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Using CRISPR to enhance T cell effector function for therapeutic applications

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ABSTRACT

T cells are critical to fight pathogenic microbes and combat malignantly transformed cells in the fight against cancer. To exert their effector function, T cells produce effector molecules, such as the pro-inflammatory cytokines IFN-γ, TNF-α and IL-2. Tumors possess many inhibitory mechanisms that dampen T cell effector function, limiting the secretion of cytotoxic molecules. As a result, the control and elimination of tumors is impaired. Through recent advances in genomic editing, T cells can now be successfully modified via CRISPR/Cas9 technology. For instance, engaging (post-)transcriptional mechanisms to enhance T cell cytokine production, the retargeting of T cell antigen specificity or rendering T cells refractive to inhibitory receptor signaling can augment T cell effector function. Therefore, CRISPR/Cas9-mediated genome editing might provide novel strategies for cancer immunotherapy. Recently, the first-in-patient clinical trial was successfully performed with CRISPR/Cas9-modified human T cell therapy. In this review, a brief overview of currently available techniques is provided, and recent advances in T cell genomic engineering for the enhancement of T cell effector function for therapeutic purposes are discussed.

1. Introduction

T cells are critical in maintaining protective immunity. As part of the adaptive immune system, T cells provide protection by eradicating infected cells and combating malignantly transformed cells. Indeed, high $CD8^+$ T cell infiltrates in renal cell carcinoma and gallbladder tumors correlate with beneficial outcomes [1,2]. To perform their effector function, T cells release effector molecules. These include granzymes, perforin and cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ [3–7]. It was shown that T cell-derived TNF-α and IFN-γ are required for T-cell mediated killing of established tumors [8]. Likewise, a high *IFNG* gene signature is associated with beneficial clinical outcomes in patients receiving immunotherapy [9,10], while copy number alterations of *IFNG* pathway genes correlate with poor immunotherapy responses [11].

Tumors possess many inhibitory mechanisms that dampen T cell effector function. Amongst others, tumor cells exploit T cell inhibitory receptors such as Programmed cell Death 1 (PD-1) by expressing their cognate ligand, i.e. Programmed Death Ligand 1 (PD-L1) [12,13], but also by downregulating antigen presentation [14–16]. As a result, T cells lose the capacity to produce effector molecules, impairing tumor control and elimination. To circumvent this, several immunotherapy strategies have been designed to optimize T cell effector function. One form of therapy employs Tumor Infiltrating Lymphocytes (TILs) [17]. To generate T cells for adoptive TIL therapy, TILs are reprogrammed to reacquire the capacity to produce effector molecules, and are expanded *in vitro* for 4–5 weeks before reinfusion into patients [17,18]. Most often, TIL therapy is used for the treatment of solid cancers [17,19], such as melanoma [20–24]. Currently, the implementation of TIL therapy is being investigated for several other types of tumors [17,25], including non-small cell lung cancer [18].

Another cellular immunotherapy approach makes use of genetically

Abbreviations: AP-1, activator protein 1; ARE, AU-rich element; ARE-Del, deletion of the 3′UTR AREs from the *Ifng/IFNG* gene; CAR, Chimeric Antigen Receptor; Cas, CRISPR-associated; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeat; CRS, cytokine release syndrome; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DGK, Diacylglycerol kinase; DHX37, DEAH-box helicase 37; FOXP3, Forkhead box P3; GATA, GATA binding protein; IFN, interferon; EBV, Epstein Barr virus; LAG-3, Lymphocyte Activating 3; IL, interleukin; NF-κB, nuclear factor of activated B cells; Pdia3, Protein Disulfide Isomerase Family A Member 3; PTPN2, Protein Tyrosine Phosphatase Non-Receptor 2; PD-1, Programmed cell Death 1; PD-L1, Programmed Death Ligand 1; RBP, RNA-binding protein; RNP, ribonuclear protein; TCR, T cell receptor; TGF, transforming growth factor; TIL, Tumor Infiltrating Lymphocyte; TLRs, Toll-like receptors; TNF, tumor necrosis factor; TRAC, TCR-α chain; TRBC, TCR-β chain; tTCR, transgenic TCR; UTR, untranslated region.

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engineered T cells. By redirecting T cell antigen specificity, T cells can be specifically targeted to cells bearing a defined (subset of) antigen(s) [26,27]. This can be achieved by the (viral) integration of a traditional T cell receptor (TCR) [28 29], or a Chimeric Antigen Receptor (CAR) [30]. CAR-T cells are equipped with a receptor comprised of the variable region of a high affinity monoclonal antibody directed against a defined tumor antigen, e.g. CD19, fused to the signaling domain of CD3ζ and one or multiple signaling domain(s) from a costimulatory receptor for optimal T cell effector function [31,32]. CAR T cell therapy is to date most successful in the treatment of lymphoid and myeloid tumors [33,34], resulting in up to 90% complete remission rates in B-ALL patients treated with CD19 CAR-T cell therapy [35]. The translation to solid tumors is not yet effective due to the lack of tumor-specific antigens that can be targeted by CAR-T cells [33]. Of note, both transgenic TCRs and CARs are being investigated that target tumor neoantigens [36–38].

T cell effector function can also be enhanced through genetic editing. Several tools are available for the genetic modification of T cells, such as transcription activator-like effector nucleases, or TALENs, zinc finger nucleases, or ZFNs, and transposon-mediated genome editing [39–43]. Recently, also the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR-associated (Cas) system was successfully applied in T cell cell-lines [44], but has also been used to genetically modify primary murine and human T cells [45–49]. Specifically, CRISPR/Cas9 has been employed to directly enhance T cell effector function [48,50–56], disable inhibitory receptor expression [45,57,66–68,58–65], or to redirect T cell antigen specificity by targeting TCRs or CARs to the endogenous TCR-α chain (*TRAC*) locus [28–30,69–71]. In fact, recently, the first in-patient trial has been performed utilizing CRISPR/Cas9-edited T cells [72]. In this review, a short overview of currently available CRISPR-based techniques for the genetic modification of T cells is provided, and recent advances in T cell genomic engineering aiming to enhance T cell effector function to improve cellular therapies are discussed.

