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The application of epigenetics in CAR-T therapy

Xinyi Zhang

Huazhong University of science and technology, Wuhan, Hubei

u201912367@hust.edu.cn

Abstract. This article reviews epigenetics (including DNA methylation, histone modification, chromatin remodeling, and non-coding RNA regulation), epigenetic phenomena in T cells, and the progress of research on CAR-T therapies modified from epigenetic regulation, with the aim of providing new insights for CAR-T to improve the safety and efficacy of clinical translation.

Keywords: CAR-T treatment, epigenetics, cancer immunotherapy, DNA methylation, T cells

1. Introduction

Epigenetics refers to the phenomena of heritable modifications in gene regulation and expression that do not entail changes to the DNA sequence. Its primary study areas include DNA methylation, histone covalent modifications, chromatin remodeling, and non-coding RNA control[1]. By integrating epigenetic molecular modifications with multiple levels of regulatory networks, such as cellular signaling networks and metabolic physiology, epigenetics actually regulates a set of expressed genes and the extent of their expression. This impacts cellular behavior and even human growth, development, and aging[2]. At the same time, epigenetics combines genetics and environment, transforming a complicated biological system (genomics, transcriptomics, etc.) into one that is not only stable but also responsive and malleable[3].

T cells are important components of the adaptive immune system. Two primary kinds of T cells are CD4 T cells that develop into helper T cells, and CD8 T cells that function as cytotoxic T lymphocytes to kill infected cells[4]. When antigenic peptide-MHC complexes attach to T cells, the effector process starts. Following activation and differentiation, T cells produce an immune response that kills target cells and depletes themselves. Additionally, memory T cells (MTC) formation is a durable defense mechanism that permits a rapid response to an antigen re-exposure[5]. Most of these biological processes that affect T cell function continues via epigenetic programs, which is regulated by genetic factors[6]. Thus, T cell epigenetic alterations have an impact on immunotherapies based on T cell engineering.

Chimeric antigen receptor T-cell (CAR-T) immunotherapy is a tumor immunotherapy approach that has gained considerable attention in recent years. CAR-T immunotherapy may target cancer cells and has demonstrated remarkable success in the treatment of cancers (particularly lymphoma). The process and foundation of CAR-T therapy is genetic engineering to create T cells with CAR structures that target tumor antigens and are then transfused back into the patient to enable the T cells to carry out their duty of locating and eliminating cancer cells[7]. Yet, many studies have demonstrated that CAR-T therapy is inefficient against solid tumors, most likely as a result of antigen escape and the tumor microenvironment, which CAR-T is unable to combat[8]. Aspects of CAR-T that hinder its translation to the clinic include off-target effects, cytokine release syndrome (CRS), and other restrictions that make it simple to relapse after treatment and cause severe toxic side effects in patients[9].

Since substantial epigenetic remodeling occurs in T cells in response to antigenic cues, as revealed by epigenomic profiling and other genetic methods, it is potential to improve the efficacy of CAR-T treatment by changing the epigenetically-regulated cellular activity. Combining the findings of the previous two years' research on enhanced CAR-T therapies utilizing epigenetic techniques, this study offers fresh perspectives to address the development and difficulties of CAR-T in clinical applications.

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2. Introduction of Epigenetics

DNA methylation, histone covalent modifications, chromatin remodeling, and non-coding RNAs involved in post-transcriptional control are all examples of epigenetic processes. Epigenetics regulates gene expression but does not alter gene sequences, and it is widely valued as it is heavily involved in the growth and development of organisms, while it is thought to play an important regulatory role in disease treatment and other aspects[10].

2.1 DNA Methylation

DNA methylation (5-mC), also known as DNA methyltransferases (DNMTs) or 5-methylcytosine, is the process through which cytosine's 5'-carbon atom receives a methyl group[11]. Based on their structure and function, eukaryotic DNA methyltransferases have been divided into three classes: Dnmt I, Dnmt II, and Dnmt III. Among them, Dnmt1 is the most prevalent DNMT in adult cells and functions at the hemimethylated CpG site of the DNA replication neostrand[12], Dnmt3a and Dnmtb are crucial for early embryonic development[13], and Dnmt2 has very little DNA methylation activity but is remarkably conserved across species[14]. Further research must be done to determine the specific role of DNA methylation. The "gene regulation model," which primarily involves transcriptional silencing such as blocking the recognition and binding of transcription factors and corresponding DNA, further influences cellular behavior and controls biological and physiological processes, is the most widely accepted theory in eukaryotes[11]. DNA methylation, which locks in the gene expression program that determines the cell lineage identity and supports the appropriate functioning of the immune system, has been demonstrated to be necessary for the epigenetic suppression of CD4 gene expression deficit in mature CD8+ T cells. However, malignant reprogramming of cells, which can cause cancer, may result from dysregulated DNA methylation around the transcriptional start site of important genes that control cell fate[15].

