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Universal CAR-T Cells: A Novel Approach to Cancer Immunotherapy

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Abstract: ***Cancer remains a leading cause of mortality globally, posing enduring challenges for clinical and economic healthcare systems alike. Conventional treatment modalities often fall short in providing durable remission, particularly in advanced-stage disease. Chimeric Antigen Receptor T cell (CAR-T cell) therapy has emerged as a promising frontier in cancer treatment. This innovative approach harnesses the power of the body's own immune system to eradicate malignant cells. However, current CAR-T cell therapies face limitations, including the risk of graft-versus-host disease (GvHD) and the challenge of manufacturing personalized treatments for each patient. This paper explores the concept of developing "universal" CAR-T cells, which could be derived from any suitable donor and administered to patients without the need for stringent HLA matching, overcoming the limitations of current therapies. Using CRISPR/Cas9-based gene editing, particularly base editing, this research aims to eliminate the expression of T cell receptors (TCR), Major Histocompatibility Complex (MHC) molecules, and Programmed Death-1 (PD-1) molecules on donor T cells, mitigating the risk of immune rejection and enhancing anti-tumor efficacy. The development of universal CAR-T cells holds the potential to revolutionize cancer treatment, offering a readily available and cost-effective immunotherapy approach.***

Keywords: Universal CAR-T cells, Cancer immunotherapy, CRISPR/Cas9, Base editing, Gene editing

1. Introduction

Deoxyribonucleic acid (DNA), the blueprint of life, holds the instructions for the development, functioning, and reproduction of all living organisms [1]. This intricate molecule comprises a double helix structure formed by two strands of nucleotides [2]. Each nucleotide consists of a sugar molecule (deoxyribose), a phosphate group, and one of four nitrogenous bases: adenine (A), thymine (T), guanine (G), and cytosine (C). These bases pair specifically, with A bonding to T and G bonding to C, constitute the paired base sequences of the DNA helix [2].

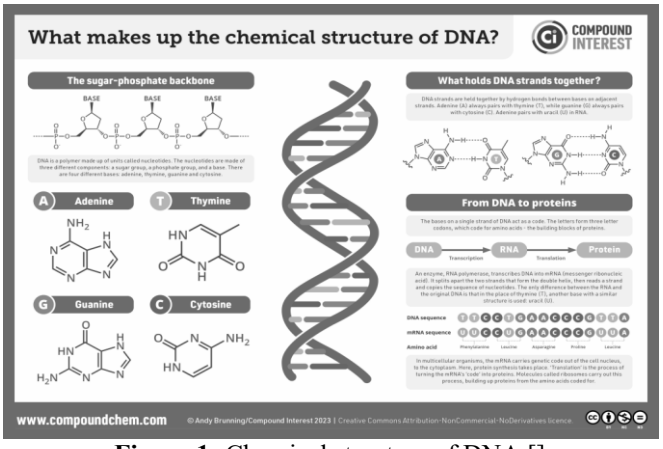


Figure 1: Chemical structure of DNA []

Long stretches of DNA are organized into chromosomes, residing within the nucleus of every cell. Within these chromosomes lie genes, the fundamental units of heredity [1]. Genes contain the instructions for building proteins, the workhorses of the cell responsible for a myriad of functions [1]. The process by which the genetic information encoded in DNA is used to synthesize proteins is known as the central dogma of molecular biology, first articulated by Francis Crick [3].

The central dogma unfolds in two major steps: transcription and translation. During transcription, the DNA sequence of a gene is copied into a messenger RNA (mRNA) molecule. This mRNA molecule then serves as a template for translation, where the sequence of nucleotides in the mRNA is decoded into a sequence of amino acids, the building blocks of proteins [1]. This intricate process highlights the crucial role of DNA and its faithful translation into functional proteins. Disruptions or errors in this process can lead to a range of diseases, including cancer.

Cancer arises from the uncontrolled growth and proliferation of abnormal cells that have acquired genetic and epigenetic alterations [4]. These cells, often originating from a single rogue cell, acquire mutations in their DNA that disrupt normal cellular processes. These mutations can be inherited or acquired throughout life due to environmental factors such as exposure to radiation or certain chemicals [5]. These alterations can activate oncogenes that promote unchecked cell growth, or the inactivation of tumor suppressor genes, genes that normally inhibit cell growth [4]. As a result, cancer cells divide uncontrollably, invading surrounding tissues and potentially spreading to distant sites in a process known as metastasis [4].

