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Review

Reprogramming the anti-tumor immune response via CRISPR genetic and epigenetic editing

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Precise clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genetic and epigenetic manipulation of the immune response has become a promising immunotherapeutic approach toward combating tumorigenesis and tumor progression. CRISPR-based immunologic reprograming in cancer therapy comprises the locus-specific enhancement of host immunity, the improvement of tumor immunogenicity, and the suppression of tumor immunoevasion. To date, the *ex vivo* re-engineering of immune cells directed to inhibit the expression of immune checkpoints or to express synthetic immune receptors (chimeric antigen receptor therapy) has shown success in some settings, such as in the treatment of melanoma, lymphoma, liver, and lung cancer. However, advancements in nuclease-deactivated CRISPR-associated nuclease-9 (dCas9)-mediated transcriptional activation or repression and Cas13-directed gene suppression present novel avenues for the development of tumor immunotherapies. In this review, the basis for development, mechanism of action, and outcomes from recently published Cas9-based clinical trial (genetic editing) and dCas9/Cas13-based pre-clinical (epigenetic editing) data are discussed. Lastly, we review cancer immunotherapy-specific considerations and barriers surrounding use of these approaches in the clinic.

The human immune response consists of a complex and diverse array of molecular and cellular processes to differentiate between self and non-self, which allows it to defend and protect the host from pathogen infection, cellular damage, or neoplastic transformation. In the context of cancer, the constant selective pressure exerted by the host immune response often results in the selection of tumor variants capable of immune evasion that enable tumor cells to survive. Broadly, these tumor evasion mechanisms can involve (1) the accumulation of suppressive cells, such as $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs), in the cellular microenvironment;[1,2] (2) down-modulation of the antigen processing and presentation pathways within the cancer cell;[3–5] (3) shedding of stress, damage, or transformation markers at the cancer cell surface, including the six UL16 binding proteins (ULBP1–ULBP6) and major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICA) and B (MICB);[6–8] (4) secretion of immunosuppressive cytokines, particularly transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), and interleukin-10 (IL-10), into the surrounding microenvironment;[9–12] and (5) upregulation of immune checkpoint ligands, especially those pertaining to the programmed cell death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4) pathways.[13–17] Although the last few decades have brought an improved understanding of the underlying molecular mechanisms of these immune evasion strategies, the development of safe and broadly effective immunotherapies that overcome these barriers and are applicable to multiple cancer types remains a formidable task. Accordingly, considerable research focus has been directed toward harnessing the high specificity of clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genetic and epigenetic editing as emerging precision therapeutics to counter the aforementioned immune evasion mechanisms and improve anti-tumor immunity.

Numerous reviews to date have explored CRISPR-mediated genetic and epigenetic editing, yet comprehensive overviews of clinical and pre-clinical CRISPR technologies in cancer immunotherapy, particularly in the emerging epigenetic editing space, remain scarce. In this review, a brief outline of the mechanisms underlying the CRISPR-Cas9 (CRISPR-associated protein 9), dCas9 (nuclease deactivated Cas9) and Cas13 processes is presented, which encompasses the most advanced and clinically relevant DNA and RNA-targeting CRISPR systems developed thus far.[18] This is then followed by an update on clinical Cas9-based and pre-clinical dCas9- and Cas13-mediated anti-tumor immunotherapies.

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Table 1. Variation in protospacer-adjacent motif sequences across major Cas9 orthologs

CRISPR nuclease	Length (aa)	Organism	PAM sequence (5′→3′)[a]	Reference
FnCas9	1,629	*Francisella novicida*	NGG	[22]
TdCas9	1,423	*Treponema denticola*	NAAAAN	[23]
SpCas9	1,368	*Streptococcus pyogenes*	NGG, NAG	[21,23–25]
St1Cas9	1,122	*Streptococcus thermophiles* 1	NNARAAW	[23,26–28]
NmCas9	1,109	*Neisseria meningitides*	NNNNGATT	[23,29,30]
SaCas9	1,053	*Staphylococcus aureus*	NNGRRN	[31,32]
CjCas9	984	*Campylobacter jejuni*	NNNVRYM	[33,34]

[a]N = A/C/T/G; W = A/T; R = A/G; V = A/G/C; Y = T/C; M = A/C.

CRISPR-CAS-BASED SYSTEMS ALLOW FOR TARGETED GENETIC AND EPIGENETIC EDITING

The CRISPR-Cas9 system

Originally identified as a key defensive mechanism against invading viruses and plasmids in prokaryotic genomes,[19,20] CRISPR-Cas systems have since been adapted for RNA-programmable genome editing.[21] The most common and best studied of the CRISPR-Cas systems are those that involve the large, multi-domain endonuclease, CRISPR-associated protein 9 (Cas9). The DNA cleavage by Cas9 requires a single-stranded short guide RNA (sgRNA), consisting of a programmable target-specific 20-nt CRISPR RNA (crRNA) base paired to a small non-coding *trans*-activating crRNA (tracrRNA). Additionally, Cas9 requires a conserved protospacer-adjacent motif (PAM) sequence for activity that maps upstream and adjacent to the crRNA-binding region. The PAM sequence varies depending on the organism of origin and affects the frequency and specificity of the editing process (Table 1). Upon sequence-specific binding between the crRNA and target DNA, the Cas9 protein is recruited to the PAM sequence via its PAM-interacting domain. After binding, the separation of the target DNA is initiated at the PAM-adjacent nucleation site and double-stranded breaks (DSBs) are produced. The DSBs can be repaired by either homology-directed repair (HDR) or non-homologous end-joining (NHEJ) (Figure 1A).[21,26] The efficiency of genome editing in this way varies greatly and is dependent on several factors, including the nature of the target and delivery method used. Notably, Mussolino et al.[35] reviewed the editing frequencies achieved in the case of the hematopoietic system and found that *ex vivo* editing is often 10%–30% efficient, whereas *in vivo* editing varies between 1% and 16%. However, with an optimized delivery system specific for the target cells, the Cas9 editing efficiency can increase up to 80%.[36,37]

The CRISPR-dCas9 system

Beyond the success of the CRISPR-Cas9 system, the creation of the nuclease-deactivated Cas9 (dCas9) variant[38] has widened the scope of CRISPR technologies into the field of epigenome engineering. The CRISPR-dCas9 system differs from the wild-type by two mutations (D10A and H840A), which inactivate Cas9's cleavage capacity, while maintaining its RNA-guided DNA-binding specificity.[38] As initially shown with engineered zinc finger proteins,[39–46] dCas9 can be fused with various effector domains to mediate precise and programmable transcriptional activation or repression, editing of epigenetic marks, and fluorescent tagging of endogenous genes, all without directly editing the genome (Figure 1B).[47–52]

