
Review

Combinatorial application of artificial intelligence and CRISPR/Cas9 on the next-generation CAR-T immunotherapy

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Abstract: Chimeric Antigen Receptor T-cell (CAR-T) therapy has emerged as one effective treatment against complex diseases, including malignancies, infectious disorders, and autoimmune conditions. However, its clinical applications remain hindered by severe side effects, including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). Recent innovations in genome editing and computational biology, specifically CRISPR/Cas9 and artificial intelligence (AI), offer promising avenues to improve CAR-T safety and efficacy. In this article, we reviewed recent advances in CAR-T therapy, highlighting the key role CRISPR/Cas9 plays in various aspects of CAR-T therapy through gene editing. We then studied and analyzed how AI leverages large datasets and its powerful learning capabilities to advance CAR-T therapies and CRISPR/Cas9. Finally, focusing on CAR-T therapy, we discussed how CRISPR/Cas9 and AI work synergistically to advance the development of CAR-T therapy, including reducing toxic side effects, improving therapeutic efficacy, sustaining long-term outcomes, discovering new therapeutic targets, and ensuring safety monitoring. By combining the predictive power of AI with the precision of CRISPR/Cas9, researchers can develop next-generation immunotherapies that are safe, effective, and tailored to the patients' requirements. This synergy can address previously untreatable diseases and reshape the landscape of precision medicine.

Keywords: CAR-T therapy; CRISPR/Cas9; artificial intelligence; genome editing; immunotherapy

1. Introduction

Immunotherapy, by enhancing both cell-mediated and humoral responses of the immune system, is considered one of the most potent treatments to fight complex diseases (cancer, infectious diseases, autoimmune diseases, etc.). As this field continues to expand rapidly, billions of dollars have been invested in exploring immune-based treatment strategies [1]. T cell-based immunotherapies, such as CAR-T immunotherapy, is at the forefront of these approaches [2]. In response to the shifting paradigm of disease treatments, the US Food and Drug Administration has approved various T cell-boosting therapies, including anti-CD19 KymriahTM (tisagenlecleucel) [3] and YescartaTM (axicabtagene ciloleucel) [4]. Unfortunately, complications and toxicities are life-threatening, preventing the widespread application of such therapies [5]. These toxicities significantly impact both the safety and acceptance of the treatment, necessitating new methods to predict, monitor, and mitigate such risks. ICANS (or simply neurotoxicity), a life-threatening neurotoxicity, is particularly concerning due to its unpredictable onset and severity. The other most frequently observed immune-mediated toxicity is CRS [6], which is a form of systemic inflammatory response syndrome that can be triggered by a variety of factors such as infections and certain drugs, including T-cell therapies [7–9].

Fortunately, we live in an era of rapid technological advancement; even more fortunately, two well-known methodologies, CRISPR-Cas9 and AI, can help manage complications such as ICANS and CRS. CRISPR/Cas9 enables researchers to modify T cells at the genetic level, enhancing their precision, durability, and anticancer efficacy. AI, on the other hand, can be employed to continuously analyze clinical and molecular data in real time, enabling early detection of adverse responses and facilitating dynamic, patient-specific treatment adjustments. For instance, predictive algorithms developed through AI can identify biomarkers of toxicity and assist clinicians in intervening before life-threatening symptoms occur [10–12]. In one notable study, CRISPR/Cas9 was used to delete the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene in CAR-T cells, and it was found that compared with conventional CART19 cells, these GM-CSF^{k/o} cells remained normal, had reduced toxicity, and enhanced anticancer effects *in vivo*, and their overall survival was improved. Therefore, the side effects of T cell-boosting therapies can be tamed by both CRISPR/Cas9 and AI. Further mechanistic understanding will likely be necessary to enable both methodologies to serve CAR-T therapy better, thus playing a broader and more decisive role.

The above descriptions address only a small part of the complex relationship between CAR-T, CRISPR/Cas9, and AI. Here, we aim to summarize this trilateral relationship more completely, hoping to promote the synergistic effect of AI and CRISPR/Cas9 so that CAR-T therapy can maximize efficacy while avoiding the toxic side effects of treatment.

2. Chimeric antigen receptor T cell immunotherapy and its complications

2.1. Chimeric antigen receptor T cell immunotherapy

The 21st century has witnessed a paradigm shift in disease treatment, especially cancer therapeutics. While conventional treatments of cancer, such as chemotherapy, radiotherapy, and targeted small-molecule drugs remain widely used [13–18], T cell-based therapies, particularly CAR-T immunotherapy, have demonstrated remarkable efficacy in clinical settings [7,19]. CAR-T cells, which are genetically engineered to express synthetic receptors targeting tumor-associated antigens, have led to durable remissions in patients with otherwise treatment-refractory malignancies. The clinical application of tumor antigen-specific T cells modified to express CARs via adoptive transfer has yielded long-lasting remission in various cancers [20], with particularly notable success against relapsed and refractory CD19⁺ B cell malignancies [21–24]. CD19, a 95-kDa type I transmembrane glycoprotein from the immunoglobulin superfamily, is broadly expressed during various stages of B cell development. This makes it an ideal target for immunotherapy against diseases such as chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), and B cell lymphomas [25]. CART19 cells express CD19-specific CARs and can thus be applied to patients with CD19⁺ lymphoma and leukemia, first against Non-Hodgkin's lymphoma, then CLL in 2011, and then ALL in 2013 [25–27]. CD19 activates the T cell receptor (TCR) signaling cascade of CAR-T cells, leading to the proliferation, cytokine production, and lysis of the target CD19⁺ B cells. CAR-T therapies against other diseases have also been successfully developed, such as autoimmune diseases [28,29] and COVID-19 [30,31], which recently posed a serious threat to human health [32–35]. The following descriptions are often in terms of cancer only, but one should bear in mind that they may also apply to other diseases.

CAR-T cells are genetically engineered T lymphocytes that target disease-related cells, e.g., cancer cells producing specific epitope antigens such as CD19 [36]. These antigens are recognized and then bounded by the CAR of CAR-T cells. Because the binding is independent of the major histocompatibility complex (MHC), CAR-T cells can be easily activated to exercise their anti-disease action. The production of CAR-T cells (Figure 1) begins with leukapheresis, namely, the isolation of patients' leukocytes from their blood. The leukocytes are “washed” to isolate T cells, from which CD4/CD8 T cells are selected using specific markers and conjugates. On the other hand, antigen-presenting cells (APCs) are isolated from the patients, purified, and treated with anti-CD3/CD28 monoclonal antibodies (mAbs). The autologous APCs activate the CD4/CD8 T cells, where the mAbs provide a co-stimulatory signal that engages the TCR for T cell activation. After expansion, T cells are isolated and subsequently transduced with the gene encoding the synthetic CAR using a retroviral vector whose U3 is partly deleted for safety concerns. However, despite this modification, retroviral delivery poses risks, including clonal proliferation, insertional mutagenesis, transcriptional inactivation, and inconsistent transgene expression [37]. Moreover, CRISPR/Cas9 has been used instead of retroviral vectors for the genomic integration of the CAR gene [38]; then, the newly created CAR-T cells are infused back into the patients to kill disease-related cells.