2.2 Histone Modifications

The main proteins that make up the nucleosome are known as histones. Due to their evolutionarily constant amino acid sequences, they are crucial to the integrity of chromosome structure[16]. The histone octamer is made up of two H2A and H2B dimers, two H3 and H4 dimers, and two H1 dimers, with H1 serving as the nucleosome's anchor and linker. Except for H1, histones have an asymmetric amino acid distribution along their peptide chains, with the C-terminus being rich in hydrophobic amino acids (such as valine and isoleucine) and the N-terminus being rich in basic amino acids (such as arginine and lysine). These amino acid regions are important locations for post-translational modifications like histone acetylation, methylation, phosphorylation, ubiquitination, etc[17]. Histone modifications influence the affinity of histones for DNA duplexes or other transcription factors for structural gene promoters. These modifications are linked to gene transcription, DNA damage repair, DNA replication, chromosome cohesion, and other processes, with histone methylation modifications having a significant impact on immune-related diseases[18]. Utx is a histone H3K27 demethylase, and research has shown that Utx KO mice show increased expression of pro-inflammatory cytokines, decreased Foxp3+ regulatory CD4+ T cells, and reduced levels of CCR4 (skin chemokine receptor), suggesting that Utx in CD4+ T cells may be involved in the regulation of the pathogenesis of (allergic contact dermatitis, ACD)[19].

2.3 Chromatin Remodeling

The mitotic cycle, cellular responses, gene expression, DNA replication and repair, chromosome condensation, and life processes like segregation and apoptosis are all governed by chromatin remodeling, which modifies the shape or location of individual nucleosomes as well as the higher structure of chromatin[20]. Tumor genesis and development are tightly correlated with the disturbance of all these basic mechanisms[21]. The ability of chromatin remodeling complexes to

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interfere with INO80 and BAF has been demonstrated to decrease the chromatin accessibility associated with depletion, hence enhancing T cell persistence and anti-tumor activities[22].

2.4 Non-coding RNA Regulation

98% of RNAs in nature are untranslated, with non-coding RNAs making up 30% to 50%. Noncoding RNAs, which regulate gene expression and protein synthesis, include short interfering RNAs, microRNAs, long noncoding RNAs, cyclic RNAs, etc [23].

2.4.1 Short interfering RNAs (siRNAs)

siRNAs are synthesized from long-stranded dsRNAs using Dicer, with a phosphate group at the 5' end and 2-3 prominent nucleotides (with hydroxyl groups) at each 3' end. In addition to RNAi gene regulation and transposon silencing methods, endogenous siRNAs can potentially play a role in the development of heterochromatin[24]. RNA interference is mostly brought on by siRNAs, which mute particular genes at the mRNA level. People have created disease-targeting strategies that connect siRNAs to CD4+ T cells based on this characteristic, with potential applications including HIV therapy[25].

2.4.2 MicroRNA(miRNA)

A class of 22 bp single-stranded RNAs known as miRNAs is mostly encoded by intergenic DNA regions, which are frequently transcribed in the opposite direction to nearby genes. miRNAs are widely expressed in animal and plant cells as RNA-protein complexes, also known as miRISCs, and are largely conserved across species (miRNA-induced silencing complexes). By encouraging the degradation or translational suppression of target gene mRNAs similar to miRNA sequences, they play a significant role in biological development by controlling gene expression as well as associated physiological processes[26, 27]. The target mitogen-activated protein kinase kinase 1 (MEK1) and extracellular signal-regulated protein kinase 1 (ERK1) are upregulated when miR-15a/16 is downregulated, altering the T cell activation process[28].