The human body is equipped with an intricate immune system to defend against harmful invaders, including bacteria, viruses, and even cancerous cells [6]. T cells, a type of white blood cell, play a pivotal role in this defense. They circulate throughout the body, monitoring cells for pathological or abnormal signals. T cells recognize foreign entities through T cell receptors (TCRs) on their surface [7]. These TCRs interact with Major Histocompatibility Complex (MHC) molecules present on the surface of other cells [2]. MHC molecules act as display cases, presenting fragments of proteins, called antigens, to T cells [7].

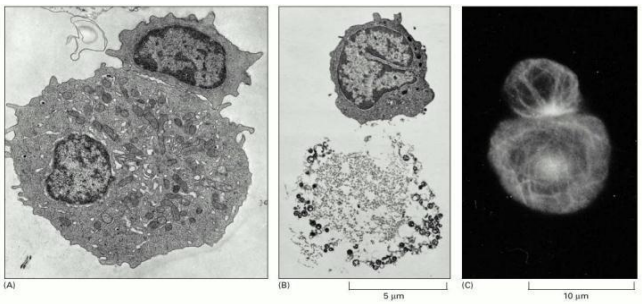


Figure 2: Effector cytotoxic T cells killing target cells in culture

When a T cell encounters an antigen presented by an MHC molecule that it recognizes as foreign, it initiates an immune response, leading to the destruction of the cell presenting the antigen.

While the immune system is generally adept at identifying and eliminating abnormal cells, cancer cells employ various strategies to evade immune surveillance [8]. One such strategy involves the PD-1/PD-L1 pathway. Programmed cell death protein 1 (PD-1, encoded by the gene PDCD1) is a protein found on the surface of activated T-cells [9]. Its counterpart, Programmed death-ligand 1 (PD-L1), is expressed on the surface of some normal cells and is often overexpressed on cancer cells [10]. The interaction between PD-1 and PD-L1 inhibits T-cell activation, preventing them from attacking the cells expressing PD-L1 [9. 10]. This mechanism, crucial for preventing autoimmune responses where T cells mistakenly attack healthy cells, is exploited by cancer cells to evade immune destruction.

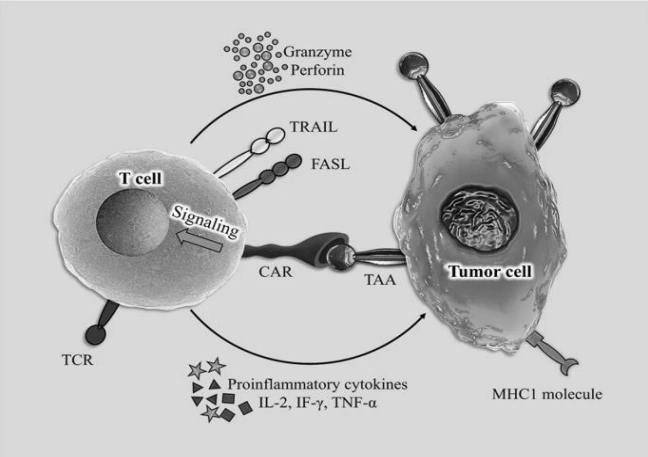


Figure 3: T cell-mediated antitumor effects by chimeric antigen receptors.

Harnessing the power of the immune system to combat cancer has led to the development of immunotherapies, a revolutionary approach to cancer treatment. Among these groundbreaking therapies, chimeric antigen receptor (CAR) T cell therapy has shown remarkable promise, particularly for hematological malignancies [11]. CAR-T cells are genetically engineered T-cells equipped with artificial receptors, known as CARs, designed to recognize specific proteins or antigens on the surface of cancer cells [12]. This targeted approach allows CAR T cells to precisely identify and eliminate cancer cells while sparing healthy tissues.

2. Background

Universal CAR-T Cell Therapy:
CAR T cells are created by extracting T cells from a patient's blood and genetically modifying them in the laboratory [11]. The gene encoding the CAR is introduced into the T cells, enabling them to express the CAR protein on their surface. These engineered CAR T cells are then expanded to large numbers in the laboratory and infused back into the patient. Once inside the patient's body, CAR T cells circulate and, upon encountering cancer cells expressing the target antigen, bind to them via their CAR. This binding triggers the activation of the CAR T cell, leading to the release of cytotoxic molecules that directly kill the cancer cell [12].

While CAR T cell therapy has shown remarkable success in treating certain types of blood cancers, it faces several limitations. One significant challenge is the risk of graft-versus-host disease (GvHD) when using T-cells from a donor (allogeneic) [13].