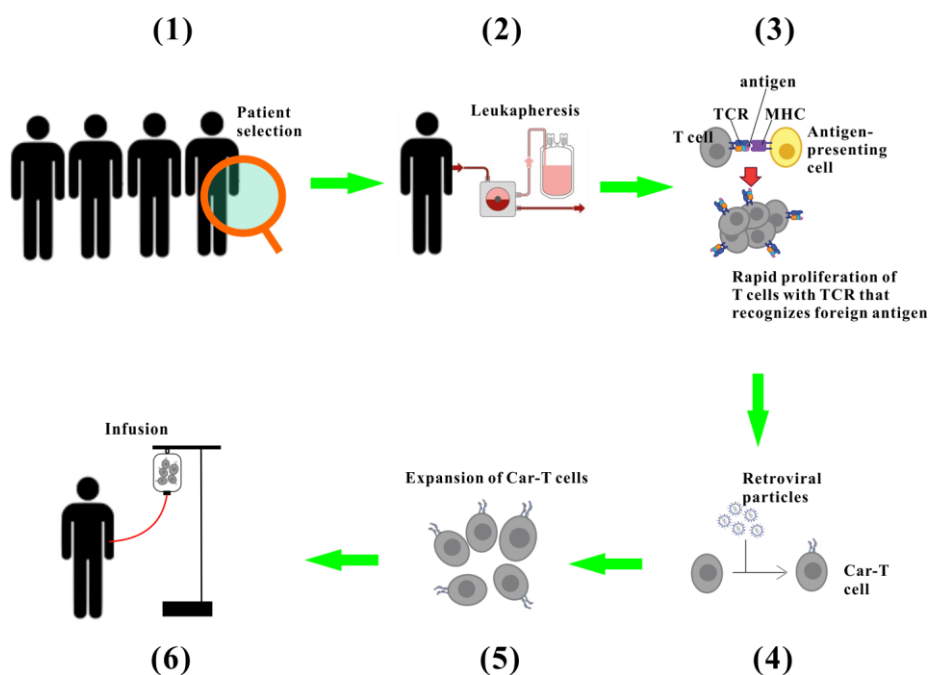


Figure 1. The production of CAR-T cells: (1) Patient selection. (2) T cell collection via leukapheresis. (3) T cell activation using antibodies/cytokines. (4) The gene encoding CAR is incorporated into the T cells via retroviral particles. (5) Clonal expansion of CAR-T cells. (6) Infusion of CAR-T cells into the patient.

Structurally, a typical CAR is composed of four key domains: The antigen-binding domain (ABD), the hinge or spacer region, the transmembrane domain, and the intracellular signaling domain (ICSD) [39]. The ABD consists of a single-chain variable fragment (scFv) containing V_L (light) and V_H (heavy) regions that are highly specific for target antigens like CD19. The V_L and V_H regions are selected for their binding ability to the target antigen. For example, the scFV specific for CD19 comprises a V_H having complementarity determining regions (CDRs) with sequences CDR1-GY-AFSSS, CDR2-YPGDED, CDR3-SLLYGDYLDY and a V_L having CDRs with sequences CDR1-SASSSVSYM, CDR2-DTSKLAS, CDR3-QQWNINPLT [40]. The linker peptide is composed of hydrophilic amino acid residues, primarily featuring glycine and serine to confer structural flexibility, as well as glutamate and lysine to improve solubility. Adjacent to the ABD, the hinge region acts as a connector bridging the extracellular receptor segment and the plasma membrane of the T cell. A well-designed hinge plays a pivotal role in optimizing receptor performance: It provides conformational flexibility to the scFv domain, minimizes steric hindrance between CAR and the target epitope, and facilitates stable immunological synapse formation. Notably, the hinge length is a critical determinant of functional efficacy, as it must be tailored to the spatial orientation and proximity of the target antigen's epitope. In general, longer (shorter) spacers bind better to membrane-proximal (-distal) epitopes, but more extended spaces have the additional advantage of increasing the flexibility of receptors. The transmembrane domain, consisting of a hydrophobic α -helix that spans the cell membrane, is essential for the overall stability of the receptor. It anchors the CAR to the plasma membrane, bridging the extracellular hinge and ABD with ICSD. The intracellular signaling domain is responsible for the signal transduction inside the T cell. After an antigen is bound to the ABD, CAR receptors cluster together and transmit an activation signal.

The ICSD in most CAR constructs is typically derived from the cytoplasmic region of CD3-zeta, owing to its critical role in native T cell activation. This domain contains immunoreceptor tyrosine-based activation motifs, which, upon phosphorylation, initiate the downstream signaling cascades necessary for full T cell activation and effector function.

Based on the ICSD, five generations of CAR-T cells were defined [41–50]. First-generation CARs included only the CD3-zeta domain, offering safety but limited functionality due to poor persistence and activation [43,44]. The second-generation CARs overcame this limitation with CD28 [45] or 4-1BB [46] added for co-stimulation. Both domains improve cytokine secretion, cell proliferation, apoptosis resistance, and *in vivo* persistence. The third-generation CARs combined CD28 with 4-1BB to augment T-cell activity; another combination was CD28 and OX40. Preclinical tests demonstrated that the third-generation CARs were more phosphorylated after binding to their target antigen, showing better *in vivo* persistence and improved effector functions [47]. However, third-generation cells have more immunologic ill effects because CARs are more activated, which has raised significant safety concerns. The fourth-generation cells are characterized by their universal cytokine killing. These cells were engineered to release a transgenic product, commonly a pro-inflammatory cytokine such as IL-12, upon activating their CAR in response to the target antigen [48]. This design not only promotes robust T cell proliferation but also facilitates the recruitment of innate immune cells into the tumor microenvironment (TME), thereby enhancing the elimination of target cells while mitigating systemic toxicity driven by excessive cytokine release [49]. In pursuit of safer and more controllable immunotherapies, fifth-generation CARs have been engineered to include additional intracellular regulatory modules. These include drug-inducible ON- and OFF-switches that enable precise modulation of CAR expression and signaling activity, enabling clinicians to either activate or deactivate CAR-T cells in a temporally and contextually controlled manner [50].

