Epigenetic Strategies to Optimize CAR-T Therapy

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Abstract. Chimeric antigen receptor T (CAR-T) cells that are obtained from the specific patient, modified genetically ex vivo, and possess the remarkable capability to identify and eradicate targeted cancer cells. These modified cells are subsequently reintroduced into the patient, effectively treating blood cancer. CAR-T therapy is approved to be applied in leukemia due to its great clinical therapeutic effect on B cell hematological malignancies. However, solid tumors are more resistant to this therapy for many reasons. The abnormal vascular structure of solid tumors hampers CAR-T cell trafficking. Various kinds of immunosuppressive cells and chemicals in the tumor microenvironment (TME) accelerates CAR-T cell exhaustion, showing poor persistence in vivo. More and more researches have demonstrated that T cell fate is strongly associated with epigenetic regulation. Epigenetic modification is not a direct addition or deletion of DNA, but a reversible method including modifications on DNA and histones, and non-coding RNA (ncRNA)-mediated regulations. The change of epigenetic landscape in CAR-T cells largely determines the therapeutic performance in vivo. This research outlines three major barriers in CAR-T therapy, including T cell exhaustion, differentiation and infiltration. Additionally, the research elucidates several promising epigenetic reprogramming strategies to reduce CAR-T cells exhaustion, modulate the cell differentiation process, and enhance their infiltration into solid tumors.

Keywords: Epigenetic, CAR-T, Exhaustion, Differentiation, Infiltration.

1. Introduction

Cancer is one of the diseases that cannot be completely cured by human beings so far, but treatments for cancer are constantly updating. With people's understanding of the mechanism of the immune system continuously deepening, immunotherapy, especially the cell therapy against tumor receives more and more research and attention. Among these viable cell therapies, chimeric antigen receptor T (CAR-T) cells therapy obtains greater recognition and application owing to its efficacy and positive clinical response against $CD19^+$ tumors in chemotherapy-refractory acute lymphoblastic leukemia. Application of second-generation CAR-T therapy for leukemia treatment was authorized by the Food and Drug Administration in 2017. In this kind of treatment, a CAR molecule is engineered onto the T cell membrane using gene editing techniques. The molecule aims to target certain antigens produced by tumor cells, enhancing the T cell's capacity to recognize and destroy tumors. Single-chain variable region (scFv), which is derived from the antibody variable heavy chain (V_H) region and variable light chain (V_L) region, aims to recognize and bind with tumor cells. V_H region and V_L region are connected by glycine and serine repeating peptides. Within the cell, intracellular T cell receptors (TCRs) based on the CD3ζ subunit with a co-stimulatory signaling domain forms the signal transduction structure. Via connection of the transmembrane protein, a complete extracellular signal reception and intracellular signal transduction device is formed, as shown in Fig. 1. However, there are still many issues needed to be resolved *in vivo*, such as T cell exhaustion, differentiation, and the immunosuppressive TME when CAR-T cells extirpate solid tumors [1, 2]. To optimize CAR-T cells, many experiments were conducted to enhance the efficacy and persistence of CAR-T cells and maintain their long-term memory in the body to prevent recurrence of tumors. Due to the development of sequencing and screening technology, the epigenetic regulation mechanism of T cells is clearer. So far, scientists have found that epigenetic regulation is pivotal for T cell proliferation and differentiation, and the epigenetic reprogramming seems to be a practical method to optimize CAR-T cells [3, 4]. This article introduces three major barriers in CAR-

T therapy, including T cell exhaustion, differentiation and infiltration, and provides epigenetic reprogramming strategies to battle with solid tumors.

