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Using CRISPR-Cas9 gene editing to engineer the next generation of CAR T cells
Využití genové editace CRISPR-Cas9 k vývoji nové generace CAR T buněk

Bachelor´s thesis

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V Prahe, 28.04.2024 Saskia Sabolová

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Abstract

Chimeric antigen receptor (CAR T) cell therapy is currently a successful treatment for hematological malignancies and is also a rapidly evolving field of research for treating solid tumors. The potential clinical expansion of this therapy depends on overcoming many obstacles, such as the persistence of CAR T cells in the hostile tumor microenvironment, induced toxicities, or the need for the transplant to be autologous. These limitations can be mitigated by CRISPR-Cas9 gene editing, which has the potential to create CAR T cells resistant to inhibition, modulate cytokine release, decrease the risk of cytokine release syndrome or neurotoxicity, and create allogeneic CAR T cells that do not cause graft-versus-host disease. Improvements in the CRISPR-Cas9 technology field, such as the development of base and prime editors, further increase safety by bypassing the dangerous double-strand break in the genome. Although many of these modifications are still subjects of research, there are a number of ongoing or already completed clinical trials that have implemented CRISPR-Cas9 technology in their CAR T cell engineering processes.

Key words: CAR T, CRISPR-Cas9, gene editing, solid tumors, inhibitory signals, toxicity, alloreactivity

Abstrakt

Chimeric antigen receptor T (CAR T) bunečná terapia je v súčasnosti úspešnou liečbou hematologických malignít a rýchlo sa rozvíja aj v oblasti výskumu liečby solídnych nádorov. Potenciálne klinické rozšírenie tejto terapie je závislé na prekonaní mnohých prekážok, ako je napríklad perzistencia CAR T buniek v mikroprostredí nádoru, spôsobená toxicita alebo potreba autológnej transplantácie. Tieto obmedzenia je možné znížiť CRISPR-Cas9 génovou editáciou, ktorá má potenciál vytvoriť bunky CAR T odolné voči inhibícii, modulovať uvoľňovanie cytokínov, znížiť riziko syndrómu uvoľňovania cytokínov alebo neurotoxicity a vytvoriť alogénne CAR T bunky, ktoré nespôsobujú ochorenie štepu proti hostiteľovi. Vylepšenia v oblasti technológie CRISPR-Cas9, ako napríklad vývoj base a prime editorov, ďalej zvyšujú bezpečnosť tým, že obchádzajú nebezpečný dvojvláknový zlom v genóme. Hoci mnohé z týchto úprav sú stále predmetom výskumu, existuje niekoľko prebiehajúcich alebo už dokončených klinických skúšok, ktoré implementovali technológiu CRISPR-Cas9 do svojich procesov pri výrobe CAR T buniek.

Kľúčové slová: CAR T, CRISPR-Cas9, genová editácia, solídne nádory, inhibičné signály, toxicita, aloreaktivita

Table of Contents

List of used abbreviations

A	adenine
BBIR	biotin-binding immunoreceptor
BCMA	B-cell maturation antigen
C	cytosine
CAR	chimeric antigen receptor
Cas9	CRISPR associated protein 9
CD	cluster of differentiation
crRNA	CRISPR RNA
CRISPR	clustered regularly interspaced short palindromic repeats
CRS	cytokine release syndrome
CTLA-4	cytotoxic T-lymphocyte antigen 4
dCas9	dead Cas9
DNA	deoxyribonucleic acid
FDA	food and drug administration
GM-CSF	granulocyte macrophage-colony stimulating factor
gRNA	guide RNA
HDR	homology-directed repair
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
ICANS	immune effector cell-associated neurotoxicity syndrome
IFN-γ	interferon gamma
IFNγR1	interferon gamma receptor 1
IL	interleukin
Indel	insertion and deletion
ITAM	immunoreceptor tyrosine-based activation motif
MHC	major histocompatibility complex
nCas9	Cas9 nickase
N	nucleotide
NHEJ	non-homologous end joining
NK cell	natural killer cell
PAM	protospacer adjacent motif
PD-1	programmed cell death protein 1
pegRNA	prime editing guide RNA
pre-crRNA	precursor CRISPR RNA
r/r	relapsed/refractory
RNA	ribonucleic acid
scFv	single-chain variable fragment
shRNA	short hairpin RNA
SUPRA CAR	split, universal, and programmable CAR
T	thymine
TAMs	tumor-associated macrophages
TCR	T cell receptor
TGF-β	transforming growth factor beta
TGFBR2	transforming growth factor beta receptor 2
Th17	T helper 17 cell
TME	tumor microenvironment
TNF-α	tumor necrosis factor alpha
TRAC	T cell receptor alpha constant
Tregs	regulatory T cells
TRUCKs	T cells redirected for universal cytokine-mediated killing
U	uracil
β2M	beta-2-microglobulin

1. Introduction

CAR T cells, a few years after their initial introduction, found their purpose in the treatment of HIV infection (Roberts et al., 1994). Currently, they are being researched and actively used for the treatment of several types of cancers. In hematological malignancies, where they target CD19 molecule or B-cell maturation antigen on the surface of B cells, CAR T cells have proven their efficiency (Zhao & Cao, 2019) and it is not surprising that their potential might extend beyond this application. Cancer has been the second most common cause of death after heart diseases in the United States, with solid cancers such as lung cancer, brain cancer, colon and prostate cancers taking the highest places in the ranking (Siegel et al., 2024). The use of CAR T cells for solid cancers comes with limitations that need to be overcome, before this treatment can be generally applicable (Marofi et al., 2021). For active enhancement of their function, many strategies have been developed that implement specific design concepts into these engineered cells, as seen in their evolution from the first generation. These include the addition of more co-stimulatory domains or the implementation of a transgene into their intracellular section, coding for a desired protein based on their target (L. Tang et al., 2023).