Locus-specific transcriptional manipulation in a guide-dependent manner was first achieved by fusing the VP64 (four copies of VP16, a herpes simplex virus transcription factor) recruiter of transcriptional activators,[53–55] as well as Krüppel-associated box (KRAB) recruiter of transcriptional repressors,[38,53] to dCas9. Since then, more complex arrays of mechanistically distinct effector domains have been described, which greatly improve the capability of dCas9 to induce transcriptional changes. This includes the VPR (tandem fusion of VP64, p65, and Rta domains[56] to generate the hybrid tripartite activator) in the case of gene activation, and the tandem fusion of KRAB with the TRD domain of MeCP2 to produce the dCas9-KRAB-MeCP2 repressor.[57]

As the arrays of effectors directly fused to dCas9 increase the size of the resulting protein, which in turn impact the expression of dCas9 and the intracellular delivery, alternative assembly methods have been developed. Notably, aptameric motifs engineered in combination with the gRNA scaffold (such as two copies of an RNA hairpin from the MS2 bacteriophage) were first described in the context of gene activation with the synergistic activation mediator (SAM) system.[58] This approach has been exploited to combine multiple activator domains derived from epigenetic enzymes, such as the catalytic domain of DNA demethylases (e.g., TET1[59,60]) or histone acetyltransferases (e.g., p300[61]), to generate high levels of gene activation. Alternatively, repetitive peptide arrays that amplify and recruit specific designer antibody-fusion proteins can be fused to dCas9, as shown in the supernova tagging (SunTag)[62] system. The dCas9-SunTag is based on single-chain variable fragment antibodies and the corresponding epitope, which offers major advantages, including high affinity and recognition of short peptide sequences. This system has previously been adapted to recruit DNA methyltransferases, including DNMT3A[63] and DNMT3L,[64] in order to induce locus-specific repression via DNA methylation, with minimal off-target binding.[65] Importantly, these next-generation dCas9 systems (SAM and SunTag) provide highly specific, effective, and tunable tools for targeted epigenetic manipulation. Lastly, the further development of these technologies to enable simultaneous expression of multiple gRNAs (multiplexed transcriptional manipulation of distinct genes)[66,67] continues to widen the scope and translatability of dCas9-based epigenetic editing.

In the arena of oncology, these dCas-based tools have demonstrated significant activation of tumor suppressor genes, such as *PTEN* (in breast cancer and melanoma),[48] *MASPIN* (in breast and lung cancers),[47,49] *REPRIMO* (in breast and gastric cancers),[47] *SARI* (in colon cancer),[68] and *DKK3* (in prostate cancer).[69] Similarly,

