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Research Progress on CRISPR/Cas9-Mediated Gene Editing Combined with CAR-T Tumor Immunotherapy

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Abstract. CAR-T cell therapy is a highly promising cancer treatment; however, its application and efficacy are limited by various factors. This paper explores the challenges faced by CAR-T cell therapy, such as antigen escape, treatment side effects, the immunosuppressive microenvironment of solid tumors, and low efficiency of cell migration and infiltration. To overcome these bottlenecks, CRISPR/Cas9 technology can be utilized to enhance CAR-T cells through gene editing, improving their anti-tumor effects. The paper details methods and applications of CRISPR/Cas9 technology in improving CAR-T therapy. Finally, the paper summarizes the current bottlenecks in the field and provides prospects for future development.

Keywords: Immunotherapy, CAR-T cell therapy, CRISPR/Cas9, Combination therapy.

1. Various Approaches to Tumor Treatment

Cancer has become a recognized global health challenge, imposing a significant social and economic burden. In 2022, there were 20 million new cases of cancer worldwide and 9.7 million deaths [1]. Generally, the occurrence of cancer is often accompanied by abnormal activation of oncogenes and persistent silencing of tumor suppressor genes [2].

Currently, cancer treatments include several approaches: traditional methods such as chemotherapy, and emerging therapies like immunotherapy and targeted therapy. Traditional treatments encompass radiation therapy, chemotherapy, and surgical resection, which were the primary strategies for combating cancer in its early stages. However, these methods are often insufficient for completely eliminating the disease, leading to high recurrence rates, drug resistance, and limited specificity with side effects [3,4]. Targeted therapy, often based on antibodies, specifically attacks cancer cells that overexpress certain antigen molecules, such as PD-1 (programmed cell death 1) [5] and PDL-1 (programmed cell death-ligand 1) [6]. Immunotherapy, often based on exogenous cells or exogenous cell factors, enhances the body’s own immune system to effectively kill tumor cells, with examples including engineered CAR-T (Chimeric Antigen Receptor T-Cell) cell therapy [7]. In recent years, immunotherapy has matured and started to be used in clinical practice. Since therapeutic agents often originate from endogenous substances in the body, immunotherapy is characterized by effective tumor cell killing, low recurrence rates, high specificity, and minimal side effects [8]. Moreover, immunotherapy has shown high recovery rates, up to 92% in patients with end-stage acute lymphoblastic leukemia, which has drawn considerable attention [7,9,10]. Additionally, emerging molecular biology technologies have been applied to enhance these therapies, with CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 being a prominent example [11,12]. Therefore, this paper will discuss the research progress on CRISPR/Cas9-mediated gene editing combined with CAR-T tumor immunotherapy, known as the "gene scissors."

2. Principles and Development of CAR-T Cell Therapy

2.1. Principles

CAR (Chimeric Antigen Receptor) is an engineered synthetic receptor designed to guide lymphocytes (most commonly T cells) to recognize and eliminate cells expressing specific target antigens. CAR-T cell therapy is a revolutionary cancer treatment method that has been shown to produce highly effective and durable clinical responses [13]. A notable milestone in this field is the unprecedented success of anti-CD19 (cluster of differentiation 19) CAR-T cell therapy in treating B-cell malignancies, which received approval from the U.S. Food and Drug Administration (FDA) in March 2017 [14,15].

The CAR binds to cell surface-expressed target antigens, leading to strong T-cell activation and a robust anti-tumor response, independent of MHC (major histocompatibility complex) receptors [16]. The critical component of CAR-T cells is the modular synthetic receptor CARs, which consists of four main parts [17]: (1) the extracellular target antigen-binding domain, which provides specificity for the target antigen; (2) the hinge region, which provides flexibility to overcome steric hindrance and aids in the adjustment of length to allow the antigen-binding domain to access the target epitope; (3) the transmembrane domain, which anchors the CAR in the T-cell membrane; (4) one or more intracellular signaling domains, which trigger endogenous immune responses in the T cells.

2.2. Development

CAR-T cell therapy has undergone continuous development and iteration, with five generations to date. First-generation CARs involve only one intracellular signaling domain, CD3ζ, while second-generation CARs include an additional co-stimulatory molecule along with CD3ζ. Third-generation CARs contain another co-stimulatory domain. The most advanced fourth-generation CAR-T cells can effectively stimulate downstream transcription factors to trigger cytokine release upon CAR detection of tumor-associated antigens (TAA) [18]. Recently, with the advancement of the CRISPR system, applying CRISPR technology to CAR-T cells to establish fifth-generation CAR-T cells has garnered widespread attention. For example, using CRISPR/Cas9 to knock out negative regulators of T-cell persistence and effector function (such as PD-1 [19], CTLA-4 [20] (cytotoxic T-lymphocyte associated protein-4), and LAG-3 [21]) has the potential to significantly enhance the therapeutic efficacy of CAR-T cells [22].

