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REVIEW

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# RNA-electroporated T cells for cancer immunotherapy

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**ABSTRACT**

Adoptive T cell therapy has proven effective against hematologic malignancies and demonstrated efficacy against a variety of solid tumors in preclinical studies and clinical trials. Nonetheless, antitumor responses against solid tumors remain modest, highlighting the need to enhance the effectiveness of this therapy. Genetic modification of T cells with RNA has been explored to enhance T-cell antigen specificity, effector function, and migration to tumor sites, thereby potentiating antitumor immunity. This review describes the rationale for RNA-electroporated T cell modifications and provides an overview of their applications in preclinical and clinical investigations for the treatment of hematologic malignancies and solid tumors.

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## Introduction

Cancer immunotherapy is a promising approach for potentiating antitumor immunity, with the ability to promote long-lasting antitumor immune responses while restricting the toxicities caused by conventional cancer treatments such as radiation and chemotherapy. Consequently, a number of immunotherapeutic strategies have been evaluated to target cancer over the past couple of decades. Such strategies have included an abundance of vaccine-based strategies to trigger cellular and humoral anti-tumor immunity, as well as antibody-based strategies to mediate complement and natural killer cell (NK)-dependent anti-tumor cytolytic activity, block inhibitory receptors, or modulate the tumor microenvironment.[1–5] However, these approaches are limited by central and peripheral immune tolerance, which prevent immune effector cells from effectively targeting tumor cells due to the lack of a T-cell repertoire to self-antigens overexpressed or aberrantly expressed by tumors. The establishment of an immune-suppressive state also constitutes a limitation to cancer immunotherapies. The use of immune checkpoint blocking antibodies, such as anti-PD1, has proven to be a successful and safe strategy for overcoming peripheral tolerance and has been shown to abrogate tumor growth and enhance antitumor immune responses in multiple preclinical as well as clinical trial studies.[6–10] As a result, immune checkpoint inhibitors have recently been FDA approved for the treatment of various cancers.

One approach to overcome central tolerance has been the transfer of potent tumor-specific effector cells into patients through adoptive cell therapy (ACT). The rationale for ACT of T cells stems from the central role of T lymphocytes in tumor antigen recognition and cell-mediated immunity. Unlike other immunotherapy modalities that rely on endogenous tumor responses, ACT is based on the isolation of T cells from the patient's tumor (tumor-infiltrating lymphocytes) or peripheral blood, *ex vivo* expansion of tumor-specific unmodified or genetically engineered lymphocytes, and infusion of these T cells back into the patient.[11,12] The feasibility and efficacy of ACT have been clearly documented in melanoma patients and a few other solid tumors, such as ovarian and colorectal cancers, as well as some B cell malignancies.[12–19] Although numerous preclinical studies have demonstrated the potential value of ACT for various cancers, only a few of them have been successfully translated into the clinic. Major hurdles to the success ACT are summarized in Figure 1. Improvements in this potentially powerful treatment intervention are therefore needed.

The advancements made in tumor antigen discovery and *ex vivo* culture of T cells have led to the development of strategies aimed at augmenting antitumor responses through the reprogramming of T cells prior to ACT.[20–42] Methods for T cell modification have included the use of viral vectors (e.g., retroviruses and lentiviruses) and non-viral vectors (e.g., electroporation (EP) and liposomes) for DNA or RNA delivery. Each of those methods presents its own advantages and disadvantages (as summarized in Table 1), which have previously been reviewed in detail by others.[43–47] Traditionally, T cell modifications have been achieved through DNA-mediated, viral vector platforms.[48,49] However, while preclinical and clinical studies have demonstrated the antitumor potency of virally engineered T cells, these studies have also highlighted substantial regulatory hurdles (e.g., clinical production of the plasmids, as they are associated with genes for antibiotic resistance; removal of viable residual packaging cells). These hurdles hinder implementation in human clinical trials and adoption into clinical practice due to the toxicities associated with long-term transgene expression or the potential presence of endotoxin in the viral vector preparation. Therefore, significant preclinical and clinical research have been dedicated to developing alternative vector systems that are not dependent on viral design, such as mRNA transfer that has proven to be safe and efficacious in both preclinical and clinical studies.[20,23,25,26]

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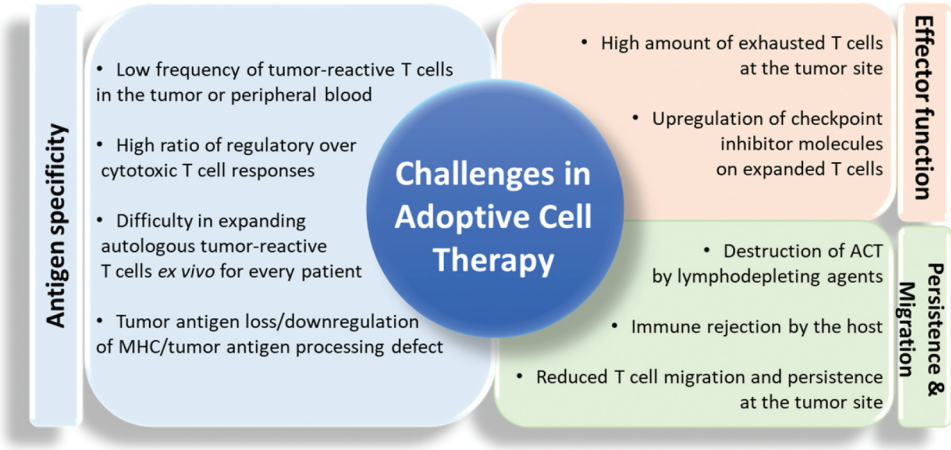



**Figure 1.** Challenges in Adoptive Cell Therapy. Illustrative figure describing the major limitations of the adoptive T cell transfer in different categories including antigen-specificity, effector T cell function and T cell persistence and migration. (ACT) adoptive cell transfer.