2.2. Cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome

Despite its clinical success, CAR-T therapy may lead to significant toxicities caused by robust immune responses, such as CRS and ICANS; these adverse events were not detected in early preclinical murine models but became apparent during human clinical trials [7]. The pathogenesis of CRS takes place when large numbers of white blood cells (including B cells, T cells, natural killer cells, macrophages, dendritic cells, and monocytes) are activated by stressed or infected cells through receptor-ligand interactions, and these immune cells release pro-inflammatory cytokines, which subsequently activate additional white blood cells, creating a positive feedback loop of pathological inflammation [8]. CRS often begins with non-specific constitutional symptoms such as fever, malaise, and loss of appetite. Severe CRS manifests as systemic inflammation, including hypoxia, hypotension, and organ dysfunction. The latter could happen secondary to hypotension/hypoxia but could also result directly from cytokine release. If CRS signs and symptoms are timely and adequately managed, then CRS would not cause organ dysfunction [51,52]; otherwise, dysfunction of renal, pulmonary, cardiac, hepatic, and gastrointestinal systems would occur. ICANS, or neurotoxicity in brief, may occur after CAR-T therapies, where activated T cells release cytokines (e.g., IL-6, TNF- α , IFN- γ), causing a cytokine storm that damages the blood-brain barrier (BBB). This allows cytokines, immune cells, and CAR-T cells to enter the brain, sparking neuroinflammation. Microglia (brain immune cells) become hyperactive, releasing toxins that harm neurons and glial cells. Astrocytes lose their protective roles, worsening nerve damage, while

cytokines disrupt neuronal communication and BBB integrity. Cerebral edema and blood clotting issues further compress brain tissue, risking severe neurological symptoms. It is a typical toxic encephalopathy manifested by symptoms such as dysphasia, somnolence, and aphasia; weakened motor skills, confusion, and word-finding difficulty could also occur. More severely, symptoms such as cerebral edema, seizures, coma, and motor weakness have been noted. Most ICANS patients were preceded by CRS, which is thus regarded as an ‘triggering event’ or ICANS co-factor, although they may occur concurrently. Both syndromes are largely reversible, which do not cause permanent disorders in nervous system [51–53].

3. CRISPR/Cas 9 in chimeric antigen receptor T cell immunotherapy

3.1. Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9

CRISPR/Cas9 derives from the immune system of bacteria. During a virus’s infection of a bacterium, some viral DNA segments are captured and incorporated into the bacterial genome as CRISPR arrays. This integration creates a genetic “memory” for the bacterium and enables it to recognize and defend against future viral infections. Owing to the memory, the bacterium produces RNA molecules that guide the Cas9 enzyme to move to and then cleave specific viral DNA sequences, which disables the virus. Researchers have repurposed the CRISPR/Cas9 bacterial immune system for genome editing in eukaryotic cells by engineering guide RNAs (gRNAs) that direct the Cas9 endonuclease to the correct genomic locus via sequence complementarity [54–57], where Cas9 induces a double-stranded break (DSB) similar to its natural mechanism in bacterial systems. Although Cas9 is the most widely utilized nuclease in genome editing, alternative enzymes such as Cpf1 (also known as Cas12a) have been employed for specific applications due to their distinct cleavage patterns and protospacer adjacent motif (PAM) sequence requirements. Following the DNA cut, the cell’s repair system can add, remove, or modify DNA sequences to achieve the desired genetic change. Figure 2 illustrates the essential action of CRISPR/Cas9.

3.2. CRISPR/Cas9 alleviating the side effects of CAR-T therapy

CRS and ICANS have become the biggest obstacles to disseminating CAR-T therapy [1]. For instance, while the interleukin-6 receptor antagonist tocilizumab has been approved for the management of CRS, no regulatory-approved therapy exists for addressing CAR-T-associated neurotoxicity such as ICANS. To alleviate the side effects, researchers are exploiting genome editing technologies to modify T cells’ genetic structure by inserting, deleting, or modifying nucleotides at specific locations. Among the genome editing technologies, CRISPR/Cas9 stands out for its superb speed, affordability, flexibility, accuracy/precision, and efficiency [58,59].

Given that monocytes and macrophages contribute to the development of CRS and neurotoxicity after CAR-T therapy, Sterner *et al.* investigated the possibility of counteracting GM-CSF to manage CART19-associated toxicities [60]. In their study, GM-CSF-deficient CART19 cells were generated by employing CRISPR/Cas9 to disrupt GM-CSF during the production of CAR-T cells. These GM-CSF^{k/o} cells retained normal proliferation and cytotoxic functionality while exhibiting enhanced anticancer efficacy *in vivo*, leading to improved overall survival in treated models. The findings underscore a promising strategy for mitigating ICANS and CRS through

targeted GM-CSF neutralization, offering dual benefits (toxicity reduction and therapeutic potentiation).

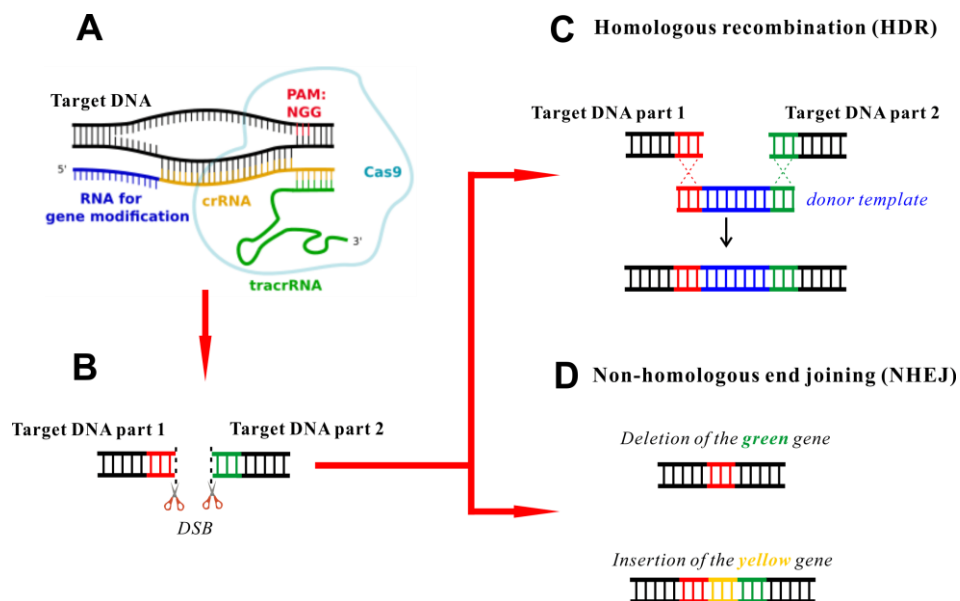


Figure 2. Essential action of CRISPR/Cas9 on a target DNA. (A) A duplex of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) acts as gRNA to guide gene modification of the target DNA at a specific DNA sequence. Cas9 binds the tracrRNA and needs a DNA binding sequence (5'NGG3') called the PAM. (B) After binding, Cas9 introduces a DSB to the target DNA (shown primarily in black, but with the fragment at the cleavage site shown in red and green), which is followed by two different kinds of gene modification. (C) Gene modification via homologous recombination (HDR). The donor template is primarily shown in blue, but with the ending fragments shown in red and green, which are homologous to the red and green fragments of the target DNA, respectively. The HDR pathway is then activated, using the donor template's sequence for precise DNA repair. (D) Gene modification via non-homologous end joining (NHEJ): Semi-random insertion-deletion mutations (indels) are introduced. For example, gene deletion involves the deletion of the “green” gene, and gene insertion involves the insertion of the “yellow” gene.

