An Optimized Workflow for Generating Anti-CD19 CAR T Cells by mRNA Electroporation

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Abstract

Chimeric antigen receptor (CAR) T-cell therapy is a revolutionary and promising approach for cancer treatment. However, its potential is limited by technical challenges and safety risks associated with viral transduction–based CAR T-cell generation workflows. Nonviral methods, such as electroporation, represent a potentially safer and less challenging approach for generating CAR T cells. Here, we describe the generation of anti-CD19 CAR T cells using the Gene Pulser™ Xcell Electroporation System in an mRNA electroporation–based CAR T-cell workflow optimized for efficiency and viability. Our workflow and optimized conditions resulted in CAR transfection efficiencies as high as ~70 and ~90% in primary and immortalized human T cells, respectively, at 6 hr after electroporation, and a viability of ~60% in primary human T cells. These data highlight the utility of the Gene Pulser Xcell Electroporation System for generating CAR T cells without the use of viral transduction.

Introduction

CAR-expressing T cells are a promising therapeutic strategy for addressing cancers in humans, as demonstrated by their success in the treatment of various human B cell lymphomas and leukemias. In this approach, patient or donor T cells are harvested, genetically reprogrammed to express a CAR that targets an antigen selectively expressed on the surface of cancer cells, and infused into a patient for therapeutic use. The binding of a CAR T cell to its ligand results in major histocompatibility complex (MHC)–independent activation and mobilization of CAR T cells against the antigen-expressing cancer cells. CAR T-cell therapy is associated with numerous successful therapeutic outcomes in patients with specific B-cell lymphomas and/or leukemias (Sterner and Sterner 2021). The best known and most clinically validated CAR T cells recognize CD19, a cell surface antigen found on normal and malignant B cells. CD19-directed CAR T cells are shown to target different leukemias and lymphomas, leading to long-term disease remission. Given these significant results, much biomedical and clinical research is currently focused on workflows leading to successful production of viable CAR T cells for therapeutic use.

To date, all U.S. Food and Drug Administration–approved CAR T cell therapies have relied on viral transduction to achieve expression of a CAR in patient T cells (National Cancer Institute, 2022). This method is used primarily because of its relatively high rate of gene transfer, scalability, and the well-studied techniques associated with viral transduction. However, this approach comes with several potential health risks, including insertional mutagenesis with oncogenic potential and other associated side effects (Vannucci et al. 2013; Zhang et al. 2017; Stock et al. 2019; Sterner and Sterner 2021).

Transiently expressed CARs produced by mRNA delivery can avoid the potential risks associated with viral transduction because transfected CAR mRNA cannot be integrated into the host genome and its expression diminishes with time due to mRNA turnover (Foster et al. 2019). In a pivotal study, Krug et al. (2014) successfully implemented mRNA transfections in a good manufacturing practice (GMP)–compliant CAR T-cell workflow. In their study, mRNA transcripts encoding CARs were delivered via electroporation into human T cells, resulting in highly functional CAR T cells. More recently, the same group implemented this mRNA electroporation workflow to produce CAR T cells at clinical scale using the Gene Pulser Xcell Electroporation System, resulting in cytolytic CAR T cells that targeted melanomas (Wiesinger et al. 2019). While primary human T cells are relatively