To date, RNA-mediated modifications of T cells have been aimed at enhancing antigenic specificity, effector function, persistence, and/or migration to tumor sites, as summarized in figure 2.[21–26,28-35,37–42,50-55] The modifications made to enhance T-cell antigenic specificity include addition of a tumor antigen-specific T cell receptor (TCR), chimeric antigen receptor (CAR), bispecific T cell engagers (BiTEs), and T cell expressing two additional receptors (TETARs). Recently, RNA-modified T cells have also been explored as biological carriers to intracranial tumors[52] as well as an effective tool for immunomodulatory agent delivery for cancer therapy.[56] The present review provides an overview of various T cell modifications achieved through RNA electroporation and their progress in the treatment of hematologic malignancies and solid tumors.

## Improvement of T-cell antigen specificity

Effective T cell-based therapies require a directed and synchronized process in order to generate an adaptive cell-mediated immune response. In order to mount an effective immune response, T cells are initially primed by tumor peptides presented by major histocompatibility complexes (MHCs) on antigen-presenting cells (APCs) in the lymph nodes. T cells recognize tumor-associated antigens or tumor-specific antigens through the TCR, a transmembrane complex composed of two subunits (α and β chains).[57] This recognition leads to the activation and migration of T cells to the tumor, consequently eliciting cytotoxic T cell functions.[57] However, most tumors are poorly immunogenic,[58–61] and the peripheral T cell repertoire typically anergic,[62] devoid of high-avidity tumor-reactive T cells[63] and favoring the differentiation of regulatory over cytotoxic T cell responses.[64] Therefore, preclinical research has focused on developing methods to reprogram T cells *ex vivo* so they can express a diverse repertoire of tumor-specific antigen recognition receptors such as TCRs or CARs. While tumor-specific αβ TCRs typically recognize processed antigens that are presented by tumor cells' MHC Class I, CARs have been designed to overcome this MHC-restricted recognition through targeting antigenic peptides expressed at the cell surface.[46]

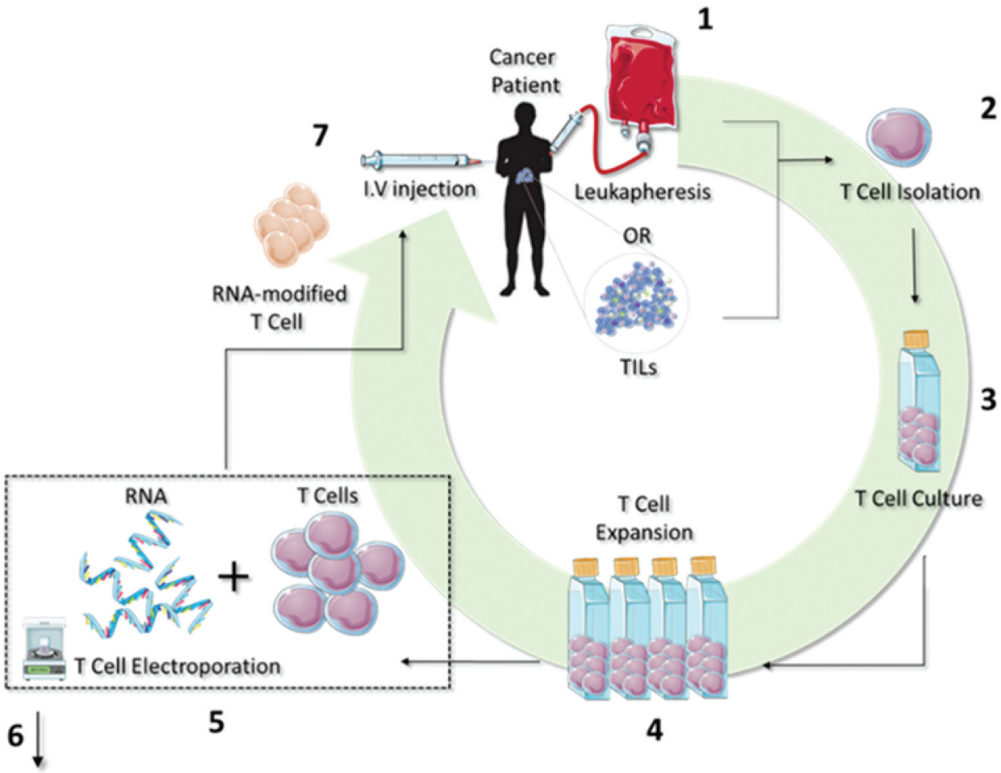
T cells engineered to express tumor-specific TCRs or CARs using viral vectors have shown promising clinical outcomes in ACT for various cancers.[48,49,65] However, this strategy has several limitations, such as "on-target" or "off-target" effects that can be potentiated by long-term transgene expression.[45–47] Therefore, methods exploring non-viral, RNA-mediated modifications of T cells have been investigated as potential, similarly efficacious, and safer alternatives to stable expression via viral vectors.[66] RNA EP has been used to enhance T-cell antigen specificity, mainly through the expression of tumor-specific TCRs, CARs, TETARs, or BiTEs.[26,32–34,67]

