REVIEW ARTICLE

CRISPR-Cas gene knockouts to optimize engineered T cells for cancer immunotherapy

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While CAR-T and tgTCR-T therapies have exhibited noteworthy and promising outcomes in hematologic and solid tumors respectively, a set of distinct challenges remains. Consequently, the quest for novel strategies has become imperative to safeguard and more effectively release the full functions of engineered T cells. These factors are intricately linked to the success of adoptive cell therapy. Recently, CRISPR-based technologies have emerged as a major breakthrough for maintaining T cell functions. These technologies have allowed the discovery of T cells' negative regulators such as specific cell-surface receptors, cell-signaling proteins, and transcription factors that are involved in the development or maintenance of T cell dysfunction. By employing a CRISPR-genic invalidation approach to target these negative regulators, it has become possible to prevent the emergence of hypofunctional T cells. This review revisits the establishment of the dysfunctional profile of T cells before delving into a comprehensive summary of recent CRISPR-gene invalidations, with each invalidation contributing to the enhancement of engineered T cells' antitumor capacities. The narrative unfolds as we explore how these advancements were discovered and identified, marking a significant advancement in the pursuit of superior adoptive cell therapy.

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BACKGROUND

Immune surveillance constitutes a powerful defense mechanism thwarting the development of cancer. Orchestrated by the coordinated actions of cells and active molecules originating from both the innate and adaptive immune systems, this surveillance mechanism detects and eliminates emerging malignant cells. However, a dynamic equilibrium between immune and transformed cells may emerge, enabling tumor cells to resist immune pressure and eventually evade elimination. This has been theorized and enunciated by the immunoediting theory [1].

Among the immune cells engaged in tumor control, T cells have emerged as pivotal actors in the eradication of cancer cells, owing to their antitumor capacities [2]. A groundbreaking strategy emerged in the 1990s with the evolution of T cell engineering, enabling cells to express receptors specifically targeting tumor antigens such as transgenic T cell receptors (tgTCR) or chimeric antigen receptors (CAR) [2]. Even though both types of receptors have shown promise, CAR-T therapy has emerged as the most advanced therapeutic strategy, particularly in refractory and relapsed hematologic malignancies, while tgTCR-T therapy has shown encouraging outcomes in solid tumors and offers a broader target repertoire [3, 4]. Progress in CAR-T therapy has led to the approval by health regulatory agencies of six medications for the treatment of B cell acute lymphoblastic leukemia, several lymphomas as well as multiple myeloma [5, 6]. However, despite the encouraging clinical efficacies that have been reported, a substantial number of patients either do not respond to the therapy or have relapses, and the treatment of solid tumors remains challenging [6, 7].

Numerous obstacles that limit the sustainable efficacy of engineered T cells have been identified so far. Hence, the emergence of resistant tumor cells, the poor infiltration of effector cells, as well as the limited persistence and lack of effector functions pose new challenges in the realm of adoptive cell therapy based on engineered T cells [4, 8]. Among these challenges, significant efforts have been directed towards mitigating the functional decline of T cells, taking into account the hostile tumor microenvironment that impedes their full potential [9].

Recently, advances in molecular engineering and immunology have facilitated the identification of relevant targets for genetic invalidation, leading to the development of next-generation engineered T cells [8, 10, 11]. To achieve this, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas approach, widely used in the last few years, has shown remarkable results in the field of cancer immunotherapy by improving T cell functions through the direct targeting of negative regulators [8].

This review delves into the induction of T cell dysfunction before examining how CRISPR-Cas genetic invalidation of genes involved in the induction or maintenance of T cell dysfunction can be harnessed to strengthen engineered T cell therapy.

INDUCTION OF T CELL DYSFUNCTION

During chronic infections and cancer, T cell functions become compromised [12]. This disablement arises from persistent exposure to antigens and the immunosuppressive tumor microenvironment, gradually resulting in the attenuation of T cells' effector capacities (Fig. 1) [11, 13–15].

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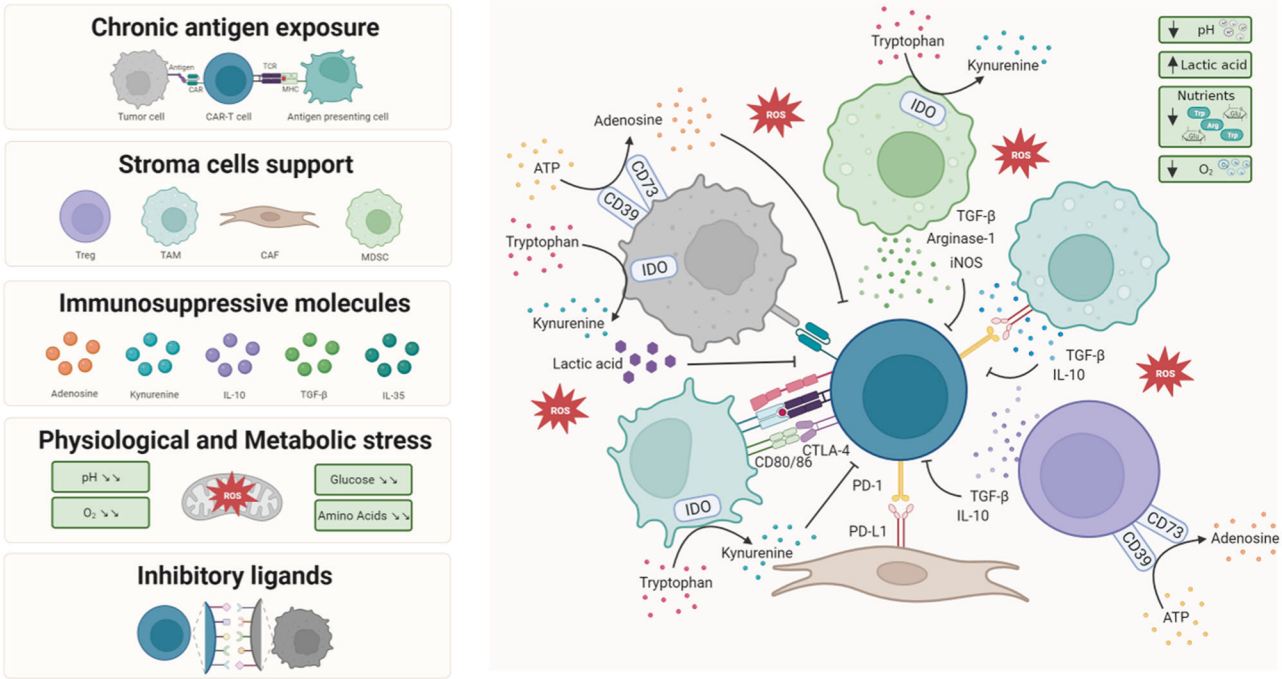