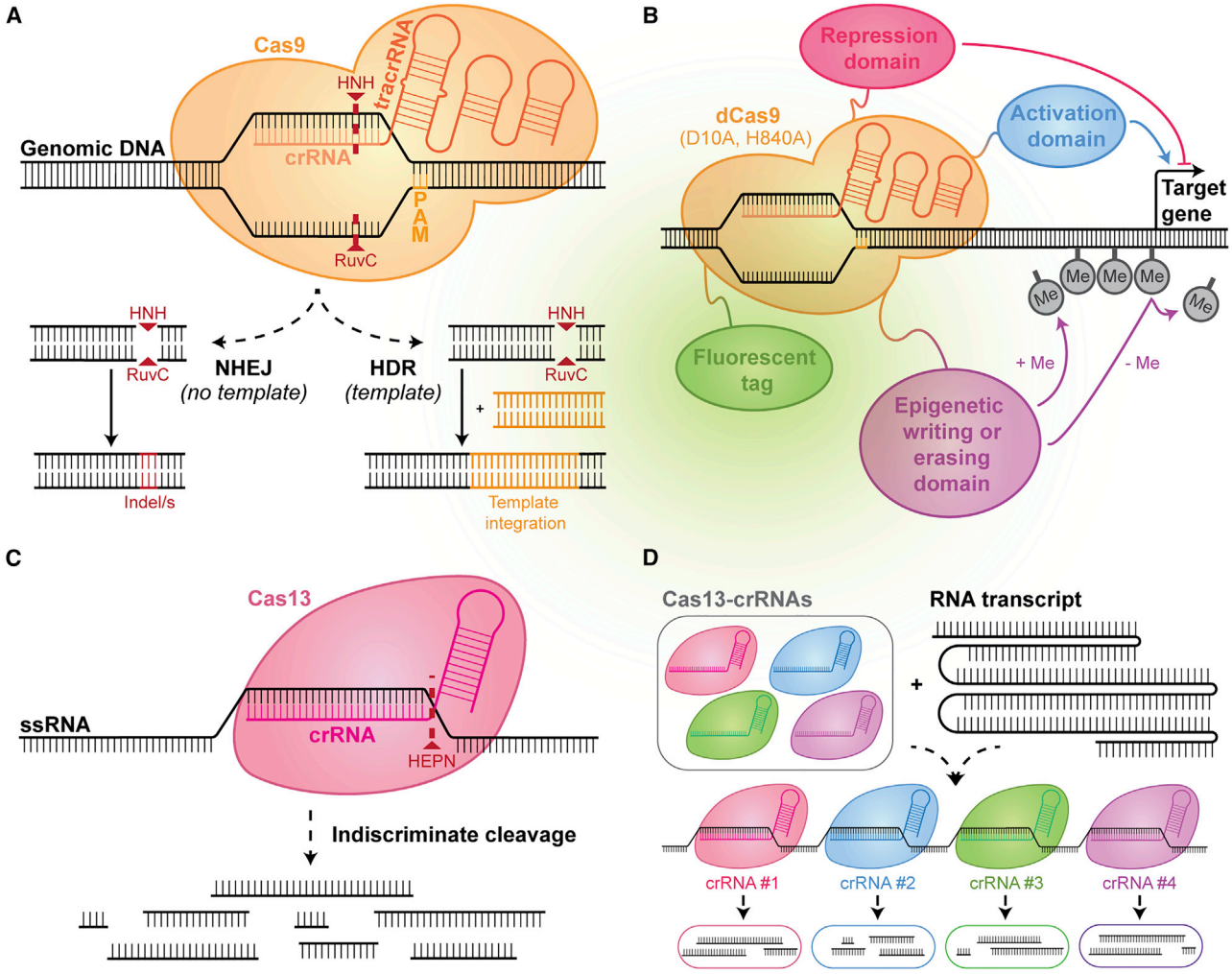

Figure 1. Mechanisms underlying CRISPR-Cas9, dCas9, and Cas13 processes

(A) A 20-nt programmable CRISPR RNA (crRNA) directs the Cas9 complex to the target DNA. Upon recognition of the protospacer-adjacent motif (PAM) sequence at the target location, the DNA strand is separated and Watson-Crick base pairing between the genomic DNA and crRNA is achieved. The HNH domain cleaves the target DNA strand complementary to the crRNA sequence, whereas the RuvC domain cleaves the non-complementary strand. If no donor-corrected template is available, non-homologous end joining (NHEJ) occurs whereby random insertions and/or deletions are incorporated, rendering the gene non-functional or disrupted. Alternatively, if a template is available, homology-directed repair (HDR) is initiated, where the provided template is inserted into the cut genomic DNA, thereby allowing for gene correction or addition. (B) Mutations (D10A, H840A) in both cleavage domains of Cas9 generates a nuclease-deactivated protein (dCas9). Despite no cleavage activity, dCas9 preserves its high binding specificity and searching capability. Therefore, fusion of dCas9 to specific domains expands its functionality to transcriptional repression (red) or activation (blue), fluorescent tagging (green), and/or epigenetic writing or erasing (purple), such as methylation (+Me), demethylation (−Me), acetylation, and chromatin reading or remodeling. (C) A 28- to 30-nt programmable crRNA directs the Cas13 complex to the target single-stranded RNA (ssRNA) transcript. Watson-Crick base pairing between the target ssRNA and crRNA initiates non-specific splicing of the target transcript, as well as any nearby transcripts, irrespective of complementarity to the crRNA. This collateral RNase activity is attributed to Cas13's promiscuous dual HEPN domains. (D) An arsenal of Cas13-crRNA complexes can be programmed to target sites across any ssRNA transcript. Cleavage of RNA in this way allows for inhibition of post-transcriptional gene expression.

dCas9-mediated oncogene suppression has been achieved successfully in colon cancer (*BRAF*,[70] *HER2*,[71] and *MYC*[72]), pancreatic cancer (*KRAS*),[70] and liver cancer (*GRN*).[73] In addition, several works outline that epigenome editing can be highly efficient, having achieved nearly complete gene repression[38] or robust (several fold) gene activation,[56] with minimal off-target effects, which mainly depend on the nature of effector domains used. For the most studied domains, such as VP64 and KRAB, off targets have been shown to be either zero or have negligible effects on non-cognate gene transcription.[74] Finally, whereas Cas9 genome engineering unavoidably results in permanent changes, epigenetic approaches are reversible, circumventing the risk of inducing sequence changes in the target DNA,[71,75] a key factor in the targeting of tumors harboring high degrees of genetic instability. Moreover, the durability of the epigenetic and

transcriptional changes induced by dCas9 editing can vary depending on the specific combination of effectors and may be dependent on the targeted loci. Thus, current research in the field of epigenome engineering faces the challenge to adapt the technology for the manipulation of different loci in diverse cell types with differing chromatin microenvironments.[76]

The CRISPR-Cas13 system

The reduced RNA-cleavage efficiency and the likelihood for off-target effects on host DNA have meant that RNA-targeting orthologs of Cas9 are unlikely to be useful in targeting RNA transcripts directly, such as non-coding RNA (ncRNA), messenger RNA (mRNA), or viral RNA genomes.[77,78] Moreover, existing RNA interference (RNAi) technologies also exhibit substantial off-target effects.[79] As such, increasing focus is being directed toward the development of novel CRISPR-based technologies utilizing RNA-specific Cas proteins. To date, the most successful of these technologies are those exploiting Cas13, of which four subtypes have been identified thus far: Cas13a (formerly known as C2c2), Cas13b, Cas13c, and Cas13d.[80] In prokaryotes, Cas13 functions as an RNA-guided RNA endonuclease and operates as a defensive mechanism specific for viral RNAs.[81] Unlike Cas9, the Cas13 effector family contains two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains as its catalytic effectors, which confer RNase activity (Figure 1C). In addition, the Cas13 protein can form a complex with multiple crRNAs to cleave at multiple locations along an RNA transcript with high specificity and offers an alternative gene knockdown method to RNAi technologies (Figure 1D).[82,83] Beyond Cas13's cleavage capability, Zhang and colleagues[84–86] have pioneered the potential of catalytically inactive Cas13 (dCas13) in several applications. In particular, dCas13 has been fused with fluorescent tags to precisely label target RNA molecules and assess RNA-specific intracellular localization. Similarly, the fusion of dCas13 with deaminase domains from adenosine deaminases specific for RNA (ADARs) has been shown to mediate precise RNA editing to alter full-length transcripts containing pathogenic mutations.

Individually and in combination, Cas9, dCas9, and Cas13 have shown great promise as therapeutic options for multiple diseases, such as cancer,[70,87,88] viral infection,[82,89,90] non-viral infection,[91,92] and autoimmunity.[93,94] In oncology, the ability of CRISPR-Cas to efficiently and specifically knock out (Cas9) or repress (dCas9 and Cas13) pro-tumorigenic genes or transcripts opens new strategies for tumor suppression.[95–97] Moreover, the same system can be used to introduce (Cas9) or activate (dCas9) important immune-related genes to directly improve the host immune response.[95,96] Consequently, the capacity of CRISPR-based systems to work individually as therapeutics and in combination with current immunotherapy strategies is developing into an emerging area of interest.

THE CRISPR-CAS9 SYSTEM DEMONSTRATES SAFETY IN ADOPTIVE T CELL IMMUNOTHERAPY

As of March 1, 2020, there have been 21 registered trials utilizing CRISPR-Cas systems aiming to genetically alter human T cells—a key adaptive immune cell type that responds specifically to antigens and is critical in host immunity (Table 2). These trials constitute approximately 48% (21/43) of all trials using CRISPR on the ClinicalTrials.gov registry. Recently, results from the first three CRISPR-Cas9 clinical trials were published by Jing et al.[98] (ClinicalTrials.gov: NCT03081715), Lu et al.[99] (ClinicalTrials.gov: NCT02793856), and Stadtmauer et al.[100] (ClinicalTrials.gov: NCT03399448). Outcomes from these trials are discussed below.