Several improvements are responsible for preventing CAR T cells from being inhibited by the patient's immune system (Ren, Liu, et al., 2017), regulating cytokine release (Zhang et al., 2022), preventing toxicity associated with this therapy and even enabling the therapy to be independent of autologous transplant (Stenger et al., 2020). All of these can potentially be achieved by implementing clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9 (CRISPR-Cas9) gene editing technology in their development process.

As CRISPR-Cas9 technology still carries many risks of undesired events related to its mechanism of action (Kosicki et al., 2018), base editors and prime editors, which bypass the introduction of dangerous double-strand breaks into the genome, are promising enhancements (Anzalone et al., 2019), further improving its safe and reliable use in CAR T cell development.

Currently, several active, ongoing or already completed clinical trials have implemented CRISPR-Cas9 gene editing in the therapeutic CAR T cell design, not only for the treatment of hematological malignancies but also for solid cancers, such as breast cancer or renal cell carcinoma (source: ClinicalTrials.gov).

1.1. Aim of the work

This thesis aims to provide the background of CAR T cell therapy as a cancer treatment, highlighting the design features and limitations currently existing in this field. The main focus is on the implementation of CRISPR-Cas9 technology in CAR T cell engineering. Its use is demonstrated by scientific research, supplemented by personal viewpoints and a critical perspective on the mentioned topics. The theoretical use of CRISPR-Cas9 is supported by an overview of selected clinical trials. Lastly, this thesis hopes to provide a future perspective on further development and enhancement of CAR T cells, with emphasis on the use of CRISPR-Cas9 technology in the process.

2. Fundamentals of CAR-T cell therapy

Chimeric antigen receptor T (CAR T) cells are created by ex-vivo modification of T cells, in which a gene coding for chimeric antigen receptor (CAR) is introduced (Eshhar et al., 1993). There are several generations of CAR T cells, with differences in their structure affecting their functions and resulting effects in the body. The further described mechanism of action also comes with unwanted side effects that might limit their use and still require further investigation, as they can lead to life-threatening states.

2.1. CAR structure and mechanism of action

A CAR generally consists of an antigen-recognizing and binding extracellular domain, most often represented by a single-chain variable fragment (scFv) derived from an antibody (Eshhar et al., 1993). This extracellular binding domain is connected through a hinge domain to a transmembrane domain. These structures play essential roles in signal transduction, where the hinge domain is crucial for regulating the signalling threshold, and the transmembrane domain contributes to the regulation of CAR signalling intensity by controlling the level of CAR expression on the cell surface (Fujiwara et al., 2020). The cytotoxicity is mediated by the intracellular domain, which consists of an immunoreceptor tyrosine-based activation motif (ITAM) – traditionally the CD3ζ chain (Bridgeman et al., 2014), along with a co-stimulatory receptor, such as 4-1BB (CD137), OX40 (CD134), CD28, ICOS (CD278), or CD40, each providing a specific characteristic to the CAR T cell (Weinkove et al., 2019). The CD3ζ domain, present in all currently used CAR-T products, has been tested against CD3δ, CD3ε, and CD3γ chains, which have shown to possess many beneficial features compared to CD3ζ, with CD3δ CARs showing the highest anti-tumor activity (Velasco Cárdenas et al., 2023). These domains should therefore be further investigated and potentially implemented in the next generation CAR constructs.

Compared to T cell receptors, which are able to recognize both surface and intracellular proteins, CARs are limited to antigens on the cell surface. This makes them less flexible but, on the other hand, more universal, as the antigen presentation is human leukocyte antigen (HLA)-independent (Sadelain et al., 2013).

Previous generations of CAR T cells, as shown in the picture below (Figure 1), differ in their intracellular signalling domains. First-generation CARs contained a single signalling

domain, typically the CD3ζ chain (Eshhar et al., 1993). In the second generation, an extra co-stimulatory domain was implemented, such as 4-1BB (CD137), which greatly improved effectiveness against leukemia (Imai et al., 2004), or the CD28 molecule, which is natural for T cell activation upon binding with its ligand molecule, B7 (Lenschow et al., 1996). The third generation is marked by adding another co-stimulatory domain, like OX40 (CD134), which provides further benefits such as reduced apoptosis and increased proliferation (Zhang et al., 2021). The combination of co-stimulatory domains provides the CAR T cell with additional positive features, but it is not always the case, as it has been shown that co-stimulation with CD28 and OX40 (CD134) led to terminal differentiation into CD56 positive cells, which easily undergo apoptosis and the anti-tumor efficacy was decreased compared to CD28 co-stimulation alone (Hombach et al., 2013). The fourth generation of CAR T cells, also known as T cells redirected for universal cytokine-mediated killing or TRUCKs, contain a transgene, often coding for cytokines such as IL-12 or IL-18, or other active molecules, such as enzymes or ligands, which can be chosen based on the target environment, for example, IL-18 producing TRUCKs targeting disialoganglioside positive cells (which are many solid cancers, for example childhood neuroblastoma or breast cancer) were highly successful (Glienke et al., 2022). Later generations of experimental split, universal, and programmable (SUPRA) CARs (Cho et al., 2018) and biotin-binding immune receptor (BBIR) CARs (Urbanska et al., 2012) differ in the extracellular domain with additional features, such as the ZipCAR containing a leucine zipper in SUPRA CARs (Cho et al., 2018). CRISPR-Cas9 system can potentially be used to integrate the transgene into the intracellular domain, as seen in the fourth-generation CARs, or in combination with features from other designs.