3. Challenges Facing CAR-T Cell Therapy

3.1. Antigen Escape

One of the most challenging limitations of CAR-T cell therapy is the resistance of tumors to monoclonal antigen-targeted CAR-T constructs. Although initially, single-antigen targeted CAR-T cells can provide high response rates, a significant portion of patients undergoing CAR-T cell therapy [23] exhibit malignant cells that show partial or complete loss of target antigen expression, a phenomenon known as antigen escape [21,24]. For instance, despite 70% of relapsed and 90% of refractory acute lymphoblastic leukemia (ALL) showing durable responses to CD19-targeted CAR-T cell therapy, recent follow-up data indicate a common disease resistance mechanism, with 30-70% of relapsed disease patients experiencing downregulation or loss of CD19 antigen after treatment [25].

3.2. Treatment-Related Side Effects

Despite CAR-T cell therapy being a revolutionary cancer treatment tool, its high toxicity and mortality rates have impeded its adoption as a first-line treatment. The selection of target antigens can result in associated toxicity; ideally, these antigens should possess characteristics such as high coverage, stability, and specificity [26]. Tumor antigens can be classified based on their expression patterns into tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs). TAAs are present not only in tumors but also in normal tissues, leading to toxicity to normal cells when CAR-T cell therapy is applied. However, due to the lack of TSAs, most current CAR-T cell therapies for solid tumors target TAAs. The limited specificity of TAAs makes off-target effects inevitable, sometimes resulting in severe adverse effects that can even be fatal [27].

In addition to the increased likelihood of off-target effects due to the lack of TSAs, in actual treatments, CAR-T cells that recognize target antigens can trigger activation-induced cytokine release or stimulate immune cells to release inflammatory cytokines. This can lead to cytokine release syndrome (CRS), neurotoxicity, and various other adverse reactions [28]. To date, the potential toxicity of CAR-T cell therapy has been most extensively characterized in patients receiving the first FDA-approved CAR-T cell therapy targeting CD19 [29]. Even in clinical trials with the highest response rates, severe, life-threatening adverse reactions occur in patients [30]. Specifically, in patients with acute lymphoblastic leukemia/lymphoblastic lymphoma (LBL) receiving CAR-T cell therapy, almost all patients exhibit at least some mild toxicities, while 23-46% of patients show severe physiological cytokine production and substantial in vivo T-cell expansion [31]. Systemic cytokine release and severe immune cell cross-activation at these toxicity levels can lead to the following toxicities in some patients: 1. CRS [32]; 2. Hemophagocytic lymphohistiocytosis (HLH) and/or macrophage activation syndrome (MAS), a severe high-inflammatory syndrome characterized by CRS, accompanied by elevated serum ferritin, hemophagocytosis, renal failure, elevated liver enzymes, splenomegaly, pulmonary edema, and/or reduced NK cell activity; and immune effector cell-associated neurotoxicity syndrome (ICANS), characterized by elevated cerebrospinal fluid cytokine levels and disruption of the blood-brain barrier [33].

3.3. Immune-Suppressive Microenvironment in Solid Tumors and T-Cell Exhaustion

The complex immune-suppressive network within the tumor microenvironment (TME) is referred to as the tumor immune-suppressive microenvironment, composed of various immune cells, secretions, and inhibitory signals that collectively promote tumor initiation and progression [32]. In the TME, suppressive immune cells, including tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs), inhibit the proliferation and effective anti-tumor responses of CAR-T cells [34]. These immune cells and tumor cells promote the production of immune-suppressive cytokines such as TGF-β (transforming growth factor-β), IL-10 (interleukin 10), and IL-4 (interleukin 4), accelerating the exhaustion of T cells and CAR-T cells. Due to increased expression of PD-1 (programmed cell death 1) on both the TME and CAR-T cells, the binding of PD-1 on CAR-T cells to PD-L1 (programmed cell death ligand 1) on tumor cells initiates inhibitory signals, leading to impaired CAR-T cell function [35] and promoting immune evasion by tumor cells [36].

