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Dominika Berdecka[a,b], Stefaan C. De Smedt[a], Winnok H. De Vos[b,#] and Kevin Braeckmans [a,#,*]

[a] Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium

[b] Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

[#]Shared senior authorship

*Correspondence: Kevin.Braeckmans@ugent.be; Tel.: +32-9-2648098; Fax: +32-9-2648189

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Abstract

Adoptive T cell transfer has demonstrated remarkable clinical success in the treatment of hematological malignancies, leading to a growing list of FDA-approved chimeric antigen receptor (CAR)-engineered T cell therapies. However, the therapeutic efficacy for solid tumors remains unsatisfactory, highlighting the need for refined T cell engineering strategies and combinatorial approaches. To date, CAR T cell manufacturing relies primarily on gammaretroviral and lentiviral vectors owing to their high transduction efficiency. However, their use is associated with safety concerns, high cost of cGMP-compliant production, regulatory hurdles and restricted cargo capacity, hindering broader application of engineered T cell therapies. To overcome these limitations, non-viral approaches, including membrane permeabilization and carrier-mediated methods, have been investigated as a more versatile and sustainable alternative for next-generation T cell engineering. Non-viral delivery methods can be designed to deliver a broad range of payload molecules, including RNA which enables more controlled and safe modulation of T cell phenotype and functionality. In this review, we provide an overview of non-viral delivery of RNA in adoptive T cell therapy. We first define the different classes of RNA therapeutics, highlighting manufacturing advancements towards their therapeutic application, after which we discuss the challenges to achieve effective RNA delivery in T cells. Next, we provide an overview of current and emerging delivery technologies for RNA transfection of T cells. Finally, we discuss ongoing preclinical and clinical investigations with RNA-modified T cells.

1 Introduction

1.1 Introduction to adoptive T cell therapy

Cancer is a complex disease characterized by the uncontrolled growth of malignant cells that have the potential to invade neighboring tissues or spread to distant sites in the body. With an estimated 19.3 million new cases in 2020, cancer ranks second among the leading causes of death worldwide, accounting annually for 10 million, or one in six, deaths [1]. Despite the tremendous progress in the field of cancer biology, the genetic and phenotypic diversity of the disease often underlies its resistance to treatment. While conventional treatment strategies, such as surgery, radiation, chemotherapy and targeted therapy have proven highly beneficial in managing primary tumors, treating metastatic or relapsed/refractory (r/r) cancers remains a significant challenge. Over the past years, immunotherapy has instigated a revolution in oncology by exploiting the inherent ability of the immune system to recognize and destroy cancer cells, and has become the fifth pillar of cancer treatment[2,3]. Several approaches to unleash natural defense responses against immune-evasive cancer cells have been exploited, including cytokine therapies, immune checkpoint inhibition, cancer vaccination and adoptive cell transfer [2,4]. In particular, antibody therapies targeting immune checkpoints such as programmed cell death protein 1 (PD-1) and its ligand (PD-L1) or cytotoxic T lymphocyte associated protein 4 (CTLA-4) have proven an effective strategy to overcome peripheral tolerance by removing the breaks on T cell activation and enhancing antigen-specific responses [5].

Given the central role of T lymphocytes in tumor antigen recognition and cell-mediated immunity, adoptive T cell transfer has emerged as an alternative treatment modality [6]. Three main adoptive T cell therapy types can be distinguished: tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR)-engineered T cells and chimeric antigen receptor (CAR)-engineered T cells [7–9]. In TIL treatment, lymphocytes that have infiltrated tumor tissue are isolated from a cancer biopsy, expanded *in vitro* and re-infused into the patient in high numbers [10]. Despite initial promising outcomes in patients with metastatic melanoma, TIL therapy has been limited by difficulties with cell isolation, insufficient expansion of cells, and modest antitumor effects due to the scarcity of tumor-reactive T cells and their limited persistence *in vivo* [11–13]. Consequently,

the focus has shifted to genetically engineered approaches, where peripheral blood lymphocytes are first isolated from blood samples in a process called leukapheresis and then reprogrammed *ex vivo* to effectively target cancer cells (**Figure 1**). Besides redirecting T cell specificity by expressing tumor antigen-specific receptors, T cells can be additionally engineered to enhance their antitumor efficacy and improve their safety for potential use in allogeneic applications. Next, the engineered T cells are expanded to achieve therapeutically required doses, while the patient undergoes a lymphodepleting chemotherapy, which eliminates endogenous T cells and increases systemic levels of T cell-stimulating cytokines, augmenting the *in vivo* expansion of subsequently transferred lymphocytes [14–16].

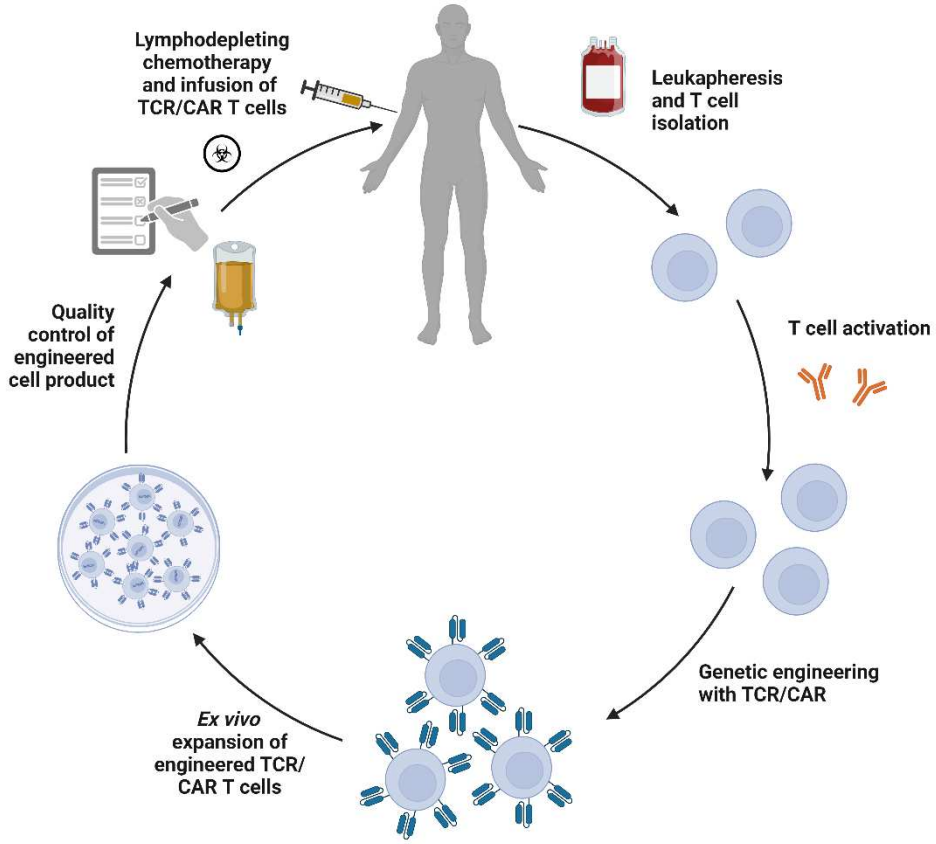


Figure 1. Schematic overview of autologous adoptive T cell therapy. Leukocytes are isolated from the patient's blood via leukapheresis and activated with anti-CD3/CD28 antibodies. Next, T cells are virally or

non-virally engineered to express, *e.g.* a T cell receptor (TCR) or chimeric antigen receptor (CAR). Modified cells are then expanded to therapeutic T cell doses and undergo a quality control. Finally, the patient receives lymphodepleting chemotherapy before being infused with an engineered T cell product.

T cell receptors are heterodimers composed of α and β chains that recognize antigens presented by the major histocompatibility complex (MHC) and subsequently associate with CD3 subunits to form a functional CD3-TCR complex and initiate T cell activation. In engineered T cell therapy, antigen-binding domains of TCR α and β chains are modified to redirect T cell specificity toward an antigen of interest (**Figure 2**) [9,17]. The repertoire of targetable antigens includes peptides derived from both intracellular and membrane proteins presented by human leukocyte antigen (HLA) class I and class II, respectively. However, since HLA encoding genes are the most polymorphic in the human genome, MHC-matching can be extremely complex and restrict the number of patients who can benefit from a given TCR-engineered T cell therapy [18]. Another challenge is α/β chain mispairing between transgenic and endogenous TCR chains, leading to nonfunctional complexes or the generation of new TCRs with autoimmune specificity [19,20]. In addition, competition with mispaired and endogenous TCRs for association with a limited amount of CD3 components may further reduce the expression of engineered TCRs [21]. Despite their ability to target both intracellular and surface antigens, the number of targets for TCR T cell therapy identified with sufficient safety and efficacy remains limited [9,22]. Most clinical trials to date have evaluated cancer-testis antigens, with New York esophageal squamous cell carcinoma 1 (NY-ESO1)-targeted T cells demonstrating objective clinical responses in patients with refractory melanoma, synovial cell sarcoma and multiple myeloma [23–25].

To overcome limitations imposed by the HLA-restriction of TCRs, synthetic CARs have been designed to direct T cell specificity to virtually any target on the surface of malignant cells independently of the MHC presentation The CAR structure has a modular design consisting of an antigen-binding domain (most often a single-chain variable fragment derived from a monoclonal antibody, scFv), hinge, transmembrane domain and intracellular signaling domain (**Figure 2**). The first generation of CAR T cells comprised an extracellular antibody scFv coupled to a CD3 ζ-signaling domain [26–28]. However, this design proved ineffective in clinical trials due to limited T cell proliferation and cytokine production [29]. This led to the incorporation of one or multiple

costimulatory molecules such as CD28, 4-1BB (CD137) or OX40 (CD134) in the second and third generation CARs, respectively, providing additional signals necessary for T cell activation [30–34]. Subsequent generations of CAR T cells feature further modifications aimed at improved anti-tumor efficacy. For instance, fourth generation (TRUCKs or armored CARs) have been engineered to release proinflammatory cytokines such as IL-12 upon CAR engagement in tumor lesions for modulating the immunosuppressive tumor microenvironment [35]. The fifth generation construct incorporated truncated cytoplasmic IL-2 receptor domain and STAT-3 binding moiety to promote activation-dependent JAK-STAT signaling and enhance cell proliferation [36].

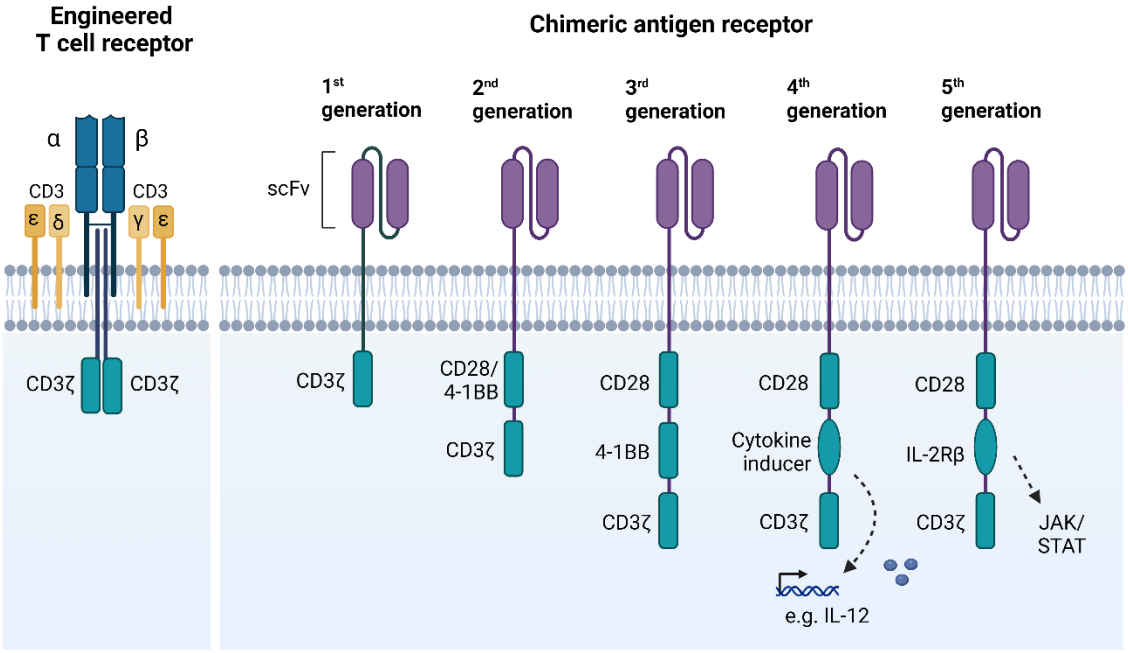


Figure 2. Schematic illustration of an engineered T cell receptor and the evolving designs of chimeric antigen receptors. The TCR complex comprises paired α and β chains which recognize antigens loaded on MHC molecules, and CD3 γ, δ, ε and ζ signaling modules. Upon peptide-MHC binding, phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3ζ chains propagates downstream signaling for T cell activation. CAR consists of an extracellular antigen-binding domain (scFv), a hinge, a transmembrane domain and cytoplasmic signaling domain. In early CAR design, the scFv domain was fused with a single CD3ζ signaling domain only. In the second and third generation CARs, one or two costimulatory domains (*e.g.,* CD28, 4-1BB) were incorporated. The fourth and fifth generation CARs are based on second generation constructs, but additionally contain an inducible cytokine cassette or IL-2 receptor β chain (IL-2R β) fragment for JAK/STAT pathway activation, respectively. scFV- single chain variable fragment.

At the time of writing, six CAR T cell therapies have been approved by the US Food and Drug Administration (FDA), all of which are based on the second generation CAR design. The first CAR T cell product was tisagenlecleucel (Kymriah®, Novartis), approved by the FDA in August 2017 for the treatment of r/r B cell acute lymphoblastic leukemia (ALL) [37]. Later that year Kite/Gilead received FDA approval for axicabtagene ciloleuce (Yescarta®) to treat diffuse B cell lymphoma [38]. These were followed by two more CD19-specific CAR T cells, namely brexucabtagene autoleucel (Tecartus®, Kite/Gilead) [39,40] and lisocabtagene maraleucel (Breyanzi®, Bristol-Myers Squibb) [41], approved for treating r/r mantle cell lymphoma and large B cell lymphoma, respectively. In April 2021 idecabtagene vicleucel (Abecma®, Bristol-Myers Squibb) became the first B cell maturation antigen (BCMA)-specific CAR T cell product approved for the treatment of multiple myeloma [42], while in February 2022 the FDA approval for the first Chinese CAR T cell therapy was obtained, ciltacabtagene autoleucel (Carvykti®, Legend Biotech/Janssen), which is also BCMA-directed for the same indication [43].

Despite the remarkable clinical success achieved in certain subsets of B cell leukemias and lymphomas, there are many barriers that limit CAR T cell therapeutic efficacy in other hematological malignancies and solid tumors [44]. A lack of durable clinical responses is attributed to insufficient engraftment and persistence of infused CAR T cells [45], or development of tumor resistance to single antigen targeting CAR constructs due to loss of target antigen expression on malignant cells, known as antigen escape [46]. For solid tumors, critical challenges include a paucity of specific target tumor antigens and limited T cell trafficking towards and into the tumor bed. In addition, the immunosuppressive tumor microenvironment (TME), characterized by upregulation of inhibitory checkpoints, such as PD-L1 and LAG-3, and the presence of multiple immunosuppressive cell populations (e.g., regulatory T cells, myeloid-derived suppressor cells, M2 macrophages) impairs T cell persistence by inducing T cell exhaustion or anergy [47,48]. Manufacturing challenges pose another barrier to autologous CAR T cell therapy. For instance, often insufficient numbers and poor quality of lymphocytes are collected from often elderly and heavily pretreated patients, which has sparked interest in allogeneic “off-the-shelf” CAR T cell development [49].

It is now widely recognized that further progress in CAR T cell therapy requires combinatorial approaches moving beyond single-target immunotherapy. Such novel engineering strategies center around multiple targeting, checkpoint blockage, *de novo* cytokine production, improved trafficking with chemokines and remote control CAR designs [50,51]. To realize such novel approaches, one critical consideration is the choice of genetic engineering tools that should offer safety, high efficiency, cargo flexibility to accommodate different types of payloads and increasingly large CAR constructs, as well as clinical scalability at low cost.