Fig. 1 Drivers of T cell dysfunction. Chronic antigen exposure and the immunosuppressive tumor microenvironment can impair T cell effector functions. By inducing tough inhibition of T cells' antitumor capacities through interactions with various immune cells and molecules, as well as modulating the overall environment, tumor cells can evade T cell immunosurveillance, ultimately leading to the failure of adoptive cell therapy. CAF, cancer-associated fibroblasts; CAR, chimeric antigen receptor; TAM, tumor-associated macrophages; Treg, regulatory T cells; MDSC, myeloid-derived suppressor cells.

The prolonged overstimulation leads to a significant impairment of T cell functions, as elucidated in numerous studies published in the early 2000s. These investigations have underscored a direct correlation between the extent of antigen stimulation and the severity of dysfunction, thus proving that such stimulation alone is adequate to precipitate the decline in effector capacities of $CD8^+$ T cells [16]. Furthermore, within the hostile tumor microenvironment, various factors contribute to the development of dysfunctional T cells. For instance, essential nutrients such as glucose and amino acids become depleted. This competition between tumor cells and T cells arises from the reliance of cancer cells on glycolysis for energy production. Through this metabolic pathway, cancer cells increase lactic acid and CO_2 production, directly impacting the survival and functions of effector T cells [17]. Consequently, the tumor's metabolic activity generates an acidic and hypoxic environment, detrimentally affecting antitumor effector T cell activity. This is notably achieved by inhibiting the PI3K/Akt/mTOR pathway and fostering the differentiation of regulatory T cells (Tregs) [17, 18]. These disruptions lead to the accumulation of immunosuppressive metabolites, including kynurenine, adenosine and reactive oxygen species (ROS), known to significantly inhibit T cell function [19–21]. Moreover, enzymes such as indoleamine 2,3-dioxygenase (IDO), whose level is elevated in patients with poor prognosis, and ectonucleotidases CD39 and CD73, are upregulated during this process, contributing to increased levels of kynurenine and adenosine respectively [20, 22, 23]. Dysfunction is further exacerbated by the presence of immunosuppressive cells and their associated cytokines that actively participate in repressing T cells [9, 11]. Indeed, tumor cells possess the ability to recruit supportive cells such as cancer-associated fibroblasts (CAF), tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), or Tregs to promote their progression. Through their intrinsic properties, immunosuppressive cells closely collaborate to drive T cell dysfunction [9, 11]. Furthermore, other immune cells originally involved in cancer cell clearance, such as antigen-presenting cells or natural killer cells, also indirectly contribute to the development of dysfunctional T cells by fostering an inflammatory environment or deviating from their initial functions [24].

CONSEQUENCES OF DYSFUNCTION IN T CELL ABILITIES

The state of dysfunction is characterized functionally and phenotypically by a progressive loss of effector functions, increased and sustained expression of inhibitory receptors, modification of metabolic programming, and an altered transcriptional and epigenetic landscapes (Fig. 2) [11, 25].

In the initial phase, interleukin (IL)-2 production is altered, gradually followed by reduced tumor necrosis factor (TNF)-α and interferon (IFN)-γ expression [26]. This reduction in the secretion of pro-inflammatory cytokines drastically alters T cell functions, leading to reduced cytotoxic and proliferative abilities [26]. Simultaneously, inhibitory receptors, initially serving as immune checkpoints to prevent overactivation, become more prominently expressed on the cell surface [27–29]. Consequently, T cell cytotoxic activity decreases as inhibitory signals are mediated through various molecular pathways (reviewed by Catakovic et al.) [15, 28]. Moreover, defective mitochondrial functions, stemming from altered mitochondrial biogenesis and mitochondrial membrane depolarization, enhance aberrant ROS production and decrease oxidative phosphorylation, impairing T cell metabolic fitness [30]. Recent data has also confirmed that the transcriptomic and epigenetic landscapes of dysfunctional T cells differ from those of effector and memory T cells [31]. This distinction could be attributed to the collaboration of transcription factor members of the TOX and NR4A families, which notably impose T cell dysfunction by driving epigenetic programming [32, 33].

To enhance the capacities of engineered T cells and overcome their limitations, various approaches have emerged and are

actively under investigation [34]. For instance, numerous studies have focused on ex vivo CAR-T manufacturing, with efforts concentrated on CAR constructs, cell culture conditions, as well as cell sources [8, 35–38]. Furthermore, the combination of immune checkpoint blockade with CAR-T cells has shown interesting results [39]. Similarly, genome editing has emerged as a highly promising tool in recent years for enhancing polyvalence and antitumor efficacy of CAR-T cells [8, 11, 38].