2.4.3 Long non-coding RNAs (long ncRNAs, lncRNAs)

80% of ncRNAs belong to the class of long noncoding RNAs (lncRNAs), which are RNA transcripts longer than 200 nucleotides and without a complete open reading frame. In addition to post-transcriptional control, gene splicing, mRNA processing, protein translation, protein localization, stem cell pluripotency, cellular structural integrity, heat shock response, and human illnesses, they also play tissue-specific roles[29]. According to one study, lncRNA-GM polarizes TH17 and prevents iTreg differentiation by reducing Foxo1 activity, demonstrating that lncRNAs can control the differentiation of T cell subpopulations in autoimmune disorders caused by T cell activation[30].

2.4.4 Cyclic RNA (circRNA)

CircRNA is a eukaryotic species-specific endogenous non-coding RNA with a closed-loop structure that is primarily formed by variable pre-mRNA shearing. By co-regulating the expression of target genes with related miRNAs and sponging miRNAs, circRNA plays a crucial biological role in the development of organisms[31]. According to one study, the circTRPS1/miR141-3p/GLS1 axis controls intracellular reactive oxygen species (ROS) homeostasis and CD8+ T cell depletion, and it has significant promise as a biomarker for BCa[32].

3. Epigenetic Mechanisms in T Cells

T cells are lymphocytes that mediate acquired immunity, which are crucial for immune system homeostasis and the body's defense against external pathogens[33]. T progenitor cells are the source of T lymphocytes, which can be present in extra-thymic organs including bone marrow and that give rise to early T lineage precursor (ETP) cells in the thymus. After TCR rearrangement, DP cells mature in the thymus to become functional antigen-specific T cells with the help of the positive

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selection of MHC molecules on the surface of stromal cells and the negative selection of neuropeptides bound to MHC. There are currently three phases in the differentiation of T cell destiny. CD4+/CD8+SP, CD4+/CD8+DP, and CD4-CD8-DN. helper cd4+ The two most prevalent subsets of mature T cells are T cells (Th) restricted by their own MHC class II molecules and cd8+ cytotoxic T cells (CTL) restricted by their own MHC class I molecules[33].

The adaptive immune system is driven by T cells. The antigenic peptide-MHC complex is specifically recognized by T cells, which are then activated and proliferate as a result of the initial signal produced by the interaction of this complex with T cells' TCR and the co-stimulatory signal produced by the interaction of CD28 and B7 family members[34]. Then, antigen and cytokine selection causes CD4 T0 cells to differentiate into several lineages, including TH1 and TH2, and CD8 T0 cells to differentiate into effector T cells (mainly CTL), in a way that is Th cell-dependent/independent. Virus-infected cell processing and antigen presentation, CTL binding to antigenic peptide MHC class I molecular complexes, CTL activation to kill target cells (secretion of granzyme/perforin and FasL/Fas), CTL dissociation, and target cell death are the four main stages of the immune response to antigenic tumor cells. After repeated antigen exposure, a portion of T cells eventually succumb to various causes of failure[35]; a portion develops into memory T cells (Tmem), which can react more quickly when exposed to same antigens the second time[36].

The intensity of the immune response and whether it manifests in response to a particular antigenic stimulus, but, differ significantly between individuals, suggesting that the immunological response is strictly regulated by the genetic background[37]. As epigenetic events in the development and growth of T cells have a significant role in the development of diseases and the development of treatment approaches, epigenetics has recently come into a clearer understanding[6]. Numerous studies have demonstrated that epigenetic changes have a significant impact on T cells[38–43]. Early T cell activation causes chromatin remodeling in the promoters of the core 3268 genes as well as alterations in the expression of several pathways that regulate metabolism, the cell cycle, inflammatory response genes, and cell survival.[38]. Also, the removal of key chromatin modification (H3K27me3) deposition by the histone demethylase KDM6B is essential for t-cell activation, and if inhibited kdm6b-dependent H3K27me3 demethylation can limit the size of virus-specific CD8+ T-cell responses and the formation of memory CD8+ T-cell populations[39]. It has also been observed that conditional DNA methyltransferase 3a (DNMT3a) knockdown T cells (KO T cells) show distinct, localized regions of hypomethylation compared to WT cells, and that these hypomethylations correspond to changes in gene expression in multiple pathways of T cell signaling and differentiation, reflecting the DNMT3a's critical role[40]. Thus early T cell lineage-specific chromatin reprogramming is closely associated with epigenetic modifications. Epigenetic regulation related to immune response such as histone methylation and acetylation can play an important role in the immune response by regulating CD4+ helper T (Th) cell function and lineage integrity and attenuating cytotoxicity of CD8 T cells through lysine methyltransferase (kmt) dot1l and histone deacetylase HDAC3, respectively[41, 42]. And Sun et al. found that histone deacetylase inhibitors (HDACi) upregulate the production of MHC-I pathway molecules and accelerate the activation of antigen-specific immune responses by tumor lysates, thus enhancing the recognition and killing activity of CTL[43]. Based on the impact of these epigenetic modifications on T-cell-mediated immune responses, studies have been conducted to develop new therapeutic strategies for certain diseases from an epigenetic perspective, such as the use of miR-200c-EpCAM axis-improved pericyte therapy (ACT)[44], DNA methylation-based PD-1 immune blockade therapy[45], and CAR-T, which is the focus of this paper immunotherapy.