2. CRISPR basics and different CRISPR/Cas9 tools available for T cells

CRISPR/Cas9-mediated gene-editing requires three components: the Cas9 DNA-nuclease, a targeting CRISPR RNA, or crRNA, and a *trans*-activating CRISPR RNA, or tracrRNA, of which the latter two can be supplied either separately or combined as a single guide RNA [73]. The targeting CRISPR RNA guides the Cas9 ribonuclear protein (RNP) complex to the cleavage site via DNA base pairing, and the *trans*-activating CRISPR RNA facilitates Cas9 activation [73]. However, Cas9-mediated DNA cleavage only occurs when the target site is directly adjacent to a 3′ protospacer-adjacent motif, or PAM [74], a 3–8 base pair DNA sequence [73,75]. The CRISPR-system can therefore be targeted to virtually any DNA region of interest.

Currently, several delivery methods are available to introduce the CRISPR/Cas9 system in T cells (Table 1). Each of them has benefits, but also drawbacks. For instance, one of the first delivery methods developed were lentiviral vectors for Cas9-delivery. These have been used to target e.g. the *CCR5* locus in human T cells, but resulted in low knock-out efficacies [76,77]. Furthermore, stable expression of gene-editing factors is not desirable as this could result in increased off-target effects [78], and viral gene transfer has posed significant risks in the past [79,80]. Furthermore, it was recently shown that the serum of a majority of healthy donors and cord bloods contains Cas9 antibodies [81,82], which could potentially trigger immune responses upon peptide presentation or secretion by stable Cas9-expressing cells. Therefore, transient expression or presence of the CRISPR/Cas9 system is preferred to prevent unwanted side effects. This can be achieved by for instance receptor-mediated Cas9 uptake through the addition of fusion tags [83], lipid-based vector delivery [84,85], or cell-membrane penetration via the addition of cell-penetrating peptides [86–88]. Of note, these novel methods have not been tested yet in T cells. Another non-integrative strategy makes use of membrane disruption. Through electroporation, Cas9 can be introduced as a plasmid, mRNA, Cas9 protein, or Cas9 RNPs [48,73,89–91]. While electroporation can be costly and laborious to optimize, several protocols describing the use thereof in T cells have been recently published [46,92,93]. Of note, while the electroporation of T cells was shown to be potentially cytotoxic, resulting in up to 95% loss of e.g. viable T cells in the first 24 h after electroporation [94], T cells retain their expansion potential after electroporation-based CRISPR/Cas9-mediated genome editing [48]. Thus, due to the transient presence of the CRISPR/Cas9 system and the retention of T cell expansion potential, electroporation-based strategies might be most optimal for the use of Cas9-mediated genome editing in T cells for therapeutic purposes.

3. Enhancing T cell effector function via CRISPR

The CRISPR/Cas9 toolbox can be used for silencing or knocking out genes [48,95,96], knocking in genes [44,90], and for the induced activation of genes in T cells [97–101]. Through these different approaches, various research questions have been investigated in T cells. These include diverse topics such as preventing HIV infection in T cells [90,92,102], regulation of T cell effector molecule production [48], TCR signaling [51,52], redirecting T cell antigen specificity [28–30,69–71], stabilization of regulatory T cell phenotypes [98], and investigating pathogenic cytokine receptor expression by T cells [96,99]. Here, I focus on the different approaches that have been taken to augment T cell effector function for immunotherapy purposes by enhancing cytokine production, blocking inhibitory receptor signaling, or redirecting T cell antigen specificity through CRISPR/Cas9 induced knock-outs.

3.1. Using CRISPR to directly augment cytokine production

The CRISPR/Cas9 system has been used to directly enhance T cell function (Table 2). The production of effector molecules by T cells is regulated on multiple levels (Fig. 1). The magnitude of antigen-specific T cell activation is determined by the integration of TCR-signaling and costimulatory signals [3,6,103]. TCR triggering results in the engagement of downstream signaling pathways and regulatory processes [104,105]. The threshold for T cell activation can be lowered via costimulatory signals, such as through the engagement of costimulatory receptors, e.g. CD28 and/or CD27 [103,106,107], triggering of cytokine receptors via cytokines such as interleukin (IL)-2 and IL-12 [108,109], or even innate-like sensing of pathogens through pattern recognition receptors [110], such as Toll-like receptors (TLRs) [6,110,111].

Table 1
CRISPR-tools.

Type	Advantages	Disadvantages	Applied in primary T cells	Ref
Lentiviral	Inclusion of selection marker	Low knock-out efficacy Genomic integration	Yes	[76,77]
Electroporation	T cells retain expansion potential Extensive protocols are available	Cytotoxic Costly	Yes	[48,73,89–91]
(Lipid) nanoparticles	Highly adaptable to specific need	Complex to engineer	No	[84,85]
Ligand fusion tags	Cell-type specific	Cells need to express receptor	No	[83]
Cell-penetrating peptides	Produced in-house	Varying quality and efficacy due to batch-to-batch differences	No	[86–88]

Table 2
Direct enhancement of T cell effector function via CRISPR/Cas9-mediated genome editing.