### *TCRs*

EP of mRNA into immune cells was first employed to modify dendritic cells (DCs) so they express tumor antigens or to functionally modulate their phenotype.[68–70] Subsequently, mRNA EP has been used to transfer full-length, tumor-specific TCR α and β chain genes into T cells in order to redirect their antigen specificity.[31,33] The impetus for reprogramming T cells' TCR mainly stemmed from the challenges in isolating and expanding autologous tumor-reactive T cells *ex vivo* for every patient receiving ACT. Additionally, the low frequency of high-affinity, antigen-specific CD8+ T cells among cancer patients' peripheral blood lymphocytes (PBLs) is a limiting factor in tumor cell killing efficiency.[71]

Zhao et al. were the first to report T cell EP with TCR RNA in human primary PBLs.[32] In this study, TCR RNA was isolated from activated PBLs stimulated with New York esophageal squamous cell cancer-1 (NY-ESO-1)-specific peptide, and TCR RNA EP was used to screen for TCR functionality prior to generating viral vector constructs. Co-culture of NY-ESO-1 TCR RNA-modified T cells with tumor cells resulted in the killing of cognate human tumor cells, thus demonstrating functional human leukocyte antigen (HLA)-A2-restricted and NY-ESO-1-specific TCR α- and β-chains.[32]

Since 2005, a handful of preclinical studies using TCR RNA-electroporated T cells against solid tumors have been reported for different types of cancer (e.g., neuroblastoma, ovarian cancer, melanoma), as shown in Table 2. These reports have




| | T cell modification | Characteristic/Purpose of Modification | Examples |
|---|---|---|---|
| Antigen Specificity | TCR | T cell receptor consists of a tumor antigen-specific αβ TCRs that typically recognize processed antigens that are presented by tumor cell MHC I. | • ErbB2 - tgTCR<br>• CEA - tgTCR<br>• NY-ESO-1 - tgTCR<br>• Gp100 - tgTCR<br>• WT1 - tgTCR |
| | CAR | Chimeric antigen receptor consists of an extracellular antigen-binding variable regions of the heavy and light chains of an antibody attached to the intracellular domain of T cell signaling molecules. | • α -CD19 • α -EGFR<br>• α -CD33 • α -FRα<br>• α -CD20 • α -GD2<br>• α -EpCAM • α -MSLN |
| | BiTEs | Bispecific T cell engagers are fusion proteins consisting of bispecific monoclonal antibodies that are designed to "bridge" tumor cells and T cells. | • Anti-CD19 |
| | TETARs | T cells expressing two additional receptors that allows for targeting distinct antigens simultaneously. | • gp100 - tgTCR + MCSP - tgTCR<br>• gp100 - tgTCR + CCT6Am - tgTCR |
| Function | | T cell modification aiming to enhance effector T cell function. | • LAT 2KR<br>• PD-1/CTLA-4 siRNA<br>• GM-CSF<br>• TLR4 |
| Migration | | T cell modification with the goal of improving T cell persistence and migration. | • CXCR2<br>• TERT<br>• TALEN<br>• CCR7<br>• CXCR4 |

**Figure 2.** RNA-electroporated T cells. 1. Peripheral blood cells (leukapheresis) or tumor infiltrating lymphocytes (TILs) are collected from cancer patients. **2**. T cells are isolated from blood or TILs by density gradient centrifugation. **3**. Isolated T cells are incubated at 37°C. **4**. T cells are expanded *in vitro* with cytokines (*e.g.*IL-2). **5**. T cells are combined with RNA and transfected with an electroporation device. **6**. Types of RNA T cell modifications. **7**. RNA-modified T cells are injected back into the same patient.

**Table 1.** Comparison of advantages and limitations of viral vectors versus RNA transfection for T cell modification.

| | Viral Vectors | RNA |
|---|---|---|
| Gene transfer efficiency | High | Variable |
| Duration of transgene expression | Long | Short |
| Packaging restrictions | Small capacity | No limitation but may require transfection optimization |
| Complexity of release testing | High | Low |
| Insertional mutagenesis risk | High | Low |
| Timing and complexity of manufacturing | High | Low |
| "On target" or "Off target" risks | High | Low |
| Possible vectors | Lentivirus Retrovirus | Electroporation liposome |

demonstrated that transfecting lymphocytes with tumor-specific TCR RNA can redirect CD8+ T cells to lyse targeted tumor cells specifically and elicit similar cytotoxic capacity as observed with retrovirally transduced T cells.[29,31,33]

In addition, T cells modified with RNA can be used to screen highly antigen–binding regions of TCRs in order to modulate and enhance tumor cell recognition by CD8+ and CD4+ T cells. In transgenic mice expressing fully human HLA-A2.1 (previously immunized with HLA-A2.1-restricted carcinoembryonic antigen (CEA) epitopes), RNAs encoding for amino acid substitutions of the TCR α- and β-chains were transfected into CD8+ and CD4+ T cells. While replacement of a single amino acid of the TCR enhanced tumor cell recognition by CD8+ T cells, dually replaced TCR amino acid positions enhanced tumor cell recognition by CD4+ T cells. In response to HLA-A2.1 and CEA-expressing human colon cancer cell lines, or genetically engineered cell lines, these TCR modifications led to functional effector T cell functions as detected by IFNγ secretion.[28]

A potential drawback to T cell TCR reprogramming is the dimer formation that can occur between endogenous and transferred TCR chains, which has shown to affect T cell specificity.[72] To overcome this challenge, Shimizu et al. evaluated the antitumor effects of γδ T cells that were modified to express an invariant natural killer T cell (iNKT) TCR (an innate lymphocyte's TCR).[37] Since γδ T cells lack α- and β-chains, the authors hypothesized that transfection of iNKT α- and β-chain mRNA into γδ T cells would prevent the formation of mispaired receptors. iNKT-TCR mRNA-transfected γδ T cells retained parental effector T cell functions and responded to both γδ T and glycolipid iNKT ligands; moreover, they showed cytotoxic T cell responses against iNKT ligand-expressing target cells and leukemia cells *in vitro*.[37] Together, these studies demonstrate that TCR RNA transfer can redirect and enhance T cell antigen specificity for tumor adoptive immunotherapy.

