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Review Article

CRISPR-Cas9-Mediated Suppression of PD-L1 as a Novel Strategy in Cancer Immunotherapy

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Abstract

The PD-1/PD-L1 checkpoint pathway is a fundamental mechanism of immune evasion, allowing tumors to suppress T-cell activity and undermine the efficacy of immunotherapy. While monoclonal antibodies such as Avelumab block this interaction and remain the clinical standard, their effectiveness is often limited by innate or acquired resistance, high costs, and inconsistent patient responses. As an alternative, CRISPR-based genomic editing offers a transformative strategy by targeting the PD-L1 gene at its source to achieve a more durable suppression. This review evaluates advanced CRISPR techniques, including CRISPR interference (CRISPRi) for transcriptional repression, and more precise Base and Prime Editing for single-nucleotide changes, that are being deployed to eliminate PD-L1 expression in models of lung cancer, hepatocellular carcinoma, and melanoma. Studies consistently demonstrate that CRISPR-mediated PD-L1 knockdown restores T-cell-mediated cytotoxicity and leads to significant inhibition of tumor growth both *in vitro* and *in vivo*. Furthermore, combining PD-L1-edited cancer cells with CAR-T cell therapy has shown enhanced anti-tumor efficacy in solid tumor models. The precision of these approaches is now being amplified by artificial intelligence (AI), which optimizes guide RNA designs to maximize on-target efficiency and minimize off-target effects. In conclusion, CRISPR-mediated PD-L1 editing, particularly when integrated with AI-driven design, lays a robust foundation for next-generation immunotherapies. Translating this promise into clinical reality, however, hinges on overcoming persistent challenges in delivery vehicle efficiency and long-term safety profiling through ongoing investigative efforts.

Keywords: CRISPR, PD-L1, Cancer Immunotherapy, CAR-T, Gene Editing, Artificial Intelligence (AI).

Introduction

Cancer remains one of the leading causes of mortality worldwide, with aggressive types such as melanoma and non-small cell lung cancer (NSCLC) exhibiting poor survival rates despite advances in conventional therapies. Limitations of standard treatments, including chemotherapy, radiotherapy, and surgery, have driven the development of innovative therapeutic strategies, among which immunotherapy has emerged as a promising approach [1,2]. Immunotherapy enhances the ability of the immune system to recognize and eliminate tumor cells; however, many cancers evade immune surveillance by exploiting the PD-1/PD-L1 inhibitory axis. PD-L1, expressed on the surface of cancer cells and certain

normal cells, binds to PD-1 on T cells, thereby dampening their effector functions and suppressing the immune response. While this pathway maintains immune homeostasis under physiological conditions, tumors can upregulate PD-L1 to escape cytotoxic T-cell-mediated destruction, facilitating tumor growth and metastasis. Elevated PD-L1 expression has been reported in various malignancies, including NSCLC, melanoma, breast cancer, gastric cancer, and Hodgkin lymphoma [3]. Therapeutic strategies targeting the PD-1/PD-L1 interaction, such as monoclonal antibodies (Avelumab, Pembrolizumab, Nivolumab), anti PD-L1 nanoparticles, and nanobodies, have demonstrated significant antitumor activity in certain patients. Nanobodies, due to their small size, enhanced tissue penetration, and high stability, offer promising advantages for tumor targeting, although patient responses remain heterogeneous [4-6]. These challenges have prompted the exploration of gene-editing technologies, particularly CRISPR-Cas9, as a precise and versatile tool for PD-L1 modulation [7].

CRISPR-Cas9, guided by specifically designed single-guide RNAs (sgRNAs), enables targeted silencing or modification of the PD-L1 gene. Preclinical studies in lung, melanoma, and gastric cancer models have shown that PD-L1 knockout enhances T-cell proliferation and effector functions, leading to reduced tumor growth. Advanced CRISPR-based tools, including CRISPR interference (CRISPRi), CRISPR activation (CRISPRa), Base Editing, and Prime Editing, provide more precise and flexible approaches to fine-tune PD-L1 expression [8]. Within the context of immunotherapy, PD-1 and PD-L1 play central roles. PD-1 is expressed on activated T cells, NK cells, and B cells, whereas PD-L1 is predominantly found on tumor cells and antigen-presenting cells (APCs). T-cell activation requires two distinct signals: antigen recognition via the T-cell receptor (TCR) and a co-stimulatory signal from APCs, which are essential for T-cell proliferation, differentiation, and survival. In the tumor microenvironment, PD-L1 engagement with PD-1 suppresses these pathways, but pharmacological inhibitors can relieve this suppression, allowing T cells to proliferate and eliminate cancer cells (Figure 1).

This review provides an overview of CRISPR-mediated PD-L1 inhibition, addressing design strategies, potential challenges, and clinical applications in immunotherapy. While previous reviews have discussed CRISPR or immunotherapy separately, this review specifically focuses on CRISPR-mediated modulation of PD-L1 in cancer therapy, highlighting advanced CRISPR tools and their integration with AI, as well as potential clinical applications. It emphasizes how gene-editing technologies can complement existing therapies and expand the therapeutic potential for cancer patients.

CRISPR Revolution: From Programmable Nucleases to Precision Genome Engineering

Genome engineering has rapidly advanced with the development of programmable nucleases, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and more recently, the CRISPR-Cas system. These tools enable precise DNA modifications with broad applications in genetic disease therapy, agriculture, and biotechnology. While ZFNs and TALENs represented pioneering breakthroughs, their complex design and high production costs limited widespread use. By contrast, CRISPR-Cas, inspired by the bacterial adaptive immune system, offers a simpler, more efficient and cost-effective strategy. Using a single guide RNA (sgRNA) to direct Cas nucleases, CRISPR facilitates multiplex gene editing, a capability previously unattainable.