Species	Target		CAR	*In vitro*									*In vivo*					Ref
	Gene	Protein		IFN-γ	TNF-α	IL-2	IL-17	CD107a	GzmA	GzmB	GzmC	GzmD	Cytotoxicity	Model	IFN-γ	IL-2	GzmB	Tumor	
Human	*DGKA/DGKZ*	DGKα/ζ	EGFRvIII	+		+							+	Glioblastoma	+		+	R	[51]
	IFNG 3′UTR	IFN-γ		+	=	=													[48]
	PTPN2	PTPN2	Mesothelin	+	=	=	+												[52]
	TGFBR2	TGFBR2		+		+			+	+			+	Lung cancer	+			R	[56]
Mouse	*Dhx37*	Dhx37		+				+		+	+	+	+	E0771-OVAMammary tumor				D	[53]
	Gata3	Gata3												B16-F10 melanoma	+	+		D	[50]
	Nr2f6	Nr2f6		+										B16-OVA melanoma				D	[55]
	Zcd3h12a	Regnase-1												B16-OVA melanoma	+	+	+	D	[54]

+, increased production; =, equal production; R, tumor regression; D, tumor outgrowth delayed. Blank indicates not reported.

Together, these stimulatory signals determine the cytokine output of T cells.

A major determinant of cytokine production is the strength of the TCR signal that T cells receive [6,104]. Unsurprisingly, T cell activation can be amplified by direct enhancement of TCR signaling. Diacylglycerol kinases (DGKs) are enzymes that catalyze diacylglycerol metabolism, and have been shown to interact with critical signaling modules downstream of the CD3 co-receptor, such as protein kinase C and Ras activating protein [112]. Through diacylglycerol phosphorylation, DGKs inhibit CD3 signaling and T cell effector function [112]. DGKs have been suggested as potential immunotherapy targets, and it was recently shown that CRISPR-mediated *DGKA* and *DGKZ* double knock-out human T cells exhibit increased *IFNG* and *IL2* transcription, enhanced IFN-γ and IL-2 production, and increased cytotoxicity *in vitro* [51]. Furthermore, in a glioblastoma xenograft model, *DGKA/Z* double knock-out human T cells expressing an EGFRvIII CAR provided complete protection against tumor outgrowth, in contrast to mice treated with WT EGFRvIII CAR T cells, where tumors initially shrunk but rapidly grew out again [51].

Another negative regulator of TCR signaling is protein tyrosine phosphatase non-receptor 2 (PTPN2) [113,114]. Human *PTPN2* knock-out T cells generated via CRISPR/Ca9s-mediated genome editing exhibit enhanced calcium flux and IFN-γ and IL-17 production upon TCR stimulation [52]. However, Ptpn2$^{-/-}$ mice exhibited CD8^{+} T cell mediated autoimmunity [113], therefore, *PTPN2* knock-out might not be optimal for CD8^{+} T cell-based therapeutic purposes.

Together, these studies indicate that while there is potential for the enhancement of T cell effector function through the direct augmentation of TCR signaling, care should be taken to prevent unbridled T cell activation that might result in pathologies. Furthermore, due to the advent of CAR T cells, where multiple signaling domains from co-stimulatory receptors can be incorporated into one signaling module for optimal antigen-specific T cell activation [31,32,115], CRISPR/Cas9-mediated enhancement of T cell effector function through the modulation of TCR signaling might be suboptimal.

While CARs have been designed for optimal signal transduction, T cell cytokine production is regulated by both transcriptional and post-transcriptional events to prevent the unwanted production and release of these potentially cytotoxic mediators (Fig. 1) [105,116,117]. On the transcriptional level, epigenetic markers present on DNA and histones, and transcription factor availability, localization and phosphorylation determine the amount of RNA that is produced [118]. For example, demethylation of the *IFNG* locus only occurs in effector and in memory T cells [119]. This allows for rapid locus accessibility by transcription factors upon T cell activation to drive the transcription of *IFNG* RNA [119].

Multiple transcription factors are important for driving the production of T cell cytokines such as IFN-γ, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) [120,121]. One of the interacting partners of NF-κB identified in a genome-wide CRISPR/Cas9 screen investigating immunotherapy targets is the DEAH-box helicase 37, or DHX37 [53]. *DHX37* expression in TILs from breast cancer patients correlates with poor outcomes [53]. Potentially, this is due to DHX37-induced T cell dysfunction. *Dhx37* knock-out murine T cells generated through CRISPR/Cas9-mediated genome editing exhibit increased expression of *Gzmc* and *Gzmd*, increased Granzyme B and IFN-γ production and increased cytotoxicity *in vitro*. Furthermore, *Dhx37* knock-out T cells delayed tumor outgrowth in a murine mammary tumor model [53].

Similarly, DNA binding by AP-1 is hindered by the nuclear zinc-finger orphan receptor NR2F6, preventing cytokine production in T cells [122]. Indeed, CRISPR/Cas9-mediated *Nr2f6* knock-out murine T cells produce more IFN-γ, and delayed tumor outgrowth in a B16-OVA model compared to WT T cells [55].

Another approach taking advantage of the transcriptional machinery is knocking out transcription factors that inhibit the production of pro-