1.2 T cell engineering with viral vectors

Currently, CAR T cell manufacturing relies on the use of gammaretroviral and lentiviral vectors that offer high transduction efficiencies and long-term stable transgene expression. Out of six FDA-approved CAR T cell products two use gammaretroviral vectors (Yescarta and Tecartus) and four utilize lentiviral vectors (Kymriah, Breyzani, Abecma, Carvykti). To generate replication-defective vectors, viral sequences coding for genes necessary for additional rounds of virion replication and packaging are removed and replaced by the transgene of interest. Necessary viral sequences encoding capsid proteins, enzymes for reverse transcription/integration and envelope glycoproteins (*i.e.,* gag, pol, env) are provided on separate plasmids [52]. Co-transfection of these plasmids with vector plasmid incorporating the gene of interest provides all the components needed to produce functional viral particles in packaging cell lines such as HEK 293T. Separation of genes required for virion formation prevents progeny virus production while allowing to generate vectors capable of infecting mammalian cells and integrating their genetic material into the host genome [52].

Gammaretroviral vectors can only transduce dividing cells, while lentiviral vectors are able to infect both dividing and non-dividing cells, though T cell activation is typically required to achieve higher gene transduction efficiencies [53]. Another difference lies in their genomic integration profiles. Gammaretroviral vectors derived from Moloney murine leukemia virus (MLV) show preferential integration near transcriptional start sites and CpG islands, including promoters and enhancers [54,55]. Such an integration profile carries a risk of oncogenic transformation due to the activation of proto-oncogenes. This concern remained theoretical until MLV use in gene therapy

for X-linked severe combined immunodeficiency (SCID-X1) resulted in leukemia development caused by activation of the LMO2 oncogene due to vector integration near LMO2 promotor, prompting careful monitoring of viral vector safety ever since [56]. Contrary to retroviruses, human immunodeficiency virus (HIV)-derived lentiviral vectors show preference to integrate in transcriptionally active regions, which is in general considered a safer genomic integration profile [57,58]. Even though insertional mutagenesis cannot be excluded, no evidence of oncogenic transformation after T cell transduction with retroviral or lentiviral vectors has been observed to date. Nonetheless, recent reports indicate that the variability of lentiviral vector integration sites in CAR T cells could influence T cell proliferation and clinical responses, highlighting the need to better understand the correlation between vector integration and therapeutic outcomes [59,60].

Viral vector production for clinical applications is performed under current Good Manufacturing Practices (cGMP) in specialized biosafety level 2 facilities and takes 2 to 3 weeks with most of the time being spent on the expansion of HEK 293T producer cells to obtain large quantities of replication-defective vectors [52]. Compared to gammaretroviral vector manufacturing, lentiviral vector production turned out more challenging to scale up due to the lack of stable vector packaging cell lines and lot-to-lot variations arising from multi-plasmid transient transfection procedures [61]. Since there is a theoretical potential for generating replication-competent retroviruses or lentiviruses (RCRs/RCLs) during vector manufacturing, the FDA requires extensive testing for RCRs/RCLs in the packaging cell lines and the purified vector product, as well as the final transduced cells before infusion into the patient [62]. In addition, the FDA recommends patient follow-up for RCRs/RCLs emergence for up to 15 years. Such complex and highly centralized manufacturing processes combined with the need for long-term safety-monitoring results in exceptionally high costs and various logistic challenges, significantly restricting patient accessibility to CAR T cell therapy. Other drawbacks associated with viral vectors are limited cargo capacity of ~8-9 kb and intrinsic risk of immunogenicity [63,64].

The disadvantages of viral vectors have prompted the development of alternative non-viral transfection approaches with a better safety profile and less manufacturing difficulties, resulting in reduced cost and regulatory hurdles, and even facilitating point-of-care CAR T cell production to shorten vein-to-vein time. These techniques will be discussed in more detail in §3.

1.3 The potential of RNA to engineer therapeutic T cells

Traditionally, T cell modifications for therapeutic applications have been achieved through permanent transgene integration mediated by viral vector transduction. However, RNA moieties have recently emerged as a powerful tool to modulate T cell efficacy in cancer immunotherapy thanks to substantial progress in RNA manufacturing and the development of novel RNA delivery technologies . For instance, T cells can be transfected with mRNA to transiently express tumor antigen-specific receptors. This offers a superior safety profile because the mRNA does not integrate into the genome [65] and avoids the risk of insertional mutagenesis. In addition, transient CAR expression in T cells decreases the risk of "on-target off-tumor" toxicity in case target antigens are also expressed in healthy tissues. On the other hand, short-term CAR expression may reduce the T cell's anti-tumor efficacy, requiring repeated administration of mRNA-modified CAR T cells. Another area of interest is gene editing with designer nucleases, where nuclease delivery in mRNA format results in a narrow time-window of enzyme expression, thus conferring greater control over potential off-target genome editing effects. In addition, RNA therapeutics can also be used to inhibit immunosuppressive receptors and to modulate cytokine expression, which may increase the T cell's anti-tumor efficacy. In the next section we will discuss the different classes of RNA molecules, followed by an overview of non-viral transfection technologies and their application in T cell engineering.

2 Classes of RNA molecules and manufacturing advancements towards clinical translation

RNA therapeutics constitute a diverse class of molecules that can regulate the expression of both protein-coding and noncoding genes by acting on proteins, transcripts and genes. A major advantage of RNA-based therapeutics is their ability to target in principle any gene of interest, many of which may be inaccessible to other drug classes like small molecules and antibodies. It was estimated that only 0.05% of the human genome has been drugged by the presently approved protein-targeted therapeutics, since most (98.5%) of the human genome consists of non-protein-coding DNA sequences [66]. In addition, 85% of human proteins remain difficult to target pharmacologically due to a lack of well-defined pockets for small molecule binding [67]. Yet,

most of the human genome is transcribed into RNA, which can be targeted by antisense oligonucleotides (ASOs), small interfering RNA (siRNAs) and microRNAs (miRNAs) based on complementary base-pairing. Thus, by acting on both conventional proteome (protein expression) and the previously undrugged transcriptome (inhibiting expression), RNA molecules can significantly broaden the range of therapeutic targets. The different categories of RNA therapeutics based on their structure and mode of action will be discussed next.

2.1 Antisense oligonucleotides

ASOs are short, synthetic, single-stranded (ss) oligonucleotides (12-25 nt) designed to specifically hybridize to a complementary endogenous pre-mRNA or mRNA through Watson-Crick base-pairing [68,69]. The main mechanism of action is the formation of DNA-RNA heteroduplexes, leading to the recruitment of endogenous RNase H and cleavage of the complexes or steric blocking of the ribosomal assembly [70,71]. In addition, ASOs can promote alternative splicing by interacting with pre-mRNAs in the nucleus [72–74]. Downregulation of the target RNA expression can be achieved by translational arrest upon binding with the 5' untranslated region (UTR) of the mRNAs, cleavage of 5' cap structures or polyadenylation changes [75–77]. Alternatively, ASO binding to upstream open reading frames (uORFs) and translation inhibitory elements (TIEs) results in increased production of specific proteins encoded by target RNAs [78,79]. Finally, ASOs can upregulate the expression of desirable proteins by binding to miRNAs or miRNA-binding sites, thus inhibiting miRNA-mediated downregulation of gene expression [80,81]. The therapeutic use of ASOs was first reported by Stephenson and Zamecnik in 1978, who demonstrated that DNA-based ASOs could inhibit Rous sarcoma virus replication *in vitro* [82]. However, these effects were not sustained *in vivo* since unmodified oligonucleotides were prone to nuclease degradation and displayed a poor target affinity. Consequently, in the third generation of ASO therapeutics, numerous chemical modifications such as nucleobase modifications, alternative backbones and bridged nucleic acids have been implemented to improve their stability, target affinity, pharmacokinetics and pharmacodynamics, as extensively reviewed elsewhere [69,83,84]. Nonetheless, delivery of ASOs remains a hurdle for their broader clinical application.

2.2 Small interfering RNA

RNA interference (RNAi) is a conserved endogenous mechanism used to defend against invading viruses and transposable elements [85]. Gene silencing can be initiated by short double-stranded (ds) RNA sequences such as siRNAs or miRNAs, which mediate sequence-specific mRNA degradation or mRNA translational repression. The endogenous siRNA pathway starts by cleaving long dsRNA molecules into 21-23 nucleotide long siRNAs by the RNase III-type enzyme Dicer. Once incorporated into a multiprotein RNA-induced silencing complex (RISC) in the cytoplasm, siRNA is unwound into the passenger (sense) strand and the guide (anti-sense) strand. The passenger strand is then degraded by Argonaute 2 (AGO2) protein, whereas the guide strand is retained to direct RISC binding to target mRNA to induce AGO2-mediated mRNA cleavage [86–89]. Finally, the sliced target mRNA is released and the activated siRNA-RISC complex can be recycled to destroy additional targets, propagating the gene silencing effect [90]. The catalytic activity of siRNA can be sustained for 3 to7 days in rapidly dividing cells, after which its concentration drops below the therapeutic threshold and repeated administration is required to achieve a persistent effect [91].

Since its first description in plants and nematodes in the 1990s [92], the RNAi mechanism has been extensively exploited in fundamental studies of gene function and in developing new therapeutics. Although the first clinical trials using unmodified siRNAs failed due to immune-related toxicities and questionable RNAi effects [93], further improvements in chemical design, sequence selection and delivery strategies opened the way for safer and more efficacious RNA compounds [94–96].

2.3 CRISPR-based gene editing

Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9, aka CRISPR-Cas9, is a part of the bacterial adaptive immune system, which has been transformed into a potent genome editing technology in eukaryotic cells [97]. The system relies on a DNA nuclease (Cas9 protein) guided by an RNA sequence that is complementary to the target DNA region (guide RNA or gRNA). In bacteria, native Cas9 requires a guide RNA composed of two associated disparate RNA molecules, being the CRISPR RNA (crRNA) which enables the recognition of the

target gene and trans-activating CRISPR RNA (tracrRNA) which facilitates crRNA maturation and Cas9 recruitment. However, for gene editing purposes, both RNA molecules can be linked into a synthetic single guide RNA (sgRNA). Upon gRNA binding to Cas9, a ribonucleoprotein (RNP) complex is formed, whereby recognition of a 20-nucleotide target sequence and protospacer adjacent motif (PAM) engages Cas9 nucleolytic activity, inducing a double-strand break (DSB) [98,99]. The latter can be repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is an error-prone process where direct rejoining of the lesion introduces small deletions or insertions, ultimately disrupting the targeted locus (gene knock-out). In contrast, HDR is a more precise mechanism that can be exploited for gene insertion or correction (gene knock-in) in the presence of a donor DNA sequence [99,100].

Over the years, the CRISPR-Cas toolbox has expanded significantly by exploitation of the natural diversity of the CRISPR systems as well as rational engineering. CRISPR-mediated genome editing capabilities were first demonstrated using type II Cas9 DNA endonuclease from the *Streptococcus pyogenes* [98]. The Cas9 nuclease consists of two catalytic domains, HNH and RuvC, which cleave the target and non-target strand, respectively. These domains can be mutated towards the development of base editors and prime editors that operate without inducing a double-strand break, thereby reducing the risk of chromosomal rearrangements [101]. Inactivation of one of the nuclease domains creates a Cas9 nickase (nCas9) which introduces single-strand cuts, offering better control over off-target effects. Alternatively, inactivation of both nuclease domains generates a dead Cas9 (dCas9), stripped of catalytic activity but still able to recognize and bind to target DNA. The latter can be exploited, for instance, in gene regulation through dCas9 fusion with transcriptional activators or repressors and in epigenetic remodeling via linking with epigenetic effector enzymes [101–103].

Unlike Cas9, most Cas12 nucleases require only crRNA to induce staggered end cuts distal from a 5’ T-rich PAM sequence. Cas12a mediates genome editing with a higher specificity than Cas9, which can be related to its lower nuclease activity [104]. In addition, its smaller size and ability to process its own guide RNAs make Cas12 an attractive candidate for multiplex gene engineering [105]. More recently discovered Cas13 nucleases have two HEPN domains and their endonuclease activity is directed toward RNA. Once bound to the target, Cas13 may display a non-specific

RNase activity by cleaving bystander RNA molecules in a non-discriminatory manner. This collateral cleavage property has been exploited in nucleic acid detection-based diagnostic technologies, simultaneously raising concerns for therapeutic applications [106]. However, a recent screening of Cas13 mutants has identified some high-fidelity variants displaying efficient RNA knockdown activity with minimal collateral damage [107].

Despite the robustness and simplicity, the therapeutic application of CRISPR-Cas systems faces challenges related to effective delivery, off-target mutagenesis, genome editing efficiency and immunogenicity. Consequently, several strategies have been developed to enhance Cas specificity. For instance, using paired Cas9 nickases instead of Cas9 nuclease significantly reduces off-target effects without sacrificing the on-target cleavage efficiency [108]. In addition, several high-fidelity Cas9 variants have been engineered by rational design or directed evolution. One example is *Sp*Cas9-HF1 harboring alanine substitution to disrupt the nonspecific contact between SpCas9 and the phosphate backbone of target DNA [109]. Other approaches rely on the modification of gRNA, including truncated gRNAs [110], engineering secondary structures [111], or addition of cytosine stretches to the 5'-end of the gRNAs as a 'safeguard' strategy [112].

Also, chemical modifications optimized for ASOs and siRNAs can be applied to gRNAs to improve their stability against enzymatic degradation, enhance on-target performance and reduce toxicity/ immune recognition. For instance, the incorporation of 2'-O-methyl-3'-phosphonoacetate at specific sites in the ribose-phosphate backbone of gRNAs can significantly reduce off-target cleavage while preserving high on-target activity [113]. Similarly, crRNA modification with bridged and locked nucleic acids broadly improves Cas9 cleavage specificity [114]. In another study, chemical modifications comprising 2'-O-methyl, 2'-O-methyl 3'phosphorothioate, or 2'-O-methyl 3'thioPACE were incorporated at both termini of sgRNAs to enhance genome editing efficiency in primary human T cells and CD34+ hematopoietic stem and progenitor cells [115]. Finally, the 5'-hydroxyl modification of gRNA generated by triphosphate group removal helps to evade innate immune responses, leading to efficient Cas RNP-mediated targeted mutagenesis in primary human CD4+ T cells [116].

CRISPR-Cas components can be delivered to cells in three formats: DNA vector (either plasmid or viral vector) encoding Cas and gRNA; mRNA encoding Cas protein with a separate guide RNA; or

mature CRISPR-Cas ribonucleoprotein. Plasmid-based delivery is a convenient strategy for the co-transfection of multiple components such as Cas, sgRNA(s) and exogenous DNA for HDR, potentially increasing genome editing efficiency [117]. However, it requires nuclear entry and translation and is associated with the risk of host genome integration and off-target effects resulting from prolonged expression [118]. In addition, exogenous DNA sensing by cellular receptors can trigger innate immune responses [119,120]. Compared to plasmids, delivery of Cas-encoding mRNA enables faster onset of genome editing as there is no need for a transcription step before translation commences in the cytoplasm. The transient nature of protein expression can be leveraged to better control the dose and duration of Cas nuclease activity, reducing off-target effects [115]. However, due to poor stability and susceptibility to enzymatic degradation, mRNA molecules require chemical modifications and carefully considered delivery mechanisms, as will be discussed further in the next section. Finally, Cas delivery in protein format offers immediate onset of gene editing. Its transient presence translates to reduced off-target effects and toxicity [121,122]. However, Cas RNP delivery can be challenging due to the large size and charge of the protein.

2.4 Aptamers

Aptamers are single-stranded oligonucleotides that can bind to various targets with high affinity and selectivity by folding into specific three-dimensional structures. They are produced *in vitro* through a controlled process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [123]. Often regarded as a chemical equivalent of antibodies, aptamers have the advantage of being relatively small, more stable, nonimmunogenic and programmable via chemical modifications and conjugation [124]. Aptamer-based therapeutics include antagonist aptamers which disrupt the interaction of disease-associated targets such as protein-protein or receptor-ligand interactions, and agonist aptamers, which can activate target receptors. Furthermore, cell type-specific aptamers serve as carriers to deliver other therapeutic agents to the target cells and tissues. Aptamer-based delivery systems include conjugates with different oligonucleotides and drugs and aptamer-decorated nanomaterials [124].