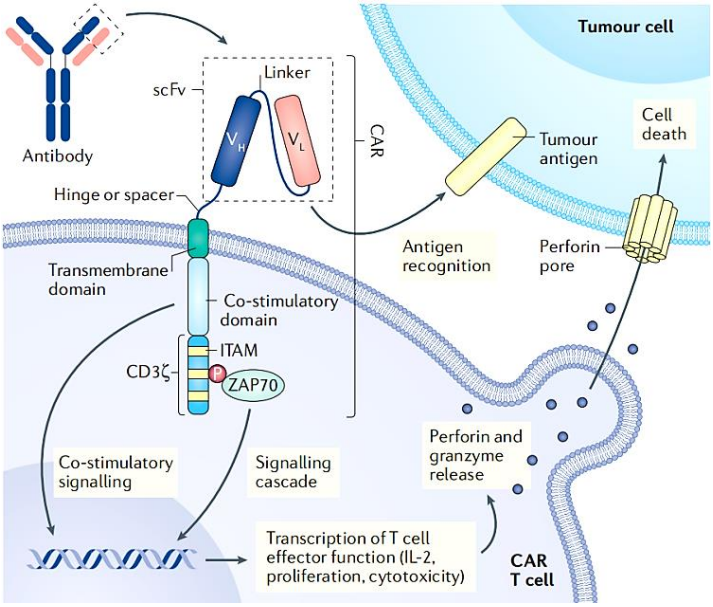


Figure 1. Structure and signaling pathways of the CAR-T cell [1]

2. Epigenetic reprogramming to break down barriers

2.1. T cell exhaustion

2.1.1 The definition of exhaustion

Exhaustion is a condition of dysfunction that T cells enter after being continuously activated by the specific antigen (Fig. 2). This state is characterized by a lack of responsiveness. In acute infection, naïve T cells proliferate and differentiate into effector phenotypes which completely eliminate pathogens and subsequently form memory T cells, while during chronic infection, rapid and complete immunity cannot be achieved. Consequently, T cells express more inhibitory receptors and show more dysfunctional because of prolonged interaction with antigens, leading to exhaustion [2].

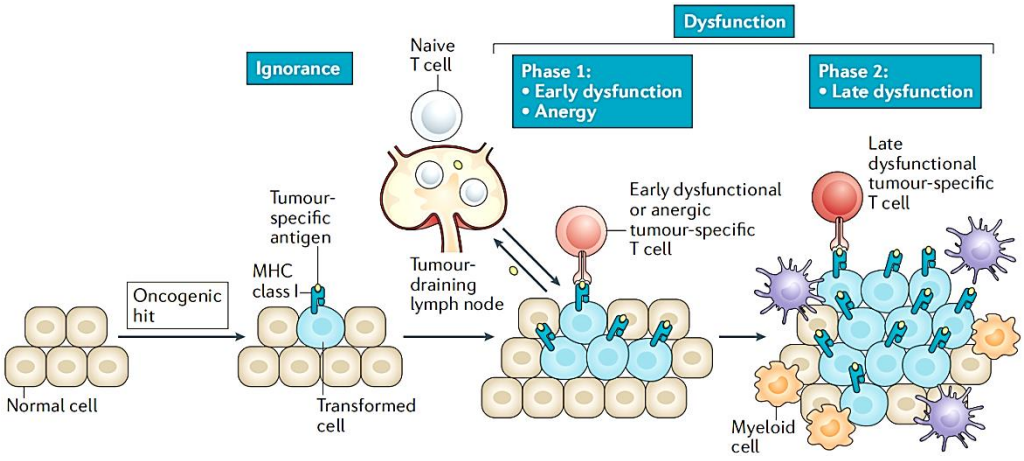


Figure 2. Process of T cell exhaustion in solid tumors [2]