CRISPR-CAS GENOME EDITING TOOL

Recent advancements in biotechnology have introduced innovative tools such as meganucleases, zinc finger nucleases, transcription activator-like effector nucleases (TALEN) or CRISPR-Cas, offering the potential to address, prevent, or mitigate some of the prevailing limitations of next-generation engineered T cells [40–42]. Among the various genome editing tools available, TALEN and CRISPR-Cas methods have demonstrated significant potential in silencing unwanted gene expression, but the CRISPR-Cas method has emerged as the most widely used strategy for genome editing, owing to its precision, flexibility, and simplicity to implement [43–45]. This technology has notably already demonstrated promising results in completed clinical trials [46, 47].

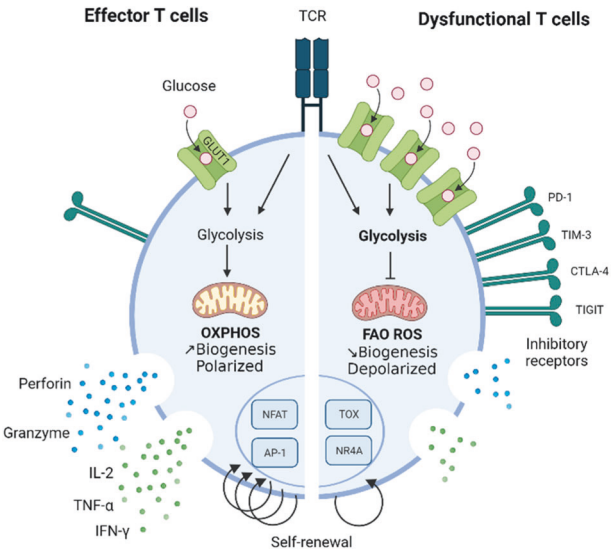

Fig. 2 T cells' loss of function. Dysfunctional T cells exhibit alterations in epigenetic, metabolic, and transcriptomic programs, resulting in a sustained upregulation of inhibitory receptors, a lack of cytokine secretion, as well as a decrease in degranulation and cell proliferation capacity. FAO, fatty acid β-oxidation; OXPHOS, mitochondrial oxidative phosphorylation; ROS, reactive oxygen species.

Facilitated by a guide RNA (gRNA) and the Cas endonuclease, the CRISPR-Cas system precisely cuts a specific gene to induce double-stranded cuts [48]. Following this phenomenon, natural error-prone non-homologous end joining (NHEJ) or homologous-directed repair (HDR) mechanisms initiate gene repair [49]. The mutagenic behavior of NHEJ can lead to deletions and insertions, resulting in the emergence of premature STOP codons or a shift in the reading frame causing the invalidation of the gene [50]. Additionally, the HDR mechanism may take place when a homologous repair DNA template is introduced (Fig. 3) [51].

Various Cas proteins have been identified for CRISPR-Cas based gene editing. Among them, Cas9 has been extensively studied and employed for its high efficiency in genome editing. However, other proteins like Cas12a, Cas13 and Cas14, have been explored as promising alternatives, as reviewed by Hillary et al. [52]. Additionally, apart from the usual double-stranded cuts induced by Cas proteins to invalidate a gene, various other innovative CRISPR-Cas approaches have also been developed. One such approach involves the use of a dead Cas (dCas) protein, a mutated Cas which can be paired with a repressor protein, which induces CRISPR interference when the gRNA is fixed to the desired gene. Another approach is the utilization of nickase Cas (nCas) proteins, where one of the two catalytic Cas sites is inactivated, enabling more precise gene disruption by targeting two sites on either side of the target gene. Furthermore, when nCas proteins are linked to specific enzymes, they can be used for base or prime editing [51].

Thus, the CRISPR-Cas tool can be harnessed to target and invalidate negative regulators of T cells' functions involved in the development and/or maintenance of their dysfunctional state [37, 38]. However, identifying compelling targets to optimize engineered T cells remains quite challenging. In pursuit of this goal, two distinct strategies have emerged.

KNOWLEDGE-BASED APPROACH

Transcriptome profiling methods have revolutionized research by providing deeper insights into complex biological phenomena [53]. Utilizing patient-derived T cells in transcriptomic studies has emerged as the strategy of choice to unravel the intricacies of T cell dysfunction. By combining this technology with immunological knowledge, a "good guess" approach has been used to identify new potential targets for optimizing engineered T cells (Table 1).

Several research teams have demonstrated the efficacy of this approach by targeting suppressive molecules directly contributing to T cell functional loss. Their investigations have highlighted cell surface proteins such as immune checkpoints (PD-1, LAG-3) or

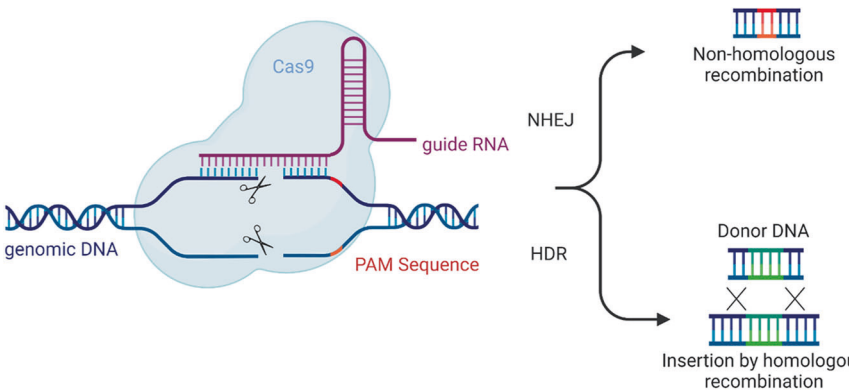

Fig. 3 CRISPR-Cas principle. The CRISPR-Cas tool is a technology that is capable of inducing targeted gene knockouts through the assistance of an endonuclease coupled with a specific guide RNA complementary to the gene requiring editing. This approach activates natural DNA repair mechanisms, often resulting in complete gene silencing.