3.4. Inefficient Migration and Infiltration of CAR-T Cells in Solid Tumors

In solid tumors, CAR-T cell therapy often yields suboptimal results due to physical barriers and an immune-suppressive TME [37]. Physical barriers, such as abnormal vascular systems, dense extracellular matrix (ECM), and interstitial fluid pressure, obstruct the infiltration and migration of CAR-T cells within solid tumors [38]. Additionally, the presence of immune-suppressive cells, regulatory cytokines, and inhibitory molecules within the TME further limits the expansion and efficacy of CAR-T cells [39].

4. Maturation and Development of the Gene Editing Technology “CRISPR/Cas9”

CRISPR has evolved from an obscure bacterial genomic element to a widely used gene editing platform over several decades. In the late 1980s, researchers first noticed some unusual bacterial elements, where repetitive units were interspersed with non-repetitive spacer sequences [40]. By the mid-2000s, scientists realized that these repetitive units—later known as CRISPR—are part of the bacterial immune system: bacteria integrate phage DNA sequences into the spacer regions to acquire resistance [41]. Protospacer adjacent motifs (PAMs) were discovered as the conserved sequences adjacent to the spacer regions required for target DNA binding [42]. The various components of the CRISPR system were isolated and further defined: CRISPR-associated (Cas) enzymes cleave DNA, CRISPR RNA (crRNA) guides Cas to the DNA target site, and trans-activating CRISPR RNA (tracrRNA) binds with crRNA, forming a protein-RNA-Cas complex [43]. In 2012, two studies demonstrated that CRISPR could programmatically target desired DNA sequences in prokaryotic cells [44]; soon after, there were reports of gene editing in eukaryotic cells [45], solidifying CRISPR’s status as a versatile gene editing tool.

CRISPR systems are classified based on Cas nucleases. The most commonly used gene editing system is CRISPR/Cas9, followed by CRISPR/Cas12a. Cas9 recognizes a PAM sequence of NGG and cuts the target DNA through a blunt double-strand break upstream of the PAM [46].

CRISPR components can be introduced into target cells via transfection or transduction. Studies have shown that high-efficiency gene editing can be achieved using lentiviral transduction of CRISPR/Cas9 [47]. However, stable integration of the Cas9 gene into the host genome may increase off-target editing and trigger unwanted immune responses. Therefore, transient expression of Cas9 protein is advantageous, especially in clinical settings. CRISPR components can be introduced as DNA, RNA, or ribonucleoprotein (RNP) pre-complexes of RNA and Cas protein. RNPs require additional incubation steps but avoid the need for transcription or translation [48]. CRISPR components are typically introduced by electroporation or liposome-mediated infection. In electroporation, a pulsed electric current creates transient pores in the cell membrane, allowing charged RNA/Cas molecules to enter the cytoplasm. Electroporation is a rapid and efficient method for CRISPR gene editing but can be toxic to cells [49]. An alternative method is liposome-mediated infection, which uses cationic lipid particles to complex with CRISPR molecules and enter cells via endocytosis. Liposome-mediated infection has lower toxicity than electroporation but lower transfection efficiency [50].

Identifying target genes that regulate T cell function and fate is a key step in applying CRISPR-Cas9. CRISPR library screening technology is a biological tool based on the CRISPR-Cas9 system used for high-throughput gene function studies. Whole-genome CRISPR-Cas9 screening can unbiasedly identify key factors in cancer cell proliferation, drug resistance, and metastasis [51]. Additionally, CRISPR screening combined with Cas9 gene editing technology can identify essential transcription factors (TFs) for T cell differentiation and functional maintenance, revealing the role of immune suppression and metabolic signaling in shaping T cell fate [52]. Furthermore, it can provide insights into checkpoint regulation of human T cell cytokine production [53] and assist in designing more effective anti-cancer and anti-infection T cells.

5. Improvements and Applications of CRISPR/Cas9 in CAR-T Cell Therapy

5.1. Universal CAR-T Cells (Allogeneic CAR-T Cell Preparation Techniques)

The clinical development of novel CAR-T cell therapies is often hindered by the low yield and poor functionality of autologous peripheral blood T cells, especially in elderly patients and those with extensive pre-treatment. Consequently, the development of allogeneic CAR-T cells has become a major focus [54]. Recently, there has been increasing interest in using non-autologous T cells for treatment. This approach involves generating universal tumor-specific T cells from T cells of healthy donors, which can be used in any patient without the need for human leukocyte antigen (HLA) matching. This strategy could reduce costs, accelerate treatment, and provide T cell products for patients who have experienced lymphopenia or severe cancer, thus lacking sufficient healthy T cells for treatment [55].