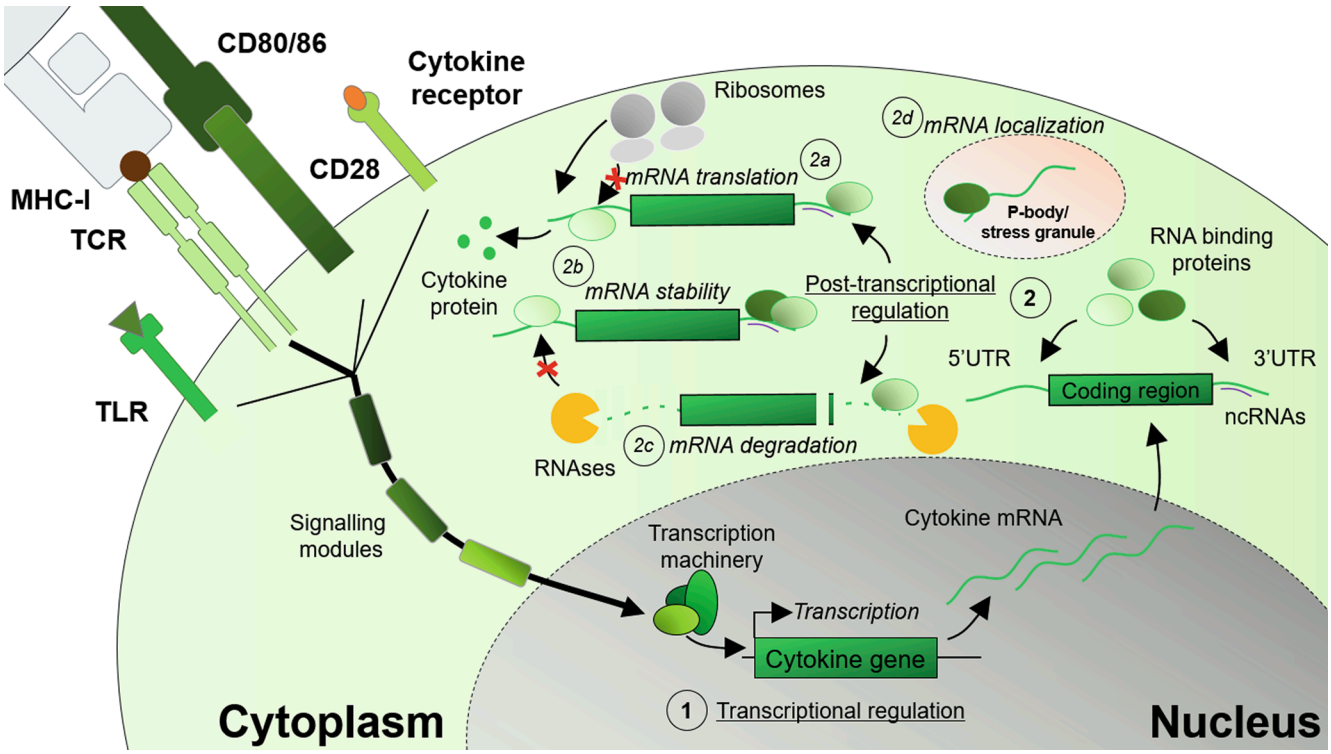

Fig. 1. Transcriptional and post-transcriptional machinery engaged during T cell activation. Integration of stimulatory signals, i.e. engagement of the TCR, triggering of co-stimulatory receptors such as CD28, sensing of pathogens through TLRs, and stimulation via cytokine gradients through cytokine receptors results in the engagement of downstream signaling cascades and the activation of transcription factors. (1) Transcriptional regulation determines the amount of cytokine mRNA that is produced. After mRNA maturation and export to the nucleus, (2) post-transcriptional mechanisms determine the protein output. RNA binding proteins and non-coding RNAs, such as microRNAs, bind to regulatory elements present in the 5′ and 3′UTR of mRNA transcripts. Depending on the binding repertoire, (2a) mRNA translation is enhanced or hampered, (2b) mRNA stability is influenced, (2c) mRNA is actively degraded by recruited RNAses, or (2d) mRNAs are targeted to a different location, i.e. stress granules or P-bodies.

inflammatory cytokines. For instance, GATA binding protein 3 (GATA3) is regarded as the master transcription factor for inducing a type 2 phenotype in T cells [123], and has been shown to significantly inhibit IFN-γ production in T cells [124]. *GATA3* was also identified as the top ranking transcription factor involved in the dysfunctional transcriptional program in T cells from melanoma patients [50]. *Gata3* knock-out murine T cells generated via CRISPR/Cas9-mediated genome editing showed enhanced capacity for the production of IFN-γ and IL-2 while less *Gata3* knock-out T cells produced IL-10 *ex vivo* in a B16-F10 melanoma model [50]. Furthermore, *Gata3* knock-out T cells also delayed tumor outgrowth [50].

Similarly, also Forkhead box P3 (FOXP3), the master regulator of the regulatory T cell transcriptional profile [125], inhibits the production of cytotoxic T cell effector molecules such as IFN-γ [126]. One of the factors involved in FOXP3 upregulation is transforming growth factor (TGF)-β [127]. CRISPR/Cas9-mediated knock-out of *TGFBR2*, one of the TGF-β receptors, in Mesothelin CAR-T cells increased *IFNG*, *GZMA* and *GZMB* expression and IFN-γ and IL-2 production *in vitro* by preventing FOXP3 upregulation and thus reducing induced regulatory T cell formation [56]. *TGFBR2* knock-out Mesothelin CAR-T cells retain their lytic capacity and exhibit enhanced *in vivo* tumor elimination compared to WT Mesothelin CAR-T cells in a murine lung cancer model [56].

Together, these reports identify several approaches through which transcriptional regulation can be exploited to enhance T cell effector function. Future research will determine whether modulation of transcription factor activity can be employed in treatment strategies.

The combined output of transcription factors determines the T cell transcriptome. However, another layer of regulation exists, determining the protein output of T cells (Fig. 1) [105,117]. After RNA splicing and nuclear export of mature mRNA molecules, post-transcriptional regulatory events determine mRNA half-life, subcellular localization and translational rate, and thus the amount of protein that is produced [116]. Post-transcriptional regulation is mediated by microRNAs, (long) non-coding RNAs, RNA-binding proteins (RBPs) and mRNA modifications [116,128–131]. In the past years, it has become increasingly clear that post-transcriptional regulation plays a major role in determining the timing and magnitude of T cell responses [48,104,105,116,117,132,133]. These processes rely, in part, on the binding of RBPs upon recognition of *cis*-elements present in mRNA transcripts [116]. These *cis*-elements are located in either the 5′ or 3′ untranslated region (UTR) of mRNA molecules [116,134]. Many cytokine genes contain at least one *cis*-regulatory element [116]. Thus, the protein production of most cytokines is, at least partially, determined by post-transcriptional events.