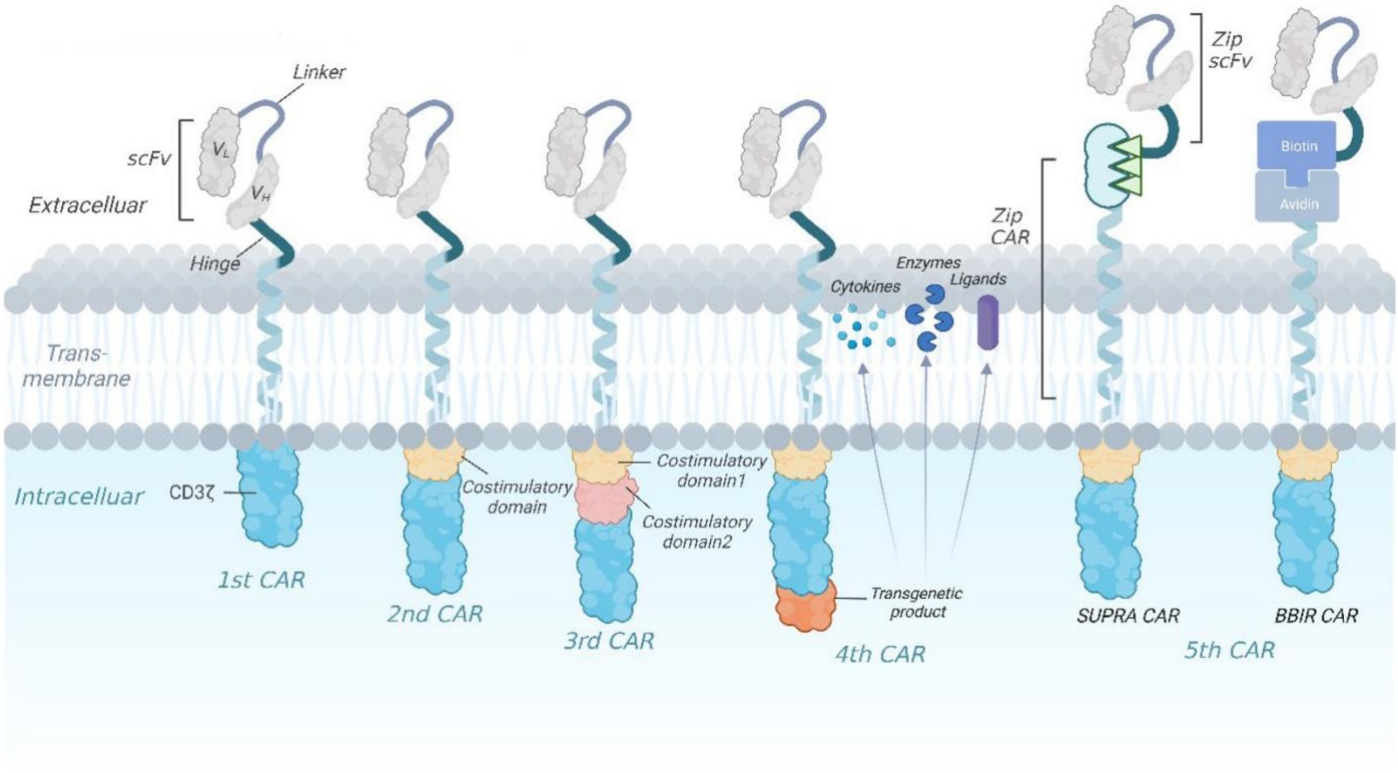

Figure 1: Different generations of CAR. Adapted and modified from Zheng et al., 2023.

2.2. Current use and limitations

In the current clinical state, B-cell malignant diseases are the primary target for CAR T cell therapy. While traditional treatments such as chemotherapy and surgery are still used for cancer patients, CAR T cells are employed in cases where these conventional treatments have not shown sufficient results and the cancer is considered relapsed or refractory (r/r) (Khan et al., 2024). To date, the FDA has approved the use of six CAR T therapy drugs, targeting CD19 for the treatment of acute lymphoblastic leukemia, non-Hodgkin lymphoma, follicular lymphoma, mantle cell lymphoma and B-cell maturation antigen (BCMA) in multiple myeloma patients (Denlinger et al., 2022). By the nature of their function, the development of new CAR T cells and the expansion of this therapy beyond the current diseases is highly dependent on the knowledge of tumor antigens.

For the treatment of solid tumors, this therapy faces many challenges, including the difficulty of trafficking CAR T cells to the tumor mass and infiltrating it, which is due to physical barriers as well as the fact that the cells have to navigate through the area in the

proximity of the tumor, the tumor micro-environment (TME), which is highly immunosuppressive. It provides inhibitory signals to the immune system through certain cytokines, myeloid-derived suppressor cells, or regulatory T cells (Tregs) that support tolerance (Wagner et al., 2020). In addition to cells, common signalling molecules found in the TME include interleukins such as IL-6 and IL-10, pro-inflammatory chemokines, tumor necrosis factor-alpha (TNF-α), and factors related to hypoxic conditions (Al-Akra et al., 2019). These above listed interleukins, together with interferon gamma (IFN-γ), are the main mediators of cytokine release syndrome (CRS), a common toxicity resulting from the vast expansion of activated T cells and can lead to life-threatening states that need to be immediately treated (Shimabukuro-Vornhagen et al., 2018). Therefore, it would be beneficial to prevent CRS by modifying the genes coding for these cytokines or their receptors in the design of CAR T cells.

Regarding the use of CAR T cell treatment for solid tumors, the potential success of the therapy highly depends on the rate of immune cell infiltration, which varies among different types of cancers. The table below (Tab. 1) provides a summarized overview of several types of solid cancers and descriptions of their infiltration by immune cells. The type of immune cell predominantly infiltrating the tumor is of high importance, as the therapy depends on the presence of cytotoxic CD8+ and helper CD4+ T cells and minimal immunosuppression mediated by Tregs and myeloid cells (W. Yang et al., 2024).