3.3. CRISPR/Cas9 enhancing CAR-T therapy

Beyond its role in minimizing CAR-T-associated toxicities, CRISPR/Cas9 technology also holds significant promise in augmenting the therapeutic efficacy and scalability of CAR-T cells. Through precise gene editing, it enables the elimination of intrinsic inhibitory pathways, streamlines the manufacturing process, lowers production costs, and broadens the applicability of CAR-T therapies to solid tumors and other non-hematologic malignancies. Immune checkpoints, including programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated protein 4, and lymphocyte activation gene 3, may limit the potency of CAR-T cells. CRISPR/Cas9 can disrupt these checkpoint genes to generate CAR-T cells resistant to immunosuppression and the subsequent

exhaustion, thereby improving their persistence and anticancer response [61,62]. By knocking out genes such as β -2 microglobulin (B2M) or TCR α constant chain (TRAC), heterologous T cells expressing foreign MHCs can be prevented from being rapidly eliminated. This improves the persistence of CAR-T cells made from T cells donated by healthy people [63]. CRISPR/Cas9 was used to remove the CD33 protein from healthy cells. This makes the presence of CD33 a distinct identifier of leukemia cells, enabling CAR-T therapy to precisely target and effectively destroy these cancer cells [64].

Eyquem *et al.* validated a “two-in-one” strategy with TCR knockout and CAR knock-in strategy. To achieve targeted disruption of *TRAC* locus and simultaneously integrate a CAR specific for CD19 under its endogenous transcriptional regulation, referred to as the TRAC-CAR strategy, the researchers used a gRNA guiding toward 5' of *TRAC* gene's first exon. In parallel, they constructed an adeno-associated virus (AAV)-based repair template containing a self-splitting P2A sequence followed by the cDNA of CAR, enabling seamless integration via homology-directed repair [65]. Cas9 mRNA and gRNA were delivered by AAV into T cells through electroporation, achieving high knockout frequencies (about 70%) with limited cell death. The knock-in efficiency was proportional to the AAV dose, exceeding 40% at a multiplicity of infection of 10^6 . This efficient targeting at the *TRAC* locus made about 95% of CAR⁺ cells TCR negative. The high selectivity of *TRAC* integration was corroborated through the genome-wide identification of AAV vector insertion sites, and no off-target hotspots were found, further validating the specificity of targeting. Compared with the uneven retrovirus-encoded CAR, *TRAC*-CAR showed uniform and consistent expression in multiple donors [65]. The validated “two-in-one” strategy is indeed promising.

CRISPR/Cas9 can target multiple genes simultaneously (multiplexed genome editing) [66]. For example, investigators deleted both *TRAC* and *TRBC* (TCR β constant region), which lessened TCR mispairing and boosted the expression of *NY-ESO-1*, a synthetic TCR transgene. Ren *et al.* developed gene-edited allogenic CAR-T cells that simultaneously lacked PD-1, MHC-I, and TCR. This was achieved through a combinatorial approach involving lentiviral transduction of the CAR construct alongside electroporation of mRNA of Cas9 and gRNAs guiding toward B2M, PD1, and TCR [67]. These CAR-T cells edited by CRISPR exhibited strong anticancer activity. In addition, T cells deficient in TCR and HLA had attenuated alloreactivity, without causing graft-versus-host disease. Notably, the *in vivo* anticancer efficacy of the edited cells was further enhanced through editing triple genes simultaneously, including targeted disruption of PD-1. These results suggest that T cells equipped with an allogenic, gene-edited CAR and lacking endogenous TCR expression can act as universal donors, offering a viable alternative to autologous T cell sources in adoptive immunotherapy [67].

Integrating synthetic biology with CRISPR/Cas9 may yield T cells that are optimally programmed, as synthetic biology has the transformative power to optimize cell function [68–70]. To expand CAR-T cells' capacities, Cho *et al.* presented a split, universal, and programmable (SUPRA) CAR that switches between targets without repetitive engineering of T cells, finely tunes the activation strength of T cells, and senses multiple antigens and logically responds to them [71]. The components of the SUPRA system were humanized to minimize potential concerns of immunogenicity. To demonstrate the broad applicability of the system, it was evaluated across two distinct cancer models and further engineered to independently modulate separate T cell subsets, thereby establishing a dual induction CAR framework. These findings exemplify that a variety of

features can be integrated to achieve versatile SUPRA CARs. This highly enhanced universal CAR-T system represents a paradigm that could revolutionize disease treatment.

3.4. CRISPR/Cas9 screening for new targets to enhance the persistence of CAR-T cells

Identifying unrecognized genes that influence CAR-T cell functionality is essential for improving their therapeutic performance in clinical settings. To enable such discoveries, researchers often utilize comprehensive small RNA (sRNA) libraries that target the entire genome rather than relying on individual sRNA constructs [72]. In recent years, genome screening frameworks utilizing CRISPR/Cas9 technology have become practical tools for identifying novel targets for cancer immunotherapy [73].

Screening for targets that regulate memory or exhaustion phenotypes. The molecular mechanisms underlying CAR-T cell differentiation and exhaustion remain insufficiently characterized. To uncover novel genetic regulators of exhaustion and memory formation, Carnevale *et al.* conducted CRISPR knockout (CRISPR-KO) screens across multiple immunosuppressive models. Their analysis identified RASA2 as a previously unrecognized target whose deletion significantly enhanced the persistence and therapeutic efficacy of CAR-T cells [74]. In a separate study, Katherine *et al.* performed two independent CRISPR-KO screens in CAR-T cells subjected to chronic antigen stimulation. Their findings revealed that cyclin C and mediator complex subunit 12 play central roles in modulating effector functions. The authors further demonstrated that these two factors form a functional mediator kinase module that amplifies CAR-T cell cytotoxic activity [75]. Belk *et al.* performed a complementary CRISPR screening across the genome (*in vitro* depletion and screening followed by *in vivo* screening) to identify a series of epigenetic regulators (e.g., cBAF and INO80) of chromatin remodeling. Genetic disruption of the cBAF complex was shown to enhance persistence of T cells, inhibit exhaustion, and enhance anticancer efficacy. In contrast, perturbation of the INO80 chromatin remodeling complex altered gene expression profiles and was found to be critical for maintaining cellular metabolic homeostasis. Moreover, deletion of the Arid1a subunit within the cBAF complex markedly extended persistence and enhanced anticancer activity following adoptive transfer of T cells [76]. Trefny *et al.* employed a whole-genome CRISPR screening approach to uncover uncharacterized regulators of exhaustion. Through next-generation sequencing of gRNAs and follow-up single-gene knockout validation, they identified SNX9 as a candidate gene associated with the exhaustion phenotype. Functional assays further demonstrated that deletion of SNX9 promoted a central memory-like profile in CAR-T cells and significantly improved their anticancer activity [77]. Zhang *et al.* developed an *ex vivo* model of anergy to replicate CAR-T cell exhaustion observed *in vivo*, and utilized CRISPR/Cas9-based screening to identify critical genes contributing to this dysfunctional state. Their study revealed that CAR-T cells lacking BATF exhibited enhanced anticancer efficacy. Furthermore, BATF deficiency conferred increased resistance to exhaustion and promoted a central memory-like phenotype, thereby improving the durability of CAR-T cell responses [78].