A critical class of *cis*-elements driving post-transcriptional regulatory events are AU-rich elements (AREs) encoded in the 3′UTR of many cytokine genes [105,116,135–139]. These AREs are critical to fine-tune the production of the prototypical T cell cytokines IFN-γ, TNF-α and IL-2 [135,139–141]. For instance, while antigen-experienced T cells express *Ifng* mRNA in a resting state, IFN-γ protein is only produced upon T cell activation [104]. Upon deletion of the 3′UTR AREs from the *Ifng* gene (ARE-Del) in a murine model, *ex vivo* resting antigen-experienced ARE-Del T cells produce IFN-γ due to a failure to keep the *Ifng* mRNA translationally quiescent [139]. Furthermore, upon deletion of the AREs in the 3′UTR of IFN-γ, ARE-Del T cells produce more IFN-γ *in vitro* due to increased mRNA half-life [139,142]. This enhanced mRNA stability leads to prolonged IFN-γ production [141], a feature that is critical for anti-tumor responses. Indeed, upon ARE-Del T cell therapy of B16-OVA melanoma-bearing mice, tumor outgrowth was significantly delayed compared to mice treated with wild type T cells [141].

The 3′UTR of the *IFNG* gene is conserved between mice and men [139]. Recently, these results were translated to human T cells [48]. By making use of CRISPR/Cas9 technology, primary human T cells with a 3′UTR ARE-deletion in the *IFNG* gene were generated [48]. Similar to their murine counterparts [141], also more human ARE-Del T cells produced IFN-γ, which was at least in part driven by increased *IFNG* mRNA stability [48]. This increased IFN-γ production capacity was also observed upon TCR engineering [48]. More MART-1 TCR-expressing ARE-Del human T cells produced IFN-γ compared to WT MART-1 TCR expressing T cells in a co-culture with patient-derived MART-1$^+$ melanoma tumor cells [48].

TNF-α production could also be enhanced by similar exploitation of post-transcriptional regulatory mechanisms. TNF-α is also (partially) regulated via the AREs present in the 3′UTR of *Tnfa*, and removal of the 3′UTR AREs from the *Tnfa* locus in mice results in constitutive TNF-α production [135]. However, this constitutive TNF-α production elicits inflammatory diseases [135], and thus might not be optimal for use in human therapies. Furthermore, given the dual role TNF-α plays in both anti-tumor responses and promoting tumor growth [143], the exploitation of TNF-α post-transcriptional regulatory mechanisms should first be thoroughly investigated prior to use in the clinic.

A more indirect approach to interfere with post-transcriptional regulation is by modulating the expression of RBPs, which govern the effect of 5′ and 3′UTR *cis*-regulatory elements. For instance, while not directly binding to the 3′UTR of *Ifng* [144], T cells from Regnase-1 knock-out mice exhibited enhanced IFN-γ production [144]. This was due to deregulation of the Roquin family of proteins [144], known RBPs which have been previously implicated in the regulation of IFN-γ production [145]. Also murine *Regnase-1* knock-out T cells generated via CRISPR/Cas9 exhibited increased IFN-γ production [54]. Additionally, also Granzyme B production was increased [54], a phenotype that was also observed in Regnase-1 knock-out mice [145].

Modulation of the post-transcriptional machinery governing cytokine production provides novel angles to enhance T cell effector function for therapeutic applications. While it has not yet been established whether the regulatory capacity of e.g. Regnase-1 is conserved in human T cells, other post-transcriptional mechanisms are conserved between mice and men and can further enhance TCR-engineered T cell effector function [48]. Potentially, this can be exploited in the future for therapeutic applications.

Together, these studies provide many opportunities for the direct augmentation of T cell effector function via CRISPR/Cas9-mediated genome editing. As regulation of T cell effector function is mediated on multiple levels [105,118], potentially, the engagement of multiple pathways can result in T cells with the most optimal T cell effector function. This, however, remains to be determined.

3.2. Disabling inhibitory receptors to enhance anti-tumor T cells

Within tumors, T cells encounter many inhibitory signals in the suppressive tumor microenvironment, including the ligands for inhibitory receptors present on T cells. To date, many T cell inhibitory receptors have been identified, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Lymphocyte Activating 3 (LAG-3) and PD-1 [146,147]. Tumors exploit these checkpoint receptors to dampen T cell effector function as a means of immune escape [12,13]. To circumvent this, inhibitory receptors are being targeted in antibody-mediated immunotherapy approaches [146]. These antibody-therapies, mostly targeting the PD-1/PD-L1 axis or CTLA-4, prevent the triggering of these receptors [12]. As a result, T cell effector function is restored, and anti-tumor responses can be mounted [148]. This type of therapy is employed in i.e. melanoma patients, resulting in an extraordinary 3-year survival of approximately 50% of patients [17]. Furthermore, clinical trials are also underway evaluating the efficacy of combined anti-PD-1/PD-L1 immunotherapy and adoptive cellular therapy with TILs [17]. CRISPR/Cas9 could also be utilized to render TILs refractive to inhibitory signals by knocking out inhibitory receptors prior to infusion. With this approach, systemic immunomodulatory antibody therapy is not required, which has been shown to be pose significant side effects [149]. A summary of the effects of CRISPR/Cas9-mediated abrogation of inhibitory receptor signaling on T cell effector function is provided in Table 3.