PD-1 knockout T cells

Many tumors, including esophageal squamous cell carcinoma (ESCC) and non-small cell lung cancer (NSCLC), express immune checkpoint PD-1 ligands (PD-L1s) that bind to PD-1 receptors on host T cells to inhibit their proliferation and cytokine production, thereby enabling immunoevasion (Figure 2).[101–103] To date, anti-PD-1 and anti-PD-L1 monoclonal antibodies have shown success in many cancers, including melanoma,[104] Hodgkin's lymphoma,[105] liver cancer,[106] and lung cancer.[107] However, given the potential of PD-1/PD-L1 inhibitor therapy to result in severe toxicity,[108,109] genetic disruption of PD-1 expression on host T cells is seen as an alternative and potentially safer immunotherapeutic avenue. Jing et al.[98] and Lu et al.[99] independently investigated the safety of PD-1 knockout T cell reinfusion in 17 patients with advanced ESCC and 12 patients with metastatic NSCLC, respectively. In both trials, adoptive cell transfer (ACT) was used, whereby peripheral blood mononuclear cells (PBMCs) were collected, followed by *ex vivo* CRISPR-Cas9-mediated PD-1 (*PDCD1* gene) knockout. The edited PBMCs were then selected, expanded, and reinfused back into each patient. In both studies, the regimen was well tolerated, with no serious (grade 3/4) treatment-related adverse events observed. No complete or partial responses were witnessed in either trial; however, approximately 35% (6/17) of ESCC patients and 18% (2/11; early withdrawal by one patient due to bacterial infection) of NSCLC patients exhibited stable disease. Results by Lu et al.[99] demonstrated that co-transfection of Cas9 and sgRNA plasmid DNA (pDNA) via electroporation resulted in a low median editing efficiency of 5.81% (range, 0.42%–24.85%) in the 12 enrolled patients. In the edited PBMC pool, a median of 99.1% of cells were CD3 positive (range, 95.9%–99.6%), with $CD3^+CD8^+$ T cells accounting for 73.5% (range, 38.5%–93.0%). Whole-genome sequencing at 100-fold coverage targeted toward 2,086 sites determined by Cas-OFFinder[110] detected no true off-target events, which constitute arguably the greatest cause for concern in CRISPR-Cas9 clinical applications. Ultimately, both trials appear to confirm that only minor (grade 1/2) adverse effects, including fatigue, fever, joint pain, and skin rash, were attributed to the treatment, suggesting that CRISPR-Cas9-mediated ACT may be safe for clinical use. Moreover, although therapeutic efficacy was not the focus of these trials, they nonetheless highlight that significant improvements, such as a substantially increased editing efficiency, greater expansion of tumor-reactive T cells, enhanced antigen specificity, and a clearer understanding of the specific T cell subtypes undergoing the editing process, are required in order for an improved patient response. Importantly, both trials support that CRISPR-Cas9-based immunotherapy warrants further clinical investigation.

Table 2. Summary of all clinical trials utilizing CRISPR-Cas technologies to target the anti-tumor immune response

Trial status	Date first posted	Identifier, ClinicalTrials.gov:	Phase	Country	Condition	Target gene	Edited cells
Completed	March 16, 2017	NCT03081715	1	China	esophageal cancer	PD-1	T cells
	June 8, 2016	NCT02793856	1	China	lung cancer	PD-1	T cells
Recruiting	February 7, 2017	NCT03044743	1, 2	China	EBV-associated cancers	PD-1	T cells
	May 25, 2017	NCT03166878	1, 2	China	leukemia/lymphoma	TCR, B2M	T cells
	January 16, 2018	NCT03398967	1, 2	China	leukemia/lymphoma	CD19 and CD20 or CD22	T cells
	June 4, 2018	NCT03545815	1	China	solid tumors	TCR, PD-1	T cells
	July 29, 2019	NCT04035434	1	United States, Australia, Germany	leukemia/lymphoma	CD19	T cells
	July 30, 2019	NCT04037566	1	China	leukemia/lymphoma	HPK1	T cells
	January 28, 2020	NCT04244656	1	United States, Australia, Canada, Spain	multiple myeloma	BCMA	T cells
	June 5, 2020	NCT04417764	1	China	liver cancer	PD-1	T cells
	June 11, 2020	NCT04426669	1, 2	United States	gastrointestinal cancer	CISH	T cells
	June 18, 2020	NCT04438083	1	United States, Australia	kidney cancer	CD70	T cells
	August 6, 2020	NCT04502446	1	United States, Australia	lymphoma	CD70	T cells
	September 21, 2020	NCT04557436	1	United Kingdom	leukemia	CD19, CD52, TCR	T cells
	November 20, 2020	NCT04637763	1	United States	lymphoma	CD19	T cells
Not yet recruiting	October 1, 2018	NCT03690011	1	United States	leukemia/lymphoma	CD7, CD28	T cells
Terminated	January 16, 2018	NCT03399448	1	United States	multiple myeloma	TCR, PD-1	T cells
Withdrawn	August 11, 2016	NCT02863913	1	China	bladder cancer	PD-1	T cells
	August 15, 2016	NCT02867332	1	China	kidney cancer	PD-1	T cells
	August 15, 2016	NCT02867345	1	China	prostate cancer	PD-1	T cells
Unknown[a]	November 20, 2018	NCT03747965	1	China	solid tumors	PD-1	T cells

Terms used in search: CRISPR, clustered regularly interspaced short palindromic repeat. PD-1, programmed cell death-1; EBV, Epstein-Barr virus; CISH, cytokine-induced SH2 protein; TCR, T cell receptor; HPK1, hematopoietic progenitor kinase 1; CD, cluster of differentiation; B2M, β_2-microglobulin; BCMA, B cell maturation antigen. Search cutoff date: March 1, 2020.

[a]Unknown studies denote those whose last known status was recruiting, not yet recruiting, or active, but had passed the completion date, and the status had not been last verified within the past 2 years.

Multiplex PD-1 and TCR knockout

Results from the first phase I clinical trial to test the safety of multiplex CRISPR-Cas9 in treating patients with refractory cancer (ClinicalTrials.gov: NCT03399448) were published in February 2020 by Stadtmauer et al.[100] Three patients were recruited and CRISPR-Cas9 was applied *ex vivo* to simultaneously disrupt expression of the *PDCD1* gene (PD-1) and the endogenous T cell receptor α/β chain genes (*TRAC*, *TRBC*), which encode the T cell surface receptor (TCR) responsible for recognition of antigenic peptides presented in the context of MHC class I and II molecules. Patient $CD3^+$ T cells were further transduced with TCRs specific for tumor antigen (NY-ESO-1) recognition to enhance anti-tumor responses. The same ACT process as above was used, with the engineered T cells being reinfused back into each patient following editing and expansion. Stable disease was seen in two patients, whereas the third patient exhibited disease progression. Importantly, no serious (grade 3/4) adverse events were caused by the treatment, with the re-infusion process and persistence of transduced cells being well tolerated by all patients. Unlike the plasmid-based delivery method used by Lu et al.,[99] Stadtmauer et al.[100] utilized electroporation of ribonucleoprotein (RNP) complexes composed of recombinant Cas9 loaded with equimolar mixtures of the three sgRNAs targeting each gene. In this case, the editing efficiency was approximately 20% for *PDCD1*, 45% for *TRAC*, and 15% for *TRBC*. Although RNP delivery shows significant editing improvements compared to plasmid-based systems,[99] incomplete editing of *TRAC*/*TRBC* genes has previously been reported to result in mispairing and/or competing for expression between the transgenic TCR and endogenous TCR.[111] Additionally, iGUIDE,[112] a variant of GUIDE-seq (genome-wide unbiased identification of DSBs enabled by sequencing),[113] determined three significant off-targets.