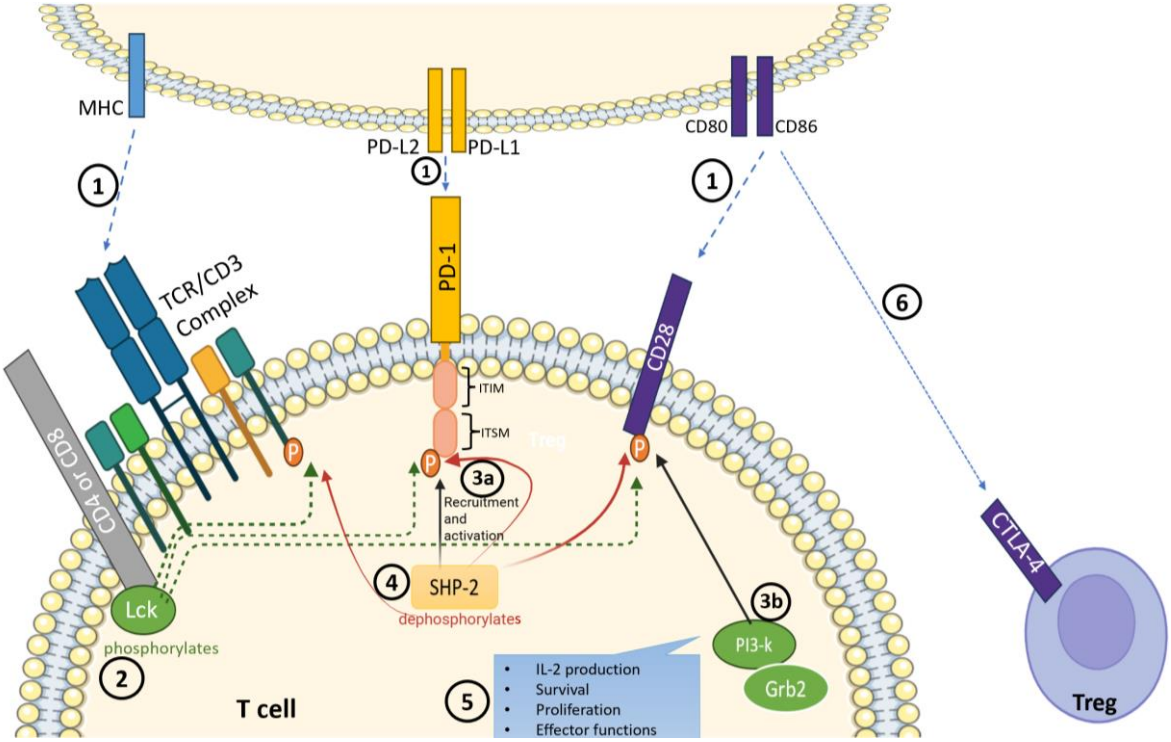


Figure 1. Signaling pathways mediated by PD-1 in T cell activation: (1) the interaction of surface receptors (PD-1, CD28, and TCR) with their corresponding ligands at the immunological synapse; (2) the phosphorylation of these receptors by the kinase Lck; (3a) the recruitment of the phosphatase SHP-2 to PD-1; (3b) the concurrent binding of PI3K and Grb2 to CD28; (4) the dephosphorylation of CD28 and CD3 by SHP-2, resulting in the suppression of stimulatory signaling; (5) the essential function of CD28 in enhancing activation and the diminished responses following the disruption of this pathway; and (6) the indirect inhibition of CD28 signaling through the competitive binding of CTLA-4 to CD80/CD86. Created by authors.

This versatility has positioned CRISPR-Cas as the leading platform in genome engineering and biomedical research [9].

Foundational Tools for Genome Engineering

ZFNs and TALENs were the first breakthroughs in genome editing, paving the way for more advanced tools like CRISPR. ZFNs require assembling multiple zinc finger proteins, each recognizing a three-nucleotide sequence (triplet), which makes their design technically challenging and prone to off-target effects. TALENs improved on this by using TALE proteins, which bind DNA with higher precision, paired with nucleases such as FokI to introduce double-strand breaks (Figure 2). Compared to ZFNs, TALENs are more flexible and easier to design, reducing the chance of targeting errors. However, both systems remain costly and labor-intensive, limiting their use, especially for applications that require dynamic gene regulation [10]. CRISPR-Cas overcomes many of these limitations. By using guide RNAs (gRNAs), which can be easily designed to match almost any DNA sequence, CRISPR allows precise and efficient targeting of genes. These gRNAs consist of two components, which in engineered systems are fused into a single guide RNA (sgRNA) to streamline the process and enhance editing efficiency. This approach has been successfully applied in cancer models, for example, to inhibit PD-L1 expression, enabling precise genome modifications without permanent disruption when desired.

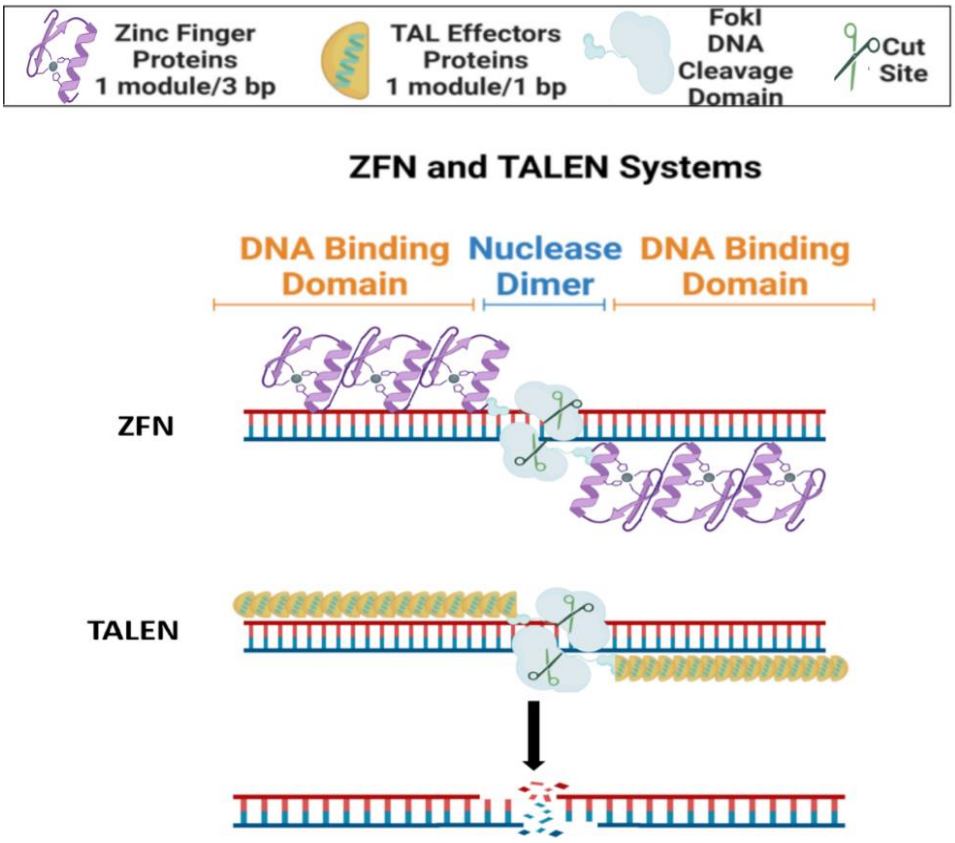


Figure 2. A schematic illustration comparing the ZFN (Zinc Finger Nucleases) and TALEN (Transcription Activator-Like Effector Nucleases) systems utilized for targeted genome editing. Each system comprises DNA-binding domains along with a nuclease domain (FokI). Created by authors

Components and Mechanisms of the CRISPR-Cas System for Targeted Gene Editing

The CRISPR-Cas system is a highly advanced and precise genome-editing platform composed of Cas nucleases and guide RNAs, designed for targeted genetic manipulation. The most widely used nuclease is Cas9, which functions as molecular scissors capable of generating site-specific double-strand breaks (DSBs) in DNA. Beyond Cas9, other Cas variants such as Cas12 and Cas13 exhibit unique properties; Cas12 cleaves DNA through a distinct mechanism, whereas Cas13 exclusively targets RNA, allowing enzyme selection to be tailored to the specific gene-editing application. The system relies on two main RNAs: CRISPR RNA (crRNA), which recognizes the target sequence, and trans-activating CRISPR RNA (tracrRNA), which associates with Cas9 to enable cleavage. In practice, these two RNAs are commonly fused into a single-guide RNA (sgRNA), which simultaneously directs Cas9 to the target locus. Accurate sgRNA design is essential to maximize editing efficiency and minimize off-target effects, involving careful selection of complementary sequences and ensuring compatibility with the Cas nuclease employed [11]. Target recognition by Cas9 requires the presence of a Protospacer Adjacent Motif (PAM), a short DNA sequence critical for nuclease binding and cleavage. Without PAM, Cas9 cannot engage or cut the DNA [12]. Upon PAM recognition, Cas9, guided by sgRNA, inspects the adjacent DNA sequence; if complementarity is confirmed, Cas9 introduces a DSB through its two nuclease domains: HNH, which cleaves the target strand, and RuvC, which cleaves the non-target strand containing the PAM (Figure 3A).