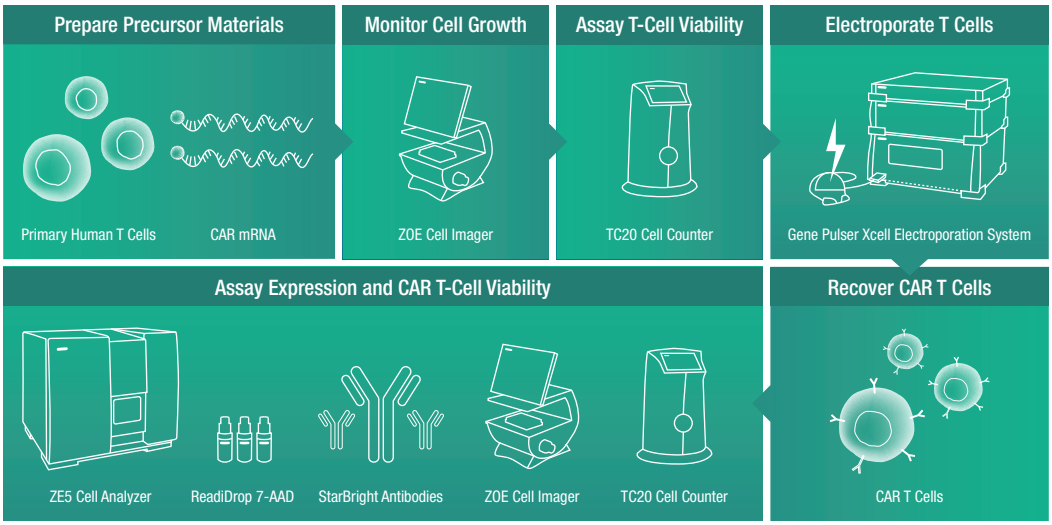

Fig. 1. CAR T-cell workflow. First, precursor materials are prepared: primary human T cells are obtained by leukapheresis and negative selection, and CAR mRNA is analyzed for quality, size, and concentration using a 2100 Bioanalyzer Instrument (Agilent Technologies; not shown). T-cell growth is checked using the ZOE Fluorescent Cell Imager, and viability is confirmed using the TC20 Automated Cell Counter. Then, the T cells are electroporated with CAR mRNA using the Gene Pulser Xcell Electroporation System. Transfection efficiency is measured with the ZOE Cell Imager using GFP. The viability of CAR T cells recovered from electroporation is then tested using Trypan Blue and the TC20 Cell Counter as well as ReadiDrop 7-AAD and the ZE5 Cell Analyzer. CAR expression is also analyzed using StarBright Antibodies with the ZE5 Cell Analyzer. CAR T cells can then be prepared for infusion in a patient. 7-AAD, 7-Aminoactinomycin D; CAR, chimeric antigen receptor; GFP, green fluorescent protein.

difficult to transfect, Wiesinger and colleagues reported a high transfection efficiency (~90%) while concurrently preserving cellular health and viability (~70%). Here, we describe the optimization of an mRNA transfection–based CAR T-cell workflow that includes the development of a CAR, optimization of an mRNA electroporation protocol using green fluorescent protein (GFP), electroporation of primary and immortalized human T cells with CAR mRNA, confirmation of GFP and CAR expression by live cell imaging and flow cytometry, and pre- and post-electroporation viability analysis by automated cell counting and flow cytometry (Figure 1).

Materials and Methods

Generation of an Anti-CD19 CAR and mRNA

The anti-CD19 CAR used in this study was designed to contain extracellular granulocyte-macrophage colony–stimulating factor (GM-CSF) leader sequence, anti-CD19 V_H and V_L chains, transmembrane and cytoplasmic CD28 domains, and a cytoplasmic CD3ζ domain. See Figure 2A for a schematic of the anti-CD19 CAR used in this study. DNA encoding this CAR was synthesized and cloned into pTwist-CMV (Twist Bioscience Corporation). We purchased a large-scale custom in vitro mRNA synthesis kit that incorporates the 5' Cap 1 structure (Aldevron). This mRNA was modified to include a more substantial poly(A) tail using *E. coli* Poly(A) Polymerase (NEB, catalog #M0276S) and purified using the Monarch RNA Cleanup Kit (NEB, #T2030S). Concentration, size, and purity of mRNA was estimated using a 2100 Bioanalyzer Instrument (Agilent Technologies, Inc., #G2939BA) in combination with an RNA 6000 Nano Kit (Agilent Technologies, #5067-1511). DasherGFP mRNA (Aldevron, #3870-0200) containing a 5' Cap 1 was used to track mRNA delivery and expression as a control. RNA was aliquoted and stored at –80°C to minimize freeze-thaw cycles.