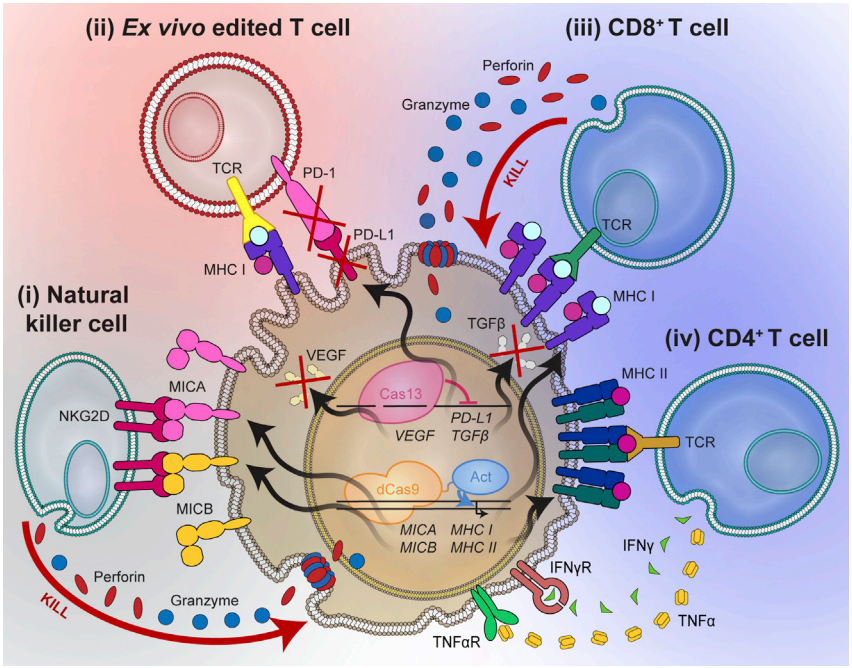

Figure 2. A combination CRISPR-Cas9 and dCas9- and Cas13-based approach to improving anti-tumor immunity

To date, immunotherapy approaches have aimed toward improving effector cells, such as T cells. However, CRISPR can also be used to counter immune evasion mechanisms within the tumor cell itself. (i) dCas9 fused to activation domains (Act) can be used to transcriptionally activate markers of cell stress and neoplastic transformation (MICA, MICB), thereby improving natural killer cell cytotoxicity (perforin, granzymes) toward the target cell. (ii) Cas9 genetic editing of host T cells *ex vivo* allows for re-engineering of the T cell receptor (TCR) and removal of immune checkpoint receptors (PD-1). Moreover, Cas13 gene inhibition can be used in combination with this approach via knockdown of corresponding immune checkpoint ligands (PD-L1) and suppressive cytokine signaling (VEGF, TGF-β). Using either of these approaches has been shown to improve the killing capacity of immune effector cells. CRISPR-dCas9 linked to Act can also be used to directly upregulate the genes responsible for antigen presentation, MHC class I (iii) and MHC class II (iv). Increased presentation of antigens improves the likelihood of recognition and elimination via $CD8^+$ T cells and $CD4^+$ T cells, particularly those re-engineered *ex vivo* to recognize specific tumor antigens.

The first, caused by the *TRAC* sgRNA, affected the transcriptional unit of chloride intracellular channel 2 (*CLIC2*) and was deemed acceptable, as it is not reported to be expressed in T cells. The remaining two off-targets, within the genes encoding zinc finger protein 609 (ZFN609) and long intergenic non-protein coding RNA 377 (LINC00377), were attributed to the *TRBC* sgRNA. Both off-targets were also deemed by the authors as acceptable due to their minimal impact on the gene (ZFN609) and unknown role to date (LINC00377). Despite the small sample size, Stadtmauer et al.[100] supported that multiplexed CRISPR-Cas9-directed ACT is feasible and appears to be safe for patients. Additionally, their trial reinforces that off-targeting continues to be an important consideration in any clinical application of CRISPR, and high-throughput sequencing technologies should be used to enable their identification.[114] Lastly, although the high editing efficiency achieved in this trial using RNP delivery verifies the established pre-clinical success of RNPs as the preferred CRISPR delivery system,[115,116] further improvements to *ex vivo* CRISPR editing are clearly needed to see an improved patient response. Namely, complete editing of *TRAC*/*TRBC* genes to eliminate the possibility of TCR mispairing, a detailed understanding of the specific T cell subsets undergoing editing, and extensive functional elucidation of off-targets and their effects (particularly, novel targets such as LINC00377) should be explored in future studies.

Using CRISPR-Cas9-based gene editing to target immune checkpoint pathways, such as the PD-1/PD-L1 axis, in combination with re-engineering the TCR for tumor specificity is expected to be an effective strategy to improve immune recognition and, ultimately, to promote the elimination of cancer cells. Moreover, use of these technologies is likely to complement and enhance patient responsiveness and/or serve as an alternative to monoclonal antibody treatment that targets immune checkpoint inhibitors (ICIs), such as PD-1 or CTLA-4, which have been effective in only a subset of cancers.[117–119] Aside from the suitable safety documented in the above CRISPR-Cas9-based anti-tumor immunotherapy clinical trials, the small sample sizes and high variability in survival of individual patients limit definitive conclusions on the efficacy of these treatments. However, with a number of current CRISPR-based trials targeted toward boosting the immune response via manipulation of immune-related genes (Table 2), more conclusive results are likely to be seen during the coming months.

EPIGENETIC EDITING BY CRISPR-dCas9 CAN IMPROVE ANTI-TUMOR HOST IMMUNITY

While major modalities of cancer immunotherapy, such as ICIs and ACT, have revolutionized cancer treatment, not all patients respond to these therapies.[107,120] Often, durable immune responses occur in patients with immunogenic ("hot") tumors, characterized by infiltrating $CD4^+$ and $CD8^+$ T cells and accumulation of pro-inflammatory mediators.[121] However, non-immunogenic ("cold") tumors that lack these components have significantly reduced response rates, although some patients still respond, and cold tumors may require conversion into a more hot-like phenotype in order to achieve a better outcome.[122] The CRISPR-dCas9-mediated epigenetic modulation of tumor immunogenicity represents one such conversion method. In this approach, dCas9-mediated transcriptional activation of pro-immunogenic genes, or repression of genes involved in tumor immunoevasion and immunosuppression, may enhance tumor immunogenicity.[122] Studies using these methods have shown success in pre-clinical testing and hold great potential for novel immunotherapeutics, as discussed below.