2.5 Messenger RNA

Messenger RNA (mRNA), first discovered by Brenner and colleagues in 1961, transfers genetic information from the DNA in the nucleus to the cytoplasmic ribosomes, where it can be translated into proteins [125]. The therapeutic potential of mRNA molecules was first realized in the 1990s, when protein expression was demonstrated by direct injection of *in vitro* transcribed (IVT) mRNA constructs [126]. In another study, Jirikowski *et al*. injected vasopressin mRNA into the hypothalamus of Brattleboro rats to induce the synthesis of vasopressin and (transiently) reverse diabetes insipidus [127]. Later, Conry *et al*. injected mRNA constructs encoding a carcinoembryonic antigen in mice to induce an anti-tumoral antibody response [128]. These early demonstrations, coupled with advancements in mRNA design and manufacturing, laid the foundation for a plethora of applications investigated today, including: (1) protein replacement therapy, where exogenous mRNA is administered to replace or supplement endogenous proteins; (2) vaccination, where mRNA encoding specific antigens is introduced to elicit an immune response against infectious diseases or cancer; (3) adoptive cell therapy, where mRNA transfection is used to alter the therapeutic cell's phenotype or function; (4) gene editing, where mRNA enables the transient expression of gene editing nucleases.

mRNA therapeutics offer several advantages compared to DNA-based strategies. First, mRNA does not need to enter the nucleus, thus circumventing the challenge of nuclear delivery and the risk of genomic integration. In addition, as the cytoplasmic site of action makes mRNA independent of cell cycle progression, it is efficacious in both mitotic and non-mitotic cells. The relatively short half-life of mRNA can be advantageous for applications that require only transient protein expression, such as expression of nucleases for gene editing, epitopes in vaccination and transposase in stable non-viral gene transfer. Finally, manufacturing of synthetic mRNA by *in vitro* transcription is relatively simple, fast, scalable, and cost-efficient.

IVT mRNA can be synthesized in a cell-free approach using a phage RNA polymerase (such as SP6, T3, or T7) and a linear DNA template in the presence of nucleotides. The IVT mRNA molecules resemble naturally occurring mature eukaryotic mRNAs and comprise five functional regions: a 5' cap, a 5' untranslated region (UTR), an open reading frame (ORF) encoding the gene of interest,

a 3’ UTR and a 3’ poly(A) tail (**Figure 3**). Each of these structural elements has been modified in recent years to enhance mRNA stability and translation efficiency or to modulate immunogenicity [129–132]. The 5’ cap structure regulates pre-mRNA splicing, nuclear export, mRNA stability against 5’-3’exonuclease-mediated degradation and translation initiation by recruiting eukaryotic initiation factor 4F (eIF4F). The natural eukaryotic 5’cap (cap-0) contains 7-methyl-guanosine connected to the 5’ nucleotide through a 5’-5’ triphosphate bridge (m7Gppp). Ribose of the first and second nucleotide can be subjected to 2’-O-methylation to generate cap-1 and cap-2, and these methylations have been found to reduce immunogenicity, indicating a role in distinguishing between self and non-self mRNA [133,134]. In contrast, unmodified mRNA or cap-0 structures can be recognized by cellular pattern recognition receptors (PRRs) such as retinoic acid-inducible gene (RIG)-like receptors (RIG-I and melanoma differentiation-associated protein 5 (MDA-5)), leading to interferon responses and mRNA degradation [130]. In addition, 5’ cap structures can be subjected to various chemo-enzymatic modifications to achieve cap analogs with high affinity for eIF4F and low susceptibility for decapping enzymes, or to modulate immunostimulation [135,136]. Currently, two methods are used to cap IVT mRNA: co-transcriptional capping (CleanCap® technology, TriLink Biotechnologies) and posttranscriptional capping (capping enzymes from vaccinia virus).

Along with the 5’ cap, the 3’ poly(A) tail regulates mRNA stability and translation efficiency by interaction with poly(A) binding proteins. The length of the poly(A) tail is usually increased to counteract the deadenylation process that eventually leads to mRNA decay, though the optimal length of the poly(A) tail remains controversial [129,137]. For instance, one study demonstrated that mRNA modification with a poly(A) tail measuring 120 nt increased mRNA stability, translation efficiency and T cell stimulatory capacity of dendritic cells, providing a potential optimization strategy for mRNA vaccine manufacturing [138].

UTRs do not encode proteins but play important roles in regulating translation efficiency, mRNA stability and subcellular localization [129,139]. The 5’ UTR is mainly involved in ribosome recruitment and the initiation of mRNA translation. A strong Kozak sequence is often incorporated after the 5’ UTR to improve translation efficiency [140,141]. The latter can also benefit from eliminating sequences that display an increased propensity towards the formation of stable secondary and tertiary structures, hindering mRNA interactions with ribosomes. The 3’ UTR contains miRNA

binding sites and governs mRNA stability and half-life [142]. For instance, removing miRNA binding sites from 3' UTR can promote encoded protein expression. Alternatively, inserting a tissue-specific miRNA binding site can increase mRNA degradation in off-target tissues upon systemic administration, reducing undesired side effects [143]. mRNA translation and half-life can be improved by the incorporation of sequences derived from endogenous long-lived mRNAs, such as alpha and beta globin [141]. Furthermore, optimization of the guanine-cytosine content results in enhanced stability and reduced immunogenicity of synthetic mRNA constructs [144].

The ORF coding the sequence of the protein of interest is the core of the IVT mRNA. One approach to increase translatability is codon optimization, where rare codons are replaced with synonymous high-frequency codons to speed up the translation. However, this strategy is controversial since codon replacement may affect protein conformation and give rise to novel peptides with unknown biological activity [145,146]. Therefore, nucleoside modification appears as the most attractive alternative. The incorporation of modified nucleotides in mRNA, such as pseudouridine (ψ), N1-methylpseudouridine ($m^1ψ$), 5-methoxyuridine (mo^5U), 2-thiouridine (s^2U), 5-methylcytidine (m^5C) and N6-methyladenosine (m^6A) suppresses the activation of TLR receptors, thereby inhibiting the innate immune responses and improving protein translation efficiency [130,133,147,148]. It is worth noting that N1-methylpseudouridine modification has been implemented in the development of both Pfizer/BioNTech (comiranty®) and Moderna Therapeutics (spikevax®) SARS-CoV-2 vaccines [149].

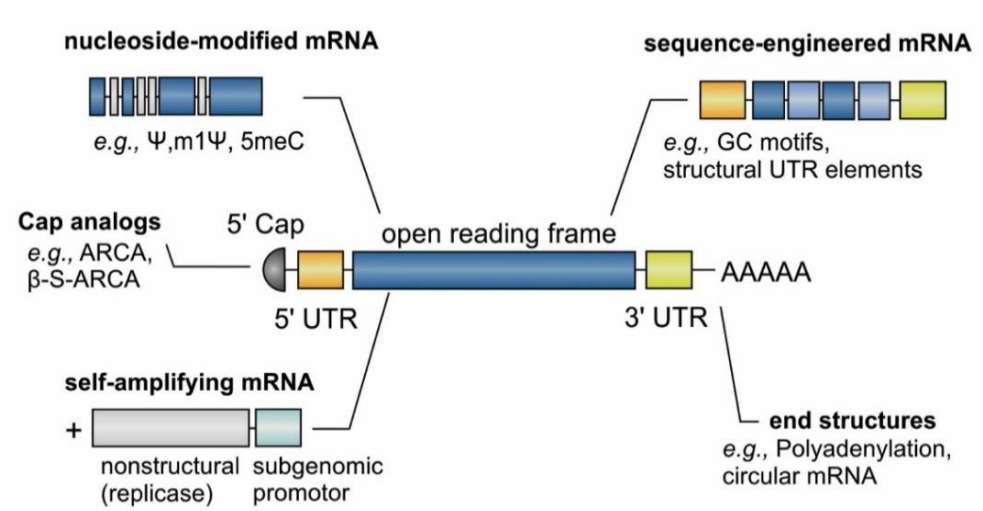


Figure 3. The structure of *in vitro* transcribed (IVT) mRNA. IVT mRNA comprises five functional regions: a 5' cap, 5' and 3' untranslated regions (UTRs), the protein-encoding open reading frame (ORF) and a 3' poly(A) tail. In recent years, each of these elements has been modified to improve mRNA stability and efficiency, or to modulate immunogenicity. Figure adapted from Verbeke *et al.*[150]

Another strategy to reduce the immunostimulatory potential of the IVT mRNA is to perform additional purification steps for removing potentially immunogenic contaminants, such as residual templates, free nucleotides and dsRNA. The most common method to purify IVT mRNA is high performance liquid chromatography (HPLC). For instance, Kariko *et al*. used reversed-phase HPLC to remove dsRNA impurities and demonstrated a remarkable increase in protein expression by 1000-fold, without inducing the production of IFNs or inflammatory cytokines [151]. However, HPLC is not suitable for large scale production of mRNA. Alternative purification methods include oligo(dT)-cellulose chromatography and RNase III specific digestion. The latter has been employed by Foster *et al*. to remove dsRNA byproducts from mRNA encoding CD19 CAR. T cells electroporated with a purified construct displayed decreased expression of checkpoint inhibitors and improved cytotoxicity in a murine leukemia model [148].

The successful development of COVID-19 mRNA vaccines has fueled further innovations in mRNA engineering aimed at increased stability and more robust expression *in vivo*. In vaccination, achieving adequate antigen expression levels for protection or immunomodulation depends on

the number of successfully delivered conventional mRNA transcripts and thus may require large doses or repeated administration. This limitation can be addressed using self-amplifying mRNA (saRNA), based on self-replicating elements derived from the alphavirus genome [152,153]. Such a construct consists of the alphavirus replication genes, while the structural elements are substituted with the selected gene of interest. As a result of their self-replicative activity, saRNAs can be delivered at lower doses than conventional mRNA to achieve comparable antigen expression [154,155]. saRNA-based SARS-CoV-2 vaccines have already shown efficiency in inducing high neutralizing antibody titers in animals [155–157] and several other candidates against infectious diseases and cancer are being tested in clinical trials [158,159]. However, the substantially larger size of self-amplifying mRNA compared to conventional mRNA (~10 kb vs. ~2-3 kb) may necessitate optimization of delivery formulations. In addition, saRNA displays a higher innate immune-stimulating activity compared to conventional mRNA [160].

3 Non-viral delivery platforms for RNA therapeutics

For T cell engineering the RNA molecules need to cross the cell membrane to gain access to the cytosol. However, their hydrophilic nature, macromolecular size and overall strong negative charge preclude cellular entry via passive diffusion. Therefore, to facilitate intracellular delivery of RNA, various non-viral strategies have been employed, which can be broadly categorized as membrane disruption-mediated and carrier-mediated methods. Membrane disruption-based technologies enhance the permeability of the plasma membrane mostly *via* physical stimuli, such as electrical fields or mechanical forces, offering direct access to the cytosol. Although considered relatively universal in terms of cell type and cargo molecules to be delivered, such physical methods are often limited to *in vitro* or *ex vivo* applications, being less suited for *in vivo* delivery. In contrast, carrier-based delivery systems designed to condense nucleic acid into compact nanoparticles can be applied both *ex vivo* and *in vivo*. However, these nano-vehicles face specific challenges related to cellular uptake and endosomal escape, as discussed in the next paragraphs. In this section, we provide an overview of both established and emerging technologies for RNA delivery. For each method, the delivery mechanism will be discussed as well as its advantages and disadvantages for therapeutic cell engineering.

3.1 Membrane-disruption based delivery methods

3.1.1 Electroporation

Electrical membrane permeabilization, or electroporation in short, is an approach in which cell exposure to high-voltage and low frequency electrical pulses induces a transient increase in plasma membrane (PM) permeability, allowing transmembrane transport of otherwise impermeant exogenous molecules (**Figure 4**). This phenomenon was first demonstrated in 1982 by Neumann *et al.*, who reported efficient transfection of pDNA into mouse lyoma cells upon application of strong electric fields [161]. Although a comprehensive understanding of the mechanisms of electroporation is still lacking, there is broad consensus that electroporation is best described by the theory of aqueous pore formation that is induced by interfering with the cellular transmembrane potential (TMP) [162–165]. According to the theory, once the applied voltage exceeds a critical threshold, PM breakdown occurs in two phases: first, water molecules start penetrating the bilayer, forming a water channel; next, the lipids adjacent to the water channel reorient toward the channel with their polar head groups, creating metastable (lasting milliseconds up to several minutes) hydrophilic pores [163,165,166]. In addition, there is increasing evidence that exposure to electric pulses may cause chemical changes to membrane lipids and modulation of protein function, contributing to the increased permeability of the lipid bilayer [163,167].

The extent of membrane permeabilization depends on the magnitude and duration of the applied electric forces [163,165]. Generally, it is believed that coverage area of pore formation is determined by pulse strength while pore size correlates with pulse duration [164]. For instance, application of sub-microsecond pulses induces many small pores (radius ~1 nm), whereas longer pulses result in less numerous but larger pores of up to tens of nm [168]. In addition, high voltage ultrashort pulses in the nanosecond range might be used to target intracellular organelles without disrupting the PM [169,170]. Pore formation is also influenced by factors such as cell size, membrane curvature, temperature, and osmotic pressure. Generally, smaller cells, such as T lymphocytes, require higher voltages than larger cells to achieve effective PM permeabilization [165,171].

Intracellular delivery of exogenous molecules is highly dependent on pore size and cargo properties, such as size, charge and conformational flexibility [164,172]. Small neutral molecules enter the cell via diffusion through the pores, while transport of charged species such as nucleic acids is facilitated by additional electrophoretic forces present during the pulse [173–175]. For instance, siRNA delivery can be mediated by a combination of electrophoretic and/ or diffusive mechanisms depending on the size and lifetime of the pores [176,177]. In contrast, transfection of large DNA plasmids is often described as a multistep process involving DNA condensation at the cell membrane, followed by endocytic internalization and a yet poorly understood step of endosomal release in the cytosol and eventual translocation to the nucleus [178–180].

To ensure successful intracellular delivery and preservation of cell viability post treatment (*i.e.,* reversible electroporation), several parameters such as field strength, pulse duration and number of pulses need to be optimized for a given combination of cell type and effector molecules [181–184]. Moreover, the composition of the electroporation buffer can be adjusted in terms of osmolarity and conductivity to balance transfection efficiency with cytotoxicity [185–188]. This flexibility, combined with high delivery efficiencies has established electroporation as one of the leading non-viral transfection technologies for both basic research and clinical applications [185].

Wide laboratory adoption of electroporation has been supported by the development of several commercial systems such as Gene Pulser™ (Bio-Rad), Nucleofector™ (Lonza), Neon™ (Invitrogen) and NEPA21 electroporator (Nepagene). Clinical manufacturing applications have been facilitated by the introduction of large-scale electroporation platforms, such as MaxCyte's ExPERT family of instruments based on flow electroporation™ technology, CliniMACS® Electroporator from Miltenyi Biotec and CTS Xenon offered by Thermo Fisher Scientific. For example, the MaxCyte GTx™ system can transfect up to 20 billion cells in less than 30 minutes. Such large volume electroporators can be coupled with modules like the CliniMACS Prodigy® platform (Miltenyi) or Cocoon® (Lonza) to assemble a fully automated and closed cGMP workflow from cell isolation/activation to genetic engineering and expansion.

Despite being the most established non-viral method for T cell transfections, electroporation comes with certain limitations, not in the least a substantial loss of cell viability post-treatment.

Cell damage can be attributed to electrolytic effects such as Joule heating, pH changes and contamination via corrosion of electrodes [164,189,190]. In addition, cell exposure to strong electric fields has been suggested to trigger lipid peroxidation, protein denaturation, generation of reactive oxygen species and DNA damage [163,167,191]. Furthermore, if the PM integrity remains compromised for extended periods of time, it may lead to severe disruption of cell homeostasis, triggering delayed cell death mechanisms [192]. Even when cells survive, they may carry persistent phenotypical alterations, leading to reduced proliferation potential and changes in signaling pathways, activation states and transcriptional responses [193–195]. For instance, in an early study by Zhang *et al*., enhanced transcriptional activity and increased expression of surface activation markers were observed in CD4 T cells treated by nucleofection [193]. Later, DiTommaso *et al.* showed that electroporation induced significant gene expression changes and aberrant cytokine secretion in primary T cells, which translated to functional deficiencies *in vivo* with electroporated T cells failing to demonstrate sustained antigen-specific effector responses and tumor control [194]. It seems, therefore, that the main challenge for electroporation-based T cell engineering lies in long-term survival and functionality rather than the initial delivery efficiency [164,196,197].