2.1.2 Alleviating CAR-T cell exhaustion through epigenetic reprogramming

PDCD1 is responsible for the expression of programmed cell death protein 1 (PD-1). PD-1 serves as an important immunological checkpoint that hampers the function of T cells by interacting with its ligands, such as PD-L1 and PD-L2, therefore controlling the degree of the immune response. Tumor cells seize on this vulnerability, so they highly express PD-L1 ligands, which inhibits cytotoxic T cell

efficacy and induces exhaustion, in order to evade the elimination by the immune system. During the transformation of immature T cells into active T cells, *PDCD1* undergoes a temporary removal of methyl groups, a process known as demethylation. The *PDCD1* demethylation is related to the intensity and duration of signaling through the T cell receptor. However, in exhausted T cells (T_{ex}), this gene is entirely demethylated, indicating that the process of methylation and demethylation in regions linked with exhaustion-related genes could potentially reverse the exhaustion state of CAR-T cells [5]. In addition, microRNAs, also known as miRNAs, have irreplaceable functions in the regulation of epigenetic modifications. Through the process of attaching themselves to the 3' untranslated regions of target messenger RNAs (mRNAs), miRNAs impede the translation of mRNAs into protein or to lead to the breakdown of mRNA, which directly influences the expression of specific genes. Li et al. demonstrated that miR-28 can inhibit the expression of immunosuppressive receptors, such as PD-1 and cytotoxic T lymphocyte antigen-4 (CTLA-4), effectively reducing T cell exhaustion in the TME [6].

Other epigenetic editing sites that could be targeted include transcriptional factors and regulators associated with exhaustion. Thymocyte selection-associated high mobility group box (TOX) is a key regulatory protein in T cell differentiation that helps keep T cells at tumor site. However, ectopic expression of TOX causes exhaustion and TOX is required for the development of T_{ex}, but not effector T cells or memory T cells. Khan et al. previously identified that TOX bound with exhaustion-associated proteins involved in DNA replication, RNA translation and processing, chromatin assembly and remodeling [7], and repressive epigenetic regulation through mass spectrometry, suggesting that the binding loci of TOX-bound complexes are possible epigenetic reprogramming sites. An example of this would be the interaction between the ORC1 (HBO1) complex and TOX via the KAT7 subunit. This complex is an acetyltransferase that is involved in the acetylation of histone H3 and H4. To counteract this, targeting the TOX-bound HBO1 complex in order to encourage the deacetylation of histone H3 and H4 may be able to reverse CAR-T cell exhaustion [8]. It is practical to alleviate CAR-T cell exhaustion by blocking the production of immunosuppressive receptors through epigenetic modification of DNA or through epigenetic regulation of non-coding RNAs. Moreover, through specifically targeting certain transcription factors and protein complexes, it is possible to alter the level of epigenetic modifications in genes, thereby effectively preventing the terminal differentiation of T cells.

2.2. T Cell differentiation and memory formation

2.2.1 T cell fate

The ability of CAR-T cells to eradicate tumors is only one factor that determines their effectiveness and another key element is their ability to proliferate and develop into memory T cells, which are able to react swiftly to eliminate infections upon exposure to the same antigen. Therefore, this part introduces the process of T cell proliferation and differentiation and find possible solutions. Within a living organism, T cells that have not been previously exposed to a specific antigen, known as naïve T (T_N) cells, undergo differentiation into effector (T_{eff}) T cells and memory T (T_{mem}) cells upon stimulation. T_{eff} cells are responsible for destroying invaders but once the pathogens are cleared, effector T cells initiate signaling pathways leading to dysfunction and apoptosis, while memory T cells still persist in the body and monitor whether the same pathogen invades again [9]. There are two main functional subgroups for memory T cells: $CD45RA^-/CCR7^+/CD62L^+$ central memory T (T_{CM}) cells which are less differentiated but less functional, and $CD45RA^-/CCR7^-/CD62L^-$ effector memory T (T_{EM}) cells which responses more quickly in peripheral tissues and more functional [10]. It is noteworthy that there is a particular subgroup named T stem memory (T_{SCM}) cells derived from resting-state naïve T cells. They possess superior proliferative and differentiation ability compared with any other memory T cell subgroups. CAR-T cells derived from T_N and T_{CM} cells performed better than those derived from T_{EM} (Fig. 3). Alvarez-Fernández et al. engineered *ex vivo* CD30-CAR-T cells derived from T_{SCM} [11]. Hodgkin lymphoma was eradicated entirely *in vivo* by these CD30-CAR-T cells, which also exhibited remarkable persistence and long-lasting immunity. Above