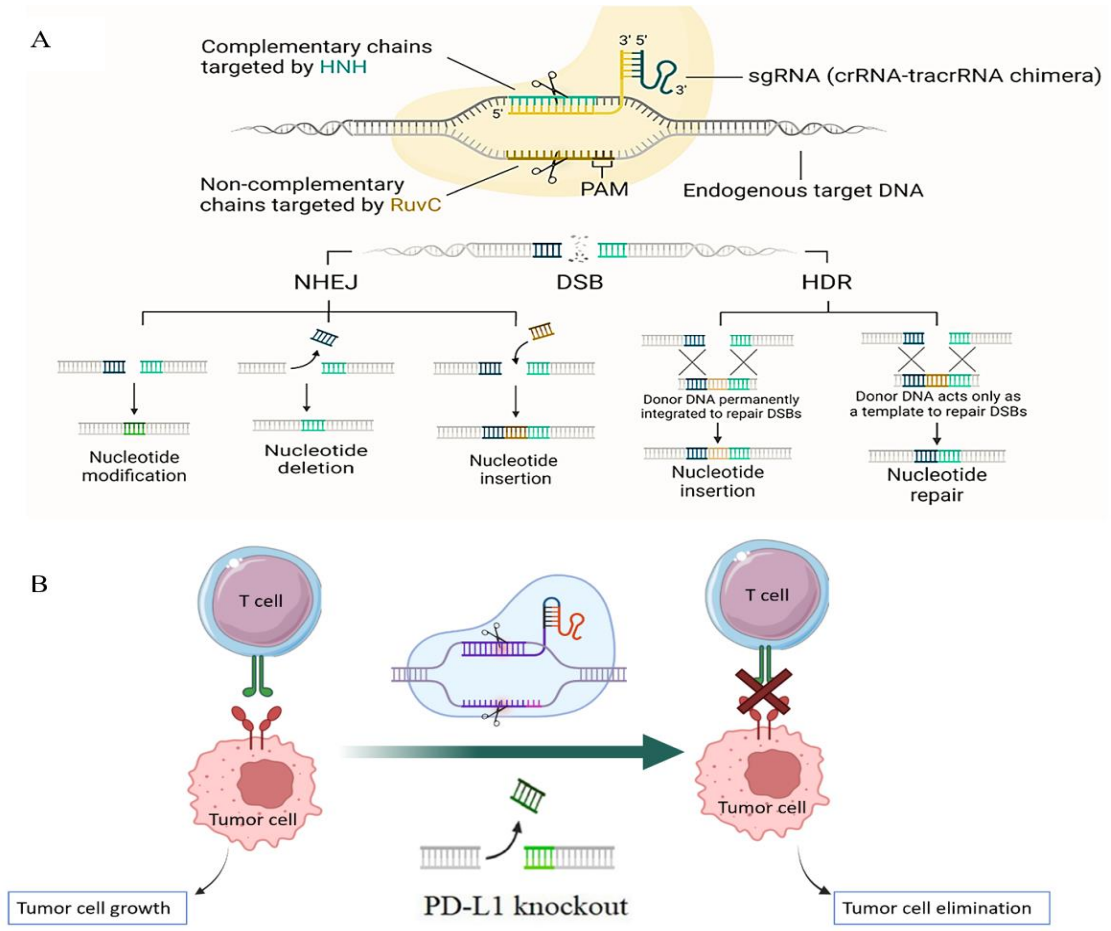


Figure 3. A) The CRISPR/Cas9 mechanism in genome editing, highlighting the DNA repair processes. In this system, the sgRNA, which comprises crRNA and tracrRNA, attaches to the target DNA sequence near the PAM region, while the Cas9 enzyme induces a double-strand break (DSB). This break can be repaired via two distinct pathways: firstly, the NHEJ (non-homologous end joining) pathway, which may result in the deletion, modification, or insertion of nucleotides; and secondly, the HDR (homologous repair) pathway, which utilizes a template to facilitate accurate repair of genetic alterations. B) A schematic illustration of the use of CRISPR/Cas9 in cancer immunotherapy. In T cells, the PD-1 gene is rendered inactive through the application of sgRNA and Cas9 (resulting in PD-L1 knockout), thereby diminishing the PD-1/PD-L1 interaction between T cells and tumor cells. This mechanism suppresses tumor cell proliferation and promotes their destruction by T cells. Created by authors

Following cleavage, DNA repair mechanisms are recruited, predominantly non-homologous end joining (NHEJ) and homology-directed repair (HDR) [13]. NHEJ is a rapid but error-prone pathway that frequently introduces small insertions or deletions (indels), making it effective for gene disruption—for instance, inactivating PD-L1. In contrast, HDR is a more precise repair pathway that incorporates a homologous DNA template to introduce defined modifications, and it is most active during the S and G2 phases of the cell cycle when DNA replication provides accessible templates.

CRISPR-Cas uses gRNAs to specifically target and disrupt PD-L1 on cancer cells. The resulting DNA break is usually repaired by NHEJ, creating mutations that silence the gene (Figure 3B). This prevents PD-L1 from binding to PD-1 on T cells, thereby restoring antitumor immunity. Studies in lung cancer and melanoma models confirm that CRISPR-mediated PD-L1 editing enhances immune responses. Depending on the strategy—

mutation with active Cas9 or epigenetic regulation with dCas9—this inhibition can be transient or permanent, highlighting CRISPR-Cas as a powerful tool in cancer immunotherapy.

Comparative Analysis of CRISPR-Based Strategies for PD-L1 Genome Editing

CRISPR-Cas9: Permanent Deletion of the PD-L1 Gene (Knockout)

CRISPR-Cas9 is a gene editing technology that employs a guide RNA (gRNA) along with the Cas9 enzyme to induce double-strand breaks (DSBs) in DNA. When focusing on PD-L1, this system can effectively silence the protein permanently by generating deletion or insertion (indel) mutations. Research has indicated that the deletion of PD-L1 through CRISPR-Cas9 improves the immune response and diminishes tumor evasion. Nevertheless, this method may lead to side effects, including off-target effects and irreversible alterations to the genome, which could potentially provoke undesirable immune reactions [14].

CRISPRi: Temporarily Suppressing PD-L1 Expression without Inducing DNA Breakage

CRISPR interference, commonly referred to as CRISPRi, represents a unique method that emphasizes the temporary control of gene expression while avoiding any permanent alterations to DNA. In this methodology, a deactivated form of the Cas9 enzyme (dCas9) is employed, which, rather than cleaving DNA, inhibits gene transcription by attaching to the promoter region of the target gene (PD-L1) and by interacting with transcriptional repressive factors such as the KRAB domain. Indeed, the KRAB domain that associates with dCas9 engages with additional proteins like KAP1 (KRAB-associated protein 1) and ultimately modulates gene expression through chromatin remodeling. This process leads to a reduction in PD-L1 expression at the RNA level and lessens the likelihood of unintended genetic damage, as it does not necessitate double-strand breaks. This feature renders CRISPRi an optimal selection for short-term experiments or therapies that necessitate reversible regulation of gene expression. For instance, this method could be employed to explore the function of PD-L1 during the initial phases of tumor development or to evaluate the consequences of temporarily inhibiting it in particular cells, thereby enabling researchers to examine biological effects without making permanent modifications to the genome. Nevertheless, the long-term viability of this approach is constrained, as the decrease in expression is influenced by various factors, including the stability of dCas9 within the cell, chromatin remodeling, and even the degradation rate of inhibitory proteins. This limitation may necessitate the implementation of advanced delivery systems, such as nanoparticles or controllable vectors, which in turn escalates the cost and complexity of the procedure. Furthermore, in comparison to methods that seek to eliminate a gene, the inhibition of gene expression through CRISPRi may prove inadequate in certain instances, potentially diminishing its efficiency and effectiveness under specific conditions [15].

Base Editing: Accurate Alteration without the Need for DNA Cleavage, C→T or A→G Transformation in the PD-L1 Gene

Base editing represents a significant advancement in CRISPR technology, enabling accurate alterations to the PD-L1 gene without necessitating the formation of double-strand breaks. This approach employs cytosine base editors (CBEs) and adenine base editors (ABEs), which can convert C to T or A to G in specific target sequences, respectively. This technique is applicable for generating inactivating point mutations in the PD-L1 gene or for modifying its promoter region to selectively diminish gene expression. The

method has garnered considerable interest, particularly in therapeutic contexts, due to its exceptional precision and marked reduction in off-target effects. Moreover, the absence of reliance on cellular DNA repair mechanisms such as NHEJ or HDR mitigates the risk of unintended alterations, which is particularly crucial in sensitive cell types like human cells. For instance, this technique can be employed to deactivate particular sites within the PD-L1 gene that are essential for the protein's functionality, thus hindering tumor immunosuppression. Nevertheless, the applicability of this technique is restricted, as it can only make targeted alterations to specific sequences and lacks efficiency when larger sequences require deletion or insertion. Moreover, the development of open editors for certain gene sequences can pose challenges, and their optimization is often a time-intensive process that relies on a comprehensive understanding of the target gene's structure. In certain cells, the effectiveness of base editing may be diminished due to epigenetic variations or restricted access to DNA [16].