A major challenge in allogeneic products is inducing graft-versus-host disease (GVHD). The mechanism of GVHD involves the recognition of “non-self” HLA molecules by the endogenous donor T cell receptors, leading to an immune response [54]. Allogeneic CAR-T cells can be derived from HLA-matched hematopoietic stem cell transplant donors, though this is less common [56]. Therefore, the focus often shifts to gene-edited cells that can be used in non-HLA-matched recipients. The theoretical basis for using these cells in non-HLA-matched subjects is to combine the CAR-T cell manufacturing process with TCR (T cell receptor) knockout. By targeting the TRAC (T cell receptor alpha constant) locus for gene knockout and using CAR as the knock-in target, CAR expression can be driven under the control of the endogenous TRAC promoter, similar to physiological TCR expression [57]. Simultaneously, knocking out the endogenous TCR eliminates the potential for GVHD and enables the production of allogeneic T cell products. This method has shown anti-leukemia efficacy in children and adults with relapsed acute lymphoblastic leukemia (ALL) [58].

5.2. Improving T Cell Dysfunction

Several factors often collectively hinder the long-term remission of CAR-T cell therapy, including issues with autologous CAR-T cell manufacturing, limited CAR-T cell expansion and/or persistence, and various intrinsic and extrinsic resistance mechanisms of T cells. To enhance the survival and functionality of CAR-T cells, various strategies have been employed, such as optimizing costimulatory domains [59], preventing tonic signaling [60], inhibiting the NR4A (Nuclear receptor 4A) transcription factor [61], blocking TOX/TOX2 (Thymocyte selection-associated high mobility group box protein), and overexpressing typical activator protein-1 (AP-1) factor c-Jun [62], but with limited success. Recent studies have shown that intermittent “rest” periods for CAR-T cells can alleviate exhaustion and enhance anti-tumor efficacy under the influence of epigenetic reprogramming. Therefore, using CRISPR/Cas9 technology to perform functional disruption or modulation of genes and epigenetic targets that enhance T cell exhaustion could potentially prevent or even reverse CAR-T cell dysfunction [20]. Targeting inhibitory receptors, transcription factors, and other mediators of CAR-T cell dysfunction through gene editing may rejuvenate the infused cell products. Utilizing CRISPR/Cas9 to remove negative regulatory factors for T cell persistence and functional efficacy (such as PD-1, CTLA-4, and LAG-3) could indeed be the first notable intervention point [63]. In addition to inhibitory receptors, disrupting apoptosis-related factor (FAS) receptor/Fas ligand interactions has been shown to reduce activation-induced cell death and enhance the anti-tumor function of CAR-T cells in vivo [64]. Finally, CRISPR/Cas9-mediated targeting of CAR-T cell diacylglycerol kinase (DGK) metabolism may confer resistance to immunosuppressive mediators in the tumor microenvironment (TME), such as TGFβ [65].

5.3. Enhancing CAR-T Cell Cytotoxicity and Reducing Immunogenicity

During the activation and expansion of CAR-T cells, the CRISPR-Cas9 system combined with transgene knock-in methods has been utilized to modulate cytokine signaling, thereby enhancing anti-tumor activity, improving T cell persistence, and reducing toxicity. Specifically, CRISPR/Cas9-based gene editing, combined with viral or non-viral DNA delivery, can achieve simultaneous biallelic or sequential gene targeting by designing T cells with expression cassettes at specific loci [66]. This technology allows the insertion of cytokine-encoding DNA cassettes into target genomic loci, placing these genes under the control of specific promoters to control expression timing. For example, IL-15 can be inserted into the IL-13 gene locus, thereby placing IL-15 expression under the control of the endogenous IL-13 promoter, which is highly active during T cell activation. This creates an inducible T cell-specific IL-15 activation switch [67]. Additionally, using CRISPR-Cas9 editing technology to remove genes encoding cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-6 [68], which are associated with neurotoxicity and cytokine release syndrome (CRS), may produce cell products with optimal efficacy and persistence while reducing adverse events related to abnormal cytokine production. Therefore, compared to traditional CD19 CAR-T cells, GM-CSF gene knockout CAR-T cells can maintain normal functionality, enhance in vivo anti-tumor activity, and effectively improve overall survival [54]. Gene knockout or ablation of the IL-6 gene may also improve CRS-like toxicity in leukemia mouse models [69].

5.4. CAR-T Cells in Solid Tumor Treatment

In the solid tumor microenvironment, persistent antigen stimulation leads to T cell exhaustion [70]. CRISPR-Cas9 can insert co-stimulatory molecules or disrupt the inhibitory molecule PD-1, thereby enhancing T cell proliferation and anti-tumor activity. In solid tumors, interaction between PD-1 and its ligands causes T cell exhaustion and inhibits CAR-T cell efficacy. Disrupting PD-1 expression in CAR-T cells via CRISPR-Cas9 can enhance their anti-tumor activity.