One of the most-studied inhibitory receptors is PD-1. The exact mode of action of inhibitory PD-1 signaling on T cell effector function remains unclear. PD-1 triggering recruits the phosphatase SHP-2 to the TCR complex [150]. SHP-2 then dephosphorylates TCR signaling molecules such as CD3ζ and ZAP70 [150]. Subsequent work showed that SHP-2 dephosphorylates the CD28 costimulatory receptor instead [151,152]. However, yet another mode of action of PD-1 triggering was identified recently [141]. PD-1 triggering also modulates the production of IFN-γ in T cells independently from blocking the CD28 signaling pathway [141]. Neither CD28 costimulation nor blocking PD-1 signaling via antibodies enhances the *Ifng* mRNA transcription of murine CD8$^+$ T cells [141]. Both rather function by acting on different post-transcriptional regulatory mechanisms [141]. Nonetheless, the therapeutic benefit of interfering with PD-1 signaling is evident [17], and therefore it is unsurprising that knocking out PD-1 via CRISPR/Cas9 technology has also been thoroughly investigated.

The effect of CRISPR/Cas9-mediated PD-1 knock-out was first assessed in primary human Epstein Barr virus (EBV)-specific T cells in healthy donors [57,58]. Knocking out PD-1 with CRISPR/Cas9 increased the production of IFN-γ, TNF-α and IL-2 and the degranulation capacity of T cell cell-lines and EBV-specific T cells from healthy donors *in vitro* [57,58]. This was replicated in T cells isolated from an EBV$^+$ gastric cancer patient, where PD-1 knock-out in patient-derived T cells increased not only inflammatory cytokine production but also resulted in the production of less IL-10 compared to wildtype T cells upon EBV peptide stimulation [57]. Similarly, also PD-1 deficient T cells generated via CRISPR/Cas9 mediated genome editing derived from a melanoma patient produced more IFN-γ upon stimulation with several melanoma-associated peptides compared to PD-1 sufficient T cells from the same patient [59]. Furthermore, human PD-1 knock-out T cells primed by patient-derived multiple-myeloma lysate-pulsed DCs exhibited increased IFN-γ and TNF-α production *in vitro* and delayed tumor outgrowth in a murine xenograft model, increasing survival [60].

Not only direct genetic editing of PD-1 results in the lack of expression thereof. Post-translational modifications of proteins can affect protein function, half-life and localization [153]. For instance, glycosylation is important for the expression of surface molecules on the cell membrane [154]. This was also shown to be the case of PD-1. Fut8, a fucosyltransferase, was identified to be important for the membrane expression of PD-1 in murine T cells via a CRISPR/Cas9-based screening [61]. Furthermore, *Fut8* knock-out murine T cells generated via CRISPR/Cas9-mediated genome editing also expressed less PD-1 *in vivo* [61]. This phenotype could be replicated via a small molecule inhibitor that inhibits the core fucosylation machinery, resulting in increased IFN-γ and IL-2 production *in vitro* and delayed tumor outgrowth in a murine B16-OVA melanoma model [61]. However, as noted, fucosylation is important for the location, function, and half-life of many proteins, and therefore, this approach might not be optimal for use in therapeutic applications.

Another immunotherapy approach is the use of cellular therapies, containing either TILs or genetically modified T cells. Indeed, CD19 CAR-T cells have been genetically manipulated with CRISPR/Cas9 to knock out PD-1 [45]. PD-1 knock-out CD19 CAR-T cells exhibited increased *in vitro* degranulation as measured by CD107a staining [45]. In a murine myeloid leukemia xenograft model, CRISPR/Cas9-generated PD-1 knock-out CD19 CAR-T cells were also able to clear the tumor, in contrast to wildtype CD19 CAR-T cells [45]. Similarly, also Glypican-3 CAR T-cells and CD133 CAR-T cells with a CRISPR/Cas9-mediated PD-1 knock-out exhibited enhanced effector function *in vitro* and prolonged survival in hepatocellular carcinoma and glioma xenograft models

Table 3
Abrogating inhibitory receptor signaling via CRISPR/Cas9-mediated genome editing to augment T cell effector function.

Target	CAR	*In vitro*								*In vivo*				Ref
		IFN-γ	TNF-α	IL-2	CD107a	GzmA	GzmB	GzmC	GzmD	Cytotoxicity	Model	IFN-γ	IL-2	GzmB	Tumor	
PD-1		+	+	+	+											[57–59]
		+	+								Melanoma				D	[60]
	CD19				+					+	Myeloid leukemia				R	[45]
	CD133	=	=	=						+	Glioma				D	[63]
	Glypican-3	+		+						+	Hepatocellular carcinoma	+	+		D	[62]
	Mesothelin	+		+						+	Breast cancer				D	[64]
CTLA-4		+	+													[66]
		+	+								Colorectal carcinoma				D	[65]
LAG-3		=		=							Raji Burkitt's lymphoma				R	[67]

+, increased production; =, equal production; R, tumor regression; D, tumor outgrowth delayed. Blank indicates not reported.

[62,63].

Also human PD-1 knock-out Mesothelin CAR-T cells exhibited enhanced *ex vivo* lysis and IFN-γ production compared to PD-1$^+$ mesothelin CAR-T cells [64]. Strikingly, PD-1 knock-out Mesothelin CAR-T cell therapy also outperformed Mesothelin CAR-T cell therapy with antibody-mediated PD-1 blockade in an *in vivo* mammary gland tumor xenograft model [64]. Of note, a xenograft model showed that PD-1 knock-out CD133 CAR-T cells do not proliferate uncontrollably *in vivo* [63].

Together, these studies show the feasibility of CRISPR/Cas9-mediated disruption of PD-1 signaling and the potent effect thereof on augmenting T cell effector function. Knocking out PD-1 can even further enhance the efficacy of CAR-T cell treatment in murine models, does not result in uncontrolled *in vivo* expansion and even outperforms antibody-mediated PD-1 blockade, providing *in vivo* evidence of the potential benefit of CRISPR-mediated augmentation of CAR-T cell function.