Prime Editing: A More Intricate form of Editing, for Accurate Deletion and Insertion of Sequences within the PD-L1 Gene

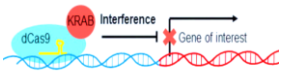
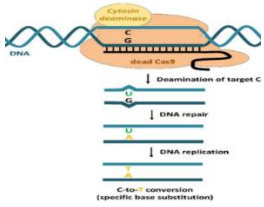
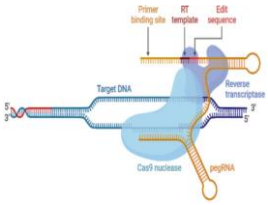
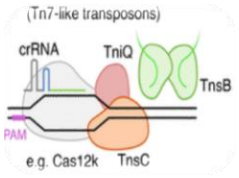
Prime Editing, recognized as one of the most sophisticated CRISPR technologies, provides significant versatility in modifying the PD-L1 gene. The system is composed of a nickase Cas9 and a reverse transcriptase enzyme that, with the aid of a specialized guide RNA (pegRNA), facilitates accurate deletion, insertion, or replacement of sequences without inducing double-strand breaks. This technique enables intricate modifications such as the insertion of premature stop codons, the removal of promoter regions, or the alteration of regulatory sequences within the PD-L1 gene, setting it apart from other approaches. For instance, it is feasible to eliminate portions of critical PD-L1 exons or to insert sequences that entirely disrupt gene expression, contingent upon the design of a suitable template for the pegRNA. This method is noteworthy because of its exceptional precision and adaptability in crafting edits, potentially playing a significant role in the development of targeted and personalized therapies for individual patients. Additional benefits include fewer side effects and the capacity to execute multiple edits, thereby reducing the likelihood of unintended alterations [17]. Nevertheless, this technology is still in its nascent stages of development, and its effectiveness across various cell types has yet to be fully refined. The design of pegRNA necessitates greater expertise and time due to its structural intricacy, which may restrict its applicability on a broader scale. Moreover, the efficient delivery of this system to target cells, particularly *in vivo*, continues to pose a substantial challenge and necessitates advancements in delivery techniques such as nanoparticles or liposomes [18].

CAST: Targeted and Broad Manipulations in the regulation of PD-L1 Expression

One of the latest developments in this domain is the application of CRISPR-associated transposase (CAST), which integrates CRISPR and transposon systems for precise genome manipulation. In this technique, Cas9 or other CRISPR proteins are linked to transposons to facilitate the insertion or deletion of larger sequences within the PD-L1 region. This method can effectively inactivate the PD-L1 gene by introducing disruptive sequences or incorporating new regulatory elements, such as transcriptional repressors. Unlike conventional techniques that depend on cellular DNA repair mechanisms, CAST operates independently, a characteristic that enhances its efficiency and accuracy in larger modifications. For instance, this approach can be utilized to insert an inhibitor gene adjacent to PD-L1 or to entirely remove its coding region, resulting in more

enduring effects compared to temporary inhibition. This technology also holds significant promise for integration with other therapeutic approaches, such as gene therapy or combination immunotherapy, potentially paving the way for new advancements in cancer treatment. Nevertheless, given the nascent stage of this technology, there is a lack of sufficient clinical data to evaluate its safety and effectiveness, necessitating further trials to validate its efficacy in practical applications (Table 1) [19].

Table 1. Comparison of CRISPR gene editing methods in regulating PD-L1 expression

Figure	Applications	Limitations	Advantages	Type of alteration	Mechanism	Technique
	Short-term studies, temporary gene regulation	Limited stability, requires repeated interventions	Non-disruptive to DNA, safe, and reversible	Transient reduction of gene expression at the RNA level	Transcriptional repression via dCas9 fused to repressor domains	**CRISPRi**
	Precise gene knockout, targeted therapy	Limited to specific base changes, complex design	High precision, reduced off-target effects	Precise, permanent nucleotide changes	Targeted conversion of specific bases (C→T or A→G)	**Base editing**
	Complex gene editing, personalized therapy	Complex design, demands additional optimization	Flexible, high precision, DSB-free	Accurate insertions, deletions, or replacements	nCas9 paired with reverse transcriptase for precise sequence modification	**Prime editing**
	Large-scale genomic modifications, combinatorial gene therapy	High complexity, insufficient clinical evidence	Independent of cellular repair, broad editing potential	Extensive, targeted genomic changes (insertions/ deletions)	Transposase-mediated genome editing integrated with a delivery system	**CAST**

Enhancing CAR-T Cell Anti-Tumor Efficacy through CRISPR-Cas9-Mediated PD-L1 Disruption

Chimeric Antigen Receptor T-cell therapy (CAR-T) represents a state-of-the-art immunotherapy that utilizes a patient's own T cells to deliver a focused strategy for combating cancer. In this method, T cells are extracted from the patient's body and genetically modified to express chimeric antigen receptors (CARs) on their surfaces.

This modification enables the T cells to identify and target specific antigens present on cancer cell surfaces. Following expansion in the laboratory, these altered cells are reintroduced into the patient's body to specifically attack the tumor. Immunotherapy utilizing engineered T cells has demonstrated considerable success in treating hematological malignancies, such as acute lymphoblastic leukemia and lymphoma; however, it encounters challenges in solid tumors due to immunosuppression linked to the PD-1/PD-L1 pathway, which restricts its effectiveness. In such scenarios, CRISPR technology has the potential to enhance the efficacy of this therapeutic approach by addressing these challenges. The integration of CRISPR-assisted PD-L1 gene inactivation with CAR T-cell therapy presents a groundbreaking and effective strategy for addressing solid tumors. By utilizing CRISPR to knock out PD-L1 in cancer cells, it is possible to dismantle the immunosuppressive barrier established by the PD-1/PD-L1 pathway, thereby enabling CAR-T cells to target the tumor more effectively. Preclinical research has demonstrated that the reduction of PD-L1 expression through CRISPR in models of lung cancer, melanoma, and hepatocellular carcinoma significantly enhances the infiltration of CAR-T cells into tumors and boosts the release of potent cytokines, including IFN-γ and TNF-α. This dual action not only curtails tumor proliferation, but also fosters a more durable immune response against tumors that are resistant to conventional immunotherapy. This innovative approach has instilled new optimism for achieving improved therapeutic results, particularly for patients who have developed resistance to previous treatment modalities. Furthermore, studies indicate that the inactivation of PD-L1 using CRISPR technology can enhance the survival rates of patients suffering from solid tumors by boosting the efficacy of CAR T-cells. Initial research has evaluated this combined approach in treating intricate cancers, including pancreatic cancer, liver cancer, and glioblastoma, with preliminary findings suggesting an extension of the immune response duration, a decrease in relapse rates, and an improvement in patients' quality of life. For instance, in preclinical models, the combination of PD-L1 knockout and engineered T cells led to a 60% reduction in tumor size and a notable increase in the survival of the subjects compared to control groups. This method can also be integrated with other immunotherapeutic strategies, such as checkpoint inhibitors, to achieve synergistic outcomes and reduce tumor resistance. In a previous study, Chamberlain *et al.* examined the decrease in PD-1 inhibitory receptor expression in tumor-infiltrating T cells (TILs) following gene editing via CRISPR-Cas9 technology. iTILs were obtained from patients suffering from various cancers, such as metastatic melanoma, head and neck cancer, thyroid cancer, and colorectal cancer. As illustrated in Figure 4, the reduction of PD-1 expression was assessed at different phases of the rapid cell proliferation (REP) process. The findings revealed that the level of PD-1 inhibition escalated over time; specifically, from day 7 to day 14, the percentage of expression reduction rose from 80.92% to 84.96%. This reduction was also preserved after the cells underwent freezing and thawing (86.4%) and achieved an average of 87.53% following restimulation with CD3/CD28 antibodies.