Table 1. Knowledge-based targets model's summary.

Protein type	Target	Method for gene knockout	Transgene	Tumor model	Assay	Impact	Ref
Cell-signaling protein	CBL-B	Viral	CEA-CAR	Solid tumor (CEA$^+$MC38)	In vivo	Effector function	[92]
	DGK	Ribonucleoprotein	EGFRvIII-CAR	Solid tumor (U87vIII)	In vitro In vivo		[67]
			NY-ESO-1-tgTCR	Solid tumor (A375P)	In vitro		
	DNMT3A		CD19-CAR	Liquid tumor (BV173)	In vivo	Effector function and persistence	[68]
			EpA2-CAR	Solid tumor (LM7)	In vitro In vivo		
			HER2-CAR				
			IL-13Rα2-CAR	Solid tumor (U373)			
	PTP1B		HER2-CAR	Solid tumor (HER2$^+$24JK)		Effector function	[70]
	PTPN2		HER2-CAR	Solid tumor (HER2$^+$E0771)	In vivo		[71]
	SUV39H1		1928z-CAR	Liquid tumor (NALM-6)	In vitro In vivo	Effector function and persistence	[69]
Cell-surface protein	Adenosine A2A receptor		Lewis-Y-CAR	Solid tumors (OVCAR-3 and MCF7)	In vitro	Effector function	[57]
	FAS/TRAC/ β_2M	Viral	CD19-CAR	Liquid tumor (CD19$^+$K562)	In vitro In vivo		[58]
	LAG-3	Ribonucleoprotein	CAR	Liquid tumor			[56]
	PDCD1		CD19-CAR	Liquid tumors (CD19$^+$K562 transduced with PD-L1)			[54]
	PDCD1/ TRAC/β_2M		EGFRvIII-CAR	Solid tumor (U87vIII)		Effector function and persistence	[55]
	PDCD1/ TRBC/β_2M		PSCA-CAR	Solid tumor (PC3)		Effector function	[60]
			CD19-CAR	Liquid tumor (NALM-6)			
	TGF-β receptor II		MESOTHELIN-CAR	Solid tumor (CRL56826 cell line transduced with TGF-β1 and OVCAR3)			[59]
Transcription factor	BATF		CD19-CAR	Liquid tumor (CD19$^+$K562)		Effector function and persistence	[61]
			HER1-CAR	Solid tumor (NCI-H226)	In vitro		
			MESOTHELIN-CAR	Solid tumor (OVCAR3 and NCI-H226)	In vitro In vivo		
	ID3		MESOTHELIN-CAR	Solid tumor (AsPC-1)	In vitro	Effector function	[62]
	IKZF3		CD133-CAR	Solid tumor (CD133$^+$U251)			[63]
			HER2-CAR	Solid tumor (MDA-MB-453)	In vitro In vivo		
	PRDM1		CD19-CAR	Liquid tumors (CD19$^+$K562 and NALM-6)		Effector function and persistence	[64]
			MART1-tgTCR	Liquid tumor (T2)	In vitro		
			MESOTHELIN-CAR	Solid tumor (MESOTHELIN$^+$K562)			
	PRDM1/ NR4A3		CD19-CAR	Liquid tumor (NALM-6)	In vivo		[65]
			MESOTHELIN-CAR	Solid tumor (AsPC1)	In vitro		
			PSMA-CAR	Solid tumor (PSMA$^+$PC3)	In vitro In vivo		
	SOX4		MESOTHELIN-CAR	Solid tumor (AsPC1)	In vitro	Effector function	[62]

receptors (A2AR, FAS, TGF-β receptor II) as attractive targets to improve T cell functions, with their deletion boosting the efficacy of engineered T cells in both hematologic and solid tumor models. These knockouts notably allowed the increase of T cell functions by decreasing the expression of inhibitory receptors, and enhancing the secretion of Granzyme B, IFN-γ and TNF-α, which allowed better tumor control [46, 54–60]. Expanding beyond cell surface targets, teams have explored intracellular targets implicated in T cell regulation, such as transcription factors [61–65]. For instance, Jung et al. utilized single-cell RNA-sequencing on autologous CAR-T infusion products administered to patients, and they identified an inverse correlation between *PRDM1* and *TCF7* gene expression [65]. Subsequent validation in chronic lymphocytic leukemia patients treated with CD19-CAR-T therapy confirmed elevated *PRDM1* gene expression in non-responders and higher *TCF7* gene expression in responders. Finally, they proved that knocking-out *PRDM1* resulted in CAR-T cells maintaining an early memory phenotype and enhanced cytotoxicity, although sustained improvement over time was not observed [65]. Yoshikawa et al., had previously observed that *PRDM1*-edited CAR-T cells expressed more TOX and exhibited comparable expression of inhibitory receptors compared to unedited CAR-T cells [64]. This observation has also been confirmed by Jung's team, who performed bulk RNA-Sequencing and identified an upregulation of exhaustion-characterized transcription factors such as NR4A and TOX members in chronically stimulated human CAR-T cells as well as mouse tumor infiltrating T cells (TILs) [65]. Building upon these findings, the team performed a double-knockout of *PRDM1* and *NR4A3* genes, thereby improving CAR-T effector functions in both solid and liquid tumor models, even after tumor rechallenge [65]. Similarly, Zhang et al. conducted an in-depth analysis of exhaustion-related gene expression in patients suffering from liver, colorectal and non-small cell lung cancer (NSCLC). By aligning this data with genes upregulated in their in vitro exhaustion model, they underscored the potential of targeting a specific transcription factor, BATF, to reinforce T cells against dysfunction. This approach not only heightened resistance to dysfunction but also amplified tumor clearance efficacy by increasing the central memory T cell subset [61]. These investigations have also piqued the interest of other research teams, leading them to target genes such as *ID3*, *IKZF3*, *TOX1/2*, or *SOX4* [32, 62, 63]. The *ID3* and *SOX4* genes have notably been shown to be co-expressed with other dysfunction signature genes and their knockouts significantly improved the potency of CAR-T cells in killing targeted tumor cells, while TOX double knockouts showed decreased expression of *NR4A1/2* and multiple inhibitory receptors genes such as *PDCD1*, *HAVCR2* and *LAG3*, as well as increased expression of granzymes in T cells [32, 62, 63]. The double knockout of *NR4A1/2* also showed improved antitumor immunity by increasing $TCF1^+$ stem-like precursors of exhausted $CD8^+$ T cells, as well as enhanced persistence in the tumor microenvironment [66]. Additionally, scrutiny has also extended to proteins involved in T cell signaling, thus unveiling promising candidates such as *DGK*, *DNMT3A*, as well as *SUV39H1* to optimize engineered T cells [62, 67–69]. For instance, *DGK* targeting has been shown to amplify T cell effector capacities by improving their proliferation and expression of effector cytokines, like IL-2 and IFN-γ, even following repeated exposure to tumor stimuli [67]. Similarly, targeting epigenetic proteins, such as DNMT3A and SUV39H1, has proven effective in preserving a memory-like phenotype, evidenced by the expression of CD62L and improvements in metabolic fitness. These cells also demonstrated prolonged and enhanced control over tumor progression, even upon subsequent tumor rechallenges [68, 69]. The knockout of some other genes, like *PTP1B* and *PTPN2*, has also demonstrated its potential in murine models to enhance T cell functions by exhibiting higher capacities of eliminating tumor cells. Both these knockouts increased not just the capacity of CAR-T cells in killing their tumor target, but also their antigen-specific activation notably by enhancing STAT5 signaling [70, 71]. Overall, edited CAR- and tgTCR-T cells in these studies exhibited increased effector cytokines, heightened proliferative and cytotoxic capacities, and diminished exhaustion markers. Some investigations even suggested potential enhancements in metabolic fitness.