## CARs

CARs consist of the extracellular antigen-binding, variable regions of the heavy and light chains of antibodies (specific to a particular antigen), a transmembrane domain, and a signaling endodomain. The addition of co-stimulatory signaling molecules can further enhance the antitumor immune responses and *in vivo* persistence of CAR T cells.[49] Importantly, CAR T cells recognize and kill tumor cells independently of MHC. This is a relevant characteristic, as many tumors present immune evasion mechanisms through which antigen processing and presentation are affected.[73] However, this feature also limits CARs to the exclusive targeting of membrane-bound antigens. A remarkable milestone in cancer immunotherapy has been evidenced by the FDA approval of an anti-CD19 autologous CAR T cell therapy for the treatment of leukemia patients who have been refractory to standard treatments or later relapsed. Although CAR T cell therapy has shown success in treating those patients, this treatment modality can also induce autoimmune responses and cytokine release syndrome, [74] which have in some cases been associated with lethal toxicity. These effects have been attributed to "off-target" tissue toxicity, which correlated with prolonged transgene expression in CAR T cells generated using viral vectors.[45,46,67] These toxicity concerns have prompted researchers to investigate the use of CAR RNA-modified T cells. A list of representative preclinical studies using RNA-based modifications of T cells for hematologic malignancies and solid tumors is shown in Tables 2 and 3.

### Hematologic malignancies

The successful application of CAR RNA-modified T cells in targeting B-cell antigens (e.g., CD19, CD20, and CD33) has been demonstrated in animal models of leukemia and lymphoma for hematologic malignancies.[21,35,38,39,41] Rabinovich et al. have shown the feasibility of generating CAR RNA-modified T cells following a simple and effective method, consisting of a vector-free tool production of mRNA from polymerase chain reaction-generated DNA templates, and tested this approach by generating CD19 CAR RNA-modified T cells that specifically targeted and lysed $CD19^+$ donor matched cells.[42]

Using bioluminescence imaging, studies have shown CAR RNA-modified T cell migration to tumor sites in mice with advanced leukemia. Following a single systemic anti-CD19 CAR RNA-transfected T cell injection in a leukemia xenograft model, these cells demonstrated efficient T cell persistence (at least 13 days post T cell infusion) and systemic migration *in vivo*.[40,54] In another study, luciferase-expressing RNA-transfected CAR T cell distribution at sites of disease recurrence remained detectable at a time of expected loss of CAR expression.[40] Importantly, CAR RNA-transfected T cells exhibited similar antitumor efficacy as viral vector-modified T cells.[40]

Other preclinical studies have used multiple CAR T cell infusions (equal doses), which resulted in tumor regression, [27,50] but these studies have been largely performed in intraperitoneal/intratumoral models and without investigation of optimal treatment regimens. Barret et al. reported that weighted dose splitting (i.e., a loading dose followed by lower maintenance doses) of anti-CD19 RNA-transfected CAR T cells and interval lymphodepletion with cyclophosphamide (an alkylating agent used as chemotherapy) resulted in increased effectiveness and long-term durable remission (> 100 days) in a disseminated model of leukemia, when compared to mice that did not receive lymphodepletion.[40] In a xenograft mouse model of primary acute myeloid leukemia (AML), Kenderian et al. showed that RNA-transfected CAR T cells against CD33 (an antigen

**Table 2. Representative preclinical studies using RNA-based modifications of T cells for solid tumors**. CAR, chimeric antigen receptor; CCT6Am, mutation of chaperonin containing TCP1, subunit 6A; CSPG4, chondroitin sulfate proteoglycan 4; EpCAM, epithelial cell adhesion molecule CD326; MCSP, melanoma-associated chondroitin sulfate proteoglycan; TCR, T cell receptor; TETARs, T cell expressing two additional receptors.