4. Epigenetic Modifications in Car-T Therapy

4.1 Car-T Cell Therapy

T cells are also crucial in the immunosurveillance of tumor cells. In recent years, scientists have extensively investigated T cell immunosurveillance and anti-tumor immune responses, leading to

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the implementation of a range of immunotherapeutic techniques[46]. And chimeric antigen receptor T cell immunotherapy, the new precision-targeted immunotherapy, has emerged as a viable treatment option. Car-T, or chimeric antigen receptor T cells, is a genetic engineering procedure in which (chimeric) antigen receptors are added to a patient's normal T cells. The T cells are stimulated to multiply in vitro and then infused back into the patient using a viral vector called CAR-T, which allows the T cells to able to perform a specific killing task[47]. When this antigen is present on the surface of tumor cells, T lymphocytes can specifically bind to the tumor syndrome cells and destroy them through cytocidal action, ultimately enabling CAR-T tumor therapy. Antigen receptor structures (scFv), spacer domains (or hinge domains), a transmembrane domain, and co-stimulatory molecular sequences (intracellular signaling sequences) are all components of CAR-T[48]. Before they can kill, CAR-T cells must first bind to the target cells and create immune synapses in the binding region. They destroy tumor cells by three main mechanisms: (1) secretion of perforin and granzyme, which directly kill tumor cells through physical action or induction of apoptosis; (2) high expression of TNF ligands on the surface, which stimulate apoptosis; and (3) secretion of specific cytokines that promote CAR-T activity and alter the tumor microenvironment, further enhancing the anti-tumor activity of CAR-T cells. The chimeric antigen receptor (CAR) is a core component of CAR-T, allowing T cells to detect tumor antigens independently of MHC (HLA), which allows CAR-modified T cells with a surface receptor TCR to recognize a larger range of targets than naive T cells[47].

CAR-T cells have shown therapeutic efficacy, especially in hematological malignancies. Three anti-cd19 CAR - t cells are currently approved by the FDA and the European Medicines Agency or are in advanced stages of development[49]. However, CAR-T cell therapies still have many limitations that must be addressed. During application of CAR-T immunotherapy, scientists have found that such treatment often becomes dysfunctional in cancer therapies, probably due to tumor evasion and drug resistance that compromise host immunity and CAR-T persistence[50]. Even after successful CAR-T cell therapy, tumor recurrence with short burden-free cycles may also cause physical and psychological damage to cancer patients[51]. Moreover, post-treatment adverse effects, such as cytokine storm and cerebral edema can be life-threatening[52]. In addition to this, the expensive cost of CAR-T treatment and its limited efficacy in solid tumors [53] likewise limit its widespread clinical use.

Epigenetic reprogramming has different effects on T cell activation, polarization, memory, and failure, and thus it may also affect the effectiveness of immunotherapy with chimeric antigen receptor T cells, and perhaps we can develop corresponding epigenetic modification methods for CAR-T cells so as to overcome the barriers limiting CAR-T cell effectiveness.

4.2 Epigenetic Modifications in CAR-T Cells

Epigenetic modifications have been shown to play a regulatory role in T cell-mediated acquired immunity. Therefore, epigenetic alterations have been performed on CAR-T cells with a view to improving their efficacy.