CRISPR SCREENING

In recent times, the utilization of CRISPR screens has witnessed a notable surge as a potent strategy for identifying target genes deemed worthy of knockout, thereby enabling large-scale genetic loss-of-function experiments [72]. This technique can be executed through either pooled or arrayed CRISPR screens [73]. In the former, a library of CRISPR gRNAs is introduced into a population of Cas9-expressing cells, each affected based on the gRNAs. In the latter, the cells are individually distributed into wells, allowing distinct gene perturbations to be discerned. After a period of cultivation to select surviving cells, edited T cells can be chosen based on the analysis of specific biomarkers or subsequent to a challenge. This challenge may involve drug treatment, virus infection, metabolic stresses, or in vitro/in vivo exhaustion protocols [72, 73]. Subsequently, the heterogeneous population of edited T cells undergoes next-generation sequencing, unveiling a comprehensive landscape of sequence expression. Finally, specific gRNAs are selected to deplete identified genes, whose inhibition confers a beneficial advantage.

This screening strategy has been extensively employed to unravel T cell biology and unearth potential targets (Table 2).

Genes such as *ARID1A*, *CBL-B*, *CUL5*, *DAP5*, *DHX37*, *FLI1*, *IKZF2*, *IRF2*, *RGS16*, *SOCS1*, *TCEB2*, and *TLE4* have been identified as functional regulators of T cells, presenting intriguing knockout targets to optimize T cell immunotherapies [74–83]. For instance, Freitas and colleagues identified *CCNC* and *MED12* genes as promising targets using chronic antigen exposure models. The specific deletion of these genes in human CAR-T cells improved their proliferative, cytokine secretion, and cytotoxic capacities. Moreover, they showed that MED12-disrupted CAR-T cells had sustained effector functions even upon chronic stimulation with a higher secretion of IL-2 and IFN-γ, a lower expression of CD39, as well as better tumor growth control. Improved antitumor responses have also been observed using a NY-ESO-1-tgTCR against NY-ESO-1^+ melanoma cells [80]. Another example is Carnevale and colleagues who proved that RASA2-edited NY-ESO-1-specific TCR T cells as well as CD19- and EphA2-edited CAR-T cells displayed enhanced in vivo antitumor capabilities across hematologic and solid tumor models, indicating improved control of tumor growth and increased survival in mice [84]. Another significant discovery was the identification of the gene *SNX9*, by Trefni et al. through CRISPR screening [85]. Their research revealed that SNX9 was co-expressed with TIM-3 among PD-1 positive TILs of patients suffering from NSCLC, and it was also highly expressed in TILs of melanoma patients with poor responses to immune checkpoint blockade therapy. By mediating CRISPR knockout of *SNX9*, the team demonstrated enhanced antitumor efficacy of NY-ESO-1-directed TCR T cells in an in vitro exhaustion model, with the enhancement being characterized by an increase in cytokine production and the level of CCR7 memory marker, as well as a decrease in the levels of TOX and NR4A1/3 transcription factors [85]. The efficacy of this approach was also evident in a CD19-CAR-T model, displaying prolonged control of tumor volume, elevated IFN-γ expression, and reduced levels of the immunosuppressive cytokine IL-10 [85]. In addition, Wei and colleagues identified *REGNASE-1* as a potential target due to its heightened expression in TILs, and its deletion improved adoptive cell transfer against both solid and liquid tumors by promoting the development of long-lived effector phenotypes [86]. Collectively, these findings underscore the burgeoning significance of CRISPR screening as a

Table 2. CRISPR screening targets model's summary.