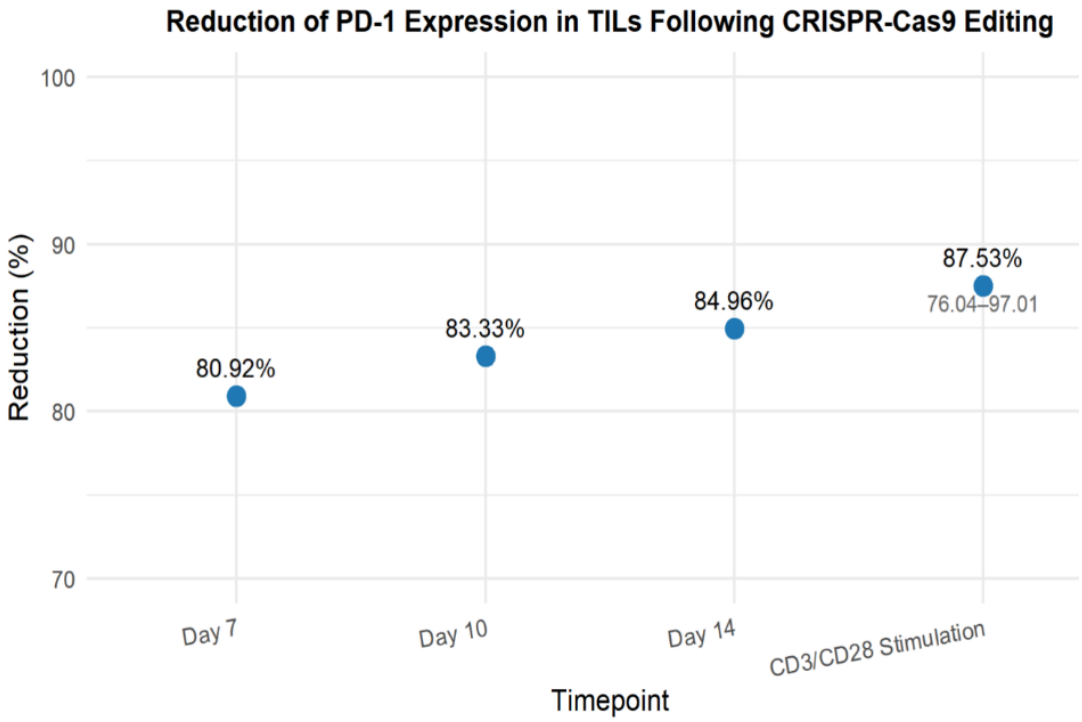


Figure 4. The reduction of PD-1 expression in tumor-infiltrating T cells (TILs) following gene editing with CRISPR-Cas9 technology. The degree of PD-1 expression downregulation was evaluated at various time intervals throughout the rapid proliferation process (REP). The most significant downregulation occurred during the CD3/CD28 stimulation phase. Created by authors

These figures demonstrate the effectiveness of CRISPR in silencing the PD-1 gene; in essence, a higher percentage signifies a greater inhibition of PD-1 expression [20].

Enhancing Cancer Immunotherapy: CRISPR-Cas9 Targeting of PD-1/PD-L1 for Improved Immune Response

Antitumor immune mechanisms vary across different cancer types, influencing tumor growth and progression in distinct ways. A crucial pathway that enables cancer cells to evade the immune system is the PD-1/PD-L1 axis, which has emerged as a significant target for immunotherapy. In recent years, CRISPR/Cas9 technology, known for its precise genome editing capabilities, has been recognized as an effective approach to modify this pathway. Research has indicated that the deletion or modification of PD-1/PD-L1 expression through gene editing can improve T-cell functionality, trigger apoptosis in cancer cells, and diminish resistance to immunotherapies. Consequently, the engineering of immune cells using CRISPR/Cas9 has greatly enhanced their capacity to confront tumor cells. Furthermore, the regulation of PD-L1 expression in cancer cells has heightened their susceptibility to targeted therapies. Despite the encouraging outcomes, challenges such as the efficiency of CRISPR delivery systems to target cells, biosafety concerns, and the potential for unintended side effects persist and necessitate further exploration. Nevertheless, recent discoveries imply that editing PD-1/PD-L1 via CRISPR/Cas9 may serve as an innovative strategy to bolster immunotherapy and address treatment resistance.

Leukemia

Leukemia is a form of blood cancer that arises from hematopoietic stem cells and is noted for its significant variety in various

disease subtypes. A research study by Chiba *et al.* examined the molecular mechanisms that regulate PD-L1 expression in adult T-cell leukemia and lymphoma. Through the use of CRISPR screening, this research revealed that multiple signals, such as the JAK/STAT, NF-κB, and MAPK pathways, are crucial in promoting PD-L1 expression [21]. Furthermore, Chen *et al.* indicated that the activation of these pathways may result in immune evasion and heightened resistance of cancer cells to immunotherapies. These discoveries could facilitate the creation of novel therapeutic approaches in the realm of adult T-cell cancers and improve the efficacy of immunotherapies [22].

Multiple Myeloma (MM)

Multiple myeloma is a malignancy of plasma cells for which no definitive treatment exists. Research has indicated that the deletion of PD-1 in cytotoxic T cells via CRISPR/Cas9 technology enhances the production of cytokines such as TNF-α and IFN-γ, promotes apoptosis in cancer cells, and ultimately slows disease progression. These discoveries have opened a new pathway for the development of CAR T cells that exhibit greater resistance to immunosuppression in patients with multiple myeloma. Furthermore, Korell *et al.* demonstrated in their research that while T cells equipped with a chimeric antigen receptor (CAR-T) targeting BCMA are effective in treating multiple myeloma, the gradual loss of these cells can result in disease relapse. Researchers employed *in vivo* CRISPR knockout screening to explore genes that influence the survival and effectiveness of CAR-T cells. The findings revealed that the inhibition of the CDKN1B gene (cyclin-dependent kinase inhibitor 1B) was a critical factor restricting the survival of CAR-T cells in later stages, and its removal resulted in enhanced cell proliferation, improved effector function, and ultimately greater tumor clearance and overall survival. These results underscore the significance of CDKN1B as a promising target for the development of more effective CAR-T therapies and highlight the utility of *in vivo* screening for identifying enhancer genes [23].

Breast Cancer

Triple-negative breast cancer (TNBC) is a specific form of breast cancer characterized by the absence of estrogen receptors (ER), progesterone receptors (PR), and the HER2 protein in cancer cells. These features render TNBC resistant to standard hormonal and targeted therapies, necessitating the development of innovative treatment approaches. Recent research has indicated that the PD-L1 protein, also referred to as B7-H1, is involved in DNA repair processes, in addition to its critical function in modulating the immune response. Xue *et al.* discovered a positive correlation between the levels of PD-L1 and DNA-PKcs (DNA repair kinases) in breast cancer, particularly in the triple-negative subtype (TNBC), suggesting that PD-L1 may assist DNA-PKcs in the repair of DNA double-strand breaks. Consequently, the downregulation of PD-L1 through CRISPR-Cas9 technology could interfere with this repair process, thereby enhancing the vulnerability of cancer cells to immunotherapies and drugs that cause DNA damage. The dual function of PD-L1 in immune evasion and DNA repair positions it as a promising target for cancer therapy. Tiwari *et al.* conducted a review on the therapeutic potential of CRISPR/Cas9 in the context of triple-negative breast cancer (TNBC). They emphasized that CRISPR/Cas9 can inhibit the progression of TNBC by targeting genes associated with drug resistance, transcriptional regulation, and epigenetic changes. The review further addressed the difficulties related to the efficient delivery of CRISPR/Cas9 to target cells, summarizing various methods including physical (microinjection and electroporation), viral

(adenoviral and lentiviral), and non-viral (liposomal and lipid nanoparticle) strategies. The authors pointed out that the absence of accurate and safe *in vivo* delivery techniques continues to be a significant obstacle to clinical application, while the combination of CRISPR with artificial intelligence and machine learning presents potential to improve therapeutic effectiveness [24]. In a study carried out by Andreu-Saumell *et al.* the influence of chimeric receptor (CAR) binding affinity on the susceptibility of CAR-T cells to inhibition by the PD-1/PD-L1 axis was examined. They proposed that the affinity between the CAR and the target antigen might dictate the sensitivity of T cells to this inhibitory mechanism. To evaluate this proposition, they engineered CAR-T cells with low-affinity (LA) and high-affinity (HA) receptors targeting the HER2 antigen and assessed their performance in various preclinical models. The findings indicated that CAR-T cells with low-affinity receptors exhibited greater sensitivity to PD-L1-mediated inhibition, whereas those with high-affinity receptors were less impacted by this inhibition. Additionally, the deletion of the PD-1 gene via CRISPR/Cas9 technology in LA CAR-T cells resulted in enhanced cytokine secretion, increased polyfunctionality *in vitro*, and improved antitumor effectiveness in animal models. These alterations were associated with a decrease in the expression of gene signatures linked to T-cell exhaustion. Conversely, the deletion of PD-1 did not significantly influence the characteristics of HA CAR-T cells [25].