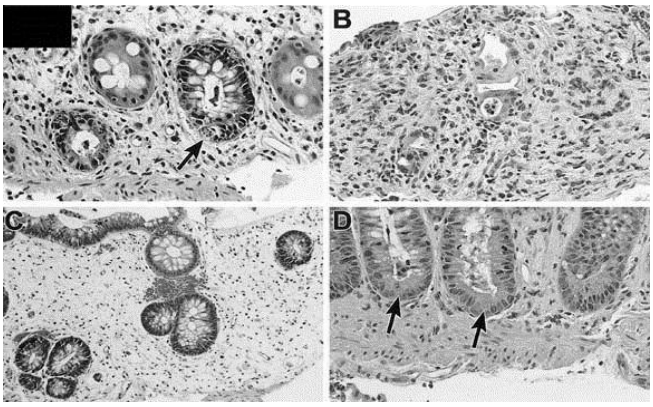


Figure 4: Histologic changes of acute and chronic graft-versus-host disease (GVHD) in the colon. A, numerous apoptotic bodies are present in the crypt epithelium (arrow), consistent with acute GVHD. B, Several crypts are present with marked damage and reactive epithelial changes as well as neutrophils in the lumen of the central crypt (cryptitis), changes consistent with acute GVHD. C, Marked crypt distortion and loss is present in this biopsy specimen, suggesting chronic GVHD. D, Paneth cell metaplasia (arrows) is another marker on chronic inflammatory disease that is suggestive of chronic GVHD in the right clinicopathologic setting.

GvHD occurs when the donor T cells recognize the patient's tissues as foreign and attack them. This serious complication stems from mismatched HLAs between the donor and the recipient [14]. HLA molecules are responsible for regulating the immune system and distinguishing between self and non-self. When donor T cells with different HLAs are introduced into a patient, they can recognize the patient's tissues as foreign and mount an immune response.

Another challenge associated with CAR T cell therapy is the manufacturing process, which is often time-consuming and costly [15]. Each batch of CAR T cells needs to be specifically engineered for each patient, using the patient's own T cells. This personalized approach, while effective, limits the availability of CAR T cell therapy and makes it inaccessible to many patients. To address these challenges,

researchers are actively exploring the development of "universal" CAR-T cells [16].

Universal CAR-T cells are a type of allogeneic CAR-T cell, meaning they are derived from a healthy donor rather than the patient themselves. The key to creating universal CAR-T cells lies in mitigating the risk of GvHD and host rejection [16]. This can be achieved through gene editing techniques, such as CRISPR/Cas9, to eliminate the expression of molecules involved in alloreactivity [17]. The objective is to modify donor T-cells to prevent them from recognizing the patient's tissues as foreign while also preventing the patient's immune system from rejecting the CAR-T product.

CRISPR/Cas9, short for Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9, is a revolutionary gene-editing tool that has transformed the field of genetic engineering [5]. Adapted from a naturally occurring defense mechanism found in bacteria, CRISPR/Cas9 allows scientists to make precise changes to the genome of living organisms, including humans [18, 19]. The CRISPR/Cas9 system comprises two main components: the Cas9 enzyme, which acts as molecular scissors to cut DNA at specific locations, and a guide RNA (gRNA), which directs the Cas9 enzyme to the desired target site in the genome. The gRNA is a short RNA molecule designed to be complementary to the target DNA sequence, ensuring that the Cas9 enzyme cuts at the correct location.

Once the Cas9 enzyme has cut the DNA at the target site, the cell's natural DNA repair mechanisms attempt to fix the break. This repair process can be exploited to introduce precise changes to the DNA sequence. For instance, by introducing a small piece of DNA with the desired sequence, scientists can trick the cell into incorporating this new sequence into the genome during the repair process. This ability to make precise changes to the genome has opened up a plethora of possibilities in various fields, including medicine, agriculture, and biotechnology.

CRISPR/Cas9 Targeting for Universal CAR-T Cells:
To create universal CAR T cells, we need to prevent the donor T cells from attacking the recipient's healthy cells while preserving their ability to target and destroy cancer cells. This can be achieved by using CRISPR/Cas9 technology to knock out or disable certain genes in the donor T cells [4]. Three genes of particular interest are CD247, TRAC, and B2M, which encode for PD-1, TCR, and MHC molecules, respectively.

Synthego is a genome engineering company that provides a platform for designing, executing, and analyzing CRISPR experiments [4]. Using Synthego's platform, researchers can identify optimal guide RNA sequences for targeting specific genes and predict the efficiency of gene editing. The platform provides information on the number of exons (coding regions) in a gene, as well as the location and sequence of potential guide RNA target sites.