Protein type	Target	Method for gene knockout	Transgene	Tumor model	Assay	Impact	Ref
Cell-signaling protein	CCNC	Viral	CD19-CAR	Liquid tumor (NALM-6)	In vitro In vivo	Effector function	[80]
			HA-28z-CAR	Liquid tumor ($GD2^+$NALM6)			
			HER32-CAR	Solid tumor (143B)			
	CUL5		CD19-CAR	Liquid tumor (NALM-6)	In vitro		[81]
	MED12		CD19-CAR	Liquid tumor (NALM-6)	In vitro In vivo		[80]
			HA-28z-CAR	Liquid tumor ($GD2^+$NALM6)			
			HER32-CAR	Solid tumor (143B)			
			NY-ESO-1-tgTCR	Solid tumor (A375)			
	RASA2		CD19-CAR	Liquid tumor (NALM-6) and solid tumor ($CD19^+$A375)		Effector function and persistence	[84]
			EphA2-CAR	Solid tumor (LM7)	In vivo		
			NY-ESO-1-tgTCR	Liquid tumor (NY-ESO-1^+NALM6 and T2) and solid tumor (A375)	In vitro In vivo		
	SNX9		CD19-CAR	Liquid tumor (Raji)	In vivo		[85]
			NY-ESO-1-tgTCR	Liquid tumor (T2)	In vitro		
Transcription factor	IKZF2		IL-13Rα2-CAR	Solid tumor (IDH1/2)	In vitro In vivo	Effector function	[79]
			HER32-CAR				
	TLE4		IL-13Rα2-CAR				
			HER32-CAR				

potent tool for identifying novel key regulators of T cell functions. These findings suggest that CRISPR screening can be employed to edit and optimize CAR- and tgTCR-T cells, thereby enhancing the efficacy of adoptive cell therapy.

TARGETS AND READOUTS

CRISPR-Cas, owing to its precise mode of action, offers a targeted modality for editing cells, focusing on membrane-bound or intracellular drivers that naturally regulate T cell effector functions. This intricate targeting activates complex biological signaling pathways crucial for effective antitumor immunity, presenting a promising avenue for optimizing engineered T cells (Fig. 4).

Within the spectrum of potential targets, specific cell surface receptors, acting as primary conduits for inhibitory signals, can be strategically deleted to mitigate the negative impacts induced by interactions with their ligands. In this context, the adenosine receptor A2AR, the TGF-β receptor II, as well as the death receptor FAS, can be effectively switched off through genic invalidation to prevent unfavorable outcomes associated with the fixation of their respective ligands [54, 56–59, 87].

Additionally, various cell-signaling proteins essential for cellular communication and specific responses, have also been targeted. Proteins such as E3 ubiquitin ligase CBL-B, crucial for post-translational modification, and RASA2, a Ras GTPase-activating protein, play pivotal roles in fundamental cellular processes [84, 88]. Moreover, epigenetic targets, including DNMT3A or ARID1A, have demonstrated significant potential in improving T cell functions, particularly considering the observed epigenetic changes during the development of dysfunctional T cells [68, 82, 89].

Furthermore, transcription factors, with their versatility in regulating multiple genes based on environmental cues, have become focal points for optimizing T cell function. For instance, *PRDM1* has been shown, through various studies, to be implicated in T cell differentiation and exhaustion [64, 65]. However, as transcription factors possess the ability to specifically regulate gene expression, their impact can lead to different outcomes as exemplified by the contrasting effects of the *BATF* and *IRF4* genes in terms of their antitumoral activities, depending on the experimental models employed [61, 90].

The selection of an ideal candidate for knockout involves a meticulous analysis of various readouts to gauge its impact on T cell survival, proliferation, fitness, and overall functionality. Parameters such as cell survival, memory phenotype, expression of activation and inhibitory molecules, proliferation ability, cytokine expression, as well as cytotoxic capacities are assessed in vitro or in vivo [57, 61, 63–65, 68, 85, 86]. Additionally, transcriptomic analysis is often employed for a comprehensive screening of genes reprogrammed after CRISPR editing, leading to further investigations providing insights into cell phenotype, as well as metabolic and epigenetic profiles [68, 80, 84].

DISCUSSION AND CONCLUSION

Engineered T cells have demonstrated success in eliciting antitumor immune responses [4, 11]. However, the rise of dysfunctional T cells poses a significant hurdle, leading to patient relapse [4, 15]. To address this major limitation, the evaluation of next-generation engineered T cells using CRISPR-Cas tool is ongoing in various studies [45]. However, selecting relevant genes for deletion poses a challenge, given the extent of their impact on cellular biology. Consequently, substantial efforts have been invested in developing new strategies to identify undesirable genes for knockout, with the aim of optimizing T cell performance.

While the knowledge-based approach laid the foundation for understanding T cell dysfunction, CRISPR screening has significantly broadened the scope. The comprehensive insights gained from this screening offer a nuanced understanding of gene functions, allowing researchers to pinpoint specific targets tailored for clinical applications. The selection process of a relevant target involves a deep evaluation of several factors to assess the impact of these genes on T cell survival, proliferation, and overall fitness. Transcriptomic analysis additionally provides valuable insights into