Recent innovations in nanotechnology and microfluidics led to the development of miniaturized electroporation systems such as micro-, nano- and microfluidic-based electroporation, offering more precise control over delivery parameters and electrode-mediated toxicities (also see section 3.1.5). For example, Cao *et al.*, reported 75% mRNA transfection efficiency in Jurkat T cells using a water-filter nanoporous membrane for a highly localized nanopore electroporation [198]. Another example is the microfluidic continuous-flow electroporation device developed by Lissandrello and colleagues for high-throughput T cell engineering, with a reported mRNA transfection efficiency of up to 95%, minimum impact on cell expansion potential and a processing rate of 20 million cells per minute [199]. More recently, the same authors reported on an optimized design for mRNA, RNP and pDNA transfection with an enhanced processing throughput of 9.6 billion cells per hour [200]. In another study by VanderBurgh *et al.,* similar efficiencies were demonstrated for mRNA transfection and CRISPR/Cas9-mediated TCR knock-out, while delivery throughput could be scaled up to 256 million cells/min [201]. When

proliferation rates were evaluated, T cells exhibited a reduced growth rate for 2 days post-electroporation, before recovering to proliferation rates comparable to control cells. For an extensive overview of such novel designs, we refer the reader to recently published reviews [202–204]. Several commercial micro/nano electroporation products are presently being developed by start-up companies, such as by CyteQuest, Kytopen and NAVAN Technologies. It will be of interest to see how these newer electroporation technologies stack up against the more established bulk electroporation devices for T cell engineering in terms of efficiency, cell viability and functionality.

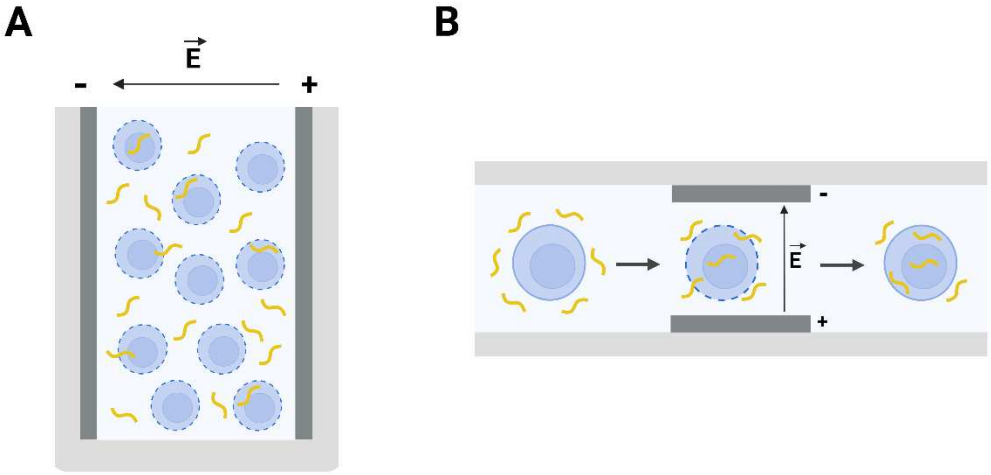


Figure 4. Schematic illustration of conventional or bulk electroporation and flow-through electroporation. (A) In traditional electroporation, cells and cargo molecules (yellow) are mixed in a conducting buffer and transferred to a cuvette with two parallel electrodes. Upon application of electrical pulses, cells become transiently permeabilized, which allows intracellular migration of cargo molecules. (B) In microfluidic flow-through electroporation, cells suspended in a conductive buffer with cargo become electropermeabilized while flowing between two plate electrodes.

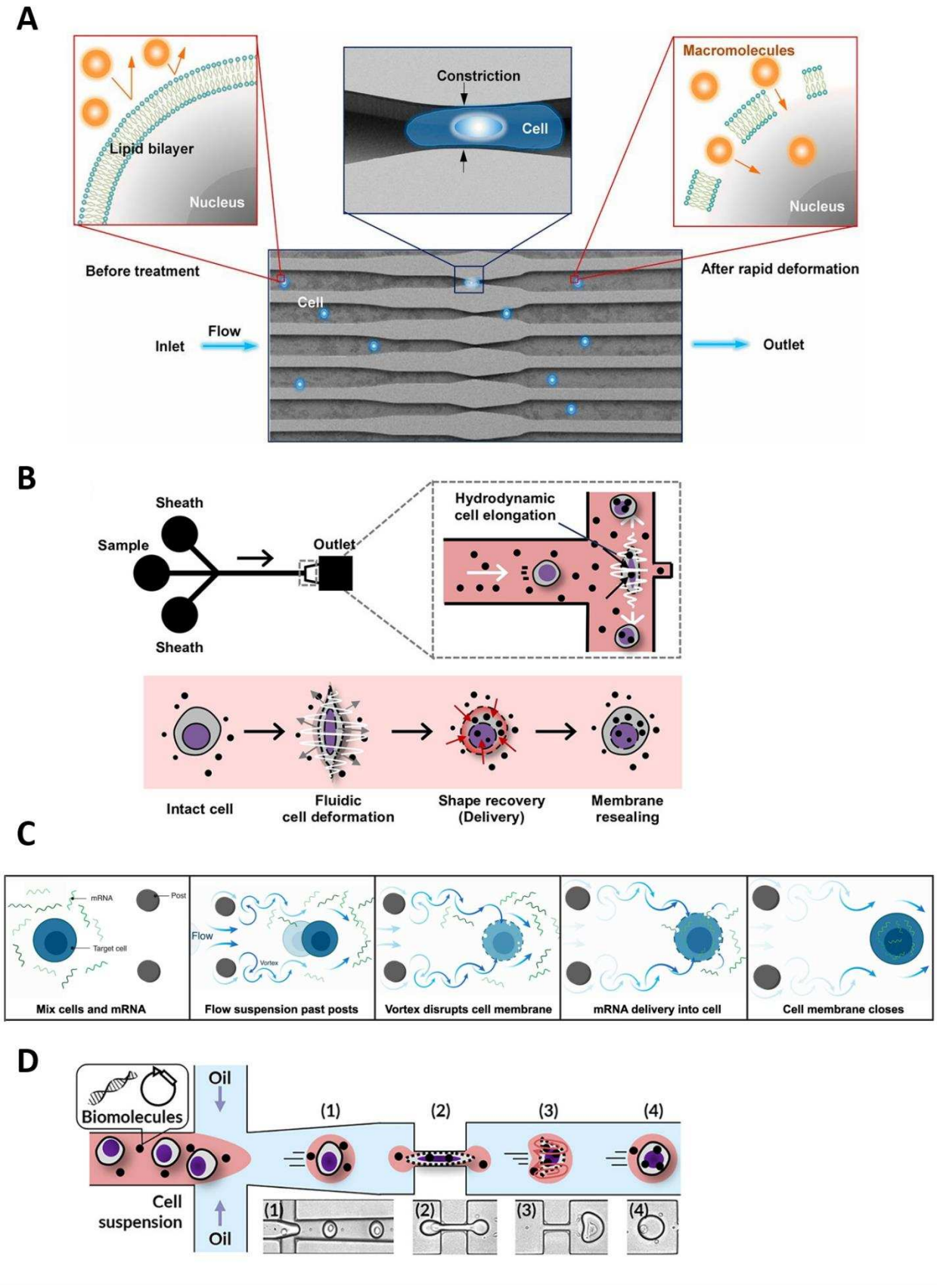
3.1.2 Microfluidic cell squeezing

As an alternative to electroporation, microfluidic platforms based on rapid mechanical deformation of cells have gained considerable attention. The original implementation of this concept, known as cell squeezing, relies on passing cells in suspension through narrow (smaller than the cell diameter) constrictions in microfluidic channels, leading to mechanical disruption of the PM and facilitating cytosolic delivery of macromolecules present in the surrounding medium

(**Figure 5A**) [205]. A major advantage of this approach is its simplicity, as it only requires a microfluidic chip, reservoirs, and a pressure regulation system to facilitate fluid flow through the chip [206]. Once microfluidic chip geometry is optimized for a given cell type, scalability through channel parallelization offers high throughput processing of up to 1 million cells per second [207–209]. Precise control over the membrane disruption process allows for high delivery efficiencies while preserving cell viability and functionality. For example, DiTommaso *et al.* reported that cell squeezing had minimal effect on T cell transcriptional responses, cytokine production *in vitro* and their therapeutic efficacy *in vivo* [194]. CellPore™ (StemCell Technologies) is a commercial device that employs CellSqueeze™ technology to deliver RNP complexes for gene editing of non-activated human T cells. Loo *et al.* fabricated a related technology in which cells are quickly squeezed and expanded through a series of constrictions. T cell transfection with mRNA via these ultra-fast physical deformations did not affect T cell proliferation capacity or expression of differentiation and exhaustion surface markers [210]. This technology is under development at the start-up company CellFe.

While clogging of microchannels with constriction sites by debris or cell clusters is a reported practical disadvantage of the cell squeeze technology, alternative microfluidic designs have emerged in which PM permeabilization is achieved by hydrodynamic forces in relatively wide channels. For instance, Kizer *et al.* developed a clogging-free cross-junction channel design where transient membrane pore formation by rapid hydrodynamic cell shearing permits both diffusive and convective delivery of external macromolecules into the cytosol [211]. The hydroporation platform achieved an mRNA transfection efficiency of ~90% with a minor effect on T cell surface antigen and mRNA expression profiles, contrary to cell treatment by electroporation (**Figure 5B**) [212]. The technology is commercialized by MxT Biotech. In another approach called 'microfluidic vortex shedding' (μVS), Jarrell *et al*. constructed a microfluidic chip with an array of equally spaced posts to generate hydrodynamic vortices, which can induce a disruption to the membrane of cells transported by the fluid flow (**Figure 5C**) [208]. In such a design, spacing between posts was approximately two times larger than the median cell diameter, increasing the tolerance for cell size variability and reducing the risk of channel clogging. The authors reported a very high processing throughput of 2 million cells per second and showed that μVS-mediated transfection

669 did not impact T cell activation state and proliferation rates for at least seven days after
670 treatment [208]. The μVS technology is presently commercialized by Indee Labs. To address the
671 problem of high cargo consumption, Joo *et al.* designed a strategy that leverages droplet
672 microfluidics with cell mechanical permeabilization (**Figure 5D**) [213]. In this approach, cells and
673 cargo macromolecules are co-encapsulated into droplets, which are then squeezed through a
674 series of narrow constrictions. Upon cell membrane disruption, intracellular delivery occurs by a
675 combination of convection and diffusion-mediated transport. While channel clogging was
676 negligible, loading into droplets significantly reduced the amount of cargo needed.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677

Figure 5. Microfluidic platforms for intracellular delivery. (A) Schematic illustration of the microfluidic cell squeezing principle. Rapid mechanical deformation of cells as they pass through a constriction smaller than their diameter generates transient disruptions in the cell membrane, allowing extracellular molecules dispersed in the surrounding medium to enter the cell. Adapted from Sharei *et al*. [205] (B) Schematic illustration of a microfluidic hydroporation channel design. Intracellular delivery is achieved by hydrodynamic cell elongation by inertial flow in a T-junction channel. Adapted from Hur *et al.* [212] (C) Schematic illustration of the microfluidic vortex shedding (μVS)-based system. Cells in suspension pass by posts that create fluid vortices, disrupting the plasma membrane. Adapted from Jarrell *et al.* [208] (D) Droplet squeezing platform. In this approach, cells are first coencapsulated with cargo molecules into water-in-oil droplets. These droplets then flow through a series of narrow constrictions to mechanically disrupt the cell membrane. With cargo molecules present in direct vicinity of membrane pores, intracellular delivery is believed to happen via convective solution exchange enhanced by recirculation flows in the droplets. An example of FITC dextran delivery in K562 cells is shown. Adapted from Joo *et al.* [213]

3.1.3 Solvent-based poration

Chemicals have also been used to permeabilize the PM. The Solupore® technology, currently commercialized by Avectas, uses a proprietary hypotonic permeabilization solution containing a low level of ethanol [214]. The cargo of interest is mixed with the permeabilization solution and applied to the cells using an atomizer. This leads to local osmotic cell swelling and reversible PM perturbation, enabling cargo molecules to enter the cell by diffusion. After a brief incubation step, a stop solution is added to facilitate membrane resealing. In the initial proof-of-concept study from 2017, O'Dea and co-workers used this technology to demonstrate successful delivery of mRNA, pDNA and proteins in various cell types, including BSA proteins in immortalized Jurkat T cells [214]. In 2021, the authors reported primary T cell engineering with CD19 CAR mRNA with an average transfection efficiency of 60% and minimal perturbation of immune gene expression and effective CAR-mediated cytotoxicity *in vitro* and *in vivo* [215]. Although little literature is available on this technology, press resources provided by the manufacturer indicate a significant potential of the Solupore® platform for T cell engineering, which is supported by its simplicity, low cost, high transfection efficiencies with possibility for multiplexing and sequential delivery, and minimal impact on cell phenotype and functionality. The current portfolio of Avectas includes a Solupore Research Grade Tool for feasibility studies and a closed, clinical-grade cell engineering

system with a processing scale of 10^8 cells. A continuous, flow-through system for allogenic cell scale manufacturing of above 10^9-10^{10} cells is currently under development.

3.1.4 Photoporation

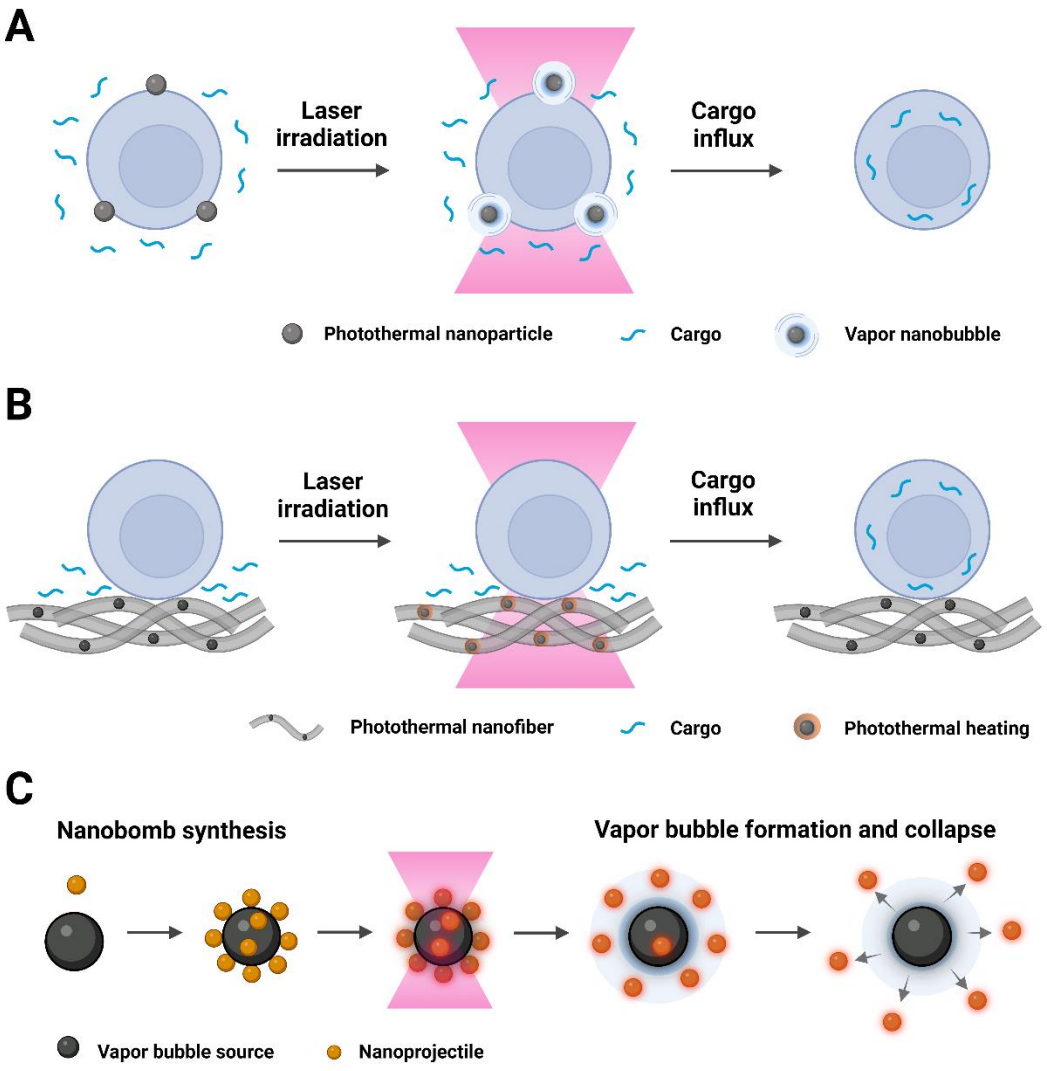
Photoporation, also termed optoporation, is a delivery technique that makes use of light energy to transiently permeabilize the cell membrane. In its original form, high-intensity femtosecond laser pulses are focused on the cell membrane to create a pore by photochemical and/or photothermal effects, allowing cytosolic entry of exogenous cargo by diffusion [216,217]. Although useful for single-cell transfections, the general utility of such an approach has been limited by its labor-intensive and inherently slow nature. To enhance photoporation throughput, the process has been combined with photothermal nanomaterials, which efficiently absorb laser light and convert this energy into photothermal effects [218]. Typically, a nanoparticle-mediated photoporation procedure starts with cell incubation with photothermal nanoparticles to let them adsorb to the cell membrane (**Figure 6A**). Attachment of NPs to the PM can be promoted by NP surface functionalization with positively charged polymers to promote electrostatic interaction or via high-affinity ligand-receptor coupling [217,219,220]. After removal of unbound NPs by a washing step, the cargo of interest is added and cells are irradiated with a laser to induce membrane permeabilization. By using photosensitizing nanomaterials, the laser energy density required for effective pore formation is substantially reduced as compared to direct laser-induced photoporation. Therefore, a wide laser beam can be used, allowing quick scanning over the cells and significantly enhancing photoporation throughput. For instance, for T cell transfection, processing rates of ~5000 cells per second were reported [220].

