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Breakthroughs and Limitations of CAR-T Cell Therapy in the Treatment of Haematological and Solid Tumours

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Abstract. Chimeric antigen receptor T-cell (CAR-T) offers a revolutionary solution to the treatment of cancer. It has demonstrated particularly high efficacy in some cancer treatments, such as haematological malignancies. However, this method still faces significant challenges. In the treatment of solid tumor, it may lead to off-target toxicity, accelerated T-cell exhaustion, and cytokine storms resulting from excessive T-cell activity. Facing the dense stroma of the tumour microenvironment(TME), the introduction of lysosomal CAR-T cells expressing PH20 or CAR-T cells targeting fibroblast activation protein breaks down the external structure of the tumour, facilitating the entry of other CAR-T cells into the tumour to carry out killing. Knocking out the MCT-1 and A2AR genes can enhance the resistance of CAR-T cells to the TME. This approach inhibits the inflow of lactate and adenosine, thereby maintaining CAR-T cell viability. Employing multi-gene knockout or base editing techniques to modify the PD-1 gene weakens the immunosuppressive effects of the TME on CAR-T cells. Overexpressing some genes in the T cells, such as c-JUN, can enhance cellular activity, enabling the cells to survive long-term within the TME and sustain their cytotoxic effects. To prevent off-target effects and uncontrolled activation of CAR-T cells, synthetic Notch circuits have been engineered. This system is a dual-antigen recognition system and can help CAR-T cell better recognise tumor cells. This article reviews the research progress in CAR-T cell therapy, methods to enhance therapeutic efficacy, and offers a perspective on future research directions.

Keywords: CAR-T cells, solid tumor, CRISPR, c-JUN, syn-notch circuits.

1. Introduction

Humans have long strived to overcome cancer. However, all treatments currently available have their own disadvantages and shortcomings. An example is immune checkpoint inhibitors, which have greatly increased the long-term survival rates of melanoma and lung cancer, though with a success rate of only about 20-30%. In the same way, although antibody-drug conjugates have the capacity to destroy tumor cells effectively, they are linked to off-target toxicity and drug resistance. Concerning mRNA vaccines, which are highly personalizable, they have to face the problem of high production costs and challenges in large-scale production. Another innovative technology that has produced impressive outcomes in treating tumors is CAR-T cell treatment, which incorporates the use of cells to treat tumors. The initial breakthrough of CAR-T therapy emerged within

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haematological oncology. A decade-long follow-up study documented sustained remission in patients with chronic lymphocytic leukaemia (CLL), while simultaneously identifying the CD4+ lineage as the primary mediator of enduring immune surveillance [1]. Compared with the complete remission (CR) rate of less than 20% achieved by conventional second-line chemotherapy, CD19-targeted CAR-T therapy has raised the remission rate to over 50%, completely redefining the treatment standard for relapsed and refractory lymphoma. The therapeutic landscape has been notably transformed by the immense promise of CAR-T cells. However, it has encountered significant obstacles in treating solid tumours. Labaniec J. et al. reviewed over 40 early-phase (Phase I/II) CAR-T clinical trials, found that the ORR for haematological malignancies typically ranges between 70% and 90%, whereas the average ORR for solid tumours (such as advanced pancreatic cancer and ovarian cancer) is only 10%–25% [2]. This is due to the highly inhibitory effect of the TME in solid tumours on CAR-T cells. This inhibitory effect stems from the TME's dense physical barriers, complex vascular system, and chemically acidic, adenosine-rich environment. To overcome these challenges, researchers have employed methods such as generating iterative CAR-T cells, gene knockout, and overexpression of the c-JUN gene. This paper aims to explore the latest strategies for CAR-T therapy in overcoming the barriers posed by solid tumours, and to provide a comprehensive comparison to identify the optimal treatment approach.