mentioned highlights the importance of T cell differentiation stage for tumor killing and long-lasting immunity, and also explains which stage of T cells should be selected and modified as CAR-T cells. Besides, engineering more stem-like CAR-T cells through epigenetic reprogramming is also a potential optimization strategy.

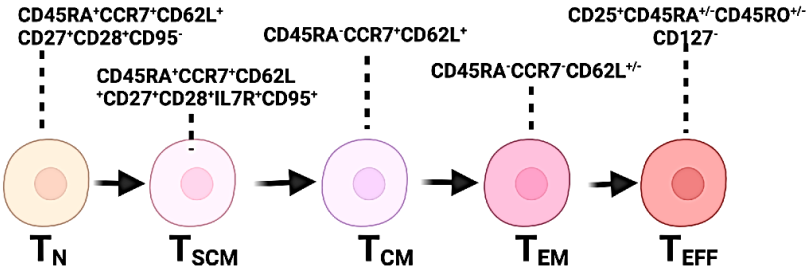


Figure 3. T cell differentiation and different types of T cells [5]

2.2.2 Regulating CAR-T cell fate through epigenetic reprogramming

Ten-Eleven Translocation (TET) protein is one type of methyltransferase and TET protein family consists of TET1, TET2 and TET3, all of which play an important role in DNA methylation. TET proteins have the ability to oxidize 5-methylcytosine (5mC) on DNA and convert it into 5-hydroxymethylcytosine (5hmC), thereby participating in the regulation of DNA methylation and demethylation process. According to Tsagaratou's article [3], abundant studies have demonstrated the importance of TET proteins in the epigenetic regulation of T cell fate, especially the TET2 protein. TET2 promotes the expression of T_{eff}-associated genes, like *Interferon-γ (IFN-γ)* and *C-C motif chemokine receptor-5 (CCR5),* contributing to memory T cell differentiation. A strong antitumor response was achieved by altering the epigenetic landscape through TET2 double knockdown, which in turn increased cell proliferation and took on more central memory-like phenotypes [12]. The transcription factor *TRF7* and *CCR7* are two examples of T_N-associated genes that are controlled by *DNA methyltransferase 3 alpha* (*DNMT3A*), one type of methyltranferase. Demethylation of these T_N-associated genes occurs when *DNMT3A* is disrupted and this leads to the emergence of more memory phenotypes and the promotion of effector T cell dedifferentiation into memory T cells, which are not normally associated with T_N.

Through directly recruiting the repressive chromatin-modifying enzymes histone deacetylase 2 (HDAC2) and H3K9 methyltransferase (H3K9MT) to specific promoters such as *interleukin2 receptor alpha (IL2RA),* it adversely regulates T_{mem}-associated genes in order to facilitate the process of differentiation. Histone H3-acetylation and decreased histone H3K9-trimethylation have been shown by Shin et al. to enhance memory T cell formation and anti-tumor activity [13]. The suppressor of variation 3-9 homolog 1 (SUV39H1) enzyme is a histone H3 methyltransferase. Its primary function is to catalyze the methylation reaction by transferring a methyl group onto the lysine-9 of histone H3. Pace et al. discovered that SUV39H1 inhibited the expression of genes associated with T_{mem} [14], while simultaneously promoting the expression of genes associated with T_{eff} and accelerating the differentiation of T cells. This discovery aligns with the recent study. Jain et al. disrupted *SUV39H1* in CAR-T cells, resulting in modified cells that possessed enhanced anti-tumor efficacy after multiple rechallenges [15].