Depending on the laser energy, PM permeabilization can be mediated by photochemical reactions, local heating, or the generation of water vapor nanobubbles (VNBs). Application of relatively low-intensity laser pulses results in photothermal heating, which induces pore formation by denaturation of integral membrane proteins or local phase transitions of the lipid bilayer [221]. When NPs are irradiated with sufficiently high laser fluences, typically with pulses shorter than 10 ns, the temperature of the NP increases quickly by several hundreds of degrees, resulting in the evaporation of the surrounding water and formation of fast-expanding vapor

738 nanobubbles. Once the thermal energy of the NPs is consumed, the VNB collapses, leading to
739 local pressure waves that generate transient disruptions in the adjacent cell membrane,
740 providing cytosolic access for external macromolecules [216,222,223].

741 The applicability of photoporation for T cell editing has been supported by a series of proof-of-
742 concept studies demonstrating successful delivery of various cargo molecules, including model
743 dextrans of up to 500 kDa, siRNA, mRNA and RNP protein complexes in both unstimulated and
744 preactivated T cells [220,224–228]. Although gold NPs have been the most used nanosensitizers, they
745 can be replaced by biocompatible and biodegradable polydopamine NPs [227]. Interestingly, the
746 polydopamine NP size can be tuned to avoid excessive cell damage and preserve T cell
747 functionality post-treatment [228]. In another approach, photothermal NPs have been incorporated
748 within electrospun nanofiber substrates (**Figure 6B**), thus avoiding direct T cell exposure to NPs,
749 and circumventing remaining safety and regulatory concerns [195]. This system was used to
750 transfect human CAR T cells with siRNA to downregulate PD-1 expression, resulting in faster
751 tumor regression in a xenograft mouse model as compared to control CAR T cells. Importantly, it
752 was shown that the functionality of T cells was better preserved as compared to electroporated
753 T cells, resulting in higher cell killing potential. To create larger pores in the cell membrane and
754 to facilitate more efficient transfection of cells with large nucleic acids like mRNA and pDNA,
755 Fraire *et al.* developed optically triggered nanobombs [229]. The nanobombs are composed of a 0.5
756 µm photothermal core particle surrounded by a corona of smaller inert nanoparticles of 0.1-0.2
757 µm (**Figure 6C**). Upon absorption of an intense nanosecond laser pulse, the smaller nanoparticles
758 are forcefully expelled by the formation of a VNB from the core particle. It was shown that these
759 nanoparticles can penetrate through the membrane of nearby cells, thus creating large PM pores
760 through which mRNA and pDNA can more easily penetrate. Being relatively gentle to cells, it was
761 demonstrated that the mRNA transfection yield of Jurkat T cells was several times higher than
762 for electroporation [229]. Photoporation with NP sensitizers and photothermal nanofibers is
763 currently being developed by the start-up company Trince, including for T cell engineering.

764 …



765

766 **Figure 6. Schematic illustration of different photoporation modalities.** (A) In standard nanoparticle-
767 mediated photoporation, cells are first mixed with photothermal NPs, such as gold or polydopamine NPs,
768 to let them adsorb to the cell surface. Next, application of pulsed laser irradiation leads to the generation
769 of transient water vapor nanobubbles around cell-bound NPs. Subsequent expansion and collapse of VNBs
770 cause mechanical membrane disruption, allowing external molecules to diffuse inside the cell. (B) In
771 photothermal electrospun nanofiber-based photoporation, photothermal iron oxide NPs are embedded
772 within nanofiber substrates fabricated by electrospinning. In this way, direct cell exposure to
773 photosensitizing nanoparticles can be eliminated, alleviating safety and regulatory concerns related to the
774 potential presence of nanomaterials in the final cell product. After T cells sedimentation on top of
775 nanofiber mesh, membrane permeabilization occurs via laser-induced photothermal heating. (C) Light-
776 triggered nano-biolistic system (nanobombs) consisting of a photothermal core particle coated with

smaller particles that act as nanoprojectiles. Upon pulsed laser irradiation, the nanobomb core heats up, evaporating the surrounding water and forming a vapor bubble. The mechanical forces emerging from vapor bubble expansion and collapse propel the nanoprojectiles through the surrounding medium and can be exploited to disrupt the plasma membrane of nearby cells.

3.1.5 Nanostructures

Nanowires, nanoneedles and nanostraws are examples of high aspect ratio nanostructures fabricated into vertically aligned arrays to mechanically disrupt cell membranes for intracellular delivery [164,230,231]. Cargo molecules can be coated at the tip of such structures or added to the cell culture medium. Alternatively, nanostraws, which are hollow versions of nanowires, are used to inject cells with cargo pumped from a fluid reservoir underneath the array. Cell interactions with nanowires rely on passive settling and adhesion, or application of an external force such as centrifugation. The exact mechanism of nanostructure-mediated penetration and intracellular delivery is a subject of ongoing debate [232,233]. It was previously proposed that in the presence of centrifugal forces, the cell membrane undergoes large-scale deformations due to the nanowire indentation, while the cell body volume does not change. In the adhesion-mediated process, cells continue to deform around the nanowires until they adhere to the substrates, inducing localized membrane tension, which eventually causes membrane rupture [232]. Penetration can be optimized by manipulation of needle geometry (density, length and diameter), surface functionalization and interfacing time. For instance, effective intracellular delivery of macromolecules into small immune cells requires nanowires that are longer, sharper and denser compared to structures suitable for larger adherent cells [234]. Transfection with siRNA-coated silicone nanowires demonstrated efficient (77%) gene silencing in resting murine CD4+ T cells without affecting cell viability and post-activation expansion rates, nor inducing innate immune responses [234]. In a follow-up study, the authors employed nanowire-based siRNA delivery to investigate the dynamic regulatory network that controls Th17 differentiation, showcasing the technology potential for efficient engineering of even unstimulated T cells without impacting their phenotype [235]. More recently, a silicone nanotube-based nanoinjection platform loaded with PCR expression cassette encoding anti-CD19 CAR was used to generate CAR T cells with an average expression efficiency of ~20% and demonstrated CAR-mediated cytotoxicity in vitro [236].

In a modified approach, the same group coupled nanotubes with a low-voltage electrical stimulation, achieving ~40% CAR gene expression in T cells (**Figure 7**) [237] and effective delivery of various molecules such as antibodies, siRNA and mRNA in fibroblasts [238]. As such, nanostructures present an attractive alternative for T cell transfections, though further research on functional consequences of such interfacing and scalable fabrication enabling high throughput treatment are still needed to validate their potential for therapeutic T cell engineering.

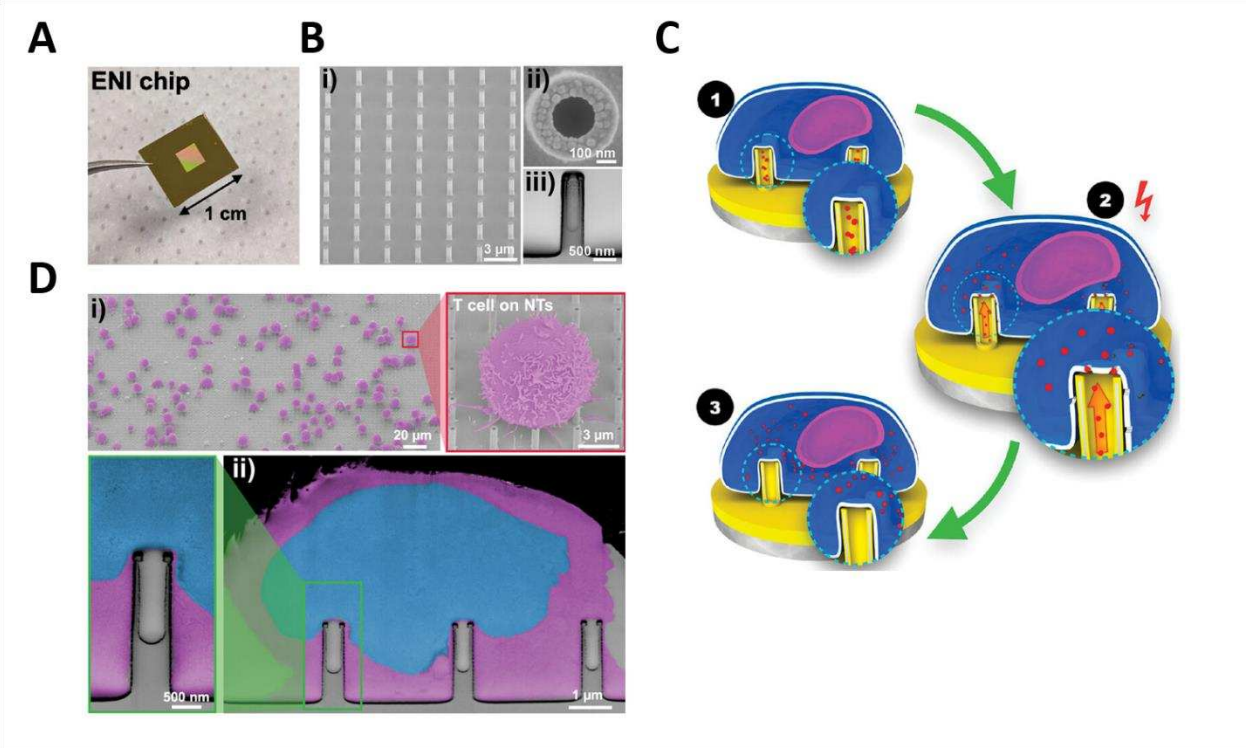


Figure 7. The electroinjection (ENI) platform for intracellular delivery. (A) The ENI chip consists of vertically configured Au-coated nanotube arrays. (B) SEM images of nanotube arrays in (i) zoom-out, (ii) top and (iii) cross-sectional views. (C) Schematic illustration of the ENI's operation mechanism. The nanotubes are first loaded with the targeted cargo molecules and T cells are seeded onto the array with centrifugation applied. Next, a series of low-voltage electric pulses is applied, leading to transient membrane permeabilization at the nanotube-cell interface and the subsequent intracellular influx of cargo molecules. (D) False-colored SEM images showing (i) the interfacial interactions between T cells and nanotube arrays and (ii) the cross-sectional profile of the nanotube-membrane interface. Adapted from Shokouhi *et al.* [237]

3.2 Carrier-mediated delivery systems

As another non-viral strategy, chemical transfection reagents can be used, which mostly rely on endocytic uptake of the complexes that are formed between the cargo RNA and transfection reagent [239,240]. However, lymphocytes are notoriously hard to transfect with conventional chemical transfection reagents such as cationic lipids and polymers. Although the exact mechanism behind this resistance is not well understood, it is most likely related to specific T cell properties, including their small size, high nucleus-to-cytoplasm ratio, nonphagocytic nature and low rates of endocytosis. For instance, it was proposed that insufficient uptake of lipoplexes can be explained by relatively low expression levels of heparan sulfate proteoglycans of which the negatively charged sulfate groups are involved in the initial binding of positively charged particles to the cell membrane [241]. To increase nanoparticle binding and uptake in lymphocytes, the nanomaterial surface can be functionalized with a receptor-specific ligand that selectively binds to T cells and induces receptor-mediated endocytosis, such as CD3, CD4, CD8, CD7 [242–247], β7 integrin [248], PD-1 immune checkpoint [241] and IL-2 receptor [249].

Another factor that can reduce the efficiency of transfection reagents is the slow acidification rate of endosomes in primary T cells, which is often needed as a release mechanism to let the RNA cargo escape the endosomes [250]. Over the years, several strategies to enhance endosomal escape have been reported, including (i) membrane destabilization and membrane fusion using fusogenic lipids and lipid-polymer nanomaterials, (ii) the proton-sponge effect in the presence of buffering polymers, where the influx of protons and chloride ions leads to osmotic endosomal swelling and rupture, (iii) pore formation via cell-penetrating peptides and (iv) photochemical and photothermal disruption [251–256]. Nevertheless, endosomal escape remains the major rate-limiting step in the delivery of RNA therapeutics by chemical transfection agents, with several studies showing that less than 2% of the internalized cargo reaches the cytoplasm [257–259]. Besides enzymatic degradation, nanoparticle excretion from the cell via exocytosis is another mechanism reducing gene delivery efficiency [258]. Also, degradation by cytoplasmic nucleases or clearance by autophagy are factors that can reduce transfection efficiency [260].

3.2.1 Lipid-based nanoparticles

Lipid-based formulations, including natural and synthetic lipids and lipid-like materials (lipidoids), represent the most widely used non-viral gene carriers. Early studies focused on cationic lipids such as DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium-propane chloride) and DOTAP (1,2-dioleoyl-3-trimethylammonium propane), which are composed of positively charged polar head groups and hydrophobic tails connected by a linker group [261,262]. In an aqueous solution, cationic lipids spontaneously self-assemble into higher-order aggregates, retaining their cationic nature in a pH-independent manner (**Figure 8**). Thanks to their cationic amino groups, they can electrostatically interact with the negatively charged phosphate groups of RNAs, leading to the formation of lipoplexes that can shield RNA from nuclease degradation. Lipoplexes obtained by mRNA complexation with cationic liposomes based on DOTMA and helper lipid DOPE were the first lipid-based delivery systems successfully employed for mRNA transfection *in vitro* in 1989 [263]. However, cationic lipoplexes have displayed limitations for in vivo applications such as high instability and rapid clearance by phagocytic cells, leading to significant toxicities and inducing proinflammatory immune responses [264–268]. As such, current research interest has shifted to lipid nanoparticles (LNPs), offering superior stability, structural plasticity and improved gene delivery efficiency [261,269,270].