2. Generational classification and characteristics of CAR-T cells

At present, there is still controversy in the academic community regarding the generational classification of CAR-T cells. Most studies divide them into 4 generations, while the latest-generation of CAR-T cells is still in basic research stage and has not yet formed a unified classification standard. There are significant differences among CAR-T cells of different generations in structural design, functional characteristics, and clinical application value, which are detailed as follows. First-generation CAR-T constructs are defined by the exclusive inclusion of the CD3ζ signaling domain. In contrast to the single-chain variable fragment (scFv), which mediates antigen recognition, CD3ζ is solely dedicated to signal transduction. Their activation is highly dependent on exogenous cytokines, with defects of insufficient T cell activation and poor persistence, thus having no clinical application value. The incorporation of costimulatory domains, specifically CD28 and CD137, distinguishes the second generation of CAR-T cells. Compared to CD3ζ, which provides the primary activation signal, CD28 and CD137 serve as costimulatory molecules to provide the secondary activation signal. This design significantly enhances the cytotoxicity and survival time of T cells, and is currently the mainstream of CAR-T cell therapy in clinical application. The third-generation CAR-T cells incorporate additional costimulatory domains (e.g., CD28+CD137) to further enhance costimulatory signals. However, some clinical studies have shown that compared with the second-generation CAR-T cells, the third-generation does not significantly improve the overall survival rate of patients, but may increase the risk of cytokine release syndrome (CRS). Its clinical application value is still controversial in the academic community, which limits its clinical transformation. The fourth-generation CAR-T cells (also referred to as "TRUCKs", T cells Redirected for Universal Cytokine Killing) are genetically engineered to express cytokine inducers and release cytokines (e.g., IL-12) into the TME in a tumor-specific manner. They enhance therapeutic efficacy by improving the TME, but their safety and long-term efficacy still need further clinical verification. The fifth-generation CAR-T cells are still in the preclinical research stage, and the strategy of activating the JAK/STAT pathway by introducing an IL-2 receptor is considered one of the most promising research directions. This method can prolong the survival time of CAR-T cells by enhancing the expression of anti-apoptotic genes (e.g., Bcl-2, Bcl-xL).

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3. Current status of clinical application of CAR-T cells

CAR-T cell therapy has achieved breakthrough progress in tumor treatment, among which CD19 is the most mature and widely used target so far. CD19 is considered an ideal target for the treatment of certain hematological malignancies, especially B-cell malignancies, and has shown significant efficacy in diseases such as B-cell acute lymphoblastic leukemia (B-ALL) and diffuse large B-cell lymphoma.

In 2012, the Children's Hospital of Philadelphia first used CD19-targeted CAR-T cells for the treatment of patients with B-cell acute lymphoblastic leukemia (B-ALL) [3]. The results indicated most patients gained morphological remission within one month, and some remained cancer-free for the subsequent 12 years, which fully demonstrated the great potential of CAR-T cell therapy in tumor treatment. At present, several CAR-T cell therapies targeting acute lymphoblastic leukemia (ALL) have been approved by the U.S. FDA and are widely used in clinical treatment.

However, CAR-T cell therapy is also accompanied by certain side effects in clinical application, the most common of which is cytokine release syndrome (CRS). It is mainly characterized by a significant elevation in serum IL-6 levels. Currently, IL-6 receptor antagonists (e.g., tocilizumab) have become the current gold standard for the treatment of CRS, which can effectively alleviate the symptoms of CRS and improve the safety of CAR-T cell therapy. Despite the remarkable success in the treatment of B-cell malignancies, CAR-T cell therapy still faces considerable challenges in the treatment of other hematological malignancies (such as acute myeloid leukemia) and solid tumors, which limit its further clinical application.4. Existing Challenges in CAR-T Cell Therapy.

4. Challenges in the treatment of cancer

4.1. Challenges in the treatment of acute myeloid leukemia (AML)

CAR-T therapy faces considerable challenges in the treatment of acute myeloid leukaemia (AML). Leukaemia stem cells differentiate from haematopoietic stem cells and progenitor cells, or originate from cells at a more advanced stage of differentiation. These cells accumulate numerous genetic mutations, such as those in FLT3 and NPM1, which regulate cell proliferation; DNMT3A, responsible for silencing and methylation; and TP53, which governs apoptosis; as well as IDH1/IDH2, ASXL1, ZRSR2 and others. This results in the presence of leukaemia cells with multiple mutation types within a single patient. Such heterogeneity significantly increases the number of potential therapeutic targets for leukaemia, making it difficult to achieve a complete cure with a single targeted drug. Consequently, there is a high probability of disease recurrence. This represents the primary challenge for CAR-T therapy in the treatment of AML.

4.2. Challenges in the treatment of solid tumors

The principal challenge in treating solid tumours is rooted in the TME. The TME exerts a triple inhibitory effect on CAR-T cells, i.e., physical, chemical, and immunological inhibition, which seriously impairs the infiltration, activation, and survival of CAR-T cells in solid tumours. Physically, first, the extracellular matrix (ECM) of the tumor is filled with high concentrations of hyaluronic acid, collagen, and fibronectin. These compounds become highly thickened to create a physical block that CAR-T cells cannot easily infiltrate; more so, the subsequent rise in interstitial fluid pressure blocks CAR-T cell access into the tumor as well. Secondly, internal vascular distortion and disorganization characterize tumors. On the one hand, such deformed blood vessels

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lead to inadequate blood circulation, and CAR-T cells cannot easily access the in-depth layers of the tumor. Conversely, the disorganized vascular endothelial cells tend to be deficient in adhesion molecules like ICAM-1 and VCAM-1 on their surface; thus, despite the ability of CAR-T cells to go to the inner tumor, they are unable to traverse through the blood vessels and get to the tumor cells.