Screening for activation, amplification, and effector function targets. Efficient expansion and sustained effector capabilities of CAR-T cells are critical for ensuring durable therapeutic responses. To support this, identifying genetic determinants that govern CAR-T cell activation, cytotoxicity, and proliferation remains a priority. Wang *et al.* performed a CRISPR-KO screen across the genome of IL13R α 2-directed CAR-T cells co-cultured with glioblastoma stem-like cells, leading to the

discovery of novel regulators of effector function [79]. In particular, knockout of TLE4 or IKZF2 enhanced cytotoxicity and decreased exhaustion, which was supported by single-cell transcriptomic analysis showing up- (down-) regulation of effector- (exhaustion-) related genes, respectively. These gene perturbations also improved the proliferation and tumor-killing capacity of CAR-T cells targeting HER2, indicating their broader applicability. In addition, the researchers screened glioblastoma stem-like cells co-cultured with CAR-T cells and discovered key genes inducing CAR-T cell resistance. Devikala *et al.* characterized therapeutically effective anticancer T cells based on four phenotypes: Genome stress, metabolic fitness, cell proliferation, and stem cell-like memory [80]. They performed a multi-type CRISPR/Cas9 screen for TCR-driven kinases and found that p38 is critical to regulate the four phenotypes. They found that the persistence and anticancer efficacy of CAR-T cells can be enhanced by drugs inhibiting p38. In addition to functional loss screening, functional gain screening has been extensively used to uncover suppressive regulators; functional gain strategies, such as CRISPR activation (CRISPRa), are also valuable for identifying genes that positively regulate CAR-T cell function. Geiger *et al.* developed a CRISPRa screening based on death guide RNA (dgRNA) in CD8⁺ T cells and identified new targets to enhance CAR-T cells' effector function [81]. The study demonstrated that PRODH2 overexpression or site-specific knock-in reprogrammed proline metabolism, an essential T cell functioning pathway, and significantly enhanced the *in vivo* anticancer efficacy of CAR-T cells across cancer models. In parallel, enforced PRODH2 expression elevated CAR-T cytotoxicity both *in vivo* and *in vitro*, induced widespread metabolic changes and gene expression changes, and improved the long-term therapeutic potential of CAR-T cells [82]. Examples of CRISPR applications in CAR-T therapy are provided in Table 1.

Table 1. CRISPR Applications in CAR-T therapy.

Background/Objective	Methods	Results/Finding	Ref
Monocytes and macrophages contribute to the development of CRS and ICANS.	Employing CRISPR/Cas9 to disrupt GM-CSF during CAR-T cell production.	GM-CSF ^{k/o} cells exhibited enhanced anticancer efficacy <i>in vivo</i> and improved survival.	[60]
CAR-T cells have low flexibility and are difficult to switch between targets.	The invention of a split, universal, and programmable (SUPRA) CAR.	SUPRA CAR-T cells were flexible and tunable (activation strength). They sensed multiple antigens and logically responded to them.	[71]
The molecular mechanisms underlying CAR-T cell differentiation and exhaustion remain insufficiently characterized.	CRISPR-KO screens were conducted across multiple immunosuppressive models.	RASA2 was identified as a previously unrecognized target whose deletion significantly enhanced the persistence and therapeutic efficacy of CAR-T cells.	[74]
Identifying genetic determinants that govern CAR-T cell activation, cytotoxicity, and proliferation remains a priority.	A CRISPRa screening was developed based on dgRNA in CD8 ⁺ T cells	New targets were identified to enhance CAR-T cells' effector function	[81]

4. Artificial Intelligence in chimeric antigen receptor T cell immunotherapy

AI refers to the simulation of human intelligence in machines designed to perform tasks that typically require human cognition, such as problem-solving, learning, perception, and decision-making. AI systems rely on algorithms; mathematical models that process data to identify patterns and make predictions. These systems are broadly categorized into narrow AI (task-specific, e.g., voice assistants) and general AI (human-level adaptability, still theoretical). Central to AI are its diverse models (Figure 3), each tailored to specific challenges. Machine learning (ML) models, like decision trees and support vector machines (SVMs), learn from labeled data to make classifications or predictions. Deep learning, a subset of machine learning, uses neural networks (NNs) with multiple layers (e.g., convolutional neural networks (CNNs) for image recognition) to handle complex data. Transformer models are widely used to analyze complex data such as natural language processing. A local server for running transformer has been established in Hugging Face library to serve models and handle inference requests (<https://huggingface.co/docs/transformers/en/index>). Unsupervised learning models, such as clustering algorithms, uncover hidden patterns in unlabeled data, while reinforcement learning (RL) models (e.g., Q-learning) optimize decisions through trial-and-error interactions with environments. Emerging architectures like generative adversarial networks (GANs) create synthetic data, and transformer-based models (e.g., BERT and GPT) revolutionize language understanding. Each model's strength lies in its design: NNs excel in non-linear pattern recognition, decision trees prioritize interpretability, and transformers leverage self-attention mechanisms for contextual analysis. These models power applications across healthcare, finance, autonomous systems, and creative industries, underscoring AI's transformative potential.

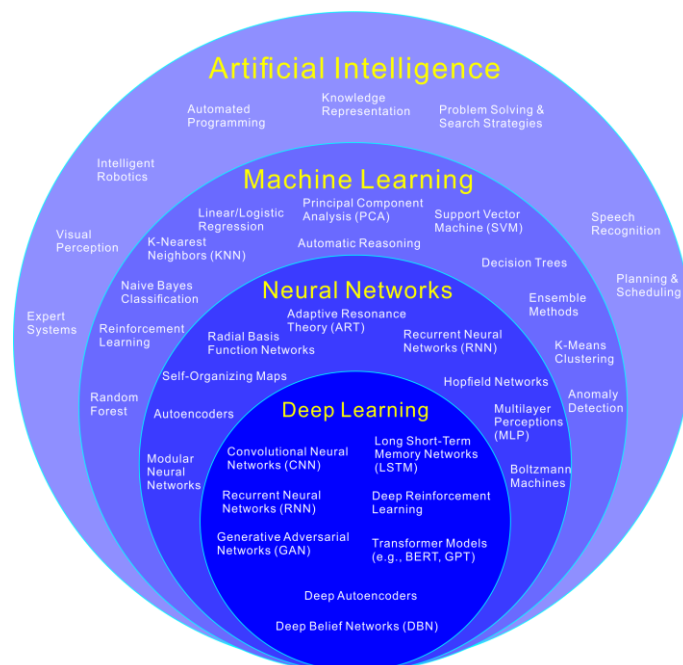


Figure 3. An overview of current AI models. Except for some general AI methods, most AI approaches fall under ML methods, including RL, decision trees, and primarily NNs. The latter encompasses various NN architectures, such as CNNs and transformers.