| Antigen target | T cell Design/ RNA | Cancer type | Major findings | Reference |
|---|---|---|---|---|
| **CSPG4** | PD1 CTLA-4 CAR | **Melanoma** | CTLA-4 and PD-1 downregulation by siRNA on the cell surface of CAR T cells boosted effector T cell functions *in vitro*. | Simon B *et al*. Exp Dermatol.[22] |
| - | PE38 | **Ovarian adenocarcinoma** | Transient transfection of primary human T cells with mRNA coding for immunotoxins e23-PE38, VEGF-PE38, and attenuated variant induced cytotoxic activity in the presence of bispecific antibody. | Eggers R *et al*. Gene Ther.[56] |
| - | GM-CSF | **Melanoma** | Activated T cells electroporated with GM-CSF RNA secreted transgene *in vitro* and prolonged overall survival in an intracranial tumor model. | Pohl-Guimaraes F *et al*. Mol Ther. [52] |
| **EpCAM** | CAR | **Peritoneal carcinomatosis** | Multiple repeated infusions delayed disease progression in tumor-bearing mice. | Ang WX *et al*. Oncotarget. [50] |
| **gp100, MCSP** | TETARs | **Melanoma** | Multi-functional T cells expressing both tumor antigen-specific CAR and TCR produced cytokines and displayed lytic activity in the presence of target cells. | Uslu U *et al*. Exp Dermatol. [23] |
| **EGFR** | CAR | **Glioblastoma** | RNA-modified CAR T cells demonstrated similar cytolytic capacity as DNA-modified CAR T cells in response to EGFR-expressing glioblastoma cells. | Caruso HG *et al*. J Immunother. [24] |
| **FRα** | CAR | **Ovarian cancer** | Codon-optimized variant CD27 co-stimulated CAR T cells facilitated the complete regression of widely disseminated human ovarian xenografts in mice and slowed the progression of solid ovarian tumor *in vivo*. | Schutsky K *et al*. Oncotarget.[25] |
| **gp100/ CCT6Am** | TETARs | **Melanoma** | Functional, dual-specific cytotoxic T lymphocyte (CTL) responses were generated against a common melanoma-antigen and an individually mutated antigen *in vitro*. | Höfflin S *et al*. Cancer Biol Ther.[26] |
| - | TLR4 | **Melanoma** | Constitutively active TLR-4 expression enhanced CTL responses and upregulation of key activation markers, pro-inflammatory cytokines, and chemokines *in vitro*. | Pato A *et al*. Clin Exp Immunol.[51] |
| **GD2** | CAR | **Neuroblastoma** | Single injection slowed the progression of disseminated disease and improved survival but did not result in long-term disease control due to limited tumor penetration. | Singh N *et al*. Cancer Immunol Res. [76] |
| **MSLN** | CAR | **Mesothelioma** | First report using matched patient tumor and lymphocytes to show that multiple injections of autologous RNA-electroporated CAR T cells could mediate tumor regression in a preclinical model. | Zhao Y *et al*. Cancer Res.[27] |
| **CEA** | TCR | **Colon cancer** | Amino acid substitutions in the antigen-binding regions of the TCR enhanced tumor cell recognition *in vitro*. | Parkhurst MR *et al*. Clin Cancer Res.[28] |
| **ErbB2, CEA** | TCR | **Ovarian and colorectal adenocarcinoma cell line** | RNA-transfected T cells displayed cytolytic activities similar to retrovirally gene-modified T cells, which lasted after 2 days of activation. | Birkholz K *et al*. Gene Ther.[29] |
| **Her-2/ neu** | CAR TCR | **Ovarian and breast cancer Melanoma** | Strong CTL responses and type-1 cytokine secretion *in vitro*. Significant inhibition of tumor growth in xenograft model *in vivo*. | Yoon SH *et al*. Cancer Gene Ther. [30] |
| **gp100** | TCR | **NY-ESO-1** | CTL responses with > 60% tumor cell killing and lytic efficiency similar to that of retrovirally transduced T cells and parental CTL clone for at least 72 hours. | Schaft N *et al*. Cancer Immunol Immunother.[31] |
| **NY-ESO-1** | | **positive melanoma and non-melanoma cell lines** | RNA electroporation of primary blood lymphocytes was used to confirm the functionality of the cloned TCR prior to viral vector transfection. | Zhao Y *et al*. J Immunol. [32] |

**Table 3. Representative preclinical studies using RNA-based modifications of T cells for hematological malignancies**. α-GalCer, α-galactosylceramide; AML, acute myeloid leukemia; BiTE, bispecific T cell engager; CAR, chimeric antigen receptor; iNKT, invariant natural killer T; LAT, linker for activation of T cell; LAT 2KR, ubiquitylation-resistant LAT; TALEN, transcription activator-like effector nuclease; TERT, telomerase reverse transcriptase; Zol, zolendronic acid.

| Antigen target | T cell Design/RNA | Cancer type | Major findings | Reference |
|---|---|---|---|---|
| **WT1** | TCR | **Leukemia** | Superior transgenic TCR expression stimulated CD8+ T cell activation and killing activity. | Campillo-Davo D *et al*. Front Immunol.[33] |
| **CD19** | BiTEs | **Leukemia** | Complete tumor remission after transfer into a leukemia mouse model. | Liu X *et al*. Blood Cancer J.[34] |
| **CD20** | CAR | **B cell lymphoma** | Well tolerated with modest, but transient, anti-tumor activity in a canine spontaneous tumor. | Panjwani MK *et al*. Mol Ther.[35] |
| **CD19** | TALEN, CAR | **Lymphoma** | Multiplex gene editing abrogated graft-versus-host reactions and rendered T cells resistant to monoclonal antibody therapy. | Poirot L *et al*. Cancer Res.[36] |
| **Zol, α-GalCer** | iNKT TCR, γδT cells | **Leukemia** | Potent bifunctional effector T cell functions *in vitro*. | Shimizu K *et al*. PLoS One.[37] |
| **CD19** | TERT, CAR | **Lymphoma** | Improved persistence and proliferation in mouse xenograft tumor models and enhanced long-term anti-tumor effects compared with CD19 viral vector-modified CAR. | Bai Y *et al*. Cell Discov.[38] |
| **CD33** | CAR | **Leukemia** | High-level gene expression with potent but self-limited activity against AML *in vivo*. | Kenderian SS *et al*. Leukemia.[21] |
| **CD19** | CAR | **Leukemia** | Compared to stable viral vector-mediated expression, multiple infusions preceded by lymphodepletion showed similar efficacy in a leukemia xenograft model, independent of co-stimulatory signaling endodomains. | Barrett DM *et al*. Hum Gene Ther.[40] |
| **NY-ESO-1, CD19** | LAT 2KR, TCR, CAR | **Leukemia/ NY-ESO-1-transduced leukemia** | Ubiquitylation-resistant LAT expression potentiated TCR- and CAR-T mediated anti-tumor responses *in vitro*. | Kunii N *et al*. Hum Gene Ther.[39] |
| **CD19** | CAR, CCR7, CXCR4 | **Leukemia/ lymphoma** | Higher cell killing, IFN-γ release with increasing amounts of mRNA, but increased activation-induced cell death. Co-transfection of second chemokine receptor transgene could promote chemotaxis. | Almåsbak H *et al*. Cytotherapy.[53] |
| **CD19** | CAR | **Leukemia** | CAR T cells rapidly migrated to distant sites of disseminated tumor, retained target-specific lytic activity, and potentiated survival in an aggressive murine leukemia xenograft model. | Barrett DM *et al*. Hum Gene Ther.[54] |
| **CD19** | CAR | **Lymphoma** | Promoted tumor growth regression in a murine xenograft lymphoma model. | Rabinovich PM *et al*. Hum Gene Ther.[41] |
| **CD19** | CAR | **Lymphoma** | CAR T cells exhibited powerful cytotoxicity against CD19+ matched donor cells *in vitro*. | Rabinovich PM *et al*. Hum Gene Ther.[42] |