Human Cell Culture, Cell Counting, and Imaging

Jurkat T cells (American Type Culture Collection, Clone E6-1) were cultured in RPMI 1640 (Thermo Fisher Scientific Inc., #21875034) containing 10% Fetal Bovine Serum (Hyclone, #SH30071.03) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, #15140122) using standard cell culture procedures. Cells were grown in suspension and passaged every 2–4 days and kept below a density of 3 x 10^6 cells/ml in culture media. Frozen human CD3+ T-cells (AllCells, product LP, CR, CD3+, NS, 25M) were thawed and expanded for 48 hr at a concentration of 1 x 10^6 cells/ml in ImmunoCult-XF T Cell Expansion Media (STEMCELL Technologies Inc., #10981) supplemented with Human Recombinant Interleuken-2 (IL-2) (STEMCELL Technologies, #78036) at a concentration of 10 ng/ml and ImmunoCult Human CD3/CD28 T Cell Activator (STEMCELL Technologies, #10991) at a concentration of 25 µl beads/ml. Cells in suspension were routinely counted using a TC20 Automated Cell Counter (Bio-Rad™ Laboratories, Inc., #1450102) and Trypan Blue (Bio-Rad, #1450021) at a 1:2 dilution. Cells were routinely examined using a ZOE Fluorescent Cell Imager (Bio-Rad, #1450031).

mRNA Transfections via Electroporation

Prior to and following electroporation, cell density and viability were determined using Trypan Blue staining in conjunction with a TC20 Automated Cell Counter. Cells were pelleted by centrifugation at 750 x g for 5–10 minutes and resuspended in Gene Pulser Electroporation Buffer (Bio-Rad, #1652677). For each electroporation, 2 x 10^6 Jurkat or primary human T cells were electroporated using the Gene Pulser Xcell Electroporation System (Bio-Rad, #1652660) in 200 µL of Gene Pulser Electroporation Buffer containing mRNA. In brief, cells were vortexed gently, loaded into 0.2 cm gap cuvettes (Bio-Rad, #1652086), and electroporated using the following conditions, which are based

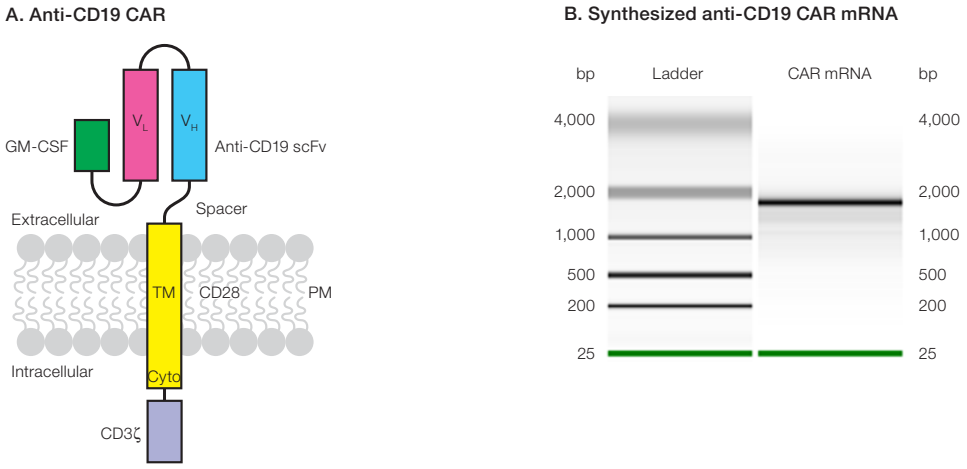

Fig. 2. Anti-CD19 CAR design and mRNA synthesis. A, schematic of anti-CD19 CAR employed in this study. The anti-CD19 CAR is expressed at the plasma membrane with an extracellular GM-CSF leader sequence, an anti-CD19 scFv domain (containing V_H and V_L domains), transmembrane and cytoplasmic CD28 domains, and an intracellular CD3ζ domain; **B**, Synthesized anti-CD19 CAR mRNA size fractioned using a 2100 Bio-Analyzer Instrument (Agilent Technologies). CAR, chimeric antigen receptor; cyto, cytoplasmic; GM-CSF, granulocyte-macrophage colony–stimulating factor; PM, plasma membrane; scFv, single-chain variable fragment; TM, transmembrane; V_H, heavy chain variable domain; V_L, light chain variable domain.

on our prior study (Wong et al., 2020): square wave, 200 V, 2 ms pulse length, 1 pulse. Following electroporation, cells were cultured in supplemented media until further analysis. Cell viability and count were assayed (as described at the beginning of this section) immediately prior to cell imaging, staining, and flow cytometry.