Less research has been performed investigating the direct knock-out of other inhibitory receptors, such as CTLA-4 and LAG-3 in T cells. CRISPR-mediated CTLA-4 knock-out has shown that CTLA-4 knock-out in human T cells increases the amount of IFN-γ and TNF-α produced *in vitro* [65,66], and prolongs survival in a murine colorectal carcinoma xenograft model [65]. Likewise, also LAG-3 knock-out human CD19 CAR-T cells provide prolonged survival in a Burkitt lymphoma xenograft model [67].

Summarizing, these studies highlight the potential of CRISPR/Cas9-mediated knock-out of inhibitory receptors such as PD-1 and CTLA-4 to render T cells refractory to inhibitory receptor signaling, potently enhancing T cell effector function *in vitro* and *in vivo*.

3.3. Redirecting T cell antigen specificity

Redirecting the antigen specificity of T cells allows for the treatment of tumors expressing defined sets of antigens. The generation and effector function of TCR transgenic T cells can be further amplified by making use of CRISPR/Cas9 technology (Table 4). This has mostly been studied in the context of traditional TCR gene modification. For instance, it was shown for MLANA (MART-1, melanoma) and HER2/neu (breast cancer) TCRs that knocking the transgenic TCR into the endogenous *TRAC* locus enhanced TCR expression compared to *TRAC* WT T cells [71]. This was even further increased when also the TCR-β chain (*TRBC)* was also knocked out [71]. Enhanced transgenic TCR expression was also shown for the *TRAC* knock-in of TCRs specific for BOB (multiple myeloma), HA-1 (multiple myeloma), MPO (myeloid leukemia) and PRAME (melanoma) peptides [28,29]. Furthermore, HA-1 TCR *TRAC* knock-in engineered T cells showed enhanced anti-tumor capacity in a multiple myeloma xenograft model [29].

Also CARs can be targeted to the *TRAC* locus after CRISPR/Cas9-mediated knock-out. CD19 CAR *TRAC* knock-in enhanced T cell effector function in a murine leukemia xenograft model, as CD19 CAR *TRAC* knock-in T cells were less vulnerable to exhaustion compared to conventional CD19 CAR-T cells [30].

Of note, several studies have also shown enhanced CMV-specific TCR expression upon knock-in into the *TRAC* locus [29,71]. While used as a negative control, this could prove beneficial, as virus-specific bystander CD8$^+$ T cells have been shown to populate tumors [69], and can be reactivated to elicit tumor control in murine models [70].

Together, these studies show the merit of using CRISPR/Cas9-mediated genome editing to enhance the anti-tumor capacity of TCR-engineered/CAR-T cells for therapeutic applications. When deemed safe for patients, this type of approach should also be considered for clinical use.

3.4. Multiplex CRISPR-editing for a multipronged approach to optimize T cell products

While CRISPR/Cas9 can be used to enhance T cell effector function

Table 4
Redirecting T cell antigen specificity by knocking in transgenic TCRs (tTCR) into the TRAC locus.

TCR/CAR	Antigen	Target gene		TRAC KI	Enhanced tTCR expression	*In vitro*				*In vivo*		Ref
		TRAC	TRBC			IFN-γ	TNF-α	IL-2	Cytotoxicity	Model	Tumor	
TCR	MLNA	Y	Y	Y	Y							[71]
	HER2/Neu	Y	Y	Y	Y							[71]
	BOB	Y			Y							[29]
	HA-1	Y	Y		Y	+			+	Multiple myeloma	D	[29]
	MPO	Y		Y	Y	=			=	Myeloid leukeumia	=	[28]
	PRAME	Y			Y							[29]
	CMV	Y			Y							[29]
	CMV	Y		Y	Y	=/+	=	=				[71]
CAR	CD19	Y		Y	Y					Leukemia	D	[30]

+, increased production; =, equal production; D, tumor outgrowth delayed; Y, yes. Blank indicates not reported.

directly (Table 2), to limit T cell dysfunction by abolishing the expression of inhibitory receptors (Table 3), or to redirect antigen specificity (Table 4), these approaches can also be multiplexed to maximize the effect thereof (Table 5). For instance, CD19 CAR-T cells that have been modified to lack expression of TRAC and PD-1 exhibited increased IFN-γ production and cytotoxicity *in vitro* compared to either TRAC-expressing or TRAC- and PD-1-expressing CD19 CAR T cells [155]. Furthermore, these PD-1$^-$TCR-α$^-$ CAR T cells provided protective immunity in a Raji xenograft model [155] and a prostate cancer model [156].

This multiplex approach can also be utilized to produce "universal T cells". Currently, approved CAR-T cell therapies are produced on a custom-made basis from patient-derived PBMCs. This is a costly and time-consuming process for patients who are at a high risk. Additionally, cellular products might not meet release criteria, even further endangering patients. One way to circumvent this would be to make use of universal T cells, which have been genetically engineered to lack expression of MHC-I molecules, such as *B2M*, and/or TCR α and/or β chains, limiting graft versus host disease and graft rejection [157]. Universal T cells can be generated via CRISPR/Cas9-mediated multiplex genome editing [158,159]. By knocking out the *TRAC* [160], or *TRAC* and *B2M* [158], high levels of CD19 CAR-expressing T cells could be generated that exhibited robust anti-tumor responses in murine xenograft models [158,160]. Furthermore, multiplex editing could also be employed to generate "off-the-shelve" inhibitory signaling-refractory CAR-T cells. For instance, CD19 CAR-T cells lacking the expression of both B2M and PD-1 have been generated [161,162] . Similarly, also EGFRvIII CAR$^+$TRAC$^-$PD-1$^-$B2M$^-$ CAR T cells were generated via CRISPR/Cas9 mediated genome editing. These cells exhibited enhanced IFN-γ and TNF-α production *in vitro,* but did not enhance survival of mice in a murine xenograft glioblastoma model compared to mice treated with WT EGFRvIII CAR-T cells [161].These studies highlight the potential of CRISPR/Cas9-mediated genome editing to supplement and further enhance T cell therapies, paving the way to an "on-the-shelf" solution for patients.