Tab. 1: Selected types of solid cancers and the description of their infiltration by T cells (W. Yang et al., 2024)

Type of solid cancer	T cell infiltration rate description
Clear cell renal cell carcinoma	High levels of CD8+ T cells
Non-small cell lung cancer	High T-cell presence, indicative of a strong immune response
Melanoma	Presence of both CD4+ and CD8+ T cells critical for antitumor activity, but Tregs inhibit effective immune response

Breast cancer	Significant T-cell presence despite traditionally being considered immunologically inactive, reflecting new insights into the TME
Ovarian cancer	Small T-cell presence with tumor-associated macrophages (TAMs) and Tregs dominating
Prostate cancer	Lower T-cell presence compared to benign conditions, reflecting enhanced immune suppression during cancer progression
Pancreatic cancer	Minimal cytotoxic T cell infiltration, with a dominance of immunosuppressive cells such as myeloid cells and macrophages
Colorectal cancer	Noticeably lower T-cell infiltration than in other solid tumors like renal clear cell, thyroid, and lung adenocarcinomas
Gastric cancer	Low T-cell infiltration with strong immunosuppressive environment

Another problem that occurs is neurotoxicity (immune effector cell-associated neurotoxicity syndrome, (ICANS), which is closely connected with CRS. This happens when the blood-brain barrier mistakenly allows cytokines and CAR T cells to enter the brain. Corticosteroids and IL-6 inhibitors are components used to manage ICANS (Gust et al., 2018). This suggests that the modification of IL-6 and other pro-inflammatory cytokine genes could modulate and decrease these toxicities.

3. CRISPR-Cas9 as a gene editing tool

CRISPR-Cas9 technology, adapted from bacterial and archaeal natural immune defence mechanism (Bhaya et al., 2011), has shown great improvements since its first demonstration as a genome editing tool in 2012 (Jinek et al., 2012). Since then, it has been restricted for use only in vitro and it was 8 years later, in 2020, when CRISPR-Cas9 was first used directly in a human body, specifically to treat Leber's congenital amaurosis 10, a rare hereditary disease of the retina (Ledford, 2020). Although its use still carries some risks of off-target effects and unexpected actions (Yan et al., 2020), further research into predicting these unsolicited events and altering its function can increase both the safety and the spectrum of its applications.

3.1. Mechanism of action

The technique of its action is originally based on the induction of a double-strand break in the genome by the CRISPR RNA (crRNA)-guided Cas9 endonuclease, which consists of two domains – RuvC and HNH. These two active sites are responsible for nicking the opposite DNA strands (Gasiunas et al., 2012). In the bacterial immunity, CRISPR array contains and stores the sequences from which a precursor crRNA (pre-crRNA) is transcribed. This pre-crRNA is enzymatically modified, which leads to a generation of crRNA. The crRNA is complementary to the nucleotide sequence called protospacer. Another component of this system is the trans-activating CRISPR RNA (tracrRNA), which is crucial for RNAse III mediated processing of the pre-crRNA (resulting in crRNA formation) and the cleaving ability of the Cas9 protein. For the purposes of gene editing, the tracrRNA and the crRNA are combined, together creating a structure called guide RNA (gRNA). This gRNA is responsible for specific sequence recognition. The recognition of the complementary nucleotide sequence is not enough for the Cas9 to act, it requires a sequence called protospacer-adjacent motif (PAM). For the typically used Cas9 variant purified from *Streptococcus pyogenes*, the PAM is very specific; it is found right next to the 3' end of the gRNA-recognised DNA sequence and its nucleotide sequence composition is NGG (any nucleotide, guanine, guanine). The PAM sequence is necessary for the engineered CRISPR-Cas9 as well as for the natural bacterial immunity mediated by CRISPR-Cas9 system, where its role consists of recognising the difference between the target sequence in CRISPR array (not being followed by PAM, therefore not cut by Cas9) and the invading organism containing the PAM that would be subsequently cleaved. The strand that is

complementary to the gRNA (crRNA) is cleaved three base pairs in the upstream direction of the PAM sequence by the HNH domain of the Cas9 protein. The other DNA strand (non-complementary strand) is cleaved at one or even more sites. These sites are located within the range of three to eight base pairs in the upstream direction of the PAM sequence and cleaved by the RuvC domain, first being acted on endonucleatically and then exonucleatically (Jinek et al., 2012).

After the introduction of a double-strand break (induced by the two endonuclease domains), the gap in the genome can be repaired either homologously or non-homologously (Haber, 2000). Non-homologous end joining (NHEJ) has been shown to be faster and is clearly favored by the cell compared to homologous recombination, with the occurrence ratio of 9:1, respectively (Mao et al., 2008). Since NHEJ generally causes random insertions and deletions (indels) (Bennett et al., 2021), homology-directed repair (HDR) is preferred when performing controlled gene editing, and several studies are exploring methods that lead to a higher occurrence of homologous repair (H. Yang et al., 2020). One such method capable of increasing the rate of HDR in the cell involves timing, as HDR is typical for the S phase (later stage) and G2 phase of the cell cycle. The timing of the delivery of Cas9 protein to the cell has shown a significant impact on HDR occurrence, increasing it to up to 33% (Lin et al., 2014).

As NHEJ causes mentioned indels, the result often causes the gene to be deactivated and so can be useful for performing a gene knock-out. A knock-out though can also be achieved by HDR, for example by providing a donor sequence, that will insert a stop codon to the targeted locus (Mali et al., 2013).

A study conducted on embryonic stem cells shows the unpredictability of reparations after a CRISPR-Cas9 induced double-strand break, revealing large alterations to the genome, which can result in serious pathological states. These findings raise concerns for the use of CRISPR-Cas9 in gene therapy, as the observed damage significantly challenges its safety and need to be addressed by further research and improvement (Kosicki et al., 2018).