CD274 (PD-1): PD-1 is a protein found on the surface of T cells that acts as an "off switch," inhibiting T cell activation [21]. Cancer cells often exploit this mechanism by overexpressing PD-L1, the ligand for PD-1, on their surface, effectively suppressing the immune response against them. By knocking out the CD274 gene, which encodes for PD-1, we can prevent the PD-1/PD-L1 interaction and enhance the ability of CAR T cells to kill cancer cells [4].

TRAC (T cell receptor alpha constant): The TCR is responsible for recognizing specific antigens presented by MHC molecules [2]. However, in the context of universal CAR T cells, TCR expression can lead to GvHD as the donor T cells may recognize the recipient's HLA molecules as foreign. Knocking out the TRAC gene, which encodes for a component of the TCR, can prevent GvHD by rendering the donor T cells incapable of recognizing the recipient's HLA molecules [20].

B2M (beta-2 microglobulin): B2M is a component of the MHC class I molecule, which is involved in presenting antigens to cytotoxic T cells. Knocking out the B2M gene prevents the assembly and expression of functional MHC class I molecules on the surface of donor T cells. This modification reduces the likelihood of the donor T cells being recognized and attacked by the recipient's immune system, thus minimizing the risk of GvHD [20].

3. Method

Base Editing: Refining Gene Editing for Universal CAR-T Cells:

While traditional CRISPR/Cas9 techniques have revolutionized gene editing, they rely on creating double-strand breaks in the DNA molecule, which can lead to unintended genetic alterations [22]. These off-target effects pose a significant concern, particularly in therapeutic applications. Base editing, a more recent advancement in CRISPR technology, offers a more precise and efficient approach to gene editing, minimizing the risk of unintended mutations.

Base editing utilizes a modified version of the Cas9 enzyme, known as dead Cas9 (dCas9), which can no longer cut DNA but retains its ability to bind to specific DNA sequences guided by gRNA [23]. Fused to dCas9 is a deaminase enzyme that can chemically modify a single DNA base, effectively converting one DNA base into another without creating a double-strand break. There are two main types of base editors:

- Cytidine Base Editors (CBEs): CBEs convert cytosine (C) to thymine (T) in the DNA sequence [23].
- Adenosine Base Editors (ABEs): ABEs convert adenine (A) to guanine (G) in the DNA sequence [24].

Base conversion offers a highly precise editing method for disrupting gene expression without the risks associated with double-strand breaks. By strategically introducing base changes at specific locations within a gene, we can disrupt its expression without altering the entire gene sequence [25, 26].

One such strategy involves targeting splice sites, the junctions between exons and introns within a gene [26, 27]. Splice sites play a crucial role in the removal of introns from pre-mRNA during RNA splicing, ensuring that only the coding regions of the gene are translated into protein. By modifying the DNA sequence at splice sites using base editors, we can disrupt the

normal splicing process, resulting in defective or incomplete protein structures.

Targeting Proteins for Universal CAR-T Cells:
To create universal CAR T cells, base editing can be employed to target the specific proteins involved in GvHD and immune evasion, including TCR, MHC molecules, and PD-1. By disrupting the expression of these proteins, we can minimize the risk of GvHD while enhancing the anti-tumor efficacy of the engineered T cells [7]. The following sections provide specific examples of how base editing can be used to target these proteins:

Targeting CD274 (PD-1): Base editing can be used to introduce mutations at splice sites within the CD274 gene, leading to the production of non-functional PD-1 protein [7]. This disruption of PD-1 expression would prevent the inhibitory interaction between PD-1 and PD-L1, allowing the engineered CAR T cells to effectively target and destroy cancer cells expressing PD-L1 [21].

Targeting TRAC (T cell receptor alpha constant): Base editing can be used to introduce mutations at splice sites within the TRAC gene, disrupting the expression of the TCR on the surface of donor T cells [7]. This modification prevents the TCR from recognizing and binding to HLA molecules on recipient cells, mitigating the risk of GvHD [26].

Targeting B2M (beta-2 microglobulin): Similar to TRAC, base editing can be employed to target splice sites within the B2M gene, disrupting the expression of functional MHC class I molecules [7]. This reduction in MHC class I expression reduces the likelihood of donor T cells being recognized as foreign by the recipient's immune system, further minimizing the risk of GvHD [26].