Cell Imaging, Staining, and Flow Cytometry

Brightfield and GFP fluorescence micrographs were collected using a ZOE Cell Imager with identical acquisition settings across all time points. To analyze electroporation efficiency and viability by flow cytometry, cells were harvested by centrifugation at 750 x g for 5–10 minutes and resuspended in an ice-cold staining buffer containing 1% Bovine Serum Albumin Solution (MilliporeSigma, #A7284) in Dulbecco's Phosphate Buffered Saline (Thermo Fisher Scientific, #14190144). For each sample, 1 x 10^6 cells were stained with 100 µl of an antibody cocktail, prepared using the same staining buffer, for 30 min at room temperature. Following staining, cells were pelleted by centrifugation, washed in 1 ml of staining buffer twice and resuspended in 0.5 ml of staining buffer. 20 µl of ReadiDrop 7-AAD (Bio-Rad, #1351102) was added immediately prior to flow cytometry analysis. Flow cytometry reagents and their dilutions are described in Table 1. Flow cytometry analysis was performed using a ZE5 Cell Analyzer (Bio-Rad, #12004279) configured with five lasers (355/405/488/561/640 nm) and controlled by Everest Software. Laser power and detector photomultiplier tube (PMT) voltage were optimized and kept identical for all experimental samples and compensation controls. Experimental data were either analyzed within Everest Software and exported to Microsoft Excel Software for presentation, or separately exported as Flow Cytometry Standard (FCS) files for offline analysis using FCS Express Software (De Novo Software).

Table 1. Reagents for flow cytometry.

Reagent	Ordering Information	Dilution
Rat Anti-Mouse CD8 Alpha: StarBright Violet 440	Bio-Rad, #MCA609SBV440	1:5
Rat Anti-Mouse CD25:StarBright UltraViolet 400	Bio-Rad, #MCA1260SBUV400	1:5
FITC-Labeled Monoclonal Anti-FMC63 scFv Antibody, Mouse IgG1 (anti-CD19 CAR antibody)	Acro Biosystems, #FM3-FY45	1:50
FITC-Labeled Human CD19 (20-291) Protein, Fc Tag	Acro Biosystems, #CD9-HF251	1:10
ReadiDrop 7-AAD	Bio-Rad, #1351102	1:25

7-AAD, 7-aminoactinomycin D; CAR, chimeric antigen receptor; Fc, fragment crystallizable region; FITC, fluorescein isothiocyanate; IgG1, immunoglobulin G1.

Results

Construction and Expression of an Anti-CD19 CAR

A schematic of the topology of the anti-CD19 CAR used in this study is provided in Figure 2A. The presence of anti-CD19 V_H and V_L chains imparts CD19-binding specificity. Plasmid DNA encoding this CAR was the basis for synthesis of mRNA transcripts, which were used for the electroporation experiments performed in this study. An example of synthesized mRNA, size-fractioned using a Bioanalyzer Instrument, is shown in Figure 2B. The prominent band corresponds to the correct molecular weight of the anti-CD19 CAR mRNA that has been 5'-capped and 3'-polyadenylated. A relatively pure mRNA sample is important for achieving optimal cellular viability and transfection efficiency. We routinely confirmed the purity and quantity of the GFP and anti-CD19 CAR mRNAs using the Bioanalyzer Instrument.

Optimization of GFP mRNA Electroporation of Human T Cells

We previously identified optimal parameters for delivery of GFP mRNA to human CD8+ T cells using the Gene Pulser Xcell Electroporation System (square wave, 200 V pulse, 2 ms pulse length, 1 pulse, 0.2 cm cuvette) (Wong et al. 2020). To verify that these conditions were sufficient for use with the CD3+ primary human T cells and mRNA constructs used in this study, we utilized the ZOE Fluorescent Cell Imager to monitor GFP expression following transfection of GFP mRNA. Samples were evaluated every hour using identical acquisition settings. GFP expression was lowest immediately after electroporation and peaked at 6 hr. Representative images acquired at 1, 2, 3, and 6 hr after electroporation are shown in Figure 3.