3.2. Enhanced CRISPR-Cas9 strategy to avoid double-strand break

As previously described, the action of CRISPR-Cas9 technology can lead to indels and undesirable effects on the genome. To overcome this obstacle, base editors and prime editors were engineered (Anzalone et al., 2019). Their mechanism of action and differences from classic CRISPR-Cas9 will be described in the following subchapters.

3.2.1. Base editing

Base editing, first introduced and described by David R. Liu and Alexis C. Komor in 2016 , uses a version of Cas9 that has been modified by introducing D10A (substitution of aspartic acid at position 10 with alanine) and H840A (substitution of histidine at position 840 with alanine) mutations (the effects of these mutations are described in the subchapter 3.2.2, in Tab. 2), rendering it catalytically dead (dCas9). This modification causes it to lose its nuclease ability but retain its DNA-binding property. This dCas9 was fused with a cytidine deaminase, capable of converting cytosine (C) to uracil (U), which pairs like thymine (T) during DNA replication, resulting in a C-G to T-A pairing conversion. Together with gRNA, it can target a specific DNA sequence and modify it without introducing a double-strand break. However, this first generation of base editors did not show promising results for human cells. The second generation incorporated uracil DNA glycosylase inhibitor to prevent the cell's natural repair processes. In the third generation of base editors, the activity of the HNH domain was restored, creating a nickase enzyme—nCas9—that cuts a single strand of DNA (Komor et al., 2016).

In 2017, base editors utilizing adenine deaminase were introduced. The deamination of adenosine results in inosine being present in the sequence, which pairs with cytidine. During reading and replication, guanosine is introduced and paired with cytidine, thus the overall process creates an A-T to G-C conversion in the genome (Gaudelli et al., 2017).

In CAR T cell development and enhancement, base editors can be used for various purposes with the benefit of not inducing a double-strand break, thereby avoiding the risk of chromosomal translocations and other safety concerns associated with the original CRISPR-Cas9 technology. The use of base editors includes knocking-out inhibitory or other genes by introducing stop codons, modulating splicing sites, converting single bases, targeting enhancers, and many other possible gene editing applications (Lahr et al., 2023). In comparison to edits made using classical CRISPR-Cas9, cytosine base editors have shown more precise gene editing abilities, fewer off-target effect risks caused by acting on the expression levels of genes adjacent to the target site, all of which demonstrate safer and more controllable manipulation using base editing (Dang et al., 2020).

3.2.2. Prime editing

Similar to base editors, prime editors use a catalytically impaired Cas9 endonuclease—a nickase—that does not create breaks in both DNA strands. Instead, the modification in the Cas9 used in this process ensures that only one strand will be disrupted. This is achieved by introducing specific mutations with two effects on the Cas9 protein, as shown in the table below (Tab. 2). The nickase is combined with a reverse transcriptase, which synthesizes DNA from an RNA template. Together with the binding spacer sequence, it forms part of the prime editor guide RNA (pegRNA) complex (Anzalone et al., 2019). This system allows for making substitutions, deletions, and insertions without the disadvantages of classical CRISPR-Cas9 and also offers the benefit of higher predictability of the result, which increases its application safety (Kim et al., 2021).

Tab. 2: Mutations of the Cas9 enzyme and their effects (Anzalone et al., 2019)

Mutation	RuvC domain	HNH domain	Enzyme cuts
D10A	inactivated	active	the gRNA complementary strand (target strand)
H840A	active	inactivated	the gRNA non-complementary strand (non-target strand)

This method further expands the possibility of reliable and predictable gene editing in the development of the next generations of CAR T cells. The further described modifications for improved function of these engineered cells potentially can be achieved by using prime editors, gaining the benefit of bypassing the double-strand break or limitations of alteration range. The picture below (Figure 2) summarizes the key differences between classical CRISPR-Cas9, base editors, and prime editors.