Women's Cancers: Cervical and Ovarian

As a novel tool, CRISPR/Cas9 technology has demonstrated considerable promise in enhancing the treatment of gynecological cancers, such as cervical and ovarian cancer. Zhen *et al.* utilized liposomes to deliver CRISPR/Cas9 targeting the E6/E7 genes of human papillomavirus (HPV) in cervical cancer, discovering that this method could bolster the antitumor immune response. They revealed that when this strategy was paired with PD-1 inhibitors, it resulted in an increase in the population of dendritic cells as well as $CD8^+$ and $CD4^+$ T lymphocytes, significantly suppressing tumor growth. These results represent a crucial advancement toward employing CRISPR/Cas9 as an innovative therapeutic approach for this prevalent cancer among women [26]. Yahata *et al.* conducted a study on ovarian cancer, demonstrating that the application of AAV-CRISPR/Cas9 to eliminate the PD-L1 gene can interfere with the expression of this protein in cancer cells, thereby enhancing antitumor immunity. Additionally, it was noted that this approach led to increased T-cell infiltration and significantly improved the survival rates of mouse models, suggesting the potential of this technology to modulate the tumor microenvironment and impede the advancement of ovarian cancer. In essence, in the context of ovarian cancer, the deletion of PD-L1 through CRISPR/Cas9 can influence the production of antitumor cytokines and chemokines, thereby hindering cancer progression. These findings have fostered optimism for the advancement of targeted therapies in this intricate disease [27].

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) ranks among the most prevalent and lethal forms of cancer globally, with various factors contributing to its development. These factors encompass viral infections such as hepatitis B and C, excessive alcohol intake, and exposure to harmful substances like aflatoxin. Research has pinpointed an intracellular protein known as the aryl hydrocarbon receptor (AhR), predominantly located in the cytoplasm and nucleus of cells; this receptor may influence liver cancer and modulate the expression of PD-L1. In the absence of a ligand (such as environmental toxins or aflatoxin), the AhR

resides in the cytoplasm. In this configuration, the AhR is associated with partner proteins, including Hsp90 (Heat Shock Protein 90), p23, and XAP2. This protein assembly stabilizes the AhR and inhibits its untimely movement to the nucleus. Upon ligand binding, the AhR undergoes a conformational change and separates from Hsp90, subsequently being transported into the nucleus. Within the nucleus, the AhR interacts with the protein ARNT (AhR Nuclear Translocator) and attaches to specific DNA regions to modulate the expression of target genes. Zhu *et al.* utilized CRISPR/Cas9 genomic screening to demonstrate that the aryl hydrocarbon receptor (AhR) is activated in response to aflatoxin (AFB1) B1, leading to an increase in PD-L1 expression. This increase contributes to the suppression of the immune response and the progression of tumors. These results indicate that the AhR/PD-L1 pathway may serve as a potential therapeutic target for aflatoxin-associated hepatocellular carcinoma (HCC) and could facilitate the development of targeted therapies [28]. Additionally, Guo *et al.* found that the inactivation of the PD-1 gene in chimeric antigen receptor T cells (CAR-T) through CRISPR/Cas9 technology can enhance the cytotoxic capabilities of these cells against HCC cells, thereby improving the antitumor response [29].

Colorectal Cancer

Colorectal cancer (CRC), recognized as a prominent gastrointestinal malignancy, is influenced by both genetic and environmental factors. Immune checkpoint inhibitors (ICIs), including anti-PD-1/PD-L1 antibodies, exhibit significant effectiveness in CRC, especially among patients with microsatellite instability (MSI) or mismatch repair deficiency (dMMR). dMMR leads to uncorrected errors in DNA replication, such as base mismatches (for instance, adenine pairing with cytosine instead of thymine), which result in the accumulation of mutations and the emergence of MSI. This phenomenon subsequently enhances the response to ICIs by producing neoantigens, abnormal proteins that are not present in normal cells but are identified as foreign by the immune system. These neoantigens, which are presented by dendritic cells, stimulate CD8+ T cells to attack cancer cells, thereby increasing the efficacy of ICIs. Nevertheless, ICIs tend to be less effective in cases that are microsatellite-stable (MSS). Tyagi *et al.* illustrated, through the use of cell models and gene expression analyses, that the aberrant activation of the Notch pathway in CRC elevates PD-L1 expression, which in turn suppresses antitumor immunity and facilitates tumor progression. Their use of Notch inhibitors confirmed this relationship, underscoring the Notch pathway as a potential therapeutic target to improve immunotherapy outcomes in MSS patients [30].

Lung Cancer

Non-small cell lung cancer (NSCLC), recognized as one of the most prevalent and lethal forms of cancer, presents considerable challenges in its treatment. Immunotherapy is regarded as the primary treatment option for these patients, as it boosts T-cell activity against cancer cells by obstructing the PD-1/PD-L1 interaction. Nevertheless, certain patients exhibit a limited response to this therapy due to mechanisms of immune evasion. To address these challenges, CRISPR/Cas9 technology has emerged as an innovative tool for direct genetic modification. In this context, numerous studies have been undertaken that concentrate on the inhibition of PD-1 and PD-L1 in both cancer and immune cells, each revealing different facets of this strategy. You Lu and his team introduced a groundbreaking method aimed at improving immunotherapy through a clinical trial. They employed CRISPR/Cas9 technology to eliminate the PD-1 gene in T cells derived from patients suffering from advanced NSCLC. The procedure entailed the extraction of T cells

from the patients, precise editing of the PD-1 gene utilizing the CRISPR/Cas9 system, and subsequent expansion of these cells in a laboratory setting. Following the expansion phase, the modified T cells were reintroduced into the patients. The findings indicated that the approach was safe, with a remarkably low incidence of off-target mutations (0.05%). However, due to the small sample size (only 12 patients), the full extent of T cell activity could not be conclusively established. Nonetheless, this research represents a significant advancement toward the clinical utilization of CRISPR/Cas9 in the field of immunotherapy [31]. Liu and his team carried out an extensive study aimed at regulating PD-L1-related immune pathways to enhance the response to immunotherapy. They employed CRISPR/Cas9 technology to concurrently target PD-L1 genes along with associated pathways such as TGF-β, both of which contribute to immunosuppression. The findings indicated that modifying these genes led to an increase in T-cell activity against lung cancer cells and heightened tumor sensitivity to immunotherapy. Additionally, they investigated the JAK-STAT pathway, which is crucial for regulating inflammatory responses and T-cell activity. The results demonstrated that manipulating this pathway using CRISPR/Cas9 could improve the effectiveness of PD-1 inhibitors in preclinical models of NSCLC. This integrated strategy opens new avenues for addressing complex resistance in lung cancer treatment [32].