Decreased persistence and killing power of CAR T cells in vivo is one of the causes of cancer recurrence, the poor effect of CAR-T therapies, and other limitations, so it is important to enhance the immune potency and duration of CAR-T cells in patients, and CAR-T therapies improved at the epigenetic level have proved promising in treatment[54-56]. It has been shown that chimeric antigen receptor T (dCAR T) cells treated with DNA methyltransferase inhibitor (DNMTi) decitabine have enhanced antitumor activity, cytokine production, and proliferation in vitro and in vivo, and that DCAR T cells have higher expression levels of genes related to memory, proliferation and cytokine production, enhancing their antitumor properties[54]. Yoshikawa et al. found that knockdown of PR domain zinc finger protein 1 (PRDM1) in less differentiated memory CAR-T cells in vivo resulted in enhanced chromatin accessibility of genes regulating memory formation and that these CAR-T cells showed improved persistence and multifunctional cytokine secretion in multiple tumor models[55]. In a patient with chronic lymphocytic leukemia, Fraietta et

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al. found that CAR-T cells with disruption of the methylcytosine dioxygenase TET2 gene gained enhanced proliferation, and knockdown of TET2 for DNA demethylation similarly demonstrated significantly improved antitumor activity of CAR-T in patients[56], so in summary, in vitro administration of decitabine, PRDM1 knockdown, and TET2 modification may all contribute to improved CAR-T immunotherapy. It has been shown that deletion of de novo DNA methyltransferase 3α (DNMT3A) in T cells expressing first- or second-generation CARs maintained cell proliferative capacity and anti-tumor response in response to sustained tumor exposure, which was associated with DNA methylation-induced epigenetic silencing of genes associated with limiting immune cell "stemness", and such DNMT3A knockout CAR- T cells exhibit an anti-depletion capacity that deserves attention[57]. Enhancing the immune function of T cells is the key to improving the efficacy of CAR-T therapy. Ding et al. found that in multifunctional CD4+ CAR-T cells obtained from transgenic T cells co-expressing CD19-CAR and mouse STAT5A (CASTAT5), CASTAT5 induced extensive chromatin remodeling in CD4+ CAR-T cells, making most non-coding genomic region accessibility alterations, thereby improving the effect on tumor infiltration while promoting the induction of antitumor CD8+ T cell responses by CD4+ CAR-T cells[58]. Thus sustained signaling transducer and activation of transcriptional activator 5 (STAT5) gives anti-tumor CD4+ T cells a desirable functional profile, which is important for the optimization of CAR-T cell therapy. In addition to this, it has been shown that estrogen receptor binding fragment-associated antigen 9 (EBAG9) inhibits cytolytic enzymes released from cytotoxic T lymphocytes, while miRNA-mediated silencing of EBAG9 enhances the effector capacity of CAR-T cells in tumor models, improves tumor eradication, promotes efficient manufacturing, and reduces therapeutic dose[59], which may be a useful strategy to improve anti-tumor efficacy.

Treatment failure or relapse due to tumor escape caused by reduced expression of target antigens has become a challenge in the field of CART therapies, and increasing the targeting of CAR-T cells is important to enhance the efficacy of this immunotherapy. Decitabine was shown to enhance the cytotoxicity of EGFR/CD44v6 CAR T cells against all uroepithelial carcinoma cell lines (UCC), and this epigenetic inhibitor-induced state of increased CAR T cell killing was associated with enhanced expression of target antigens (EGFR and CD44v6) and T cell ligands PD-L1, PD-L2, ICAM -1 or CD95, while it does not contribute to the killing profile of benign uroepithelial cells (HBLAK) [60], thus Decitabine in combination with CAR-T has important implications for tumor-specific killing in bladder cancer and may help to mitigate the off-target effects of CAR-T. Target antigen density is closely related to the efficacy of CART therapies, and Yang et al. demonstrated through both in vitro and in vivo experiments that domestic histone deacetylase inhibitor (HDACi) Chidamide may regulate post-transcriptional modifications affecting protein distribution by inhibiting histone deacetylase, upregulating the expression of CD22 on the B-cell tumor cell surface expression of CD22, thereby enhancing the efficacy of CD22 CART[61]. It was also found that tazemetostat, an inhibitor of methyltransferase Zeste homolog 2 enhancer (EZH2), triggers immune response of GD2.CAR-T cells by upregulating the dialdehyde glycoside (GD2) on the surface of low-antigen-expressing tumor cells to sufficient levels[62], thus epigenetic drugs can enhance the sensitivity of CAR-T therapy targeting.