Also the direct enhancement of T cell effector function can be combined in a multiplexed CRISPR-approach. For instance, Protein Disulfide Isomerase Family A Member 3 (Pdia3) was identified in an *in vivo* CRISPR/Cas9 screen to regulate T cell effector function in a glioblastoma model [163]. *Pdia3* knock-out combined with EGFRvIII CAR *TRAC* knock-in via CRISPR/Cas9-mediated genome editing lowered the TCR threshold required for T cell activation, resulting in higher IFN-γ production upon TCR activation compared to Pdia3$^+$ T cells [163]. EGFRvIII CAR *TRAC* knock-in Pdia3$^-$ T cells also protected against tumor outgrowth in a glioblastoma model [163].

Recently, CRISPR/Cas9-modified T cells have been applied as a therapeutic agent [72]. While TIL therapy is highly successful for the treatment of melanoma [17], attempts are still being made to further enhance T cell therapy. As previously discussed, one possibility currently under investigation is the combination of TIL therapy and anti-PD-1 therapy. Also the recent first-in-patient-trial where CRISPR/Cas9-mediated gene-edited T cells were administered investigated the targeting of PD-1. Stadtmauer et al. (2020) utilized CRISPR/Cas9 gene editing to abolish the expression of PD-1 and to target a NY-ESO-1 TCR to the *TRAC* locus in human T cells [72]. These multiplex-edited T cells were administered to 3 patients with refractory advanced cancer, and none of the patients experienced cytokine release syndrome (CRS) [72]. CRS is one of the main adverse events reported during TIL and CAR-T cell therapy [164,165], with up to 100 percent of patients treated with CD19 CAR-T cells experiencing CRS [165]. While symptoms of CRS can be mild, including mild flu-like symptoms, CRS can also result in severe life-threatening inflammatory conditions [165]. None of the three patients receiving CRISPR/Cas9-mediated gene edited T cells experienced CRS [72], highlighting the safety of this treatment. Furthermore, at the end of the trial, 2/3 patients achieved stable disease [72].

Summarizing, multiplexed CRISPR-approaches to augment T cell effector function is feasible and has even been employed as a therapeutic application. While this trial was only the first of its kind, the results are hopeful, and indicate that CRISPR/Cas9-mediated genome editing has a place in augmenting the efficacy of T cell therapies.

4. Conclusion and outlook

Since the introduction of the CRISPR/Cas9 toolbox, genome editing in T cells has taken flight. Through Cas9-mediated genome editing, the manipulation of large numbers of cells is feasible, a feature that is

Table 5
Multiplex CRISPR/Cas9 approaches to maximize T cell effector function.

TCR/CAR	Antigen	Target gene					*In vitro*							*In vivo*		Ref
		TRAC	TRBC	B2M	PD-1	Pdia3	IFN-γ	TNF-α	IL-2	GzmA	GzmB	GzmC	Cytotoxicity	Model	Tumor	
CAR	CD19	Y		Y			=		=				=	Leukemia	D	[158]
		Y		Y	Y		+		+				+	Raji Burkitt's lymphoma	R	[155]
		Y		Y	Y									Raji Burkitt's lymphoma	R	[156]
	EGFRvIII	Y		Y	Y		+	+						Glioblastoma	=	[161]
		Y				Y	+			+	+	+		Glioblastoma	D	[163]
TCR	NY-ESO-1	Y	Y		Y											[72]

+, increased production; =, equal production; D, tumor outgrowth delayed; Y, yes. Blank indicates not reported.

critical for therapeutic applications. With the novel opportunities provided by the ease of CRISPR/Cas9-mediated genome editing, new ideas have been explored to enhance T cell effector function directly, prevent T cell dysfunction through interfering with inhibitory receptor signaling and redirect T cell antigen specificity. Together, these approaches have led to T cells with a redirected antigen-specificity and enhanced effector function, as measured by cytokine production, granular content, cytotoxicity or protection in murine models. These advances have led to the recent first-in-patient clinical trial with CRISPR/Cas9-modified human T cells. While only three patients were treated, the adoptive therapy with genetically engineered T cells was deemed safe, providing the first results for the clinical use of CIRPS/Cas9-engineered T cells in patients. Nonetheless, rigorous testing is necessary to determine the efficacy and safety of CRISPR/Cas9-enhanced T cells for adoptive therapy, with a focus on safety. In particular, off-target genetic editing, in-patient T cell expansion and survival, and the potential induction of CRS need to be thoroughly investigated. Lastly, while the current CRISPR/Cas9 methods are suitable for the manipulation of small numbers of cells, the editing of the large numbers of cells required to for patient treatment might still be sub-optimal. Thus, for the clinical use of CRISPR/Cas9-mediated genome editing in for instance TIL-products, novel CRISPR/Cas9 approaches should be developed. Once these hurdles have been overcome, multiplex approaches incorporating both the maximization of T cell effector function and the use of antigen-specificity redirection via transgenic TCRs or CARs for optimized T cell activation and targeting with the CRISPR/Cas9 toolbox will allow for the production of universal T cells with augmented effector function for therapeutic applications.

Ethics approval and consent to participate

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Consent for publication

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JJFH conceived the manuscript topic, performed literature research, and wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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