expressed on malignant and normal myeloid cells) elicited potent antitumor activity *in vitro* and resulted in leukemia eradication and long-term survival *in vivo*.[21]

Promising outcomes of CAR T cell therapy in murine models of hematologic malignancies have led to the development of RNA-transfected CD20-specific CAR T cells for the treatment of dogs with spontaneous B cell lymphoma.[35] In this report, the authors demonstrated that canine CD20-ζ CAR was efficiently and transiently expressed in T cells after mRNA electroporation. Such treatment proved to be safe and efficacious, as demonstrated by effector T cell cytokine production and tumor cell killing *in vitro*, as well as modest and transient antitumor activity in a canine patient.

### Solid tumors

The therapeutic potential of RNA-modified T cells has been demonstrated in many solid tumors, including mesothelioma, ovarian cancer, neuroblastoma, glioblastoma, and peritoneal carcinomatosis.[20,24,25,27,30,50,75] Unlike for blood-borne tumors, the success of RNA-transfected CAR T cells has not yet been extrapolated to solid cancers, mainly due to physical barriers impeding T cell tumor penetration, immunosuppressive tumor microenvironments, and limited T cell persistence at the tumor sites.[50,76] In an initial study, Yoon et al. showed the therapeutic potential of CAR T cells generated by electroporation of PBLs with RNA encoding for anti-human epidermal growth factor receptor 2 (Her-2/neu) in a human ovarian carcinoma xenograft model.[30] Anti-Her-2/neu CAR T cells elicited potent immune responses against cognate antigen-overexpressing tumor cells *in vitro* and resulted in strong inhibition of tumor growth *in vivo*.[30] Similarly, Singh and colleagues demonstrated that a single intratumoral injection of autologous RNA-transfected CAR T cells directed against the neuroblastoma antigen disialoganglioside achieved antitumor effects and control of tumor growth in an orthotopic flank xenograft model of neuroblastoma.[75]

In another study, Caruso et al. evaluated RNA-modified CAR T cells against epidermal growth factor receptor (EGFR), a TAA overexpressed in adult primary glioblastoma.[24] Following electroporation of human lymphocytes with EGFR-specific CAR RNA, T cells were exposed to cytokines or stimulated with EGFR-expressing parental mouse cells. While anti-EGFR CAR T cells specifically targeted tumor cells and secreted signature Th1 cytokines, the addition of cytokines in co-culture with cognate antigen-expressing tumor or normal cells accelerated transfected RNA decay, thereby reducing effector T cell function.[24] Together, these findings indicate that RNA-modified CAR T cells allow for "on-target" immune responses and the prevention of putative "off-target" toxicities that could be associated with viral vector-based T cell modifications.

A major concern regarding the clinical application of RNA-modified T cells has been the transient CAR expression on T cells due to physiologic decay over time, which could abrogate their capacity to target tumor cells and, therefore, reduce antitumor responses. To overcome this obstacle, studies have been applying multiple and weighted dosing strategies to avoid cancer relapse.[20,25,27,75] For instance, in a preclinical model of mesothelioma, Zhao et al. tested RNA-transfected CAR T cells against mesothelin (MSLN), an antigen overexpressed in ovarian and pancreatic cancer as well as mesothelioma.[27] Sequential injections of autologous RNA-transfected MSLN CAR T cells promoted the dramatic regression of advanced flank and disseminated matched patient mesothelioma growth *in vivo*.[27] Moreover, phase I clinical trial employing mRNA-modified T cells for the treatment of MSLN-expressing tumors demonstrated the safety and feasibility of multiple injections of RNA-transfected MSLN-specific CAR T cells in most patients (NCT01355965).[20] A single patient was reported to have clinical anaphylaxis, which was most likely due to the generation of IgE antibodies specific to the murine CAR construct.[20] Together, these studies suggest that T cells engineered to express powerful activation domains through RNA electroporation represent an effective approach for adoptive cell transfer, without the associated safety concerns of integrating viral vectors.

### TETARs

RNA-mediated T cell engineering has been mostly used to target a single TAA or TSA in order to improve antigen specificity. While this approach often leads to tumor regression, relapse is likely to occur due to: 1) immunoediting mechanisms triggered in the tumor microenvironment, 2) tumor antigen processing defect, 3) antigen loss, and/or 4) downregulation of MHC, [77,78] among other factors. These tumor escape mechanisms highlight the therapeutic limitations likely to be encountered while targeting single antigens.