Chemically and immunologically, the TME is also characterized by high levels of lactate (generated by glycolysis) and adenosine (free when tumor cells are necrotized). In the case of CAR-T cells, the extracellular concentrations of lactate are higher than intracellular levels; a concentration gradient is formed as a result; therefore, excess lactate flows into the cells via MCT-1. The alkalized intracellular milieu suppresses nuclear translocation and activity of the important transcription factor NF-κB, resulting in a defect in the production of cytotoxic factors and T-cell activity and functional capacity. At the same time, elevated extracellular levels of adenosine have a high affinity for the A2A receptor on CAR-T cell surfaces. The adenosine-A2AR-cAMP-Csk pathway leads to the inhibition of Lck, which further inhibits CAR-T cell activity.

Besides the triple inhibitory effect of the TME, CAR-T cell therapy of solid tumors also has other issues: first, the high heterogeneity of solid tumor antigens causes partial elimination of tumors and a high recurrence rate; second, due to the constant stimulation of tumor antigens by the TME, CAR-T cells become quickly exhausted, which leads to lower cytotoxicity; third, CAR-T cells have a higher off-target toxicity in solid tumors because certain tumor-specific antigens are also expressed on normal tissues, causing damage to normal organs.

5. Solutions to overcome challenges

5.1. Addressing vascular and ECM barriers in the TME

Fourth-generation CAR-T cells at the physiological level are a solution to vascular challenges. NFAT promoter controls the gene (IFN-γ), which is the payload gene. Only when CAR-T cells finish identifying tumour cells is the NFAT promoter activated. This results in the quick and effective synthesis of IFN-γ. Enhanced expression of ICAM-1 and VCAM-1 on vascular epithelial cells is triggered by intense IFN-γ exposure, an event that promotes the subsequent adherence of CAR-T lymphocytes to the endothelium [4].

The barrier presented by the ECM can be tackled in two ways. The first is to use lysosomal CAR-T cells to express PH20 (hyaluronidase), degrading existing hyaluronan; the second is to deploy CAR-T cells that target FAP (fibroblast activation protein) and kill cancer-associated fibroblasts (CAFs) [5, 6].

5.2. Improving CAR-T cell activity against suppression of TME

At the chemical and immunological levels, the most effective approach to addressing the issues associated with MCT-1 and A2AR is to silence the respective genes. The other available solution is to use a target inhibitor at the same time with cell injection. But the inhibitory effect of the target inhibitor decreases with depth of penetration into the tumor and eventually vanishes. In order to deal with this problem, several doses are needed. The treatment procedure is not only complex, but the therapeutic efficacy is less than that of the knock-out of the respective target gene.

Previously, it was thought that knockout of PD-1 might also help to reverse CAR-T cell exhaustion. However, the other receptors on the T-cell surface, including Tim-3 and Lag-3, are compensatorily expressed at high levels when PD-1 is knocked out. Even though these T-cells are highly activated and have a high cytotoxicity in the initial stages, they hasten to undergo their direct

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slide into a terminal differentiation and exhaustion state [7]. This suggests that single-gene knockout is hard to efficiently address the issue of CAR-T cell exhaustion, and multi-gene editing can be a more efficient approach.

5.3. Multi-gene editing technology and safety optimization

Several genes have to be knocked out concurrently to mitigate several of the weaknesses of CAR-T cells. The University of Pennsylvania lab of Carl June was the first to test CAR-T cells with three simultaneously engineered gene loci (TRAC, TRBC, and PDCD1) in three patients with advanced cancer [8]. The modified CAR-T cells were maintained in the bodies of the patients for up to nine months, indicating the technical viability of multi-gene-edited CAR-T cells.

Nevertheless, there are high risks associated with simultaneous knockout of several genes. The rate of chromosomal translocations is high when CRISPR technology is used to knock out several genes at once. On the contrary, translocation is virtually absent in single-target editing. In dual-target editing, the incidence of translocations will rise, and with three targets, it will rise to an alarming rate of 1%-5%. The rate of chromosomal translocations is directly proportional to the gene knockouts [9]. The result of this genomic instability of T-cells is the enhanced danger of malignant transformation.

Deaminase-mediated base editing has become the focus of many studies to address these problems. This technique has a much lower chance of chromosomal translocations than CRISPR-Cas9 and can even physically exclude the possibility in some instances. Chromosome translocations in CAR-T cells treated with this technique are 100 times fewer than in CRISPR, and this significantly enhances the safety of multi-gene-modified CAR-T cells [10].