Pro-immunogenic transcriptional activation

In a seminal 2019 publication by Wang et al.,[123] a multiplexed dCas9-SAM system was harnessed to induce a genome-scale simultaneous upregulation of endogenous pro-immunogenic genes in triple-negative breast cancer (TNBC) E0771 cells, in order to improve tumor immunogenicity. Given their role as inducers of T cell proliferation and activation, a particular focus was directed toward the upregulation of *CD70*, *CD80*, *CD86*, *IFNα4*, *IFNβ1*, and *IFNγ* genes. CD70, present on antigen-presenting cells (APCs; dendritic cells, B cells, and macrophages) and some T cells, is the cognate ligand for the tumor necrosis factor (TNF) receptor family member CD27 on T cells, and it provides an essential co-stimulatory signal for T cell activation.[124,125] Similarly present on APCs and some T cells, CD80 and CD86 are cognate ligands for the co-stimulatory receptor CD28 on T cells and also play a critical role in T cell activation.[126,127] Type I (α and β) and type II (γ) interferons (IFNs) are potent cytokines that bind to the ubiquitously expressed IFNα receptor (IFNαR) and IFNγR, respectively. Most cell types can produce IFNβ, whereas IFNα is predominantly produced by plasmacytoid dendritic cells and IFNγ is chiefly secreted by activated T cells and natural killer (NK) cells. Together, type I and II IFN signaling promotes innate and adaptive immunity in a variety of ways, including activation of antigen presentation and chemokine production, enhanced antibody generation, and stimulation of T cell and NK cell cytotoxicity.[128,129] The transcriptional upregulation mediated by dCas9-SAM resulted in amplified presentation of a model antigen (ovalbumin) *in vitro*. Moreover, the edited E0771 cells transplanted into syngeneic immunocompetent (C57BL/6J) mice demonstrated significantly reduced tumor volume *in vivo* compared to both immunodeficient (nude and *Rag*-deficient) mice and $CD4^+$ and $CD8^+$ T cell-depleted immunocompetent mice. Additionally, Wang et al.[123] combined their activation system with anti-CTLA4 monoclonal antibodies, which substantially increased the efficacy of anti-CTLA4 therapy, with complete tumor regression of established tumors. As expected, the increased tumor immunogenicity via dCas9-mediated transcriptional activation of pro-immunogenic genes increased $CD4^+$ and $CD8^+$ T cell infiltration into the tumor, thereby improving anti-tumor immunity. The findings were also supported by Liu et al.'s[130] earlier work from 2017, where HeLa cells edited using the same dCas9-SAM system to overexpress the *IFNγ* gene exhibited enhanced apoptosis, inhibited proliferation, and overall reduced tumor volume when implanted in immunodeficient (severe combined immunodeficiency [SCID]) mice. Collectively, both works provide a proof of concept that manipulating the transcriptome of tumors in favor of a pro-immunogenic phenotype can greatly improve the anti-tumor immune response and the response to ICIs. Moreover, the activation of endogenous cytokines, such as IFNγ, within the tumor is likely to be an excellent starting point for this novel approach of enhancing tumor immunogenicity.

Immunosuppressive pathway inhibition

The TGF-β signaling axis has been of interest owing to its dual role in both tumor suppression and progression.[131,132] When functioning as an oncogenic activator, TGF-β signaling induces an immunosuppressive response,[133] which is further potentiated by reciprocal positive regulatory interactions with Notch and Hippo signaling.[134] Recently, microRNA-524 (miR-524) has been shown to silence the TGF-β, Notch, and Hippo pro-tumorigenic signaling pathways simultaneously by suppression of *SMAD2*, *HES1*, and *TEAD1* genes, respectively, making it an attractive target for cancer immunotherapy.[135] As such, in 2019, Liu et al.[136] aimed to explore the potential of dCas9-VP64-mediated miRNA-524 transcriptional activation as a cancer immunotherapy strategy to inhibit TGF-β/Notch/Hippo signaling *in vivo*. To this end, a pH-responsive multistage delivery nanoparticle (MDNP) was developed to deliver pDNA encoding the CRISPR-dCas9 system and sgRNA biomolecular components targeting the miR-524 locus. The systemic injection of MDNP/dCas9-miR-524 into tumor-bearing immunodeficient (nude) mice with TNBC MDA-MD-231 xenograft evidenced reduced *SMAD2*, *HES1*, and *TEAD1* gene and protein expression, significantly inhibiting tumor growth and higher levels of tumor apoptosis. Although the direct impact of miR-524 activation on tumor immunogenicity was not explored in this study, inhibition of the TGF-β axis is well recognized to improve immune cell infiltration as a consequence of the reduction in immunosuppression, tumor migration, and angiogenesis.[137,138] Altogether, Liu et al.'s[136] work provides a strong rationale for further exploration of miR-524 in cancer immunotherapy and highlights the potential of CRISPR-dCas9 technologies to inhibit immunosuppressive pathways for an improved anti-tumor response.

Although not applied in pre-clinical studies, a number of early works also suggest that dCas9-based methods can help circumvent other major tumor immunoevasion mechanisms. For instance, impairment of Treg immunosuppressive function by dCas9-KRAB-mediated forkhead box P3 (FoxP3) transcriptional repression,[139,140] dCas9-SAM-mediated enhancement of neoplastic transformation markers, such as MICA,[141,142] and dCas9-KRAB-directed repression of immunosuppressive cytokine receptors[143] have demonstrated that dCas9-mediated epigenetic engineering has potential in multiple facets of cancer immunotherapy. Ultimately, dCas9-mediated epigenetic editing to improve tumor immunogenicity and/or boost the host's anti-tumor immune response opens new therapeutic avenues, particularly in combination with ICI or ACT therapies (Figure 2).

THE CRISPR-CAS13 SYSTEM IS A NOVEL GENE INTERFERENCE TOOL IN CANCER IMMUNOTHERAPY

Significant attention has been directed toward applying CRISPR-Cas13 (RNA-targeting) technology as a diagnostic in the detection of low-frequency cancer somatic mutations[144] and in RNA editing.[85,145] Additionally, the CRISPR-Cas13 system has demonstrated highly efficient and specific knockdown of oncogenic mutant drivers,[146] gene fusions,[88] and ncRNA[147] transcripts *in vitro* with minimal off-targets, highlighting its potential for use as an RNA-based therapeutic tool. More recently, the potential of Cas13-based gene suppression in cancer immunotherapy has drawn attention due to its growing success in pre-clinical studies, as explored below.