Recent investigations have developed the use of T cells expressing two additional receptors (TETARs) as a new therapeutic strategy to target two antigens simultaneously. Through combining two separate antigen receptors on the same T cell, this strategy has the potential to overcome immune escape due to single antigen loss. Hofflin et al. reported that activated human T cells could be effectively modified to express distinct TCRs directed against a common melanoma antigen and a patient-specific, individually mutated antigen. These dual-specific T cells specifically lysed target cells loaded with each of their cognate antigens *in vitro*, with some observed reciprocal inhibition.[26] In another study, Uslu et al. generated RNA-modified CD8+ TETARs that expressed both a CAR and TCR simultaneously, each receptor being specific for a distinct common melanoma antigen. Importantly, compared to viral vector-modified CAR and TCR T cells, these TETARs showed enhanced effector T cell functions without reciprocal inhibition.[23] Through functionally combining antigen-specific CARs and TCRs, RNA-modified TETARs could circumvent tumor immune escape, offering a promising alternative in T cell design for ACT therapy.

### BiTEs

Bispecific T cell engagers (BiTEs) are fusion proteins consisting of bispecific monoclonal antibodies (mAbs) that are designed to "bridge" tumor cells and T cells.[79] This engagement facilitates the direct interaction between T cells and tumor antigens loaded on MHC. In xenograft animal models, BiTEs that have

been stably transduced in human T cells have shown feasibility and efficacy, although permanent BiTE expression on T cells has the potential to elicit cytokine storms and promote B cell aplasia.[80,81] To circumvent these potential toxicities, Liu et al. reported that BiTE T cells transfected with RNA encoding for an mAb targeting CD19 specifically secreted functional BiTEs and conferred specific tumor cell killing *in vitro* and improved overall survival *in vivo*.[34] Thus, BiTE RNA-modified T cells may represent an alternative to overcoming challenges of systemic BiTE injection while preventing toxicities resulting from current viral vector-mediated BiTE T cell therapies.

## Improvement of T cell function

Effective antitumor T cell function involves a plethora of steps needed for the elimination of targeted cancer cells. T cells must first recognize tumor-specific antigens presented on APCs in the lymph nodes, upregulate activation markers and co-stimulatory molecules, proliferate extensively *in vivo*, and traffic to the tumor site while retaining their antitumor functions in a highly immunosuppressive tumor microenvironment. In physiological conditions, cytokines and chemokines tightly coordinate and regulate appropriate effector T cell functions. However, in cancer patients, tumor-reactive T cells are typically found at low frequency in the circulation and often fail to perform their inherent effector functions due to the upregulation of checkpoint inhibitor molecules (e.g., CTLA-4, PD-1) on their surface, downregulation of MHC Class I expression on tumor cells, secretion of anti-inflammatory molecules (e.g., TGFβ, IL-10), [77,82–85] and/or insufficient T cell trafficking to the tumor sites.[86,87]

Due to the low frequency of tumor-reactive T cells present in the peripheral blood of cancer patients, enrichment of antigen-specific T cells is essential in ACT manufacturing. Our group has demonstrated that RNA electroporation selectively targets gene expression to actively dividing T cells and, thus, transfection of reporter genes such as GFP can be used to isolate and expand antigen-specific T cells responding to antigens of interest for use in ACT.[55]

Based on recent data reporting the direct effects of toll-like receptor (TLR) ligands on effector T cell function, [88] Pato et al. electroporated tumor-infiltrating lymphocytes from melanoma patients with a constitutively activated TLR-4 encoding mRNA, which prolonged the expression of activation markers, induced the secretion of cytokines and chemokines, and increased effector T cell function.[51] In another study, Kunni et al. replaced the integral membrane adapter molecule linker for activation of T cells (LAT), known to play a central role in the activation of primary human CD4+ and CD8+ T cells, with ubiquitylation-resistant LAT (LAT 2KR). This substitution resulted in enhanced T cell signaling, proliferation, and Th1 cytokine secretion. By transfecting anti-CD19-ζ CAR T cells or anti-NY-ESO-1 TCR T cells with LAT 2KR mRNA, the authors demonstrated increased CAR and TCR T cell-mediated antitumor efficacy *in vitro*.[89] A recent major obstacle in the efficiency of engineered T cells against solid tumors is the upregulation of T-cell inhibitory receptors (e.g., PD-1 and CTLA-4), resulting in limited antitumor response. By simultaneously electroporating T cells with siRNA and mRNA encoding for PD-1/CTLA-4 and CAR specific for chondroitin sulfate proteoglycan 4, respectively, Simon et al. demonstrated superior *in vitro* CAR T cell functions against melanoma cells compared with CAR T cell alone.[22] Overall, these studies demonstrate that various RNA-mediated modifications can be employed to enhance T cell effector functions.

## Improvement of T cell migration and persistence

The *ex vivo* expansion of antigen-specific T cells for ACT often results in highly activated or exhausted T cell phenotypes, [90,91] leading to reduced T cell trafficking and persistence at the tumor site[92] and limiting clinical efficacy. Our group has previously demonstrated that the electroporation of antigen-specific T cells with RNA encoding for chemokine receptor CXCR2 leads to the efficient migration of these modified T cells toward glioma-secreted CXCR2-specific ligands *in vitro* and *in vivo*, [55] showing the possibility of using RNA modifications to enhance the chemotactic functions of T cells.

