and effective vehicle for donor template delivery. Furthermore, AAV can also be adopted to safely deliver CRISPR nucleases.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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