The advent of CAR-T cell therapy has changed the face of clinical treatment for relapsed and refractory pre-B acute lymphoblastic leukemia (B-ALL) and lymphoma. Although curative responses have been reported, the long-term cure rate remains below 50%. The efficacy of CAR-T is limited by the diverse mechanisms of resistance of tumor cells to CAR-T[51]. It was shown that TNF-related apoptosis-inducing ligand (TRAIL) on CTL is involved in CAR-T immunotherapy in b-cell precursor acute lymphoblastic leukemia (BCP-ALL) through its death receptors (DR4 and DR5), where hypermethylation of CpG islands in the promoter region of DR4 and DR5 genes was significantly associated with gene and cell surface expression levels and TRAIL sensitivity. It may be one of the mechanisms of TRAIL resistance in leukemic cells, which provides important inspiration for the improvement of clinical therapies with CAR-T in BCP-ALL[63].

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The current challenges of CAR-T for solid tumor hair treatment such as a lack of tumor-specific antigens and immunosuppression of tumor microenvironment (TME)[64] have made the progress of CAR-T on solid tumors slow. In contrast, the modification of CAR-T by epigenetic modifications may give a ray of hope for the advancement of CAR-T therapy in solid tumors. It has been shown that in vitro treatment of CAR-T cells with short-chain fatty acids (SCFAs) valerate and butyrate increases the function of mTOR as a central sensor of cellular metabolism and inhibits class I histone deacetylase activity, and this reprogramming leads to increased production of effector molecules such as CD25, IFN-γ and TNF-α and significantly enhances the role of ROR1-targeted CAR T cells in the synthesis anti-tumor activity in mouse melanoma and pancreatic cancer models[65]. Sulejmani et al. found that inhibition of histone lysine demethylase 1A (KDM1A) induced TP53-mediated transcriptional activation of genes including FAS, which in turn released an antigen-independent killing mechanism through the FAS-FASL axis, making L1CAM-directed CAR T cell therapy to increase the efficacy of neuroblastoma[66]. In addition, Lei et al. found that in vitro low doses of the deacetylase inhibitor SAHA could upregulate B7-H3 expression in several solid cancer cells and downregulate immunosuppressive molecules in T cells, thereby enhancing the ability of B7-H3 CAR-T to selectively kill cancer cell lines expressing B7-H3, greatly increasing the antitumor activity of CAR-T therapy, which is important for the treatment of solid tumors significance[67].

Notably, Garcia-Prieto et al. observed that DNA methylation at loci associated with complete response (CR) affected the efficacy of CAR-T19 cellular immunotherapy in patients with B-cell malignancies, such as 5'-UTR CpG hypermethylation of FOXN3 (a candidate tumor suppressor for T-cell tumors) leading to its transcriptional downregulation, reducing the organism's anti-tumor capacit[68]. In addition, they suggested specific methylation sites that might be used to assess the effect of immunotherapy, with important implications for the selection of clinical treatment strategies for patients with B-cell malignancies.

Conclusion

This paper briefly describes the T cell and immune response process, as well as epigenetic remodeling in T cell status and function, ranging from epigenetic concepts represented by DNA methylation, histone modification, chromatin remodeling, and non-coding RNA, to research progress and application prospects of epigenetic modifications in T & CAR-T cells. CAR-T cell therapies incorporating epigenetic manipulation have shown enhanced efficacy in vitro, and these epigenetic modifications can also ameliorate the limitations of CAR-T, e.g., T cell failure and tumor antigen escape. However, no specific and effective targeted improvement strategies have been found for the two significant drawbacks of CAR-T, namely off-target effect and cytokine storm. Therefore, further studies are needed. However, the combination of Decitabine and CAR-T therapy in UCC improved the killing efficacy of cancer cells with less side-effects on benign HBLAK cells[60]. Thus, it may enhance the specific killing ability of CAR-T through epigenetic influence and reduce the harm caused by the off-target effect to the organism.

In summary, for improving the efficacy of CAR-T cell therapy, the possible role of epigenetic mechanisms needs to be further explored.

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