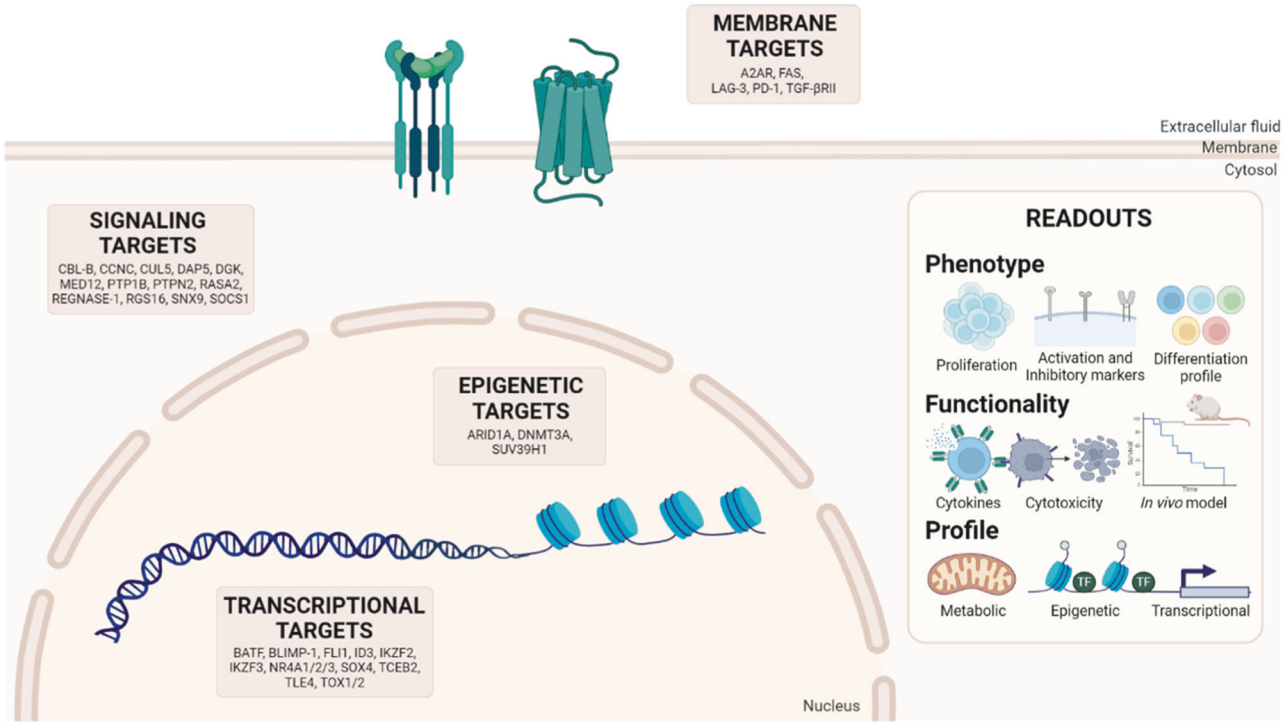

Fig. 4 Summary of CRISPR-Cas targeted genes and readouts used to improve T cell effector functions in the field of cancer immunotherapy.

the post-CRISPR editing reprogramming of genes. This approach notably led to the understanding of T cell fate in cancer, which is crucial for the identification of reprogrammable determinants for cancer immunity [91]. For instance, Wu et al. identified BHLHE40 as a key differentiation checkpoint between the subsets of exhausted T cells by using in vitro chronic stimulation model, while Zhou's team highlighted the gene regulatory network implicated in the effector function capacities of exhausted T cells through in vivo single-cell CRISPR screens [91, 92].

Nonetheless, due to the diversity of the readouts used, interstudy analyses are not relevant. In addition, even though some genes highlighted in CRISPR screens, such as *CBL-B*, *TCEB2*, or *RASA2*, have also been independently corroborated by prior research using knowledge-based or screening approaches, thereby showcasing the reliability of CRISPR screens, discrepancy remains in the identification of interesting targets to knockout [78, 84, 93]. This can be due to the different screening models used, and also the tumor antigen targeted, as this would significantly affect the induction of T cell dysfunction. Hence, due to the tumor microenvironment and the expression profile of the antigen being variable according to the therapeutic indications, it might be interesting to compare the different knockouts in the different conditions to potentially find the best targets to improve T cell functions. As an example, Larson's team identified the IFN-γ receptor pathway affecting the sensitivity of solid tumor cells, but not liquid tumor cells, to CAR-T cells [94]. Finally, apart from finding the best genes to knockout, the design of the CAR might also be taken into consideration, as this would significantly affect T cell susceptibility of dysfunction. For instance, it has been shown that CAR-T containing 4-1BB costimulation domain were a better option to prevent exhaustion domain, but they were less efficient than CAR-T containing CD28 to target tumor expressing few antigens [95]. Hence, there is no one-size-fits-all response regarding the choice of the targets to knockout.

In the realm of the clinical application for CRISPR-Cas targets, the prospect is promising, as evidenced by the success of CRISPR-Cas in pre-clinical studies and ongoing clinical trials (Tables 1 to 3) [45].

Studies concerning the therapeutic applications of CRISPR-engineered T cells primarily aim to facilitate allogeneic transfer and prevent fratricide of CAR-T cells by targeting TCR, MHC-I, MHC-II, or proteins like CD70. Some investigations have chosen to focus on alternative proteins such as PD-1, TGF-β receptor II, and Regnase-1 to reinvigorate T cells, counteract tumor microenvironment suppression, and enhance cell proliferation [96]. For instance, Lu and colleagues conducted a trial (NCT02793856) demonstrating the safety and feasibility of PD-1 knockout in T cells from metastatic NSCLC patients. In their study, adverse events were limited to grade I or II, and edited-T cells were detectable post-infusion [47]. Similarly, Carl June's team demonstrated the feasibility of this approach in a trial (NCT03399448) involving patients with advanced refractory myeloma and metastatic sarcoma, with durable and well-tolerated engraftment being observed [46].

However, while the CRISPR-Cas system serves as a potent tool to make precise modifications to the genome, it is not always error-free [97, 98]. CRISPR-Cas systems may edit genomic sequences similar to the targeted gene, leading to unintended DNA cleavage at off-target locations [99]. As a result, chromosomal aberrations or unintended generation of structural variants might be safety concerns when developing CRISPR-Cas editing [100, 101]. To overcome this phenomenon, CRISPR-base and CRISPR-prime editing could serve as interesting alternatives, as they can induce point mutations without a double-strand DNA break [102]. Moreover, the implementation of rigorous quality control should always be assessed to evaluate the safety of the CRISPR-products. Furthermore, beyond safety concerns, CRISPR-Cas technology also encounters other challenges affecting its effectiveness, such as optimizing gRNA specificity and stability, enhancing Cas protein efficiency, refining delivery methods, addressing cell proliferation, and considering genomic context [103]. However, despite taking these parameters into consideration, achieving a near-perfect knockout is often unattainable, resulting in a heterogeneity of edited cells. This variability, along with the occurrence of monoallelic and biallelic CRISPR modifications, may impact knockout effectiveness. Consequently, the acceptable proportion

Table 3. CRISPR-Cas clinical trials undertaken to optimize engineered T cells.