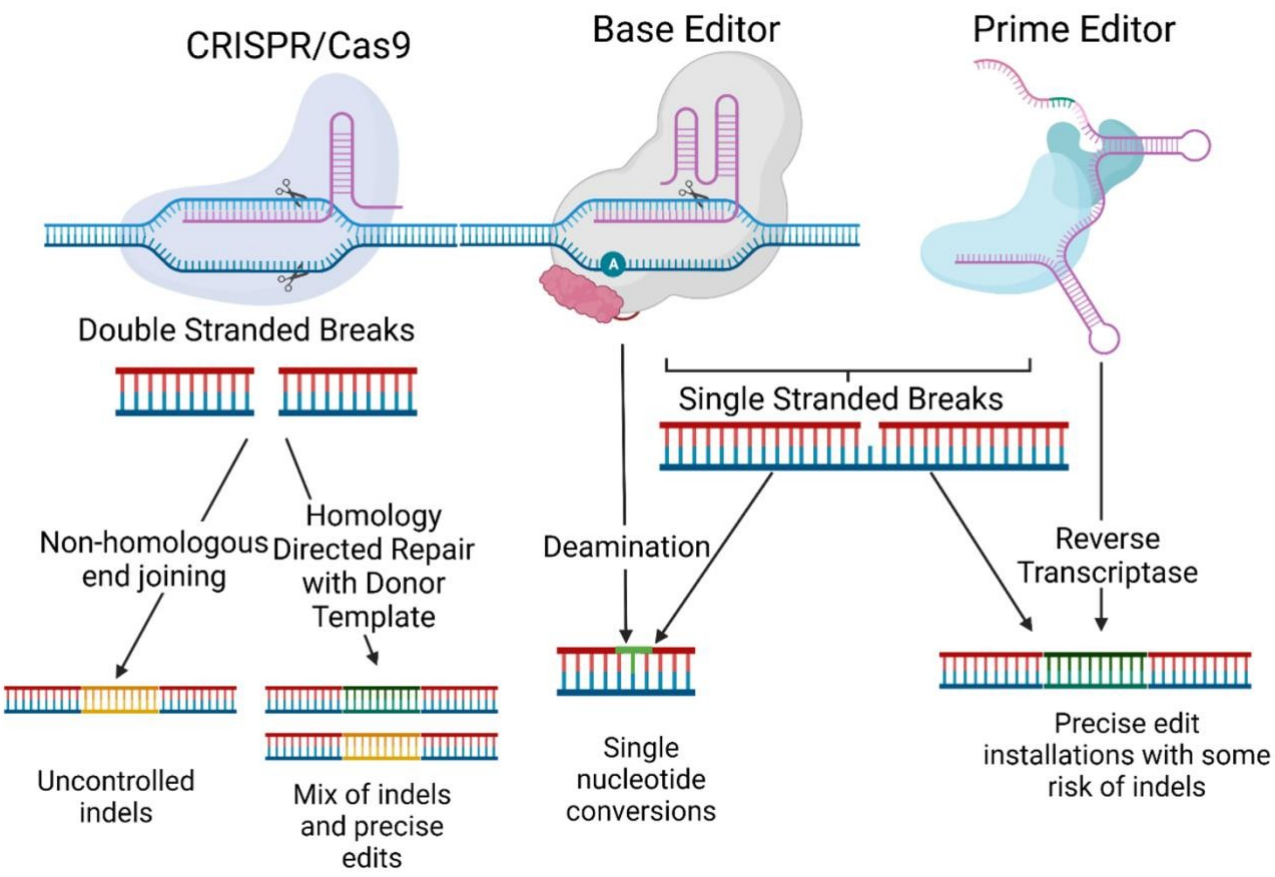

Figure 2: Differences in function among CRISPR-Cas9, base editors, and prime editors. Adapted from Brooks et al., 2023.

4. CRISPR-Cas9 in CAR-T cell engineering

Gene editing, by CRISPR-Cas9 and its adaptations such as base and prime editing, can be used in CAR T cell development for many purposes, such as decreasing inhibition, modulating cytokine release in CRS, or creating allogeneic "off-the-shelf" products, as described in the following subchapters.

4.1. Disruption of inhibitory signals

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) receptors are present on monocytes (X. B. Wang et al., 2002) and various types of solid tumor cells (Contardi et al., 2005). Programmed cell death-1 (PD-1) receptors are found on activated T lymphocytes, B lymphocytes and myeloid cells (Freeman et al., 2000). They both play a crucial role in mediating T cell inhibition (Chemnitz et al., 2004). For that reason, they have been targets for cancer therapy for many years, showing promising results, as demonstrated when these receptors were blocked using the monoclonal antibodies Nivolumab and Ipilimumab in patients with melanoma (Rotte, 2019).

The effectiveness of blocking these inhibitory signalling pathways has also been demonstrated through RNA interference. Specifically, one study conducted by Condomines et al. in 2015 has put into comparison CAR T cells with intracellular CD3ζ chain, CD28 co-stimulation and anti-CD19 CAR against CAR T cells also expressing CD3ζ chain, but in these case, the co-stimulatory domain was CD80 – a CTLA-4 ligand. They revealed that CARs without the CD80 co-stimaulation had increased tumor responses and the regression in mice was much higher. They then suppressed the activity of the CTLA-4 protein with anti-CTLA-4 short hairpin RNA (shRNA) and the CAR T cells showed different anti-tumor capabilities based on their design, where the enhancement in anti-tumor activity and expansion was predominantly observed in CAR-T cells expressing the CD3ζ chain with CD80 co-stimulation and did not affect the CD28 co-stimulated CAR T cells. The results show that the cells expressing CD80 were inhibited by binding to the CTLA-4 after their activation. (Condomines et al., 2015). The downregulation of inhibitory pathways has been researched in the treatment of solid cancers such as melanoma, where the environmental suppression of immune processes is a major limitation. By using RNA interference, the suppression of CTLA-4 and PD-1 results in a positive increase in the cytotoxicity and secretion of cytokines of these engineered CAR-T cells (Simon et al., 2018).

Targeting the PD1 pathway has shown promising results in enhancing the activity of CAR T cells against solid tumors. In a study by Liu et al., PD1CD28 engineered cells had a design feature that changed the inhibitory signal upon surface PD-1 activation to a stimulatory one. They fused the PD-1 extracellular domain with the cytoplasmic and transmembrane portions of CD28, creating the PD1CD28 receptor. These CAR T cells showed a higher rate of tumor infiltration and overall effectiveness compared to either classical CAR T cells alone or in combination with PD1-blocking antibodies (Liu et al., 2016). This suggests that genetic modification of CAR T cells to modulate inhibitory pathways might be more effective than blocking these pathways with antibodies. In 2017, it was demonstrated that CAR T cells with three simultaneous, CRISPR-Cas9 induced knock-outs of the T cell receptor (TCR), beta-2-microglobulin (β2M), and PD-1 genes showed enhanced responses against tumors, particularly against those markedly expressing PD-1 ligand (PD-L1). Among other beneficial features these cells gained from the other two modifications, this fact provides proof that the specific enhancement was the result of disruption in the PD-1/PD-L1 inhibitory pathway (Ren, Liu, et al., 2017). Enhancement in effectiveness against tumors, specifically increased degranulation and cytolytic activity in vitro and improved tumor clearance in vivo was also found and credited to this pathway disruption by CRISPR-Cas9 (Rupp et al., 2017). The knock-down or knock-out of CTLA-4 by CRISPR-Cas9 could also be a promising target, as this receptor is also present on T cells and contributes to their inhibition (Ren, Zhang, et al., 2017). By CRISPR-Cas9 induced knocking-out of CTLA-4 on cytotoxic T lymphocytes (CTL), these cells showed increased cytotoxicity against colon cancer cells, they promoted apoptosis of cancer cell line and had increased production of TNF-α and IFN-γ (Shi et al., 2017).