Overall, it is essential to understand how to drive CAR-T cells to have more memory phenotypes while exerting tumor-eradicating functions. The ability to monitor and destroy tumor cells in the body for an extended period of time is an important objective of the CAR-T therapy and epigenetic remodeling in T cell differentiation has become a key to accomplish this. The stage of CAR-T cells differentiation can be efficiently regulated through epigenetic modification of DNA or histones, which makes CAR-T cells more stem-like *in vivo*.

2.3. The TME and T cell infiltration

2.3.1 Immunosuppressive effect of TME

The tumor microenvironment encompasses the intricate surroundings surrounding tumor cells. It is composed of extracellular matrix (ECM), proteins, hormones, and dissolved chemicals and the TME is of paramount importance in tumor progression, metastasis, development, and response to treatment (Fig. 4). When T lymphocytes infiltrate into tumors, they are referred to as TILs, which stands for tumor infiltrating lymphocytes. T cell infiltration is the process by which T lymphocytes enter tissues or organs after passing through the walls of blood vessels. Extensive research has established that the TME significantly impedes the function of T cells. The abnormal vascular system is the first obstacle to CAR-T cell infiltration. In contrast to the regular distribution of blood vessels in normal tissues, tumor-generated blood vessels are abnormal and intricate, preventing CAR-T cells from entering the tumor site and posing a barrier to their transportation. The second obstacle is the tumor mechanics and the stiffness of ECM. The ECM is composed of collagen, elastin, proteoglycans, fibronectin, laminins, and other proteins. The interactions between these proteins create a physically dense barrier that prevents CAR-T cells from infiltrating. In addition, actin and myosin-mediated interactions between stromal cells and cancer cells generate forces that increase the rigidity of the ECM [7]. The third obstacle comes from the body's immune regulatory mechanism. Regulatory T (T_{reg}) cells, originally called suppressor T cells, have vital functions in immune balance and immune tolerance inside the body. Nevertheless, within the TME, tumor cells evade immune system assault by capitalizing on the immunosuppressive functions of regulatory T cells. For example, the combination of chemokine receptor CXCR3 with its ligands CXCL10 that produced by endothelial cells, contributes to the T cells infiltration, while $Foxp3^+$ regualr T cells suppress the production of CXCL10. Regulatory T cells-secreted cytokines such as TGF-β, IL-10, and IL-35, which suppress antitumour immunity by inhibiting the functions of antigen presenting cells (APCs) like DCs, $CD4^+$ T helper (T_h) cells, and the production of tumor-specific CD8+ cytotoxic T lymphocytes (CTLs) [16]. Based on the information provided, the goal of epigenetic reprogramming is to enhance the migration of CAR-T cells to the tumor site and to utilize these cells to fight tumors within the TME.

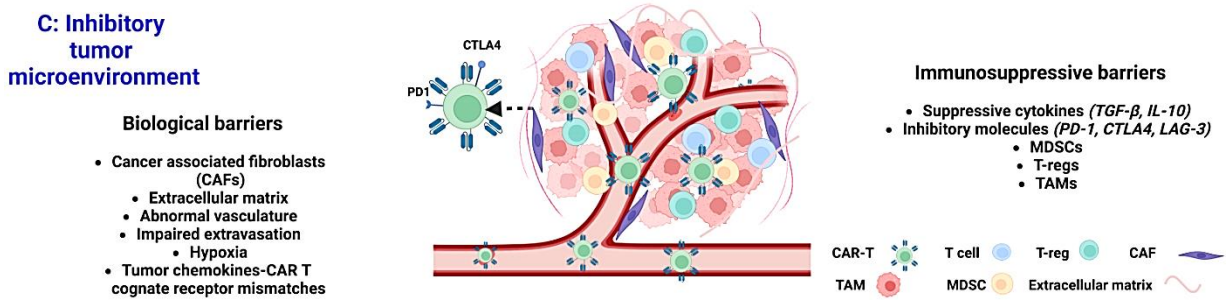


Figure 4. The immunosuppressive tumor microenvironment [5]