PD-L1 disruption

As discussed above, CRISPR-mediated PD-1 knockout T cells have shown acceptable toxicity profiles, yet their efficacy remains limited. Moreover, although PD-1 knockout therapies largely focus on T cells, other important immune effectors, such as NK cells, are also inhibited by the PD-1/PD-L1 axis.[148] Therefore, as opposed to targeting PD-1 expression on specific cell types, Zhang et al.[149] explored the capability of CRISPR-Cas13a to silence PD-L1 expression at the tumor cell surface to improve overall anti-tumor immunity. To this end, pDNA encoding Cas13a and crRNAs targeting PD-L1 transcripts were systemically delivered *in vivo* using a pH and hydrogen peroxide (H_2O_2)-responsive dual-locking nanoparticle (DLNP). Following injection of DLNP/Cas13-crRNA into melanoma B16F10-bearing immunocompetent mice, significant tumor growth suppression and improved survival was observed. Furthermore, a significant reduction in TGF-β and elevation in IFNγ, TNF-α (predominantly secreted by macrophages and promotes T cell activation), IL-2 (secreted by activated T cells and promotes T cell proliferation), and IL-12 (secreted by activated APCs to activate NK cells and induce T cell differentiation) levels were seen in treated tumors, suggesting activation of anti-tumor immunity. Knockdown of PD-L1 by Cas13 also increased the number of $CD8^+$ tumor-infiltrating T cells, reduced the number of myeloid-derived suppressor cells (MDSCs), and induced a tumor-associated macrophage polarization from a tumor-promoting M2-like ($CD206^{hi}CD11b^+F4/80^+$) to a more anti-tumor M1-like ($CD80^{hi}CD11b^+F4/80^+$) phenotype in the tumor microenvironment. This work suggests that Cas13a can successfully suppress tumor PD-L1 expression and aid in eliciting an effective anti-tumor immune response (Figure 2).

VEGF pathway disruption

The VEGF/VEGF receptor pathway constitutes one of the most promising therapeutic targets due to its significant immunosuppressive role in the tumor and surrounding microenvironment. Previously, obstruction of VEGF receptor 2 (VEGFR2)[150] and its downstream signaling pathways, BCL-2 and Survivin,[151] via small-molecule inhibitors or neutralizing antibodies has shown success in blocking tumor growth and prolonging survival. However, limitations, such as temporary efficacy, treatment resistance, and adverse complications, have justifiably limited their clinical use. With this in mind, Fan et al.[152] explored the capacity of the Cas13a system to target VEGFR2, BCL-2, and Survivin transcripts simultaneously in tumor cells. Plasmid DNA containing Cas13a and crRNA tandem sequences designed to target VEGFR2/BCL-2/Survivin transcripts were encapsulated in a dual-component liposome system coated in VEGFR2 monoclonal antibodies. The Cas13a/liposome was then perfused via intravesical administration into bladder cancer 5637 cell-bearing immunodeficient (nude) mice, resulting in significantly reduced transcription levels of VEGFR2/BCL-2/Survivin and inhibited tumor growth. It is well established that inhibition of the VEGF/VEGFR axis reduces angiogenesis and Treg/MDSC accumulation, thereby promoting immune cell infiltration into the tumor.[153–155] These findings therefore support that Cas13a represents an emerging precision medicine platform for the inhibition of tumor growth by targeting pro-oncogenic signaling pathways, such as VEGF/VEGFR, in a highly selective manner.

More recent studies have shown that dCas13-fusion (RNA editing) proteins can also achieve efficient knockdown of endogenous RNA transcripts by catalyzing the demethylation of m^6A (*N*6-methyladenosine),[156] or by the degradation of m^6A-marked RNA,[157] resulting in gene suppression. These early works suggest that dCas13-based technologies targeted toward m^6A modifications may offer an alternative to Cas13-mediated alteration of post-transcriptional RNA fate. Although more investigation is required to explore the efficacy and safety of Cas13 in cancer immunotherapy, studies support that Cas13 has the potential to function as a therapeutic through its targeted manipulation of tumor immunogenicity (Figure 2).

CONSIDERATIONS FOR CRISPR-BASED CANCER IMMUNOTHERAPY

To date, many reviews have discussed the key considerations surrounding use of CRISPR in treating human diseases. For further details on the concerns surrounding use of CRISPR-based therapeutics in a general sense, we refer to excellent previously published reviews.[74,158–161] In this section, however, we focus specifically on the concerns associated with CRISPR use in cancer immunotherapy.

Pre-existing CRISPR immunity

Given that CRISPR systems are derived from common human pathogens, such as *Streptococcus pyogenes* and *Staphylococcus aureus*, the presence of pre-existing immunity from prior exposure remains a major concern in the safety and efficacy of these technologies. Principally, in the context of cancer immunotherapy where potent immune activation is the goal, the extent of anti-Cas antibodies and/or T cells that may interfere with CRISPR-based treatment needs to be elucidated.[119,162] Recently, three studies have shown that both antibodies and T cells against *Streptococcus pyogenes* Cas9 (SpCas9) and *Staphylococcus aureus* Cas9 (SaCas9) are indeed present in many individuals, supporting that further investigation is required. First, Simhadri et al.[163] highlighted that approximately 2.5% and 10% of donors (a predominantly white cohort from the United States) tested positive for anti-SpCas9 and anti-SaCas9 antibodies in human sera, respectively, by enzyme-linked immunosorbent assay (ELISA). Second, Wagner et al.[164] showed using flow cytometry that approximately 96% of donors (ethnicity unknown) had SpCas9-reactive peripheral $CD4^+$ and $CD8^+$ T cells as measured by CD137 expression. Lastly, Charlesworth et al.[165] identified using PBMCs that approximately 58% and 78% of donors (a predominantly white cohort from the United States) had antibodies against SpCas9 and SaCas9 detected using ELISA, respectively. Moreover, approximately 67% and 78% of donors were positive for SpCas9- and SaCas9-reactive $CD4^+/CD8^+$ T cells, respectively, as confirmed by IFNγ enzyme-linked immunospot (ELISpot), intracellular cytokine staining (ICS), and/or surface expression of CD137 or CD154. Although these studies complement one another in highlighting the prevalence of pre-existing immunity to Cas9, further research is still required to elucidate whether the presence of anti-Cas antibodies or anti-Cas T cells are at biologically

relevant levels and to what extent they may impact CRISPR-based therapy.