A typical LNP formulation consists of ionizable or cationic lipids, neutral helper lipids, cholesterol and polyethylene glycol (PEG)- lipid (**Figure 8**). Ionizable lipids are positively charged at acidic pH to condense RNAs during LNP formulation but have a neutral charge at physiological pH to minimize toxicity during systemic delivery [262,270–272]. As such, nanoparticles formulated with pH-responsive lipids demonstrate superior biocompatibility, with prolonged circulation time and reduced off-target accumulation. Following cellular uptake, ionizable lipids can be protonated in the acidic endosomes and interact with anionic endosomal phospholipids to destabilize endosomal membranes and facilitate RNA release in the cytosol. Notable examples of ionizable lipids are the DLin-MC3-DMA (MC3) lipid included in the formulation of Onpattro® (patisiran), which is the first-ever FDA-approved siRNA drug for the treatment of hereditary transthyretin amyloidosis polyneuropathies [273], and biodegradable ionizable lipids, SM-102 and ALC-0315 used

for the formulation of COVID-19 LNP-mRNA vaccines from Moderna (Spikevax®) and Pfizer/BioNTech (Comirnaty®), respectively [261].

Helper lipids such as DSPC (1,2-distearoyl-sn-glycero-3- phosphocholine) or DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) can stabilize the membrane structure of LNPs and facilitate endosomal escape [274]. The cholesterol fraction regulates membrane rigidity and fluidity, promoting particle stabilization by inserting into the inter-phospholipid spaces [275]. In addition, incorporating cholesterol increases the LNP half-life in circulation by reducing the protein binding ability and plays a key role in cell transfection, potentially by promoting membrane fusion and endosomal escape. Although most LNPs are formulated with unmodified cholesterol, hydroxycholesterol substitution has been recently shown to improve by a factor of two mRNA delivery to primary human T cells *ex vivo* without altering LNP stability [276].

The hydrophilic PEG-lipid fraction is the lowest of all LNP components (~1-2 mole percentage) but has a considerable impact on their physicochemical properties (size, polydispersity and surface charge), shaping LNP pharmacokinetics upon systemic administration [277,278]. Incorporation of PEG-lipids increases LNP colloidal stability and prolongs their blood circulation time by reducing serum protein opsonization and clearance by the mononuclear phagocyte system *in vivo*. However, PEGylation can also hinder cellular internalization and endosomal escape, thus limiting nucleic acid delivery. In addition, PEGylated LNPs can be rapidly cleared from circulation upon repeated administration, as a consequence of antibody-mediated immune responses against the PEG component - commonly referred to as the accelerated blood clearance (ABC) phenomenon [279,280]. One strategy to address these challenges is to use diffusible or fast-shedding PEG-lipids with a short bilayer anchor, such as PEG2000-DMG (1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000) or PEG2000-c-DMG (PEG-carbamate-1,2-dimyristoyl-sn-glycerol) [278].

As mentioned before, the surface of LNPs can be decorated with specific targeting ligands to direct their cell-specific uptake. For example, the Peer group developed a customizable LNP platform for targeted *in vivo* siRNA delivery in lymphocytes [281]. In this work, LNPs are non-covalently coated with targeting antibodies via a recombinant membrane-anchored lipoprotein

that is incorporated in the lipid bilayer and interacts with the antibody Fc domain. Using targeting antibodies directed against CD3, CD4 and CD25, the authors demonstrated efficient delivery and silencing in different murine T cell subsets. In another study, the same research group showed effective lymphocyte targeting with a pan leukocyte β7 integrin [248].

Current state-of-the-art LNP fabrication strategies rely on microfluidic rapid mixing of the organic phase containing the lipids and mRNA dispersed in the aqueous phase, offering high encapsulation efficiency and good batch-to-batch reproducibility [282,283]. Such manufacturing process can be scaled-up to meet clinical scale demands, as best exemplified by unprecedently large and rapid rollout of COVID-19 LNP-mRNA vaccines.

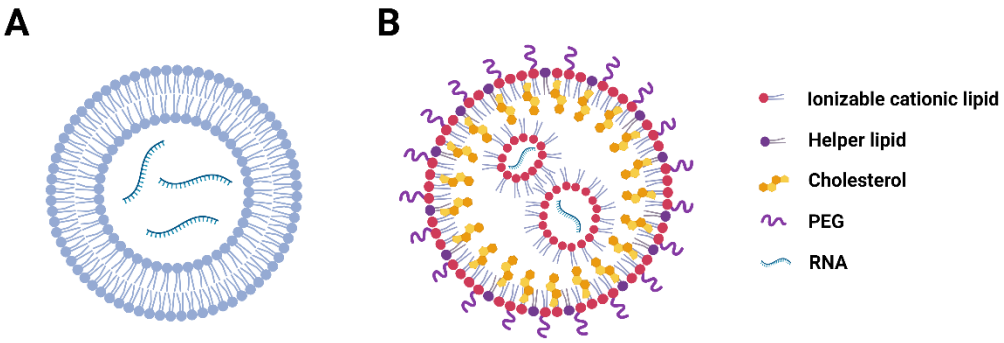


Figure 8. Schematic representation of lipid-based carriers for RNA delivery in T cells. (A) Liposomes consist of a lipid layer and an aqueous core. (B) LNPs are composed of multiple lipid layers and a densely packed core encapsulating the nucleic acid cargo. A typical LNP formulation consists of ionizable cationic lipids, neutral helper lipids, cholesterol and polyethylene glycol (PEG)- lipids.

3.2.2 Polymer-based nanoparticles

Polymer compounds and their derivatives represent another class of materials explored for gene delivery. Such carriers typically rely on cationic polymers able to complex negatively charged nucleic acids, forming so-called polyplexes. One of the most widely studied polymeric materials is polyethyleneimine (PEI) which offers high transfection efficiencies thanks to its high buffering capacity below physiological pH. Once internalized, protonation of PEI amine groups causes

928 osmotic swelling and endosomal rupture leading to endosomal escape via the proton sponge
929 effect [284]. However, since unmodified PEI is highly toxic and nonbiodegradable, several strategies,
930 such as shielding or copolymerization, have been proposed to increase its biocompatibility. For
931 instance, PEG-grafted-PEI copolymers have been used to transfect siRNA to primary T cells *in*
932 *vitro* [285] and deliver mRNA to immune cells in the lungs [286]. Alternatively, poly(2-(dimethylamino
933 ethyl methacrylate (PDMAEMA) is a water-soluble cationic polymer known for its pH- and
934 temperature-responsive properties [287]. In 2012, Schallon *et al.* reported on PDMAEMA-based
935 star-shaped nanoparticles for siRNA delivery in primary human T cells, reaching around 40% CD4
936 silencing [288]. Later, Olden *et al.* evaluated different pHEMA-g-pDMAEMA polymer architectures
937 for mRNA transfection in T cells, reporting transfection efficiencies of up to 50% and 25% in the
938 Jurkat T cell line and primary human T cells, respectively [289]. The authors identified reduced
939 cellular uptake and slower endosomal acidification as the major barriers to carrier-mediated T
940 cell transfections [250].

941 Another interesting class of polymers are biodegradable poly(beta-amino ester)s (PBAEs),
942 synthesized by conjugating amine monomers to diacrylates [290]. The Stephan's group published a
943 series of studies on T cell-targeted gene nanocarriers comprising of i) PBAE polymer matrix to
944 condense the nucleic acid, ii) negatively charged polyglutamic acid (PGA) coating to reduce off-
945 target binding and iii) surface-anchored targeting ligands (**Figure 9**). First, the authors explored
946 anti-CD3 antibody-conjugated NPs to deliver a CD19 CAR encoding pDNA and PiggyBac
947 transposase to circulating T cells [291]. Such particles enabled specific adsorption to 34% of the
948 circulating lymphocytes, while persistent CAR gene expression was observed in up to 4% of cells.
949 Next, PBAE-based nanocarriers proved suited for *ex vivo* T cell engineering with mRNA as well [292].
950 Transfection of mRNA encoding megaTAL nuclease targeting the TRAC locus resulted in an
951 average TCR knock-out of ~60%. In addition, the authors demonstrated transfection of mRNA
952 encoding FoxO1 transcription factor to promote the generation of central memory T cells which
953 are characterized by superior anti-tumor efficacy [292]. In another study, the same lab reported on
954 mRNA transfection for *in situ* T cell engineering (**Figure 9**) [293]. Here CD3- or CD8-targeted PBAE
955 nanoparticles loaded with 1928z CAR encoding mRNA were shown to reprogram ~10% of the
956 circulating T cells. In a mouse model of human leukemia, repeated infusions of these polymer

carriers proved effective in controlling tumor progression, resulting in a 26-fold reduced tumor burden after three weeks of therapy compared to controls.

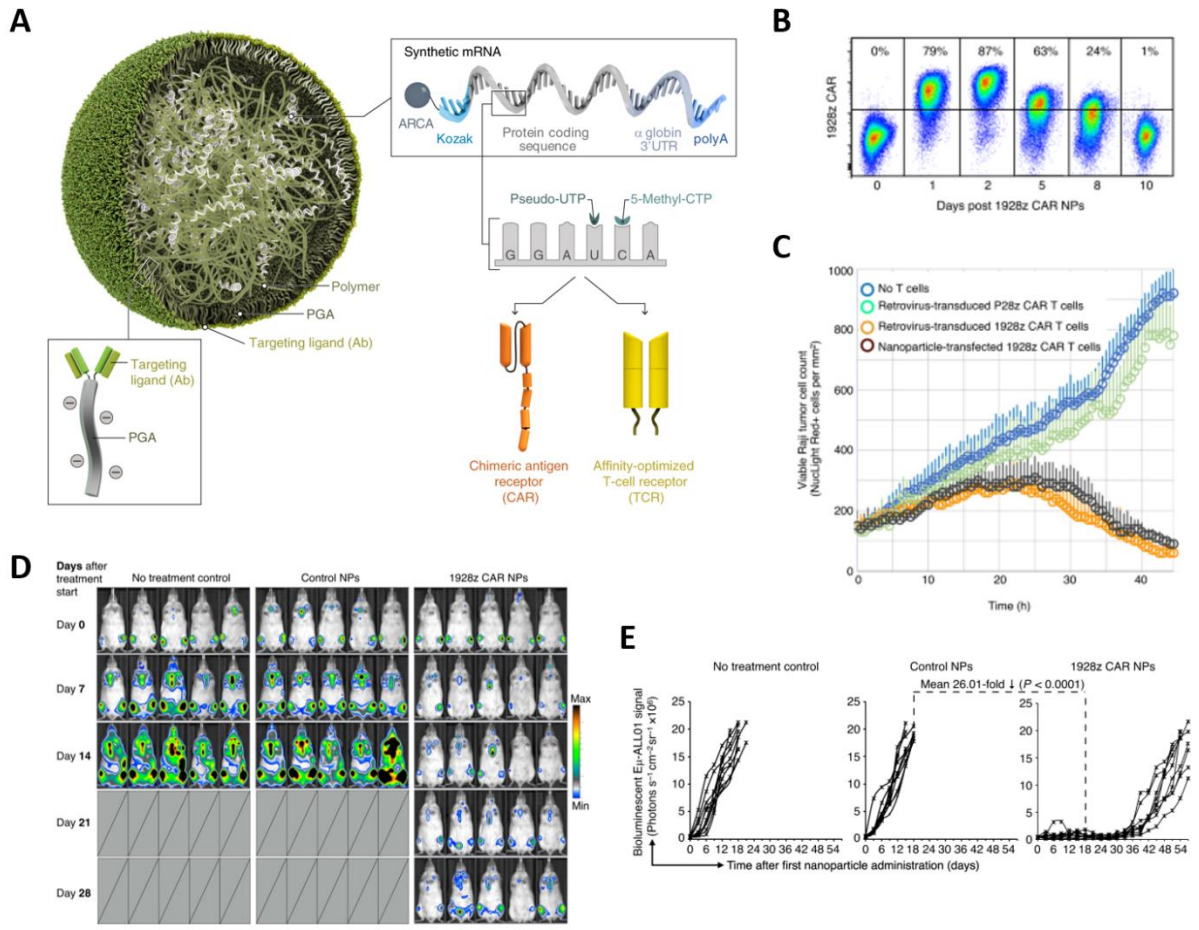


Figure 9. Polymeric nanoparticles for CAR mRNA delivery in T cells. (A) Schematic illustration of CD8-targeting poly(beta-amino ester) (PBAE) nanocarriers encapsulating IVT mRNA. (B) Nanoparticles mediated efficient but transient T cell transfection with CD19 CAR *in vitro*. (C) NP-transfected CAR T cells demonstrated comparable to retrovirally-transduced cells lysis activity against Raji lymphoma cells. (D-E) In a mouse model of B cell acute lymphoblastic leukemia, multiple infusions of CD19 CAR mRNA nanocarriers effectively controlled tumor progression, reducing tumor burden and prolonging average host survival time. Compilation of results adapted from Parayath *et al*.[293]

4 Applications of RNA therapeutics in T cell engineering

4.1 Engineering cancer specific T cells

T cells engineered to express tumor specific TCRs and CARs using viral vectors have shown considerable clinical success in adoptive cell therapy for various cancers. Among non-viral approaches, most preclinical and clinical studies have used electroporation for transfecting T cells with mRNA encoding for chimeric antigen receptors.

B cell malignancies were the first hematological malignancies to be effectively targeted with CAR T cells directed against CD19 surface antigen. In 2006, Rabinovich and colleagues were the first to generate CD19 CAR T cells by IVT mRNA electroporation, demonstrating their target-specific cytotoxicity *in vitro* [294]. In 2009, the same group showed that such CD19 mRNA modified CD3+CD8+ T cells could inhibit tumor progression in a humanized mouse model of Daudi lymphoma [295]. Barrett *et al*. evaluated the cytotoxic potential of CD19-mRNA redirected T cells in a xenograft model of acute lymphoblastic leukemia (ALL), demonstrating T cell migration to distant sites of disseminated tumor with preserved lytic activity and prolonged mice survival [296]. In another study, the authors proposed an optimized protocol based on multiple CD19 mRNA CAR T cell infusions combined with interval lymphodepletion to achieve antitumor efficacy comparable to that mediated by lentiviral-generated stable CAR T cells [297]. Building upon the preclinical success of mRNA-engineered CD19 CAR T cells, as well as the clinical success of lentiviral CD19 CAR T cells in leukemia, the University of Pennsylvania opened a clinical trial in 2014 using CD19-targeted mRNA-engineered T cells in patients with relapsed of refractory classical Hodgkin's lymphoma (NCT02277522 and NCT02624258; **Table 1**). This lymphoma is characterized by scant CD19-negative Hodgkin and Reed-Sternberg (HRS) cells within an immunosuppressive tumor microenvironment, which poses limitations for approaches directly targeting antigens expressed on HRS cells [298]. Instead, CAR T cells were targeted against CD19+ B cells in the tumor microenvironment and putative circulating CD19+ HRS cells to disrupt the immunosuppressive milieu, indirectly affecting HRS cell survival. Among four patients administered with mRNA CAR T cells, one patient achieved transient complete response, one

showed partial response, one showed stable disease and one progressed. Owing to the transient CAR mRNA expression, the therapy was well tolerated, with no severe toxicity reported [298].

Beyond CD19, other targets for hematological malignancies have been investigated as well. For instance, Panjwani *et al.* reported on the successful development of canine CD20 mRNA CAR T cells, which induced modest and transient antitumor activity in a dog with relapsed B cell lymphoma [299]. Since a subset of patients who relapse after CD19 CAR T cell therapy demonstrated outgrowth of CD19-negative tumor cells, Köksal *et al*. evaluated CD37 as an alternative target for CAR-based therapy of B-cell non-Hodgkin lymphoma [300]. *In vitro* comparison between CD37-targeting and CD19-directed mRNA CAR T cells showed a similar killing efficacy towards human Burkitt's lymphoma cell line BL41 and diffuse large B cell lymphoma cell line U-2932. In addition, CD37 CAR T cells proved as potent as CD19 counterparts in controlling tumor growth in a murine BL-41 xenograft model and outperformed CD19 CAR T cells in treating mice engrafted with U-2932 tumors that contained a CD19-negative population [300]. Compared to B-cell malignancies for which CAR T cell products have already been approved, identifying a target for CAR T cell therapy in myeloid malignancies such as acute myeloid leukemia (AML) has proven particularly challenging. Since surface antigens expressed on AML cells are usually shared with normal hematopoietic progenitors, targeting them can lead to significant on-target off-tumor toxicity. CD33 and CD123 represent the most commonly investigated markers for CAR T cell engineering in AML treatment. When Kenderian *et al*. evaluated lentiviral CD33 CAR T cells in a xenograft mouse model of AML, effective anti-tumor responses were accompanied by significant hematopoietic toxicity [301]. The authors subsequently generated mRNA modified CD33 CAR T cells that displayed potent but transient anti-leukemic activity, thus avoiding previously seen myelotoxicity. Similar findings were reported for lentivirally transduced CD123-redirected CAR T cells in preclinical AML models, with efficient leukemia eradication coming at the cost of severe hematologic toxicity [302]. The same group then assessed mRNA modified CD123 CAR T cells in a MOLM14 xenograft model, demonstrating rapid AML clearance and remission for >6 months [303]. In a pilot clinical trial at the University of Pennsylvania (NCT02623582; **Table 1**), mRNA CD123 CAR T cells were tested in patients with relapsed/refractory AML, with the primary objective of showing safety [304]. Although the therapy was proven safe, no anti-tumor efficacy could be

demonstrated. The team reported manufacturing difficulties due to the poor quality of patient T cells and a lack of persistence of administered CAR T cells. However, a sufficiently safe profile was established in this study, allowing to proceed with clinical testing of CD123 CAR T cells generated with lentiviral vectors.