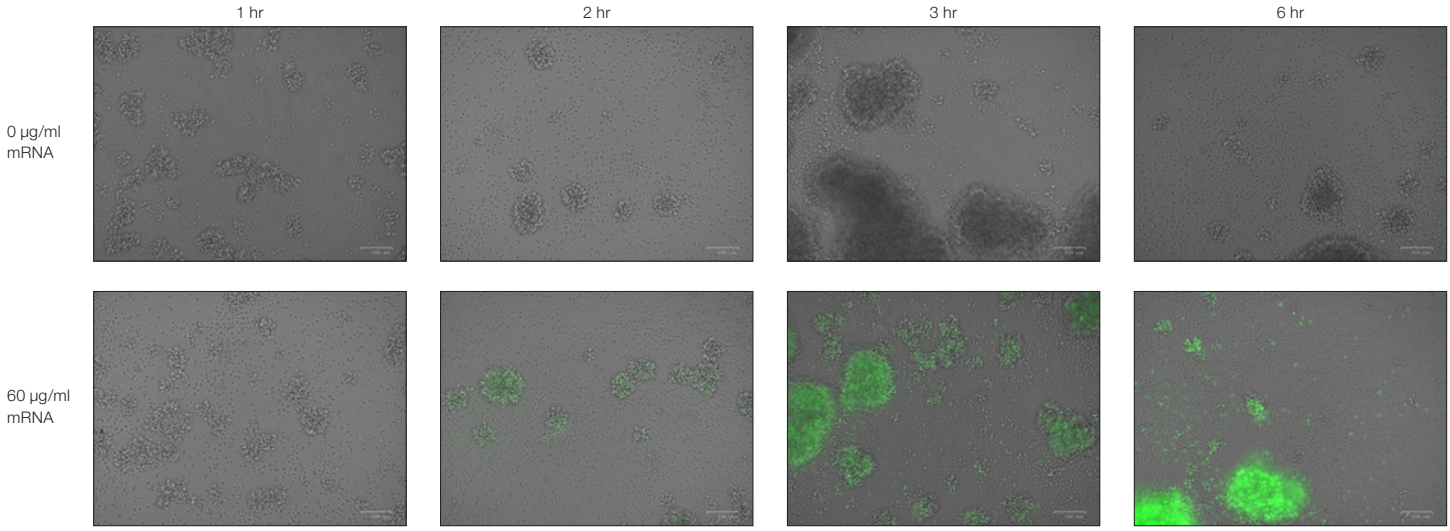

Fig. 3. GFP expression in primary human T cells after electroporation. Merged brightfield and GFP ZOE Imager micrographs of primary human T cells mock-transfected or transfected with 60 µg/ml GFP mRNA and imaged at 1, 2, 3, and 6 hr after electroporation. Scale bar represents 100 µm. GFP, green fluorescent protein.

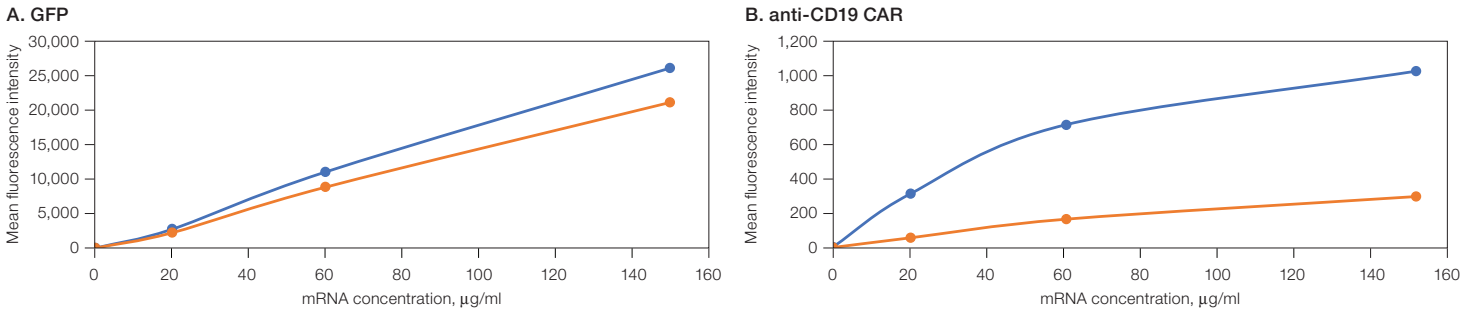

Fig. 4. Optimization of mRNA electroporation in Jurkat T cells. A, measurements of GFP expression intensity from GFP mRNA dose-response experiment; **B**, measurements of anti-CD19 CAR immunolabeling intensity from anti-CD19 CAR mRNA dose-response experiment. Expression was assayed 6 hr (—) and 24 hr (—) after electroporation. CAR, chimeric antigen receptor; GFP, green fluorescent protein.