Ultimately, if in healthy hosts the potential exists for an anti-CRISPR immune response to impede the efficacy of CRISPR-based therapeutics, this is likely to be further exacerbated in patients undergoing CRISPR/ICI or CRISPR/ACT combination cancer immunotherapy where effector immune cells are in an activated state. In order to circumvent this issue for Cas9 genetic editing, it has been proposed that transient Cas9 expression *ex vivo* outside of direct immune contact and reinfusion into the host once the Cas9 protein has been cleared may be a solution.[165] This may help to explain why CRISPR-based clinical trials to date—all using *ex vivo* editing—have not shown adverse Cas9-reactive T cell responses. However, the likelihood of producing anti-Cas9 memory T cells during the *ex vivo* editing process is still unknown, and moreover this *ex vivo* method cannot be applied to dCas9 or Cas13 systems where *in vivo* use is vital. In these cases, preclinical assessment of Cas immunogenicity or specific delivery methods that do not allow for CRISPR-immune interactions until cargo release may be required. Examples of successful delivery systems designed with this is mind include the MDNP[136] and DLNP[149] methods discussed in the pre-clinical dCas9 and Cas13 sections, respectively. These delivery systems are also necessary to ensure that the CRISPR cargo is delivered directly to the target tissue. For instance, the release of CRISPR-dCas9 with sgRNAs targeting pro-immunogenic genes in unintended tissues may elicit an adverse pro-inflammatory and/or autoimmune response. Ultimately, averting an anti-CRISPR immune response will likely require a joint approach of checking for pre-existing immunity and selecting for tissue-specific, multi-layered, non-immunogenic delivery systems.

Retroactivity in multiplex CRISPR applications

One of the key benefits of CRISPR-based systems is their capability to multiplex crRNAs and, thus, to regulate multiple targets simultaneously. For cancer immunotherapy, it is highly likely that clinical benefit will only be achieved through multiplexing, as multiple genetic elements require editing. However, as the number of target sites increases, there is also a subsequent increase in the competition between crRNAs to guide the Cas protein to their target site. Competition between crRNAs, termed retroactivity,[166] results in a decrease in the performance of CRISPR-Cas and severely hinders the ability to predict crRNA targeting efficiency. This presents a major obstacle in cancer immunotherapy, as it is essential that maximum gene editing and transcriptional regulation is achieved. In other contexts, retroactivity has been mitigated by using conditional crRNAs, whose activity is dependent on the presence or absence of an RNA trigger.[167] However, this is unlikely to serve in cancer immunotherapy, as simultaneous targeting is required. An alternative method more applicable to overcoming retroactivity in cancer immunotherapy lies in the exploitation of target hierarchy. In this approach, targets of high priority are allocated multiple crRNAs, while lower priority targets are allocated only a single crRNA.[66] This method may ensure that all sites are targeted, but those of high importance are emphasized. Furthermore, it is still unclear as to how many crRNAs can be expressed *in vivo* for an effective multiplexing strategy, while maintaining editing efficacy at all target sites. This knowledge is fundamental to achieving the desired effectiveness of CRISPR-based therapies. In the case of cancer immunotherapy, it is expected that not all target sites will have the same impact on tumor immunogenicity or immune effector cytotoxicity. Also, inherent genetic and/or epigenetic differences between patients at specific target sites is highly likely. Therefore, effective multiplexed strategies that target these different loci is essential.

Altogether, if pre-existing CRISPR immunity and retroactivity can be mitigated via improved delivery systems and crRNA prioritization/customization to particular patients, then CRISPR-based cancer immunotherapy may yet develop to become an effective clinical treatment.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The ability of CRISPR-based technologies to efficiently modify specific loci at the genetic and epigenetic level provides novel opportunities to reprogram the immune response for improved tumor elimination. To date, CRISPR-Cas9 genetic editing has successfully improved the killing capacity of T cells via the elimination and/or modification of key cell surface molecules, such as PD-1 and the TCR. However, although shown to be safe when used *ex vivo*, Cas9's potential to bind to non-target DNA sequences (off-targets), thereby causing permanent genomic instability or disruption of otherwise normal host genes, has consistently remained a major concern.[168,169] Alternatively, the development of dCas9- and Cas13-based systems that work transiently, are reversible, and eliminate the risk of long-lasting consequences are expected to be safer, and therefore more clinically relevant, for *in vivo* use. Technologies such as dCas9 in particular show significant advantages, as it mediates straightforward upregulation or downregulation of transcription and editing of epigenetic marks within the same platform, which significantly expand its applications in cancer therapeutics. Moreover, these systems are increasingly being developed as inducible structures allowing for precise control of epigenetic editing at will.[170] Still, safe and precise delivery mechanisms are yet to be addressed in a comprehensive way, and the potential for adverse immune reactions challenge clinical CRISPR applications. Altogether, based on safety and versatility, epigenetic editing platforms such as dCas9 and Cas13 are likely to overtake Cas9 genetic editing platforms in clinical applications.

Future applications of CRISPR-based technologies in the immunotherapy field are likely to expand heavily on epigenetic editing by dCas9 or Cas13. Epigenetically reprogramming immune evasion mechanisms, such as rescuing MHC class I/II expression, halting MICA/B shedding, and repressing the activation of suppressive cytokines, is an attractive therapeutic avenue, as doing so has the potential to greatly improve host immunity. Furthermore, the expanding knowledge of the role of distinct immune checkpoint inhibitors will offer grounds for the identification of additional checkpoint molecules

to be edited in multiplexed CRISPR applications. Selective epigenetic targeting of immune checkpoints in effector immune cells or at the tumor cell surface to improve *in vivo* immune activation and efficacy is likely to improve patient outcomes in the clinic. Other potential applications may involve the epigenetic manipulation of cells used in ACT aiming at enhancing intra-tumoral recruitment and persistence of these cells through the upregulation of relevant chemokine receptors, such as CXCR2 and CXCR3, which greatly improve T cell localization and migration to tumors.[171–173] In principle, this strategy could be combined with epigenetic upregulation of the genes encoding for the corresponding chemokine ligands on tumor cells, thus facilitating the treatment of cold-like tumors, which are poorly amenable by current immunotherapies. Similarly, recent studies have highlighted the complexity in the development of exhausted T cells and NK cells (failure to produce cytokines, lack of proliferation, and high expression of inhibitory receptors),[174–177] with significant epigenetic changes identified in chronically stimulated NK cells[178] and T cells that failed to respond to anti-PD-1 therapy.[179,180] Reinvigoration of exhausted and dysfunctional cell subsets using targeted epigenetic CRISPR editing of key genes, such as TCF1 (transcription factor encoded by *TCF7* crucial for T cell persistence and NK cell survival),[181–183] may assist in improving patient responses to ICI therapies and overall anti-tumor immunity. Importantly, the extent to which CRISPR-based technologies can alter the tumor landscape is still unknown; however, through multiplexed approaches, it is hopeful that durable immune responses can be achieved. Alhough in its infancy, precise epigenetic editing by dCas9 and Cas13 is likely to become a powerful tool in both basic scientific research and clinical application, especially when combined with Cas9-mediated genetic editing.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.A., P.B., and S.G.; writing – original draft, E.A., S.T., R.D., and J.C.; writing – review & editing, E.A., P.B., R.D., J.C., S.G., and A.K.N.; figures, E.A.; funding acquisition, P.B.; supervision, P.B., and S.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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