Limitations of CAR-T therapy can be overcome using AI models, which is transforming biomedical research [16,83–88]. AI can help predict efficacy, pathological responses, and patient outcomes of immunotherapy. In particular, it can help identify the best immunotherapy method, which is challenging using traditional tools that are time-consuming and labor-intensive [89]. AI offers powerful tools for optimizing CAR-T cell therapy through advanced analysis of Big multi-omics data. By integrating tumor-specific antigens, gene expression profiles, and molecular signatures, AI algorithms can systematically prioritize therapeutic targets with the highest likelihood of clinical efficacy. This facilitates the personalized design of CAR constructs tailored to a patient's unique cancer characteristics, including the identification of cancer-associated antigens and prediction of neoantigen candidates, ultimately enhancing therapeutic precision while minimizing off-target effects and adverse events. In addition, AI-based predictive models can forecast patients' individual responses to CAR-T therapy by synthesizing genomic, immunological, and clinical parameters, helping clinicians make informed decisions. AI-aided drug repurposing can uncover compounds that potentiate CAR-T cell activity, while combinatorial optimization models can propose synergistic treatment regimens involving immunomodulators. AI-enabled monitoring platforms track the performance and health of artificial intelligence systems, covering AI infrastructure (AIOps), AI models (Observability), and the impact of AI on brand reputation in various contexts, such as search engines and social media. Tools like Datadog (<https://www.datadoghq.com/>), Dynatrace (<https://www.dynatrace.com/>), and AIOps platforms (such as those from Gartner) are used for infrastructure monitoring, while platforms like Evidently AI (<https://www.evidentlyai.com/>) and Eden AI (<https://www.edenai.co/>) focus on model evaluation and observability. Brand monitoring tools, including Authoritas and AI Product Rankings, focus on how AI search and social platforms represent brands. They can dynamically assess patients' responses to immunotherapy by integrating data on cytokine fluctuations, immune cell kinetics, and clinical indicators. These systems can provide early warnings of adverse immune reactions and recommend real-time adjustments to treatment strategies, thereby improving clinical decision-making and patient safety [90]. Examples of AI enhanced CAR-T therapy are provided in Table 2.

While certain AI-driven strategies have yet to be fully implemented in clinical CAR-T therapy, they hold significant promise for enhancing its design and effectiveness.

(1) AI technologies can contribute to the rational design of novel CAR constructs and facilitate the *in silico* prediction of their therapeutic potential. Moreover, by analyzing high-dimensional data generated from single-cell RNA sequencing and T cell receptor sequencing, AI models can uncover critical genetic signatures linked to patient response and treatment outcomes [91]. ML predicted patients' responses based on individual characteristics and helped with personalized treatment strategies [91].

(2) AI can exert a deeper influence upon CAR-T therapy through spatial transcriptomics, which links gene expression with intra-tumor spatial location, thus providing a new perspective to understand CAR-T cells' interaction with the TME. Although CAR-T spatial transcriptomics has not been widely studied, it links gene expression with intra-tumor spatial location, thus providing a new perspective to understand CAR-T cells' interaction with the TME [92]. Given that AI has become essential in spatial transcriptomic analysis, AI would exert a deeper influence upon CAR-T therapy through spatial transcriptomics.

(3) AI can predict the binding specificity of CARs to antigens. Predicting TCR-antigen pairs is one of the most challenging tasks in modern immunology. To address this challenge, an AI model

based on transfer learning, called pMTnet, was developed to predict the specificity of binding between TCRs and antigens presented by class I MHC, revealing unique insights into TCR-peptide-MHC interactions [93]. Accurate predictions can be achieved using only the sequence of antigen, the sequence of TCR (CDR3 β), and class I MHC alleles. These approaches may help generate CARs whose specificity and affinity of binding are higher, thereby decreasing the possibility of off targeting and improving therapeutics.

(4) AI-driven predictive models enable the discovery of key biomarkers and relevant attributes that predict CAR-T treatment outcomes. These sophisticated AI models can be built using a variety of dataset biomarkers, including tumor mutational burden, signaling pathways, tumor cell types, and gene expression in the TME to predict the progression of disease, the chance of survival, and the response to CAR-T treatment. Specific biomarkers such as C-reactive protein, lactate dehydrogenase, and platelet levels have been linked to the effectiveness of CAR-T therapy [94].

(5) AI can improve image analysis and thus benefit CAR-T diagnosis and treatment. Radiomics, such as computed tomography, positron emission tomography, and magnetic resonance imaging, can predict CAR-T treatment outcomes. By leveraging AI-based algorithms, sensitive and subtle image patterns that are not discernible to the naked eye can be detected to distinguish treatment responders from non-responders. Accurate prediction of invasive image features by non-invasive methods can be used to improve interventions for CAR-T cell therapy. The challenge in building AI-based CAR-T prediction models is the need for large-scale omics and clinical datasets, which require the development of multi-omics datasets to implement AI methods. Radiomics and other cancer imaging data need to be generated to predict imaging features associated with CAR-T treatment response using ML techniques [42].

Table 2. AI Applications in CAR-T therapy.

Background/Objective	Methods	Results/Finding	Ref
CAR-T manufacturing requires strict controls at every stage to ensure consistent products and patient safety.	AI-based CAR-T platform was established in an intelligent hospital.	Integrated with hospital IT systems, an intelligent CAR-T platform uses AI to autonomously creates patient-specific therapies, leveraging Industry 4.0 automation.	[11]
Linking CAR-T synapse structure (the specialized interface that forms between a CAR-T cell and a cancer cell when they come into contact) and function to patient outcomes.	Using an innovative ML system, researchers analyzed vast collections of CAR-T cell synapse images. This approach improved the accuracy of synapse measurements and sped up processing. NNs automatically detected critical biological patterns across multiple experimental datasets.	Assessing the structural stability of CAR-T cell synapses helps predict treatment effectiveness against cancer. This enables doctors to match patients with tailored therapies.	[89]