2.3.2 Enhance CAR-T cell infiltration through epigenetic reprogramming

Chemokine receptor CXCR3-mediated immune response is recognized as an important pathway to recruit immune cells. However, the low-level expression of CXCR3 ligands CXCL9 and CXCL greatly blocks this signaling pathway. More precisely, genes associated with the expression of CXCL9 and CXCL10 are suppressed through epigenetic mechanisms involving DNA methyltransferase 1 (DNMT1)-mediated DNA methylation and zeste homologue 2 (EZH2), which is a subunit of the polycomb repressive complex 2 (PRC2) and possesses H3K27me3 activity [17]. Consistent with this discovery, Wang et al. also proved that CAT-T cells, when exposed to a low dosage of decitabine [18], an inhibitor of DNMT1, released substantial CXCL9 and other chemokines that facilitated the migration of lymphocytes. Furthermore, the CAR-T cells that were treated with low doses of decitabine demonstrated powerful anti-tumor efficacy, as evidenced by increased expression of T_{mem}-related genes and decreased expression of T_{ex}-related genes *in vivo*.

Another strategy is related to the metabolism. Since cell canceration is accompanied by changes in metabolic patterns, various properties within the tumor microenvironment, such as pH, oxygen content, and glucose concentration, are distinctly different from the normal steady-state environment. This will affect the original metabolic mechanism of the TILs. Consequently, looking for metabolism-related genes and other substances that have an effect on T cells' function has also become epigenetic targeting sites. HER2-CAR-T cells overexpression of miR-143 is able to promote metabolic reprogramming which pertains to the process of glucose absorption and the prevention of glycolysis by inhibiting the glucose transporter 1 (Glut1), and improve anti-tumor efficacy in the esophageal cancer model. But indoleamine-2,3-dioxygenase (IDO) suppresses miR-143 expression. Both miR-448 and miR-153 impair IDO's function and research have identified their immune enhancing effects on T cells [7].

Overall, epigenetic reprogramming is crucial for improving the ability of CAR-T cells to migrate and locate themselves precisely at the tumor site, given the intricate structure of solid tumors. Within the context of the TME, it is significant to address the immunosuppressive effects caused by T_{reg} cells and to modify metabolic pathways through epigenetic control.

3. Conclusion

The purpose of this research is to demonstrate the promising potential of applying epigenetic reprogramming strategies which aims to enhance the efficacy of CAR-T treatment. Limiting protein translation through the utilizing of ncRNAs, silencing immunological checkpoint-related genes through DNA methylation, and targeting epigenetic modification complexes that are highly related to CAR-T cell exhaustion, significantly increase the persistence of CAR-T cells *in vivo*. Furthermore, CAR-T cells derived from T_{mem} cells with more stem-like characteristics, such as T_{SCM} and T_{CM} cells, exhibit greater longevity compared to those derived from T cells with less memory phenotypes, such as T_{EM} cells. It is possible to promote the formation of memory T cells by disrupting genes that are responsible for promoting CAR-T cells differentiation and suppressing the expression of genes linked with T_{mem}. Certain medications, such as decitabine, have the ability to decrease the activity of enzymes that are responsible for inhibitory epigenetic alterations. This means that they can counteract the immunosuppressive circumstances that are present within tumors and boost the infiltration of CAR-T cells. This inhibition results in the release of chemokines, which in turn facilitates the accumulation of CAR-T cells. In addition, metabolism reprogramming is an important factor in the enhancement of CAR-T cell persistence *in vivo*. In CAR-T cells, it has been established that the manipulation of metabolic pathways by epigenetic regulation can result in favorable effects. Consequently, as more researches are conducted, additional sites for epigenetic modification will be uncovered. This will bring more promising ideas and effective ways for epigenetically reprogramming CAR-T cells to combat solid tumors.

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