Melanoma

Melanoma is recognized as one of the most aggressive forms of skin cancer, arising from melanocytes, and poses significant treatment challenges due to its propensity for metastasis. Immunotherapy utilizing immune inhibitors, such as PD-1/PD-L1 inhibitors, which enhance the immune response, is established as a standard approach for advanced melanoma; however, the rate of patient response to this therapy is frequently limited. The CRISPR/Cas9 technology, a novel gene editing tool, enables precise targeting of the PD-L1 and PD-1 genes, potentially enhancing the efficacy of immunotherapy and mitigating treatment resistance by boosting T-cell activity. In a clinical trial, Stadtmauer *et al.* employed CRISPR/Cas9 technology to eliminate the PDCD1 gene, which is responsible for encoding the PD-1 protein, as well as the TRAC and TRBC genes, in tumor-infiltrating lymphocytes (TILs) derived from patients suffering from melanoma and other advanced malignancies. The findings indicated that the approach was safe, evidenced by a low incidence of off-target mutations; however, the clinical efficacy could not be thoroughly assessed due to the limited sample size of patients involved [33]. In a separate investigation, Su *et al.* found that the inactivation of the PD-1 gene through CRISPR/Cas9 in primary T cells from melanoma patients led to a significant increase in IFN-γ production and enhanced cytotoxicity against melanoma cells. In this research, the T cells of patients were also stimulated with melanoma-associated peptides, resulting in a more robust immune response, as the presentation of these peptides effectively activated the T cells toward tumor antigens, ultimately enhancing the anti-cancer activity of these cells. This underscores the method's potential to improve immunotherapy for melanoma treatment. Nevertheless, additional research is required to facilitate broader clinical application.

Targeting the PD-1/PD-L1 Axis: A Comprehensive Review of Inhibitory Strategies across Cancer Types

To evaluate the impact of PD-1/PD-L1 pathway inhibition across various tumor types, the findings documented in existing literature have been compiled and summarized in an overview. The graph

depicted in Figure 5 illustrates the outcomes of independent studies where the CRISPR-Cas9 system was employed to directly target the PD-1 or PD-L1 genes. In the triple-negative breast cancer (TNBC) model using MDA-MB-231 cells, a complete eradication of PD-L1 expression was noted [34]. In lung adenocarcinoma cell lines A549 and H1975, a reduction exceeding 90% in PD-L1 expression at both RNA and protein levels has been documented [35].

In the glioblastoma U87 cell model, HDR-based editing achieved a 64% decrease in PD-L1 expression [36].

Furthermore, in T cells derived from patients with non-small cell lung cancer (NSCLC), PD-1 gene editing was conducted with an average efficiency of 5.8%, resulting in a 46.3% reduction in PD-1 surface expression. These results underscore the significant potential of the CRISPR-Cas9 system in modulating tumor immune evasion pathways and offering innovative strategies for cancer immunotherapy.

Engineering the Immune Response: The Role of CRISPR-Cas9 in Modulating Immune Checkpoints

According to the information presented in the dataset, the expression levels of 16 immune suppressor genes are illustrated under both control and treatment conditions (CRISPR-Cas9-edited) as shown in Figure 6. Significant downregulation occurred in key immune suppressor genes such as PDCD1 (PD-1), CTLA4, and LAG3 following the editing process, whereas genes like CD274 (PD-L1), PDCD1LG2 (the equivalent of PD-L2), BTLA, and other checkpoint genes exhibited an upregulation. This concurrent downregulation and upregulation may suggest a compensatory mechanism or a shift in the expression patterns of other checkpoints after the knockout of PD-1/PD-L1.

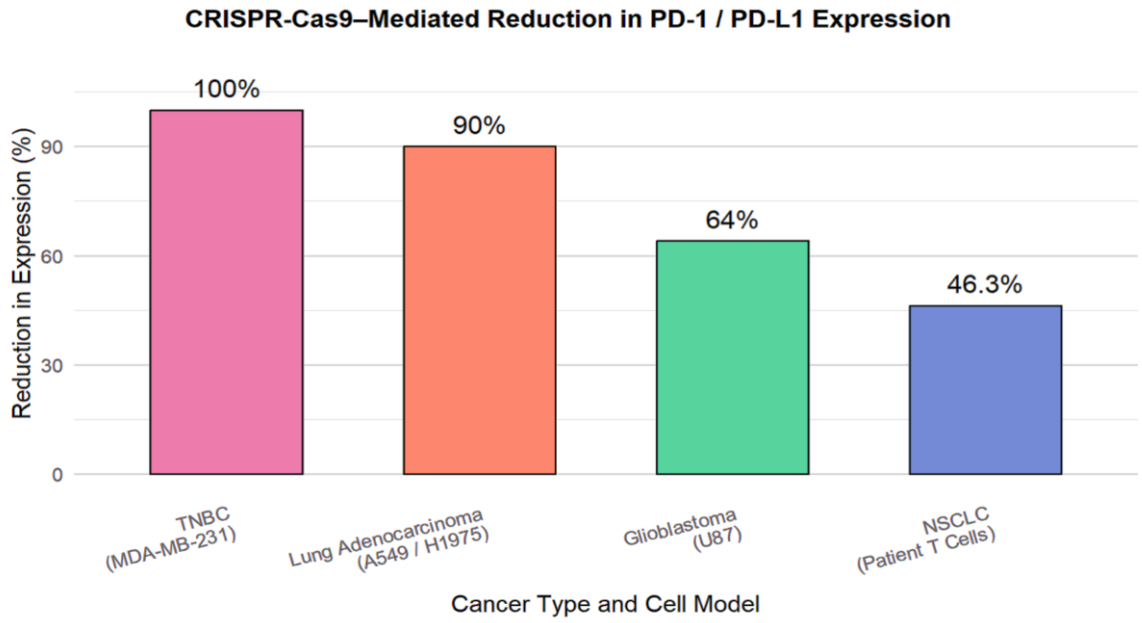


Figure 5. The degree of decrease in PD-1 or PD-L1 gene expression following the implementation of CRISPR-Cas9 technology across various cancer cell models. The most significant reduction was noted in the TNBC model, while the least was found in T cells derived from NSCLC patients. These findings illustrate the capability of CRISPR to obstruct tumor immune evasion pathways. Created by authors

In essence, while the elimination of the PD-1/PD-L1 axis removes the suppression of immune cells via this pathway, tumor cells might still evade immune detection by activating alternative inhibitory pathways.

These results highlight the necessity for a more thorough examination of the regulatory pathways involved in immune evasion when optimizing gene-edited CAR-T therapy, to avert the development of secondary resistance mechanisms beyond the inactivation of PD-1/PD-L1 [37,38].

Novel Approaches to Overcome Treatment Resistance in Oncology

In a study conducted, Abounar *et al.* employed CRISPR/Cas9 technology to silence two critical genes, PD-L1 and KRAS, in lung cancer cells simultaneously [35]. Initially, gRNAs were designed to facilitate precise editing of specific gene regions. Subsequently, A549 cells, which serve as a common model for non-small cell lung cancer (NSCLC), were transfected with the CRISPR system. The findings of this study indicated that the concurrent inhibition of PD-L1 and KRAS resulted in reduced cell proliferation, an increased rate of apoptosis (programmed cell death), and heightened sensitivity of the cells to the drug cisplatin. These results are significant for two reasons: Firstly, the inhibition of PD-L1 disrupts the immunosuppressive pathway, thereby enhancing the activity of immune cells against the tumor. Secondly, the inhibition of KRAS, an oncogene prevalent in numerous tumors, improves the efficacy of drug therapy by suppressing cancer cell growth. This study effectively illustrates that the strategic application of CRISPR/Cas9 to target essential pathways associated with tumor cell growth and immune evasion can address one of the most critical therapeutic challenges, specifically chemotherapy resistance. In fact, the genetic silencing of PD-L1 not only boosts immunotherapy, but also lays the groundwork for improved responses to traditional therapies.