Continued on next page

Background/Objective	Methods	Results/Finding	Ref
Systematically analyzing CAR-driven immune responses enables quantitative modeling and predictable optimization of therapeutic designs.	Researchers tested 200 out of 2,400 possible CAR designs combining 13 signaling motifs. ML then predicted which untested combinations would be functionally effective.	T cells engineered with enhanced-signaling receptors demonstrated superior tumor control in mouse models compared to standard designs.	[95]
Distinguishing CAR-T cells from regular blood cells using imaging alone is impossible based solely on their natural appearance or morphology, because CAR-T cells are blood cells. They look identical under standard light microscopes.	Researchers assembled a curated dataset of 500 fluorescence-stained CAR-T microscopy images and developed RCMNet--a hybrid CNN-Transformer model--to significantly improve image classification accuracy.	RCMNet accurately distinguished CAR-T cells from other cell types, demonstrating its potential value for AI-assisted diagnostics.	[96]
CAR signaling was difficult to optimize.	An AI-based tool called CAR-Toner was developed.	CAR-Toner scores CAR signal strength via positively charged patches to optimize design.	[97]

5. Artificial Intelligence and CRISPR/Cas9 in CAR-T cell enhancement

Integrating AI and CRISPR/Cas9 genome editing would advance CAR-T therapy tremendously. AI methods, especially ML and DL models, have significantly advanced multiple stages of genome engineering by enabling higher accuracy, reduced off-target effects, and improved editing efficiency [98]. In genome editing, most AI models are based on CNNs [99,100], recurrent neural networks (RNNs) [101,102], and RL [103,104]. CNNs have been used to guide CRISPR/Cas9 for the optimal targeting of DNA sequences [99,100]. These models are capable of extracting intricate patterns from genomic sequences, enabling accurate identification of optimal editing sites while minimizing the risk of off-target activity. When applied to CAR-T therapy, CNNs can analyze large genomic datasets to identify potential CAR-T cell targets with high specificity. This reduces the likelihood of accidental gene editing, thereby improving treatment safety. RNNs are designed to recognize patterns in data sequences, so they are suitable for tasks involving sequence information, such as gene sequences. By learning the dependencies among the nucleotides, they have been successful to forecast the efficiency and precision of CRISPR/Cas9 editing. When applied to CAR-T cell therapy [101,102], RNNs can more accurately predict gene editing results by considering the sequential nature of DNA. This helps design gRNAs that target specific cancer-related genes while leaving other gene regions unaffected.

RL involves training an algorithm to make decisions by rewarding it with expected results. In the context of gene editing, RL can be applied to systematically optimize the CRISPR experimental

design by probing diverse editing strategies and iteratively learning which approaches yield the most effective and reliable outcomes. Upon implementation in CAR-T therapy [104], RL can facilitate end-to-end optimization of the whole workflow (identifying target → designing gRNA → validating gRNA) of the production of CAR-T cells. RL-based models can iteratively refine each stage to enhance therapeutic efficacy while simultaneously reducing the risk of treatment-associated toxicity [105].

Despite the widespread adoption of CRISPR/Cas9 technology across both academic research and pharmaceutical development, its successful application remains critically dependent on two key parameters: On-target activity and off-target specificity [99,106]. Multiple computational algorithms have been developed to evaluate CRISPR-Cas9 activity and off-target specificity through *in silico* scoring frameworks, prediction models, and gRNA design tools have been developed to make CRISPR gene editing research and facilitate experiments more convenient and precise [107]. ML/DL-based algorithms are effective and reliable for computing scores of on-target efficacy and scores of off-target risks of gRNAs. These algorithms are typically trained on extensive datasets derived from CRISPR-KO experiments across multiple cell types, enabling them to generate reliable activity scores for candidate gRNAs [106]. With the increasing number of CRISPR gene editing datasets generated by CRISPR practitioners worldwide, data-driven ML/DL algorithms have been increasingly used to predict the activity of gRNA and its specificity, and the prediction results are getting closer and closer to experimental observations. For example, ML/DL predictors based on clinical/experimental data are more affordable and competent [99] compared with experimental methods to detect off-target, such as GUIDE-seq [108], HTGTS [109], BLESS [110] and IDLV [111].

A DL framework known as DeepCRISPR has been developed to concurrently predict both the on-target CRISPR-KO efficacy of sRNAs and whole-genome off-target cleavage map of sRNAs. This model integrates on- and off-target prediction tasks within a unified and hybrid architecture based on a deep neural network (DNN), enabling joint optimization during training and inference [112]. A deep unsupervised representation learning technique [113] was employed to autonomously extract latent features from genome-wide unlabeled sRNA sequences, enabling the model to capture meaningful structural and functional patterns without the need for manual annotation. Labeled sRNAs are then used for supervised DNN fine-tuning to optimize the learning model further. DeepCRISPR has merits as follows.

(1) By incorporating epigenetic profiles across diverse cell types, the model constructs a united representation of characteristics of genomic regions, enabling the integration of heterogeneous datasets derived from multiple experimental conditions and cellular contexts. This approach enhances its generalizability and predictive performance when applied to unseen cell types [113].

(2) The model learns from many genome-wide unlabeled sRNAs to automatically generate a parent network that generates high-level feature representations for on-target and off-target design of gRNAs. In this way, DeepCRISPR fine tunes the design of sRNA for coding regions of the genome, as well as non-coding regions, by pre-training (unsupervised) of whole-genome sequences of sRNA. Through comparative analyses between models trained with and without this pre-training strategy, the authors demonstrated the improved performance and predictive power conferred by incorporating unsupervised learning.

(3) A specialized strategy of data augmentation is employed to synthesize novel sRNA sequences harboring biologically relevant labels, thereby expanding the pool of annotated training data for predicting the target sRNA. Empirical studies have demonstrated that this approach enhances predictive accuracy and contributes to increased model robustness during training.

(4) It subsequently optimizes the pre-trained network based on the labeled sRNAs, thereby enhancing predictive accuracy even in scenarios with limited annotated samples.

(5) It effectively mitigates the issue of data imbalance commonly encountered during the prediction of off-target sites by integrating an effective and guided strategy of sampling into the training pipeline.

(6) It automatically identifies sequence and epigenetic features. With limited training samples, the model learns which features are essential in optimizing sRNA design, and the identified features can be used to optimize sRNA design further. This helps with an efficient and data-driven explanation of the mechanisms of on- (off-) target of CRISPR. Important examples showing CAR-T enhancement by combining AI with CRISPR/Cas9 are provided in Table 3.

Table 3. AI and CRISPR/Cas9 in CAR-T cell enhancement.

Background/Objective	Methods	Results/Finding	Ref
CRISPR/Cas9 application depends critically on on-target activity and off-target specificity, but it is difficult to consider both simultaneously.	DeepCRISPR was developed to simultaneously predict two critical outcomes for any sRNA: (1) Its on-target CRISPR-KO efficacy, and (2) a genome-wide map of all potential off-target cleavage sites.	This model uses a single hybrid DNN architecture to simultaneously handle both on-target efficacy and off-target cleavage predictions. By unifying these tasks, the system enables joint optimization during model training and real-time inference.	[112]
Genome-wide unlabeled sRNA sequences for CAR-T remain difficult to characterize.	Deep unsupervised learning autonomously identified latent features in sRNA sequences.	The AI model first captured general patterns about sRNA behavior (structure/function) using raw, unlabeled data. Then, researchers fine-tuned it with known examples (labeled sRNAs) to make it highly accurate for specific prediction tasks.	[113]

6. Conclusions

The synergy of CRISPR/Cas9 and AI is advancing CAR-T therapy by enabling precise, personalized treatments (Figure 4). Key strategies include: (1) AI-driven target identification via multi-omics analysis to pinpoint disease genes and optimize CRISPR edits; (2) AI-enhanced CRISPR design for efficient, specific gene editing; (3) personalized CAR-T engineering using patient-specific data; (4) AI-automated CRISPR screens to discover therapeutic targets; (5) AI-optimized CRISPR delivery systems; and (6) AI-predicted safety profiles for reduced off-target risks.