Despite the genetic stability of engineered T cells generated using viral vectors, these T cells can become exhausted by the time sufficient number of transfectants are obtained due to the long-term selection process involved in the utilization of those vectors. This process can result in T cell telomere shortening, inefficient proliferation, and short-term persistence *in vivo*.[93] Consequently, transient RNA T cell modifications have been used to complement viral vector-mediated CAR and TCR modifications in order to enhance the persistence of transferred T cells.[36,38,53] By transfecting anti-CD19-expressing CAR T cells with RNA encoding for telomerase reverse transcriptase (TERT), a major protein directly associated with cellular senescence, Bai et al. have demonstrated that TERT CD19 CAR T cells display transiently enhanced telomerase activity, increased telomere length, and decreased senescence. Notably, these cells displayed long-term antitumor immune responses *in vivo*.[38]

The destruction of transferred T cells by lymphodepleting agents, allogeneic/"off-the-shelf" manufacturing, or immune rejection by the host have created major concerns about the utilization of ACT in cancer patients.[94] By deleting T cells' αβ TCR [which mediates graft-versus-host disease (GvHD)] using transcription activator-like effector nuclease (TALEN) and CD52, a protein targeted by the monoclonal antibody alemtuzumab, Poirot et al. hypothesized that functional allogeneic antigen-specific T cells could be manufactured, while avoiding GvHD responses and rendering T cells resistant to destruction by alemtuzumab.[36] Upon exposure to alemtuzumab, CD52 and TALEN RNA-co-transfected CD19 CAR T cells displayed efficient CD19+ tumor cell killing compared to unmodified CD19 CAR T cells.[36] Together, these studies demonstrate the utility of RNA transfection as a simple and efficient method to enhance the persistence and migration of tumor-reactive T cells.

## Improvement of RNA stability and duration

Upon tumor antigen recognition, activated T cells undergo homeostatic proliferation as well as RNA metabolism and degradation; notably, RNA expression declines more rapidly

*in vivo* than *in vitro*, potentially hindering the long-term therapeutic effects of RNA-modified T cells. While transient transgene expression may be desirable in certain cases, it could also be a limitation for prolonged therapeutic delivery. Therefore, researchers have been exploring methods to improve stability and duration of transgene expression following RNA electroporation in T cells.

The *in vitro* synthesis and transfection efficiency of mRNA have been shown to depend on its structure (e.g., the capping nucleotide and poly (A) tail).[42] To improve the stability of mRNAs encoding CARs, Zhao et al. have tested the insertion of an α-globin 3′ untranslated region (prolonged (150A) poly (A) sequence) and an anti-reverse cap analogue or cap1 structure.[27] While these modifications led to increased transgene expression efficiency, no effects on transgene expression length were observed.[27] Furthermore, to reduce potential harmful "off-target" translation products and avoid immune rejection of engineered T cells, IVT RNA has been codon optimized by removing the open reading frame (ORF).[25] For instance, compared to parental FRα-specific CAR RNA T cells, optimized CAR RNA T cells demonstrated similar immunoreactivity, cytolytic potential, and antitumor function.[25] Therefore, while the limited duration of transgene expression following mRNA transfection may allow for safety when using RNA-modified T cells alone or in combination with current viral vector-based T cell engineering, there may be a need to optimize the RNA construct.

## Conclusion and future directions

*Ex vivo* T cell expansion has permitted the manipulation of T cells prior to ACT for cancer, and extensive investigations have been performed on RNA-mediated T cell modifications to improve T-cell antigen specificity, trafficking, and functions. More recently, RNA-electroporated T cells have proven to be an effective tool for immunomodulatory agent delivery for cancer therapy.[52,56] Compared to viral vectors, clinical-grade synthetic mRNA can easily be generated in large quantities and in a cell-free process, allowing for minimal costs and regulations. Several RNA-modified T cell therapy approaches are currently being tested in clinical trials for patients with advanced malignancies. In addition, methods to improve RNA stability, purity, and duration have been investigated in order to enhance the therapeutic efficacy of those modified T cells. An optimal treatment regimen remains to be determined, including dose and number of T cell injections, T cell population to transfect, and type of RNA used for T cell modifications in order to achieve optimal therapeutic effects.

Although ACT using RNA-modified T cells has proven to be a promising therapeutic tool for hematologic malignancies, developments are still needed for its application in the treatment of solid tumors, mostly due to the lack of target tumor antigens that are not expressed by normal tissues, tumor localization, and immunosuppressive microenvironment. While unprecedented durable response rates have been observed with ACT, most patients do not benefit from the treatment (primary resistance, e.g., due to the absence of tumor antigens, such as in pancreatic cancer), and some responders relapse after a period of response (acquired resistance, e.g., due to the development of mechanisms to avoid antigen presentation on surface-restricted MHC). To overcome these obstacles, combinatorial approaches with other types of treatment, such as chemo-, radio-, and targeted therapies, have been explored.[95–102] In addition, suppressive cytokines/agents and cell populations such as myeloid-derived suppressor cells and regulatory T cells present in the tumor microenvironment can induce immunosuppression, thereby blocking T cell function.[103,104] Therefore, combining ACT therapy with other strategies that inhibit negative regulators such as PD-1/PD-L1, or reverse regulatory T cell/myeloid-derived suppressor cell-mediated immunosuppression, may induce potentially profound immune antitumor responses.[105–110] Furthermore, the combination of T cell ACT with other forms of immune cell therapies has also yielded very promising results in preclinical studies.[111,112] Finally, for those combinatorial treatments to be implemented on a large scale for various cancer patient populations, issues pertaining to scale-up, automation, commercialization, intellectual property, costs/insurance reimbursements, and regulatory hurdles unique to cell therapy will need to be addressed.[1,113,114]

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## Disclosure of potential conflicts of interest

DAM holds ownership interest (including patents) in iOncologi, Inc., and DAM has patented immunotherapy-related technology that has been licensed by Annias Immunotherapeutics, Inc.; Celldex Therapeutics, Inc.; and Immunomic Therapeutics, Inc. FPG and LBH declare no conflicts of interest.

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