Alongside the CTLA-4 and PD-1 knock-outs, the deactivation of TGF-β receptor II (TGFBR2) has also been investigated (N. Tang et al., 2020). Transforming growth factor-beta (TGF-β) plays a dual role in tumor dynamics by suppressing immune responses, particularly by inhibiting the activity and proliferation of CTLs, which are crucial for eliminating tumor cells and also influencing the differentiation and function of other immune cells, such as by generating Tregs and causing induction of Th17 cells (L. Yang et al., 2010). This finding suggests, that modulating the TGF-β release or its binding to respective receptor could increase cytotoxic activity of CAR T cells and prevent more inhibition and tolerance through mentioned generation of Tregs that TGF-β promotes. Tang et al. investigated the results of knocking-out TGFBR2 in CAR-T cells using CRISPR-Cas9 and found, that it enhances their tumor-fighting capabilities, both in vitro and in vivo, by improving tumor lysis, cytokine release, and survival within the TME, all of which are crucial for their success. This modification showed to prevent

the conversion of CAR T cells into a less effective regulatory phenotype, which normally occurs under the influence of TGF-β1 (also binds and signals through TGFBR2), maintain their effector functions against repeated tumor challenges, and increase the proportion of memory T cells, suggesting a potential for lasting antitumor immunity (N. Tang et al., 2020). Consequently, the results suggest that TGFBR2 knock-out presents a promising strategy to overcome the immunosuppressive effects of TGF-β in cancer treatment.

The CRISPR-Cas9 knock-out of these genes can be performed by various methods; for example, introducing a premature stop codon using base editors, bypassing the risky double-strand break, allows for better regulation of the editing outcome (Kuscu et al., 2017).

4.2. Toxicity reduction

Although CAR T cell therapy holds significant positive impact, it has been linked to many unwanted and dangerous toxic events (Bonifant et al., 2016). These toxicities are mainly CRS and neurologic toxicity, with the risk being significantly higher in patients with more severe cancer (Brudno & Kochenderfer, 2019).

CRS is characterized by the secretion of a variety of cytokines, notably TNF-α, IL-1, IL-6, and IFN-γ (Elsawa et al., 2011), as well as IL-10 (R. Wang et al., 2011), which mediate the clinical symptoms. It causes multiple life-threatening complications, such as high fever and organ failure (X. Li et al., 2021). These molecules and their receptors could therefore be further researched for knock-down induced by CRISPR-Cas9. The effectiveness of reducing key CRS mediators has been demonstrated by the simultaneous knock-down of IL-6 and IFN-γ in the CAR T cells, which surprisingly reduced the production of other cytokines such as IL-2, TNF-α, and IL-10 as well, without compromising the cancer cell-killing ability but with a reduction in CRS toxicity (Zhang et al., 2022).

Although diminishing the IFN-γ pathway might seem like a promising solution against CRS, it still needs further research to find the balance for the ideal outcome when it comes to CAR T cell design and its effect in the body. A study focusing on the role of the IFN-γ receptor 1 (IFNγR1) showed that a knock-out of this receptor in the cells of glioblastoma—a type of solid tumor—led to a reduction in critical features such as adhesion and binding for CAR-T cells (Larson et al., 2022) which demonstrates the importance of this pathway in the treatment of solid tumors. It has also been shown that CD4 positive CAR T cells are capable of remote

targeting and elimination of tumor cells, with IFN-γ being the mediator of this distant killing ability (Boulch et al., 2023). IFN-γ is also abundantly present in the TME, from which it signals for cytotoxic action to the CAR T cells (mediated mainly by CD8 positive CAR T cells) and promotes increased major histocompatibility complex I (MHC I) expression on tumor cells, which plays an important role in immune recognition (Boulch et al., 2021).

Besides the above-described cytokines, editing of the gene coding for granulocyte-macrophage colony-stimulating factor (GM-CSF) resulting in the knock-out of this gene by CRISPR-Cas9 in a study focusing on the management of toxicity of CAR T cells has shown that these cells have reduced secretion of this pro-inflammatory cytokine and increased control over tumor growth in patients with severe cancer (Sterner et al., 2019).

Toxicity reduction and prevention of CRS and ICANS still require a lot of insightful research, as cytotoxic actions themselves are key events in tumor eradication. The cytokines that are tightly connected to CRS have other roles as well in the fight against cancer cells (as demonstrated by IFN-γ), some of which might be beneficial, and therefore it is first important to thoroughly investigate their effects outside of CRS and then find a balance to prevent toxicity-related events but maintain the effective target-killing ability.

4.3. Alloreactivity reduction

The need for CAR T cells to be autologous deprives patients of the benefits that allogeneic donors offer, as cells collected from a healthy individual could help with many current problems, such as low lymphocyte yield or impaired function of T cells from patients (Mehta et al., 2021). Graft-versus-host disease limits the use of allogeneic cells and is caused by the T cells isolated from the donor containing their native endogenous TCR, which recognizes the tissues of the recipient patient as foreign, resulting in an immune attack (Sanber et al., 2021). This finding brings the opportunity for TCR disruption to be a tool for reducing alloreactivity and enabling the allogeneic transplant of CAR T cells.