In cancer, AI identifies neoantigens and immunosuppressive tumor microenvironment (TME) factors, while CRISPR edits CAR-T cells to target tumors and resist TME suppression. For autoimmune diseases, AI pinpoints pathogenic mutations corrected by CRISPR, and in infectious diseases, AI identifies pathogen vulnerabilities targeted by CRISPR-edited immune cells. This integration accelerates safe, effective therapies for previously untreatable conditions, ushering in a

new era of precision medicine. However, this remarkable synergy does face limitations due to data scarcity and model interpretability. First, training AI requires high-quality, diverse datasets (e.g., patient genomics and tumor-immune interactions), but such data are often limited and biased, reducing model generalizability [114]. Second, AI struggles to capture the dynamic complexity of immune systems, as oversimplified models may fail to predict patient-specific responses to therapies like checkpoint inhibitors. Additionally, “black-box” algorithms lack transparency, hindering clinical trust and adoption. Overcoming these requires interdisciplinary collaboration to refine models, enhance data diversity, and align AI tools with clinical needs while addressing ethical and regulatory frameworks. Advances in explainable AI (XAI) [115] will enhance model transparency, fostering clinician trust. Additionally, generative AI [116] may design novel immunotherapeutic agents by simulating immune-tumor interactions. Again, success hinges on high-quality, diverse datasets and collaboration between computational scientists and clinicians to validate AI-driven insights in prospective trials.

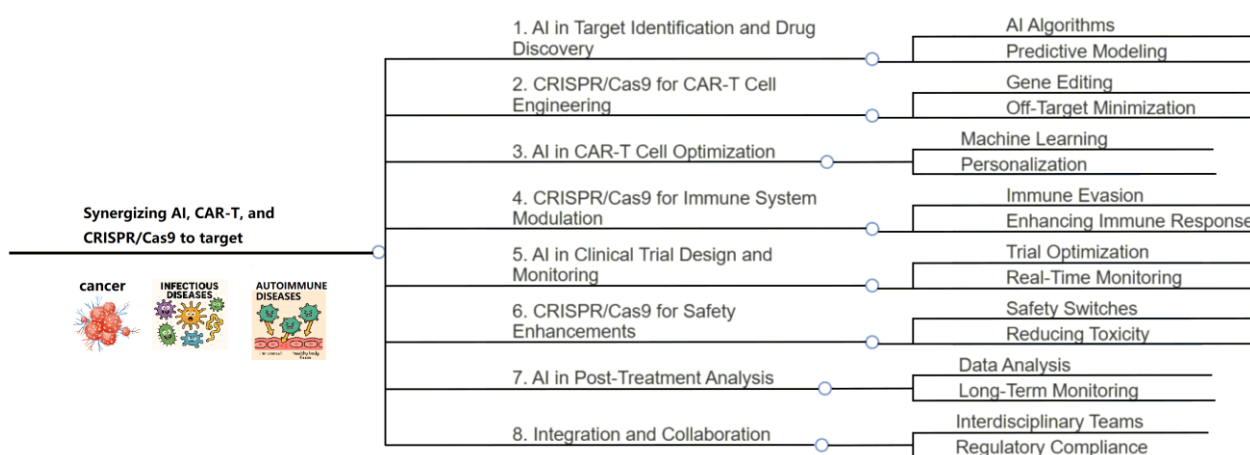


Figure 4. The roadmap to synergize AI, CAR-T, and CRISPR/Cas9.

The roadmap to synergize AI, CAR-T, and CRISPR/Cas9 is shown in Figure 4. (1) AI facilitates the identification of therapeutic targets by analyzing complex multi-omics datasets, including genomic, proteomic, and clinical data, and can predict the effectiveness and safety profiles of CAR-T cells through modeling their interactions with tumor cells and the immune microenvironment. (2) CRISPR/Cas9 enables precise genomic modifications in T cells to enhance their cytotoxic potential, such as by knocking out receptors of inhibitory natures (e.g., PD-1) or inserting persistence-enhancing transgenes. AI-guided gRNA design further improves editing precision by minimizing the chances of off-targeting, thereby supporting safer and more accurate gene engineering. (3) ML/DL-based AI models can be trained on clinical trial data to optimize CAR-T cell design, including the choice of antigen targets, co-stimulatory domains, and cytokine profiles. AI can help tailor CAR-T therapies to individual patients by predicting the best combination of genetic modifications based on the patient’s unique cancer profile. (4) CRISPR/Cas9 enables the genetic reprogramming of CAR-T cells to improve their tumor recognition and cytolytic capabilities while promoting immune evasion, thereby reducing the likelihood of host-mediated rejection and enhancing persistence *in vivo*. (5) AI can optimize clinical trial design by identifying patient subgroups most likely to benefit from CAR-T therapy and selecting outcome measures that

maximize statistical power and clinical relevance. AI will monitor patients in real-time during CAR-T therapy, predicting and managing adverse effects like CRS and ICANS. (6) CRISPR/Cas9 can be employed to incorporate inducible switches ensuring safety into CAR-T cells, enabling clinicians to modulate or terminate therapeutic activity in response to adverse events. Additionally, precise genome editing can attenuate off-tumor toxicity by minimizing CAR-T cell interactions with healthy tissues, while preserving their cytotoxic efficacy against malignant targets. (7) AI can analyze post-treatment data to identify biomarkers of response and resistance, guiding the advancement of CAR-T therapies into the next generation. It can also monitor long-term outcomes and side effects, improving the effectiveness and security of future therapies (8) The synergism may foster collaboration between AI experts, immunologists, geneticists, and clinicians to integrate these technologies effectively. It also ensures that the combined use of AI, CAR-T, and CRISPR/Cas9 complies with regulatory standards and ethical guidelines.

Author contributions

Conceptualization, D.Z. and G.W.; methodology, J.C. and G.W.; validation, J.C. and D.Z.; formal analysis, J.C. and D.Z.; investigation, J.C. and D.Z.; resources, D.Z. and G.W.; data curation, J.C. and D.Z.; writing—original draft preparation, J.C. and G.W.; writing—review and editing, D.Z. and G.W.; visualization, D.Z.; supervision, G.W.; project administration, G.W.; funding acquisition, G.W.

Use of Generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest in this paper.

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