4. Results

Analyzing the figures provided:

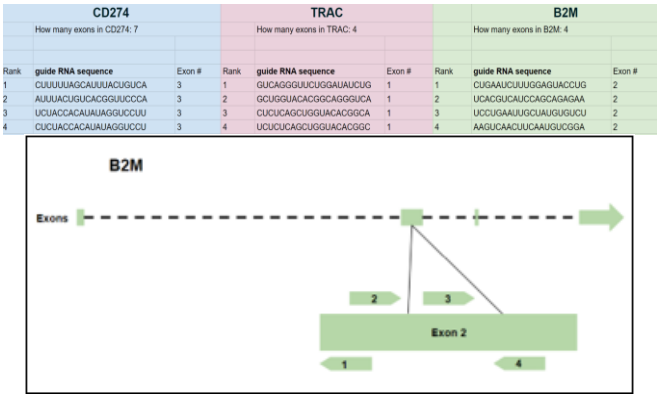
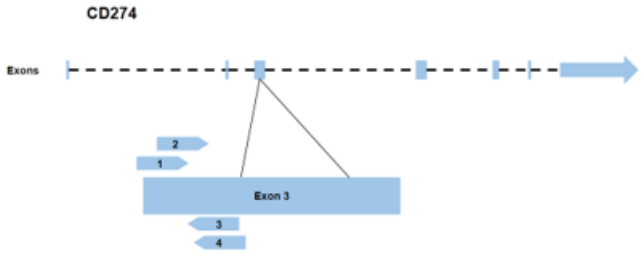
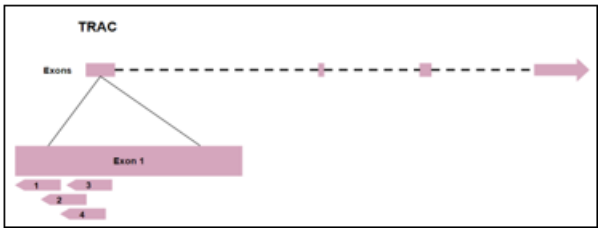


Figure 5: This figure shows a table with three columns representing three genes: CD274, TRAC, and B2M. For each gene, the table lists the number of exons, the guide RNA sequences for the top 4 ranked targets, and the corresponding exon number targeted. The guide RNA sequences are designed to target specific regions within the gene, typically near the beginning to ensure disruption of protein production.



Figures 6: These figures visually represent the exon structure of each gene and the location of the guide RNA target sites within the exons [8]. For instance, Figure 2 shows that the B2M gene has 4 exons, and the top-ranked guide RNA target site is located within Exon 2. These visualizations aid in understanding the spatial relationship between the guide RNA target sites and the overall gene structure



Figures 7: These tables present detailed information on potential target sites within each gene for base editing using CBE and ABE techniques [7]. The tables list the exon number, the specific splice site being targeted (donor or acceptor), the protospacer sequence (the sequence targeted by the guide RNA), the PAM sequence (required for Cas9 binding), the appropriate enzyme (CBE or ABE), and the predicted cDNA disruption (a measure of editing efficiency). For example, Figure 5 shows that targeting the 3' acceptor site of Exon 2 in the CD274 gene using a CBE with the specified protospacer sequence is predicted to disrupt cDNA by 0.967, indicating a high editing efficiency.

5. Discussion

These findings have significant implications for developing universal CAR T cell therapy. Targeting specific splice sites within the CD274, TRAC, and B2M genes using base editing offers a precise and efficient way to eliminate the expression of proteins involved in GvHD and immune evasion. Disrupting PD-1 expression enhances the ability of CAR T cells to overcome the immunosuppressive tumor microenvironment, leading to more effective tumor cell killing. Simultaneously, knocking out TCR and MHC class I expression mitigates the risk of GvHD, enabling the use of allogeneic donor T cells without the need for stringent HLA matching.

However, this approach also presents potential challenges and limitations. While base editing offers greater nucleotide-level specificity compared to traditional CRISPR/Cas9, the risk of off-target effects, though minimized, is not entirely eliminated. Unintended genetic alterations could have unforeseen consequences, potentially leading to the development of other diseases, including cancer [28]. Therefore, comprehensive off-target analysis and rigorous safety testing are paramount before clinical application.

6. Summary and Conclusion

The development of universal CAR T cell therapy holds immense promise for cancer treatment. By leveraging the precision of base editing to disrupt the expression of TCR, MHC molecules, and PD-1, we can create readily available CAR T cells without donor–recipient HLA matching that are readily available to patients without the need for HLA matching. This approach may substantially alter the clinical landscape of cancer immunotherapy, making this life-saving treatment more accessible, affordable, and applicable to a broader range of cancers.

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