A CRISPR-Cas9 mediated knock-out of genes coding for the TCRβ chain, necessary for the functional assembly of the TCR, was investigated by Stenger et al., showing that these cells were capable of robust proliferation, activation, and activity against leukemia, along with a significant reduction in alloreactivity. Despite the benefits that TCR-knocked-out CAR T cells provide, the study also showed that co-expressing the endogenous TCR with the CD19 molecule has a critical, positive effect on the longevity of these cells and control over leukemia

in vivo (Stenger et al., 2020). Disruption of the TCR has also been performed by placing the CD19 CAR gene into the T cell receptor α constant (TRAC) locus using CRISPR-Cas9, resulting in many beneficial features these cells gained in comparison to generic CAR T cells, such as uniformity in CAR expression across all engineered cells, effector T cell differentiation and exhaustion delay, and importantly, precise control over the insertion of the CAR gene into the TRAC locus using CRISPR-Cas9 instead of a γ-retroviral vector, which led to TCR function loss and decreased risk of alloreactivity (Eyquem et al., 2017).

Another possible approach to reducing alloreactivity is by targeting the HLA molecules. Knocking-out HLA-I and HLA-II and introducing HLA-E gene expression, together with elimination of the TCR, showed to produce cells resistant to natural killer (NK) cell rejection and therefore increase the safety regarding alloreactivity (W. Li et al., 2022). Also, by eliminating β2M, a crucial component of HLA-I proteins, it is possible to significantly prevent the rapid destruction of allogeneic CAR T cells that display foreign HLA-I molecules (Razeghian et al., 2021).

5. Clinical studies

Below (Tab. 3) is an overview of selected clinical trials using CRISPR-Cas9 technology in CAR T cell engineering. The data have been retrieved from ClinicalTrials.gov, with a respective registration number provided in the table for access to detailed information.

Tab. 3: Overview of selected clinical trials, their respective ID number and description or therapeutic CAR T cell design. Source: clinicaltrials.gov.

Clinical trial registration number (ID)	Targeted diseases	Status	CAR T design
NCT04502446	r/r T cell lymphoma and B cell malignancies	Active, phase 1	Anti-CD70 allogeneic CRISPR-Cas9-engineered T cells
NCT04244656	r/r multiple myeloma	Active, phase 1	Anti-BCMA allogeneic CRISPR-Cas9-engineered T cells
NCT04438083	Advanced, r/r renal cell carcinoma	Active, phase 1	Anti-CD70 allogeneic CRISPR-Cas9-engineered T cells
NCT04557436	r/r B cell acute lymphoblastic leukemia	Completed	CRISPR-Cas9 TCR α chain knock-out allogeneic anti-CD19 CAR T cells
NCT05812326	Advanced breast cancer	Completed	CRISPR-Cas9 PD-1 knock-out anti-MUC1 CAR T cells
NCT04035434	r/r B cell malignancies	Active, phase 2	Anti-CD19 allogeneic CRISPR-Cas9-engineered T cells

In the first mentioned clinical trial that has been completed (ID: NCT04557436), six children with r/r acute lymphoblastic leukemia were treated with allogeneic CAR T cells, that had the genes for α chain of the TCR and CD52 disrupted using CRISPR-Cas9 to prevent them from causing graft-versus-host disease. Four treated children experienced significant remission after successful expansion of CAR T cells, two of the six patients were treated for moderate CRS and one developed grade four neurotoxic complications. Overall, the study was successful, demonstrating the expected side effects and proven safety and efficacy of the engineered cells used in the trial, except for one of the patients, that developed graft-versus-host disease on the skin, which was eventually resolved (Ottaviano et al., 2022).

The second mentioned completed trial (ID: NCT05812326), has not, to this date, published the results of this clinical trial.

6. Conclusion and future prospects

With cancer being the second leading cause of death in the United States, it is crucial to continue developing new drugs and therapies to increase the survival rates of patients. Where traditional treatments fail, CAR T-cell therapy emerges as a possible solution. These engineered therapeutic cells hold significant potential for treating malignant diseases due to their versatility. Although FDA approval has so far been granted only to CAR products targeting CD19 and BCMA for hematological cancers, their efficacy against solid tumors is being thoroughly researched, with active investigations into overcoming limitations such as inhibitory signals in the TME.

Supported by current research, I have emphasized how CRISPR-Cas9 technology provides an incredibly useful tool for modifying CAR T cells to enhance their tumor-killing ability, persistence, and to reduce the risk of apoptosis or graft-versus-host disease in allogeneic transplants. The high precision of CAR gene insertion, for example in the TRAC locus, provides the cells with beneficial features regarding alloreactivity and consistent levels of CAR expression across the engineered cells. CRISPR-Cas9 has also proven effective in generating cells resistant to PD-1/PD-L1 inhibition and in disrupting CTLA-4 or TGF-β receptors in CAR T cells, all aimed at fighting immunosuppression in the TME. Knock-outs of IL-6, β2M, or IFN-γ have also been described, as these cytokines are primary components of CRS. However, eliminating IFN-γ (and possibly other cytokines) signalling might not result in solely positive effects. To make CAR T cells more readily available by decreasing the time between collection and infusion into the same patient, and to circumvent issues such as low lymphocyte numbers or decreased fitness of the patients' T cells, allogeneic transplants could be possible by creating CAR T cells with knock-outs in the TCR alpha or beta chain, HLA, or B2M.

For future research, other cytokines or their receptors involved in toxicity related to CAR T-cell therapy should be explored for disruption. Conversely, CRISPR-Cas9, and possibly prime editors, could be used for controlled insertions of genes coding for certain cytokines that are pro-inflammatory or anti-inflammatory; however, their effects will need to be thoroughly investigated. Another possible enhancement is the spatiotemporal control of CAR T cells, for example by placing the CAR gene under a specific promoter, depending on the conditions in which they are supposed to act, to prevent off-target effects. We have seen similar control over expression depending on the space (providing specific signals) in which CAR T cells operate in SynNotch CARs (Morsut et al., 2016). Given the numerous components their function relies

on, I believe we will see significant improvements in the actions of CAR T cells in future generations, also leveraging the benefits that CRISPR-Cas9 offers.

7. List of references

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