B-cell maturation antigen (BCMA) is the most common target for CAR T cell therapy in multiple myeloma (MM). Li *et al*. reported on the development of Descartes-08, an autologous CD8+ T cell-only product modified with anti-BCMA CAR mRNA, demonstrating potent cytolytic activity in MM cells and prolonged host survival in a mouse model of disseminated human myeloma [305]. Preliminary results from the phase I/II clinical trial of Descartes-8 in relapsed/ refractory myeloma patients (NCT03448978) indicated good tolerability and durable responses. A phase II clinical trial (NCT04436029) has been initiated to evaluate Descartes-11, a humanized version of Descartes-8, as a consolidative therapy in patients with newly diagnosed, high risk multiple myeloma who have residual disease after induction therapy. Interestingly, BCMA-targeting CAR T cells are also being evaluated in phase I/II clinical trial (NCT04146051) in patients with generalized myasthenia gravis, a neuromuscular autoimmune disease driven by self-reactive antibodies produced by plasma cells. According to a recently published update, Descartes-8 infusions were safe and well-tolerated, resulting in clinically meaningful improvements in disease severity for up to nine months [306].

Beyond targeting hematological malignancies, IVT mRNA-modified CAR T cells have been widely investigated for the treatment of solid tumors, where T cells face additional physical and immune hurdles that impede T cell tumor penetration and persistence at the tumor sites. These include vascular and stromal barriers, tumor antigen heterogeneity and nutrient-poor and immunosuppressive milieu. Some early studies focused on mesothelioma, a type of malignant tumor that occurs in tissues lining the heart, stomach and lungs. Zhao *et al*. designed IVT mRNA CARs targeting mesothelin, a tumor associated antigen (TAA) overexpressed in mesothelioma, ovarian and pancreatic cancers [307]. The authors demonstrated that repeated (intratumor) administration of mRNA-modified mesothelin CAR T cells markedly reduced flank mesothelioma tumors in a mouse model. In addition, similar anti-tumor efficacy was observed in a disseminated

intraperitoneal tumor model established with patient-derived mesothelioma and treated by multiple injections of autologous anti-mesothelin CAR T cells, suggesting that autologous T cells can be effectively redirected against TAAs using IVT mRNA. Based on this work, two clinical studies were initiated to evaluate the safety and feasibility of mesothelin-directed mRNA CAR T cell therapy in patients with malignant pleural mesothelioma (NCT01355965) and metastatic pancreatic cancer (NCT01897415). Preliminary analysis of four patients showed that the approach was well tolerated, except for one patient, who developed severe anaphylactic shock after the third CAR T cell infusion received after a four-week treatment interruption [308]. It was hypothesized that the anaphylactic event resulted from the induction of IgE antibodies against murine sequences in the CAR construct. The authors adjusted the schedule of infusions, avoiding breaks longer than 10 days in order to prevent further anaphylactic incidences. In a follow-up study, Beatty *et al*. reported on the efficacy of the mesothelin-targeted mRNA CAR T cells in two patients, including the one who had experienced anaphylactic shock [309]. Both patients demonstrated a partial response, with evidence of humoral epitope spreading, suggesting the induction of an adaptive immune response. In 2018, Beatty *et al*. published follow-up results from six patients with metastatic pancreatic ductal adenocarcinoma [310]. None of the patients experienced cytokine release syndrome or neurologic symptoms, nor were dose-limiting toxicities observed. The best overall response achieved with a total of 9 doses of mRNA CAR T cells was stable disease in two patients. One other patient showed a reduction of liver lesions but no effect on the primary pancreatic tumor, suggesting distinct biology between the primary and metastatic disease. The therapy induced a spreading antibody response with increased production of antibodies against multiple proteins, including immunomodulatory molecules such as PD-1, PD-L1 and BCMA. The authors proposed that mesothelin-directed CAR T cells may serve as a probing tool to investigate the immunobiology of pancreatic tumors and guide further development of effective T cell therapies for this condition.

Another target investigated for peritoneal tumors is epithelial cell adhesion molecule (EpCAM), expressed on the normal epithelium and upregulated in peritoneal carcinomatosis from gastrointestinal and gynecological malignancies. Ang *et al*. evaluated EpCAM mRNA CAR T cells in peritoneal dissemination mouse models of human ovarian and colorectal cancers,

demonstrating that repeated injections of CAR T delayed tumor growth and prolonged mice survival but were unable to eradicate the disease [311].

GD2 ganglioside and glypican 2 (GPC2) are examples of tumor associated antigens studied for central nervous system tumors. Singh *et al*. compared the efficacy of mRNA-modified and lentivirally-modified GD2 CAR T cells in local and disseminated xenograft models of neuroblastoma [312]. While intratumoral injection of mRNA GD2 CAR T cells in a localized model resulted in tumor regression, multiple infusions in a disseminated model slowed disease progression and improved survival but could not achieve long-term disease control. Histologic examination showed that, unlike permanently-modified cells, mRNA GD2 CAR T cells were unable to penetrate the tumor environment, implicating that the transient nature of mRNA expression would require local delivery to realize mRNA CAR T cell therapeutic potential. More recently, Foster *et al*. developed GPC2-directed mRNA CAR T cells demonstrating significant cytotoxicity in GPC2-expressing medulloblastoma and high-grade glioblastoma cell lines *in vitro* [313]. In addition, repeated locoregional delivery of mRNA GPC2 CAR T cells induced tumor regression in an orthotopic medulloblastoma model and prolonged mice survival in a thalamic diffuse midline glioma xenograft model.

Several TAAs have been investigated for T cell therapy of melanoma, including vascular endothelial growth factor receptor 2 (VEGFR2), gp100 and melanoma-associated chondroitin sulfate proteoglycan (MCSP; or chondroitin sulfate proteoglycan 4, CSPG4) [314–317]. Inoo *et al*. reported that triple administration of mRNA VEGFR2 CAR T cells in a B16-BL6 murine melanoma model achieved similar tumor growth inhibition as a single transfer of retrovirally-transduced CAR T cells [318]. Another strategy is to use T cells expressing two additional receptors (TETARs) that hold the potential to overcome immune escape due to single antigen loss. Hofflin *et al*. reported on developing mRNA-modified T cells targeting gp100 and a patient-specific, individually mutated antigen [314]. These dual-CAR T cells demonstrated specific lytic activity towards target cells loaded with each of their cognate antigens *in vitro*. Uslu *et al*. generated mRNA CD8+ TETARs co-expressing a CAR specific for MCSP antigen and a TCR specific for gp100 antigen, showing antigen-specific cytokine production and killing capacity against A375M and

Mel526 melanoma cell lines [315]. Of note, TETARs stimulated with both cognate antigens displayed higher cytolytic potential compared to a mixture of monospecific T cells transfected with either a CAR or TCR, indicating that TETARs were indeed able to recognize and target both antigens at the same time.

Hepatocyte growth factor receptor (c-Met) is a TAA expressed in various solid tumors. mRNA-modified c-MET CAR T cells have been evaluated in two clinical trials for the treatment of breast cancer and melanoma (NCT01837602, NCT03060356). Tchou *et al*. first demonstrated that mRNA c-MET CAR T cells elicited potent cytolytic effects in human breast cancer cell lines BT20 and TB129, and suppressed tumor growth in a murine model of human ovarian cancer [319]. Next, a phase 0 study was initiated to evaluate intratumoral administration of mRNA c-MET CAR T cells in patients with metastatic breast cancer. The treatment was well-tolerated, without significant side effects, but no clinical responses were observed. Histologic examination of excised tumors revealed extensive tumor necrosis, loss of c-MET immunoreactivity and macrophage infiltration, suggesting an inflammatory response evoked by the treatment [319]. Based on these observations, a phase I study (NCT03060356) was launched to evaluate intravenously administered mRNA c-MET CAR T cells in patients with malignant melanoma and metastatic breast cancer [320]. Treatment was safe, with only grade 1 or 2 adverse events observed, but no CRS or grade 3 toxicities. Out of 7 patients, four achieved stable disease while three experienced disease progression. The authors hypothesized that the lack of treatment response could be related to limited trafficking to tumor sites since no mRNA signal was detected in post-infusion tumor tissue.

Although *ex vivo* electroporation remains the most advanced non-viral strategy for therapeutic T cell engineering, alternative approaches based on lipid and polymer nanoformulations have been recently explored for *in vitro* and *in vivo* lymphocyte transfection. For instance, Billingsley *et al*. synthesized a library of 24 ionizable lipids and formulated them into LNPs [321]. The top-performing formulation was then used for CAR mRNA transfection in primary human T cells, achieving CAR expression levels comparable to electroporation and potent cytolytic activity against Nalm-6 acute lymphoblastic leukemia cells *in vitro*. Rurik *et al*. reported on the *in vivo* generation of

antifibrotic CAR T cells as a therapeutic strategy for cardiac injury [322]. The authors designed CD5-targeted LNPs to deliver mRNA encoding a CAR against fibroblast activation protein (FAP). 48h after intravenous administration of LNPs in a mouse model of hypertensive cardiac injury, FAB CAR expression was found in ~20% of splenic T cells. Mice treated with such *in vivo* produced CAR T cells displayed reduced fibrosis and marked cardiac function improvements. In another study by Stephan's lab, CD3-targeted PBAE nanocarriers loaded with IVT mRNA encoding specific CAR or TCR transgenes were used to reprogram T lymphocytes *in situ* [293]. Nanoparticle-transfected CAR T cells showed effector cytokine secretion and antigen-specific lysis of target cancer cells at levels comparable to those of virally transduced T cells. In murine models of human leukemia, prostate cancer and hepatitis B-induced hepatocellular carcinoma, repeated infusions of these polymer NPs programmed sufficient antigen-specific T cells to induce disease regression at levels similar to bolus administration of *ex vivo* engineered lymphocytes. If successful, *in situ* T cell reprogramming could potentially overcome the current limitations of the complex, lengthy and expensive *ex vivo* manufacturing process, making T cell therapies more accessible to patients.

An overview of clinical trials employing IVT mRNA in adoptive T cell therapy is provided in **Table 1**.

4.2 Gene editing for enhancing T cell function

In addition to introducing exogenous receptors, recent advances in gene editing technologies have opened new avenues to generate T cells with improved phenotypical characteristics, enhanced anti-tumor efficacy and the potential to be used in allogeneic applications. As discussed earlier, CRISPR-Cas9 components can be delivered to cells in various formats, such as plasmid DNA, mRNA and gRNA, or RNP complexes. In particular, delivery of mRNA encoding Cas9 nuclease alongside gRNA alleviates the risk of potential genome integration and, thanks to its transient expression profile, reduces off-target effect probability. Therefore, we mainly focus on studies describing nuclease delivery in such mRNA format. For a more comprehensive overview of CRISPR-Cas applications in T cell engineering, we refer the reader to recently published reviews [323–327].

One widely investigated area is to use CRISPR-Cas technology to replace endogenous T-cell receptors with transgenic TCRs to avoid competition in signaling and mispairing between native and transduced TCRs. This strategy can be further extended to generate “off-the-shelf” allogenic CAR T cell products. Since manufacturing of autologous T cell therapies is often hampered by low yield and poor functionality of lymphocytes collected from elderly and heavily-pretreated patients, collection of allogeneic, healthy donor leukocytes represents an attractive alternative route to produce ”universal” tumor-specific T cells with optimized persistence and anti-tumor efficacy [323,326]. However, one major challenge to allogenic transplantation is the induction of graft-versus-host-disease (GvHD), where donor lymphocyte TCRs recognize surface antigens of the patient as foreign (non-self), eliciting an immune response. In addition, alloantigens expressed on transplanted cells, such as human leukocyte antigen (HLA-1), may provoke unwanted host immune responses (allorejection). Therefore, CRISPR-Cas9-mediated knock-out of endogenous TCRs and HLA-1 molecules could improve the compatibility of allogeneic CAR T cells. In particular, the T-cell receptor α constant (TRAC) locus has been extensively investigated as a suitable target for combined gene knock-out and CAR knock-in. More specifically, placing the CAR transgene under the control of the endogenous TRAC promotor could drive robust CAR expression comparable to physiological TCR expression levels while simultaneously disrupting the endogenous TCR to eliminate GvHD concerns. For instance, Eyquem *et al*. electroporated Cas9 mRNA and sgRNA to target the TRAC locus and disrupt native TCR expression[328]. Subsequent transduction with an AAV vector encoding CD19 CAR DNA was used to induce CAR expression under the transcriptional control of the TRAC promotor. Directing CD19 CAR to the TRAC locus resulted in uniform CAR expression, reduced tonic signaling and delayed T cell differentiation and exhaustion. In a mouse model of acute lymphoblastic leukemia, TRAC-CAR T cells demonstrated potent anti-tumor responses and prolonged median host survival, outperforming conventional retrovirally transduced CARs, with and without TCR knock-out. A similar strategy exploiting cellular homology-directed repair (HDR) mechanism was reported by MacLeod *et at*. who combined an engineered homing nuclease and an AAV donor template for HDR-mediated insertion of the CD19 CAR transgene into the native TCR locus [329].

In another study, Georgiadis *et al*. employed a CD19 CAR lentiviral vector with a TRAC-targeting sgRNA sequence incorporated into the 3' long terminal repeat to mitigate potential interference effects [330]. Pre-transduced T cells were electroporated with Cas9 mRNA to induce the TRAC locus cleavage and subsequently enriched into a highly homogenous CD19+TCRαβ- population by magnetic depletion of residual TCRαβ+ cells. In a mouse model of human Daudi B cell leukemia, TCR-negative CD19 CAR T cells demonstrated effective tumor eradication without xenoreactive GvHD and reduced expression of exhaustion markers compared with conventional TCR-expressing CD19 CAR T cells. In 2022, the same group reported on the results of a phase I clinical trial (NCT04557436) of allogeneic CRISPR-engineered CD19 CAR T cells for the treatment of children with refractory B cell leukemia [331]. Lymphocytes collected from healthy adult donors were transduced with a CAR19 lentiviral vector incorporating CRISPR guide sequences targeting TRAC and CD52 loci, whose disruption upon Cas9 mRNA delivery by electroporation was intended to prevent GvHD and confer resistance to alemtuzumab used during lymphodepletion. The primary goal of the TT52CAR19 T cell application was to secure molecular remission ahead of programmed allogeneic stem cell transplantation (SCT). Four of six CAR-infused children exhibited cell expansion and achieved remission by day 28, after which they received allo-SCT. Two patients later relapsed and two remained in ongoing remission. Despite reported toxicities, primary safety objectives were met, providing early-stage evidence of feasibility and therapeutic potential of CRISPR-engineered immunotherapy.

To the best of our knowledge, no studies have yet reported on nanoformulation-mediated delivery of Cas9 mRNA specifically to T cells. However, other preclinical and clinical studies have already indicated the potential of nanoparticle-based transfection of gene-editing nucleases. For example, lipid NPs encapsulating Cas9 mRNA and sgRNA targeting transthyretin have been evaluated in a phase I clinical trial for *in vivo* gene editing in patients with hereditary transthyretin amyloidosis (NCT04601051) [332]. In a preclinical study by Moffett *et al*., polymeric NPs carrying mRNA encoding megaTAL nuclease targeting the TRAC locus demonstrated efficient TCR knockout in ~60% of T cells [292]. NP-mediated gene editing did not affect the efficiency of subsequent lentiviral transduction or the functionality of programmed CAR T cells.