NCT Number	Target	Transgene	Tumor model	Phase	Results	Status
NCT03399448	TRAC/TRBC/ PDCD1	NY-ESO-1-tgTCR	Multiple Myeloma, Melanoma, Synovial Sarcoma, Myxoid/Round Cell Liposarcoma	I	Safe and feasible approach, with great engineered-T cell persistence	Completed
NCT05722418	TRAC/β_2M	BCMA-CAR	Relapsed/Refractory Multiple Myeloma	I	-	Recruiting
NCT06128044	β_2M/PDCD1	CCL-1-CAR	Relapsed/Refractory Acute Myeloid Leukemia	I	-	Recruiting
NCT04037566	HPK1	CD19-CAR	Relapsed/Refractory Leukemia or Lymphoma	I	-	Recruiting
NCT04637763	TRAC/PDCD1	CD19-CAR	Relapsed/Refractory B Cell Non-Hodgkin Lymphoma	I	-	Recruiting
NCT04557436	TRAC/TRBC/CD52	CD19-CAR	Relapsed/Refractory B Cell Acute Lymphoblastic Leukemia	I	-	Active
NCT03545815	TRAC/PDCD1	MESOTHELIN-CAR	Mesothelin-Positive Multiple Solid Tumors	I	-	Completed
NCT03398967	TRAC/CD52	CD19/CD20-CAR and CD19/CD22-CAR	Relapsed/Refractory Leukemia or Lymphoma	I/II	-	Unknown
NCT04244656	TRAC/β_2M	BCMA-CAR	Relapsed/Refractory Multiple Myeloma	I	-	Active
NCT03747965	PDCD1	MESOTHELIN-CAR	Mesothelin-Positive Solid Tumors	I	Safe and feasible approach	Completed
NCT04035434	TRAC/β_2M	CD19-CAR	Relapsed/Refractory B Cell Malignancies	I	-	Recruiting
NCT04438083	TRAC/β_2M/CD70	CD70-CAR	Relapsed/Refractory Renel Cell Carcinoma	I	Safe, with encouraging antitumor activity	Active
NCT03166878	TRAC/β_2M	CD19-CAR	Relapsed/Refractory Leukemia or Lymphoma	I/II	-	Completed
NCT05795595	TRAC/β_2M/TGF-βRII/ Regnase-1/CD70	CD70-CAR	Relapsed/Refractory Solid Tumors	I/II	-	Recruiting
NCT05643742	TRAC/β_2M/TGF-βRII/ Regnase-1/CD70	CD19-CAR	Relapsed/Refractory Leukemia or Lymphoma	I/II	-	Recruiting
NCT04502446	TRAC/β_2M/CD70	CD70-CAR	Relapsed/Refractory T or B Cell Malignancies	I	-	Recruiting
NCT04976218	TGF-β receptor II	EGFR-CAR	Advanced EGFR-Positive Solid Tumors	I	-	Recruiting
NCT05812326	PDCD1	MUC1-CAR	MUC1-Positive Advanced Breast Cancer	I/II	-	Completed
NCT05631912	TRAC	CD19-CAR	Relapsed/Refractory B Cell Non-Hodgkin Lymphoma	I/II	-	Recruiting
NCT06014073	TRAC/POWER3	CD19-CAR	Relapsed/Refractory B Cell Non-Hodgkin Lymphoma	I/II	-	Recruiting

of edited cells may vary on the therapeutic applications, and may require cell sorting as is the case in allogeneic settings [104].

Although this review has primarily focused on the tumor microenvironment, it is essential to recognize that various other mechanisms also limit the full potential of engineered T cells. These include obstacles such as compromised cell infiltration into tumors, antigen heterogeneity, evasion of tumor targets, poor persistence or fratricide of engineered T cells, allogeneic rejection, and associated toxicities [103]. In this context, CRISPR-Cas has demonstrated and may continue to demonstrate additional potential. As an example, to improve the persistence of engineered T cells, transgenic cytokine expression has emerged as an intriguing strategy, as a few of cytokines have been shown to be beneficial in promoting their expansion and persistence capacities [105]. It has been observed in various studies that the induction of not only IL-7 and CCL19, but also IL-15 and IL-21 inductions enhanced the survival and effector functions of CAR-T cells [106, 107]. Hence, it could be of interest to validate this approach by employing the CRISPR-Cas tool to optimize engineered T cells. Moreover, in the effort to enhance T cell trafficking into tumors, some researchers have developed T cells expressing chemokine receptors of interest [108]. Several teams have already shown the potential of this approach by notably integrating various receptors such as CXCR1/2/4/6, CCR2/4/8 and CX3CR1 through viral transduction, as summarized in the review of Foeng et al. [108]. Besides, Marson's team also showed the possibility of this approach by incorporating CXCR4 receptors into T cells using the CRISPR-Cas system [109]. Moreover, on account of all genetic modifications that might be performed on T cells, induced pluripotent stem cells approach could be an interesting alternative as it allows us to perform multiple modifications and selections at the same time, while preserving similar therapeutic outcomes to primary T cells approach [110, 111].

In summary, thanks to its numerous benefits and possibility of improvement, the CRISPR-Cas system is definitively a promising tool for optimizing and developing the next-generation of engineered T cells.

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ADDITIONAL INFORMATION

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