Additionally, disrupting both TCR and PD-1 can significantly improve CAR-T cell efficacy and reduce autoimmune responses [71]. Overexpression of certain genes, such as runx3, can accelerate T cell migration to tumor sites, which is also applicable to CAR-T therapy [72]. Modifying CAR-T cells with migration markers such as IGAT4, CXCR3, and CXCR1 can improve T cell infiltration [73].

Another approach to enhance CAR-T cells is to enforce the expression of anti-inflammatory cytokines. In melanoma, CAR-T cells can kill tumor-infiltrating lymphocytes that fail to respond to treatment under IL-2 signaling. Overexpression of the p40 subunit of IL-23 in CAR-T cells can enhance their anti-tumor activity in pancreatic cancer models, showing strong expansion capabilities and reduced cell apoptosis, with no significant side effects [74].

5.5. CRISPR Screening of T Cells

T cell therapy has shown impressive results in combating cancer, viruses, and inflammatory diseases. However, their fate and function are largely dependent on the microenvironment. Whole-genome CRISPR-Cas9 screening helps identify key regulators of T cell fate and function. It is also a valuable gene editing tool for determining cell fate, function, and differentiation [75]. CRISPR-based screening technology has driven major discoveries in cell biology and virus-host interactions [76]. This technology has been applied to both in vivo and in vitro T cell lines and primary cells, including whole-genome, metabolic, and transcriptomic screenings. CRISPR-Cas9 screening focused on T cells can be applied to various diseases, not limited to blood and solid cancers/infectious diseases, inflammation, and resistance factors in immunotherapy. It also helps identify pathways that lead to drug resistance in anti-tumor processes [77] and facilitates large-scale exploration of phenotype-related genes [78].

6. Bottlenecks and Prospects

Although the CRISPR-Cas9 system is a highly promising gene editing tool for CAR-T cell therapy, there are some limitations in its clinical application [79]. Firstly, off-target effects are a significant concern. The non-targeting of Cas9 is influenced by DNA topology, transcription, and replication processes, and there are currently no precise tools to predict off-target events. The gold standard for detecting off-target effects is targeted deep sequencing. Secondly, CRISPR-Cas9 is not suitable for all genes and cell types; some cells are difficult to edit, and certain gene regions present greater challenges.

Additionally, occasional chromosomal losses also impact clinical applications [80]. Despite CRISPR-Cas9 being used in Phase I clinical trials for various cancers, enhancing T cell therapies, Cas9-induced chromosomal deletions can impair T cell survival and proliferation. CRISPR-Cas9 targeting TCR chain genes can cause chromosomal truncations, leading to carcinogenic risks and cell death. Chromosomal structural variations can persist in the host for weeks or even months, threatening genomic integrity. Efficient CRISPR-Cas9 toolkits have eliminated chromosomal translocations and viral vector integrations in mouse models [81].

CRISPR-Cas9 genome editing allows T cells to better adapt to specific microenvironments, offering opportunities for advanced T cell therapies. The successful application of CRISPR-Cas9 technology has also driven the development of related tools, such as genome editing tools, gene expression regulation tools (CRISPRa, CRISPRi), and precision editing technologies. These developments will continually address the challenges of combining CRISPR/Cas9 with CAR-T tumor immunotherapy [82].

For example, combining high-throughput techniques, CRISPR-Cas9 screening, single-cell sequencing, and bioinformatics analysis is crucial for in vivo gene editing and screening. Modular pooled gene editing screening platforms (ModPoKI) enable rapid combinations of different gene editing setups, identifying new gene combinations that extend T cell lifespan and enhance anti-cancer efficacy [83]. Research has shown that TFAP4 can improve the adaptability of long-term stimulated CAR-T cells. Non-viral knockout of the BATF-TFAP4 combination significantly enhances engineered T cell capabilities. Combining single-cell sequencing with direct open reading frame capture can examine nearly 12,000 full-length genes driving TCR-induced proliferation, identifying key drivers of T cell secretion of pro-inflammatory cytokines, and providing opportunities for clinical transplantation [84].

7. Conclusion

Overall, given the complexity of diseases and the diversity of genetic backgrounds, using CRISPR-Cas9 technology to personalize treatments by modifying specific genes and cells can significantly improve therapeutic outcomes and enhance patients' quality of life. However, there are challenges to address, including the high cost of such therapies, the confirmation of long-term efficacy, and the ethical issues associated with gene editing technologies in humans. Further efforts are needed to ensure that more patients benefit from CAR-T cell therapy.

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