Apart from endogenous TCRs, gene editing has been employed to disrupt inhibitory signals that contribute to T cell exhaustion and reduced antitumor efficacy. Many studies have focused on deleting immune checkpoint receptors, such as programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4). Beane *et al*. reported on PD-1 disruption in melanoma tumor-infiltrating lymphocytes (TILs) via electroporation of zinc finger nuclease mRNA, demonstrating their improved *in vitro* effector function and an increased polyfunctional cytokine profile [333].

CRISPR-Cas9 has also been used as an efficient strategy for simultaneous editing of multiple gene loci. Ren *et al*. used Cas9 mRNA electroporation to generate universal CAR T cells with enhanced resistance to apoptosis by disruption of endogenous TCR, HLA-I and CD95/Fas death receptor [334]. These triple-negative CAR T cells displayed increased expansion, prolonged survival in the peripheral blood and enhanced tumor control efficacy in a Nalm6 leukemia model. In another study, the same group reported on TRAC, β-2-microglobulin (B2M) and PD-1 disruption in lentivirally transduced CD-19 or prostate stem cell antigen (PSCA) CAR T cells to eliminate GvHD and host-versus-graft effects, and to increase CAR T cell activity [335]. HLA-I and TCR double negative T cells showed reduced alloreactivity compared to a single TCR- knockout, while additional disruption of PD-1 resulted in enhanced antitumor activity in a Nalm6-PD-L1 leukemia model, as evidenced by quicker elimination of leukemia cells.

Beyond Cas9, other CRISPR variants have also been explored for multiplex gene editing in T cells. Dai *et al*. used a tracrRNA-independent Cas12a/ Cpf1 nuclease to demonstrate CD22 CAR integration into the TRAC locus combined with PD-1 knock-out [336]. Compared to Cas9-edited cells, Cpf1-modified CD22 CAR T cells displayed similar cytokine production and cancer cell killing but reduced expression of exhaustion markers. Webber *et al*. reported on the application of CRISPR base editors delivered by mRNA electroporation to knock-out TRAC, B2M and PD-1 for allogeneic CAR T cell generation [337]. Cell modification with base editors reduced DSB induction and translocation frequency compared to Cas9 nuclease-mediated engineering. In addition, the authors noted higher rates of nontarget editing and indel formation when using the RNP format instead of mRNA. In another study by Gaudelli *et al*., base editors were used to target TRAC, B2M

and class II transactivator (CIITA) to reduce the expression of the endogenous TCR and MHC class I and II machinery [338].

Altogether, these studies highlight the potential of mRNA based gene editing technologies to improve the overall efficacy of T cell therapies. Some strategies combining viral vector CAR transduction with TALEN or CRISPR-Cas-enabled modifications have already entered clinical evaluation (**Table 2**), as reviewed in more detail elsewhere [323,325,326,339].

4.3 Other strategies to modulate T cell functionality

Effective anti-tumor T cell responses depend on multiple steps such as recognition of tumor-specific antigens, upregulation of activation markers and co-stimulatory molecules, *in vivo* proliferation, trafficking to the tumor site and preserving effector functions in a highly immunosuppressive tumor milieu. Upregulation of inhibitory receptors, downregulation of MHC class I expression on tumor cells and secretion of anti-inflammatory molecules can all contribute to T cell dysfunction, which can be mitigated by approaches based on immunomodulation with cytokines and co-stimulatory ligands and receptors. For instance, mRNA transfection can be employed to temporarily equip T cells with stimulatory receptors, enabling transient activation of inflammatory signaling. Pato *et al*. electroporated TILs from melanoma patients with mRNA encoding constitutively active TLR4 (caTLR4), which resulted in upregulation of CD25 and 4-1BB, increased IFNγ secretion and enhanced anti-melanoma cytolytic activity *in vitro* [340]. Similar responses were observed by Levin *et al*. upon TIL electroporation with caCD40 mRNA [341].

Furthermore, mRNA delivery has been leveraged to provide a transient and localized stimulation with membrane-bound cytokines, circumventing severe toxicities related to high-dose systemic administration. Weinstein-Marom *et al*. reported on electroporation of mRNA encoding membrane-anchored variants of IL-2, IL-12 and IL-15 in human CD8+ T cells and melanoma TILs [342,343]. Membrane-associated cytokines bound to their corresponding surface receptors mainly in *cis*, thus confining a stimulatory effect to the transfected cells only. The engineered cytokines were found to support the *ex vivo* proliferation of activated T cells to a similar extent as their soluble counterparts. Co-delivery of cytokine mRNA with mRNA encoding for caTLR4 and/or caCD40 mRNA induced IFN gamma secretion, upregulation of T cell activation markers (CD25,

CD69, 4-1BB and OX40) and improved the cytotoxicity of TILs against autologous melanoma cells *in vitro* [343]. Etxeberria *et al*. engineered tumor-specific CD8+ T cells to transiently express IL-12 and CD137 (4-1BB) ligand, showing that intratumoral injection of such modified cells led to epitope spreading and regression of both injected and distant lesions in solid tumor models [344]. In addition, patient-derived TILs electroporated with IL-12 mRNA demonstrated significant IFN gamma production and anti-tumor efficacy in a patient-derived xenograft mouse model of endometrial cancer, supporting the clinical feasibility of such an approach. In another study, the same group reported on intracavitary administration of IL-12 mRNA-engineered T cells to eradicate peritoneal metastasis in mouse models [345]. Transient IL-12 expression contributed to a favorable reprogramming of immune cells in the tumor microenvironment, prolonged *in vivo* persistence of transferred T cells and development of more durable immunity after primary tumor eradication.

Beyond mRNA transfection, other strategies to modulate T cell signaling towards improved survival and antitumor efficacy rely on the application of agonistic and antagonistic aptamers as an alternative to antibodies. Agonistic aptamers can be selected to specifically bind to T cell surface receptors, promote crosslinking and trigger downstream signaling cascades, inducing T cell proliferation and effector function against tumor cells. For example, McNamara *et al*. developed a multivalent aptamer that binds to 4-1BB costimulatory receptor [346]. The aptamer co-stimulated CD8+ T cell activation *in vitro*, as evidenced by enhanced proliferation and IFN gamma secretion in suboptimally stimulated cultures and mediated tumor rejection in mice with efficacy similar to that of an anti-4-1BB monoclonal antibody. Pastor *et al*. generated dimer anti-CD28 agonistic aptamers, demonstrating strong costimulatory activity surpassing that of monoclonal antibodies *in vitro* and potent adjuvant effects enhancing cellular and humoral responses in the context of tumor vaccination [347].

Antagonistic aptamers that can block the interaction between a receptor and its ligand represent yet another strategy to disrupt inhibitory signals contributing to T cell functional impairment in cancer. Santulli-Marotto *et al*. first developed an aptamer against CTLA-4 immune checkpoint, showing that aptamer tetramerization was required to enhance antitumor effects [348]. In another

study, Berezhnoy *et al*. selected an IL-10 receptor (IL-10R) blocking aptamer to disrupt IL-10-mediated immune suppression [349]. The authors reported that systemic administration of IL-10R antagonist inhibited tumor growth in a mouse model to an extent comparable to that of an anti-IL-10R antibody. Other groups explored aptamer use in combinatorial approaches to block multiple immune checkpoints such as PD-1 and T cell immunoglobulin mucin receptor 3 (TIM-3) towards synergistic inhibition of non-overlapping immunosuppressive pathways. For instance, Gefen *et al*. selected a trimeric TIM-3 aptamer that blocked the interaction of TIM-3 with its ligand galectin-9, reduced cell death and promoted T cell proliferation and cytokine secretion *in vitro* [350]. In colon tumor-bearing mice, systemic administration of TIM-3 aptamer delayed tumor growth more effectively than a monoclonal antibody. In addition, aptamer delivery in combination with PD-1 antibody demonstrated a synergistic effect translating to significantly prolonged mice survival.

Finally, aptamers have been used as ligands for targeted intracellular delivery of siRNA mediated by its receptor internalization upon crosslinking. For instance, it has been previously shown that inhibition of IL-2 or mammalian target of rapamycin (mTOR) signaling promotes the development of long-lasting memory T cells [349,351]. Rajagopalan *et al*. used a 4-1BB-binding aptamer conjugated with siRNA against CD25 (IL-2 receptor alpha) to attenuate IL-2 signaling in CD8+ T cells [352]. Systemic administration of this conjugate demonstrated specific downregulation of CD25 in 4-1BB-expressing CD8+ T cells promoting the acquisition of memory phenotype and potentiated vaccine-induced antitumor response in a breast carcinoma model. Alternatively, Berezhnoy *et al*. utilized 4-1BB aptamer coupled with mTOR complex 1-specific siRNA to enhance T cell differentiation into memory cells by inhibiting mTOR signaling [353]. Hermann *et al*. employed a CTLA-4 aptamer to deliver siRNA against immunosuppressive transcription factor STAT-3, whose silencing showed a reduction in tumor-associated regulatory T cells and potentiated antitumor effects in various mouse models [354]. As an alternative strategy, Manrique-Rincon *et al*. used a forkhead box P3 (FOXP3)-specific small antisense RNA conjugated to a CD28-targeting aptamer to inhibit the immunosuppressive phenotype of regulatory T cells and potentiate vaccine responses in a murine melanoma model [355].

Finally, other strategies to augment the therapeutic efficacy of T cell-based immunotherapies have focused on improving homing and T cell persistence at tumor sites. For instance, Mitchell *et al*. showed that electroporation of antigen-specific T cells with mRNA encoding chemokine receptor CXCR2 promoted their migration towards glioma-secreted CXCR-2 specific ligands *in vitro* and *in vivo* [356]. Similarly, Almåsbak *et al*. reported on co-electroporation of mRNA encoding CD19 CAR and chemokine receptors CXCR4 and CCR7 for improved chemotaxis of CAR T cells [357]. Bai *et al*. electroporated CD19 CAR T cells with mRNA encoding for telomerase reverse transcriptase (TERT), demonstrating transiently enhanced telomerase activity and delayed replicative senescence, which translated to improved persistence and long-term anti-tumor efficacy in a mouse xenograft model of B-cell malignancy [358].

Together, these studies demonstrate the utility of RNA therapeutics to enhance T cell functionality towards more efficacious treatment modalities.

5 Conclusions ant outlook

Despite remarkable progress seen in CAR T cell therapy in the last decade, several limitations remain to be addressed to move beyond the treatment of specific hematological malignancies and to make it more accessible to a broader population of patients. To mitigate toxicities and unleash CAR T cell potential for solid tumors, more sophisticated engineering approaches will be required to modulate multiple T cell phenotypical characteristics beyond single antigen-specificity. Most likely, such novel designs will necessitate simultaneous introduction and disruption of multiple genes to acquire multi-antigen specificity, reduce GvHD and HvG effects by removing endogenous TCRs and HLAs and overcome TME-imposed immunosuppression by disruption of negative regulators of T cell activation. These new editing strategies must come hand in hand with developing suitable transfection technologies capable of accommodating evolving CAR constructs, genome editing components and/or complimentary molecules to modulate T cell functionality upon re-infusion in patients. While viral vectors are still used the most for T cell engineering due to their high efficiency, they come with several safety and practical concerns, such as limited cargo capacity, high cost, specialized facility requirements and

regulatory hurdles. Therefore, much research has been devoted to non-viral transfection technologies compatible with the manufacturing of next-generation T cell therapies. Electroporation is the most investigated and clinically advanced non-viral technology, offering high transfection efficiencies, cargo flexibility and compatibility with clinical-grade cell manufacturing systems. However, since it is often associated with substantial cytotoxicity and reduced functionality, alternative physical and carrier-mediated approaches are actively explored, with a focus on preserving cell viability and long-term functionality. Nanostructure arrays, photoporation, chemical poration and microfluidic platforms are all being commercialized, although the latter two have advanced the furthest towards clinical evaluation. Also polymeric but especially lipid based carriers are making rapid progress for T cell engineering, with a promising future towards *in vivo* T cell reprogramming, thus eliminating the need for T cell isolation and *ex vivo* manipulation.

Modification of T cells with IVT mRNA to express specific tumor antigens has demonstrated good tolerability, even though the therapeutic efficacy was limited in multiple clinical trials. Due to the transient expression of CAR mRNA only lasting up to a few days, repeated administration of CAR T cells is required to achieve meaningful anti-tumor responses. Nonetheless, the superior safety profile of mRNA-engineered T cells offers the opportunity to evaluate the safety of uninvestigated CAR designs before more permanent DNA-based CAR therapies are used for long-term expression. In case of severe adverse events, transient mRNA expression allows to rapidly cease the treatment.

Besides redirecting T cell specificity, genome editing with CRISPR-Cas holds great promise to advance the field, offering high gene-editing efficiency, versatility, and relative simplicity. Delivering Cas nuclease in mRNA format reduces the probability of off-target editing events. Finally, RNA molecules showed the potential to transiently modulate T cell phenotype, for instance, by silencing immune checkpoint receptors or upregulating expression of cytokines to enhance T cell proliferation and persistence upon adoptive cell transfer. Taken together, these studies demonstrate that either alone or more likely in combination with DNA-based permanent changes, RNA molecules will play a significant role in shaping next-generation T cell therapies.

1388

Table 1. Clinical trials using IVT mRNA for adoptive T cell therapy

mRNA	Indication	Sponsor	Opened	Status	ClinicalTrial.gov identifier
anti-CD19 CAR	Hodgkin's lymphoma	University of Pennsylvania	2014	Terminated	NCT02277522
anti-CD123 CAR	Acute myeloid leukemia	University of Pennsylvania	2015	Terminated	NCT02623582
anti-c-MET CAR	Malignant melanoma, breast cancer	University of Pennsylvania	2017	Terminated	NCT03060356
Anti-mutant TGFβII TCR	Metastatic colorectal cancer	Oslo University Hospital	2018	Terminated	NCT03431311
anti-mesothelin CAR	Malignant pleural mesothelioma	University of Pennsylvania	2011	Completed	NCT01355965
anti-mesothelin CAR	Metastatic pancreatic ductal adenocarcinoma	University of Pennsylvania	2013	Completed	NCT01897415
anti-mesothelin CAR	Metastatic breast cancer, triple negative breast cancer	University of Pennsylvania	2013	Completed	NCT01837602
anti-BCMA CAR	Multiple myeloma	Cartesian Therapeutics	2018	Completed	NCT03448978
anti-BCMA CAR	Multiple myeloma	Cartesian Therapeutics	2020	Completed	NCT04436029
anti-BCMA CAR	Multiple myeloma	Cartesian Therapeutics	2019	Active	NCT03994705
anti-BCMA CAR	Multiple myeloma	Cartesian Therapeutics	2021	Active	NCT04816526

1389

Table 2. Clinical trials using electroporation to deliver mRNA encoding gene editing nucleases in T cell therapy

Nuclease (mRNA)/ Target knock-out	Indication	Sponsor	Opened	Status	ClinicalTrial.gov identifier
TALEN mRNA TRAC and CD52 KO	B cell acute lymphoblastic leukemia; Pediatric patients	Institut de Recherches Internationales Servier, France	2016	Completed	NCT02808442
TALEN mRNA TRAC and CD52 KO	B cell acute lymphoblastic leukemia; Adult patients	Institut de Recherches Internationales Servier, France	2016	Completed	NCT02746952
Zinc finger nuclease mRNA CCR5 KO	HIV-1	University of Pennsylvania	2015	Completed	NCT02388594
TALEN mRNA CD52 and PD-1 KO	Multiple myeloma	Cellectis S.A.	2019	Active	NCT04142619
TALEN mRNA CD52 and PD-1 KO	B cell acute lymphoblastic leukemia	Cellectis S.A.	2019	Active	NCT04150497
CRISPR/Cas9 mRNA TRAC KO	Acute myeloid leukemia	Cellectis S.A.	2017	Active	NCT03190278
CRISPR/Cas9 mRNA HPK1 KO	B cell malignancies	Xijing Hospital, China	2019	Active	NCT04037566
CRISPR/Cas9 mRNA CISH KO	Metastatic gastrointestinal epithelial cancer	Intima Bioscience, Inc.	2020	Active	NCT04426669

1390

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