6. Cutting-edge research directions of CAR-T cells

6.1. Syn-notch circuits

Syn-Notch Circuits is a cell surface receptor system that replicates the natural Notch signaling pathway. It is made up of tripartite architecture: a recognition domain, a cleavage site, and a transcription regulatory part. The main distinctive characteristic of it is that the extracellular signal is translated into intracellular gene expression change by direct cell contact with its neighbors. Upon recognizing a certain antigen by the extracellular domain, the receptor changes its conformation. This transformation triggers the proteases to cut the core Notch domain, freeing the corresponding cytokines. The secreted cytokines induce the expression of certain target genes (including the CAR gene). After the CAR attaches itself to the particular antigen, it starts to induce T-cell activation. This occurs because the activation of CAR-T cells is fully triggered only when both antigens are present.

This technical approach can further enhance the tumor-targeting efficacy of CAR-T cells against malignant tissues. By utilising AND gate logic, the attack of CAR-T cells on non-tumour tissues were reduced, thereby mitigating unintended cytotoxicity associated with CAR-T cells. Simultaneously, adding Syn-Notch circuits helps maintain the activity of CAR-T cells, ensuring they don't quickly become inactive and cause exhaustion. However, its complex structure may make cell engineering harder and reduce CAR-T stability, and its long-term safety in living things still needs testing in more lab and clinical trials.

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6.2. C-JUN overexpression

As a key constituent, c-JUN is integral to the AP-1 family of transcription factors. It has a potent influence on T-cell proliferation and functional expression. It is able to form homodimers with itself or heterodimers with c-FOS. These dimers attach to the particular sites in the DNA, causing T-cell immune responses (generation of IFN-γ). The dimers formed between C-JUN are, however, not the only ligands of these particular DNA sites. There are other rival ligands like BATF and IRF4.

The three factors have an interesting connection. At the initial phases, when their levels are low, they cooperate to activate T-cells. During middle life, their functions and roles begin to separate. The role of c-JUN is short-term, quick, and effective killing without taking into consideration the consequences. It does this by sacrificing its own life span for maximum combat effectiveness. Conversely, BATF and IRF4 roles are more geared towards the enhanced differentiation of T cells. They make sure that they last as long as possible in difficult environments at the cost of fighting ability. As such, in the short run, the three relations are mutually reinforcing. In the long run, they are competing, as the number of binding sites on DNA is limited.

Through its closed structure, the microenvironment of solid tumors harbors a high number of antigens. The concentration of antigens is high, which gives sustained stimulation of T cells. The continuing presence of T cells in such an environment causes these cells to experience continuous intense stimulation. This results in the massive expression of the BATF and IRF4. At the same time, a sustained, unremitting stimulation enlists the E3 ubiquitin ligases, which selectively identify and destroy the c-Jun protein. In addition, chronic stimulation as well as the high expression of BATF inhibits c-Jun transcription. The more one factor increases, the more the other decreases, which leaves c-Jun in a total downfall.

To address T cell dysfunction and uncontrolled overactivation, c-Jun overexpression has been developed as an optimized genetic modification strategy. In this approach, lentiviral or retroviral vectors are utilized to integrate CAR and c-Jun genes into a single expression plasmid. This design enables simultaneous expression of the CAR structure and the c-Jun protein in modified T cells. Furthermore, the application of an exogenous promoter guarantees stable, continuous, and independent c-Jun expression in engineered CAR-T cells.

7. Conclusion

Substantial advances have been made in the use of CAR-T cells against blood malignancies—most notably those of B-cell origin—establishing this modality as an encouraging treatment strategy within the broader field of cancer therapy. Three major challenges are presently associated with CAR-T cell therapy, namely, limited survival capacity within the TME, lack of cytotoxicity, and lack of selectivity. CAR-T cells should be able to survive in the unfavorable TME, which can be addressed through silencing the genes of the respective receptors. The exhaustion-related genes should be counteracted, and c-Jun expression should be boosted to help T cells sustain high-intensity cytotoxicity. Nevertheless, too much cytotoxicity may result in off-target toxicity, cytokine storms, and carcinogenicity. As such, their selectivity and controllability need to be enhanced. It can make sure that CAR-T cells have high specificity to tumor cells by designing logic gates like Syn-Notch circuits.

Cutting-edge strategies including multi-gene editing, Syn-Notch genetic circuits, and c-Jun overexpression provide innovative solutions to overcome the bottlenecks of conventional CAR-T therapy. Nevertheless, several challenges remain unresolved, such as the risk of chromosomal translocation caused by multi-gene editing and the long-term safety concerns of exogenous gene

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overexpression. Future research directions include optimizing the safety of gene-editing techniques, screening highly specific combinatorial target antigens to enhance tumor-targeting accuracy, and developing personalized treatment regimens tailored to individual tumor characteristics. These advancements will promote the broader clinical translation and application of CAR-T therapy, offering improved therapeutic prospects for cancer patients.

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