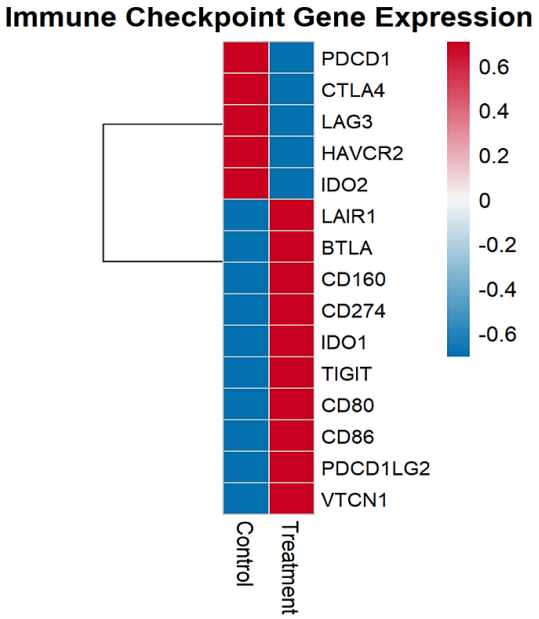


Figure 6. A heatmap illustrating the comparison of immune checkpoint gene expression in TIL cells treated with CRISPR versus control. The color red signifies heightened expression, whereas blue denotes reduced expression. Notable inhibitory genes, including PD-1, CTLA-4, and LAG-3, exhibited downregulation in the treatment cohort, while the expression levels of other genes, such as PD-L1 and BTLA, were found to be upregulated. Created by authors

The Role of Artificial Intelligence in gRNA Design

Artificial intelligence (AI) has emerged as a powerful tool for the rational design of guide RNAs (gRNAs) intended for precise genome editing, including the targeting of clinically relevant genes such as PD-L1. The selection of an optimal gRNA is a critical step, as the efficiency associated with different sequences can vary considerably; some gRNAs exhibit high cleavage activity, whereas others may perform suboptimally or lead to unintended off-target modifications. To tackle these issues, advanced computational platforms utilizing machine learning (ML) and deep learning (DL) techniques have been developed to systematically analyze large genomic datasets and predict gRNAs with the highest on-target activity and the least off-target risks. These platforms make use of experimentally validated resources, including DeepHF, GenomeCRISPR, and CHANGE-seq, and employ a range of predictive architectures. For instance, deep neural networks (DNNs) are adept at capturing complex sequence-function relationships within DNA, enabling accurate efficiency predictions [9]. Convolutional neural networks (CNNs), which are optimized for identifying sequence patterns, further enhance predictive accuracy by uncovering subtle features associated with functional gRNAs. The combination of such algorithmic frameworks facilitates the careful and efficient selection of gRNAs, thereby improving the specificity and efficacy of CRISPR-mediated genome engineering while minimizing unintended genomic alterations [39,40].

As a practical example, the modification of the PD-L1 gene using CRISPR/Cas9 involved two distinct single-guide RNAs (sgRNAs), designated as g82 and g165, in conjunction with a homology-directed repair (HDR) template. In this setup (Figure 7), sgRNA g82 is engineered to target a position 82 base pairs downstream from the start of exon 3, while sgRNA g165 is aimed at a site 165 base pairs from the same exon.

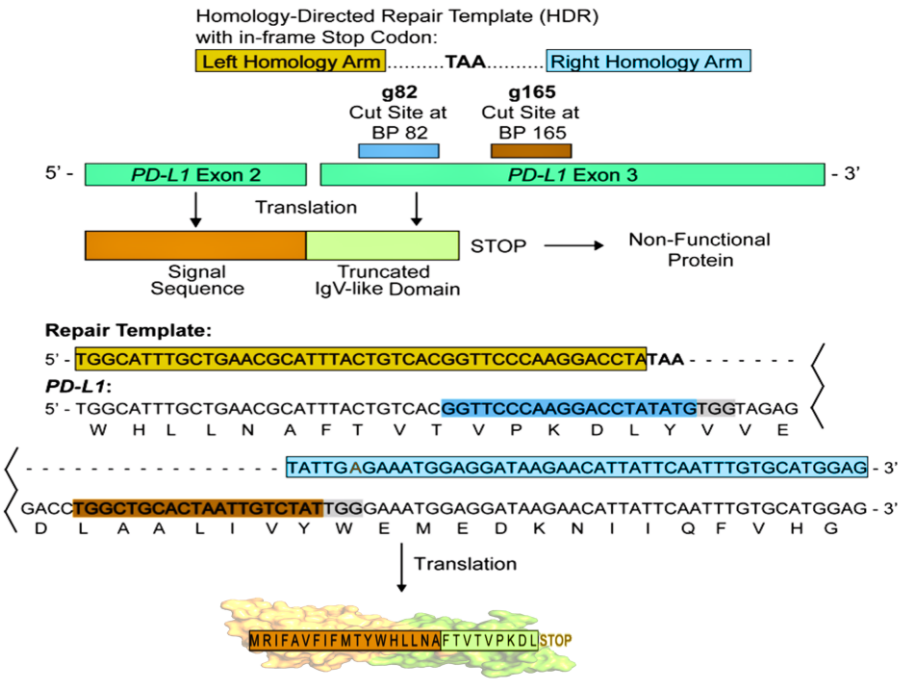


Figure 7. The introduction of a premature stop codon into the PD-L1 gene via homology-directed repair (HDR) undermines the proper functionality of the resultant PD-L1 protein. This modification reduces PD-L1/PD-1 interactions, consequently boosting immune system activity. The lower panel displays the exact nucleotide sequence that corresponds to the schematic illustration presented above. Created by authors

The HDR template was meticulously designed to insert an in-frame stop codon (TAA) into the target sequence. Its left (orange) and right (light blue) homology arms are aligned with the cleavage sites of g82 and g165, respectively, which are spaced 45 bp apart. The incorporation of the TAA codon leads to premature termination of translation, resulting in a truncated PD-L1 protein that lacks essential structural domains required for immune checkpoint signaling. Consequently, the modified protein is incapable of inhibiting immune activity. Given that PD-L1 is frequently overexpressed in tumor cells, thereby facilitating immune evasion and supporting tumor survival, its functional inactivation holds considerable potential in cancer immunotherapy, particularly by enhancing T cell-mediated anti-tumor responses. The selection and optimization of these sgRNAs can be improved through the application of artificial intelligence-driven bioinformatics tools to maximize on-target efficiency while reducing off-target effects [36].

Conclusion

Accumulating evidence highlights CRISPR/Cas9 as one of the most potent gene-editing platforms, possessing considerable potential to address therapy resistance across various cancer types. The precise targeting of the PD-1/PD-L1 axis through CRISPR-based methodologies not only reinstates the immune competence of T cells, but also amplifies tumor sensitivity to complementary treatment approaches such as chemotherapy and immunotherapy. Furthermore, the genetic modification of CAR-T cells by disrupting PD-L1 or PD-1 genes has shown remarkable effectiveness in preclinical investigations, resulting in significant tumor growth inhibition and extended survival in animal models. Recent developments have further expanded the possibilities for this strategy. The combination of CRISPR with nanobody-based delivery systems and artificial intelligence-driven design tools presents a promising avenue toward optimized, highly specific cancer therapies. Nanobodies, due to their diminutive size and excellent tissue penetration, serve as an ideal vehicles for tumor-targeted CRISPR delivery. Concurrently, machine learning algorithms can be utilized to create patient-specific gRNA sequences that maximize on-target efficiency while minimizing off-target effects. Such integrated approaches facilitate the creation of safer, more effective, and personalized therapeutic regimens, with the potential not only to surmount tumor resistance, but also to reduce treatment-related adverse effects. Importantly, translating CRISPR-based PD-L1 therapies into the clinic requires careful attention to biosafety and ethical concerns. Off-target effects, immune reactions against Cas9, and challenges in precise delivery remain key obstacles, despite improvements with high-fidelity Cas9 and AI-optimized gRNAs. Ethically, the permanent nature of genomic edits demands fully informed consent and strict use in somatic cells only. Ensuring fair access, transparent reporting, and long-term patient monitoring is essential for the safe and responsible integration of these therapies into cancer treatment.

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Ethical Approval

Not applicable.

Consent to Participate

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Data Availability Statement

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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