Table 2. Transfection efficiency in Jurkat T cells.

Time after Electroporation, hr	mRNA, µg/ml	GFP-Positive Cells, %	CAR-Positive Cells, %	
			Anti-CD19 CAR	CD19-FITC
6	0	0.00	0.00	0.04
	20	97.19	88.23	83.14
	60	98.13	89.37	86.85
	150	98.05	84.25	79.28
24	0	0.02	0.01	0.07
	20	97.30	34.83	14.20
	60	97.36	73.60	63.50
	150	97.96	71.73	65.18

CAR, chimeric antigen receptor; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate.

We optimized the concentration of mRNA used in electroporations, as well as the length of time required after electroporation, for optimal protein expression in human T cells. We carried out a dose-response experiment in human leukemic Jurkat T cells to examine the impact of increasing concentrations of GFP-encoding mRNA (20, 60, or 150 µg/ml) on GFP expression assayed at 6 and 24 hr after electroporation. We observed a dose-dependent response in GFP expression (reported as mean GFP fluorescence intensity, as measured by flow cytometry) at both 6 and 24 hr after electroporation (Figure 4A, Table 2). Interestingly, all tested concentrations of GFP mRNA resulted in greater than 97% positivity rate for GFP expression at both time points, suggesting that we had achieved optimal GFP transfection efficiency under our experimental conditions.

Optimization of Anti-CD19 CAR mRNA Electroporation of Human T Cells

Following the same approach we applied earlier, we performed a dose-response experiment examining the impact of increasing amounts of anti-CD19 CAR-encoding mRNA (20, 60, or 150 µg/ml) on anti-CD19 CAR expression in Jurkat T cells. In these experiments, we employed parallel confirmatory detection methods in conjunction with analysis by flow cytometry (Table 2):

- To confirm surface expression of the anti-CD19 CAR, we employed anti-CD19 CAR surface immunolabeling using an anti-CD19 CAR antibody
- To confirm that the anti-CD19 CAR was retaining anti-CD19 reactivity (i.e., that the anti-CD19 CAR was functional), we stained cells with FITC-conjugated recombinant CD19 protein

Further, we analyzed the extent of staining at 6 and 24 hr after electroporation. Consistent with our GFP mRNA results, we saw a dose-dependent response in anti-CD19 CAR expression (reported as mean fluorescence intensity), detected via either staining method, at both 6 and 24 hr after electroporation (Figure 4B). Additionally, at 6 hr after electroporation, we did not observe a substantial dose-dependent response in the total number of anti-CD19 CAR-positive cells (Table 2); all tested concentrations of anti-CD19 CAR mRNA resulted in greater than 79% positivity for CAR expression. This suggests that we had achieved maximal anti-CD19 CAR transfection efficiency at this time point with our experimental conditions. This result was generally similar at 24 hr after electroporation, although 20 µg/ml mRNA resulted in substantially fewer CAR-positive cells (14–35%) than both 60 and 150 µg/ml mRNA (64–74% CAR-positive cells) (Table 2). Further, all mRNA doses resulted in higher expression at 6 hr after electroporation than at 24 hr after electroporation (Table 2). Based on these results, we elected to transfect anti-CD19 CAR mRNA at a concentration of 150 µg/ml for the remainder of the experiments in this study, expecting that this would result in the highest level of anti-CD19 CAR expression.

Generation of Primary Human Anti-CD19 CAR T Cells by mRNA Electroporation

Using the optimized conditions described above, we next examined the expression of anti-CD19 CAR in primary human T cells following electroporation-based mRNA transfections. As T-cell activation improves transfection efficiency (Zhang et al. 2018), we activated and expanded primary CD3+ primary human T cells with IL-2 and anti-CD3/CD28 beads prior to electroporation. At 6 and 24 hr after electroporation, samples were analyzed for surface expression of anti-CD19 CAR using the same approach used in Jurkat T cells (that is, staining with an anti-CD19 CAR antibody and recombinant CD19 protein). We additionally identified CD8+ primary T cells using CD8 immunolabeling. In these experiments, cytotoxic CD8+ T cells demonstrated similar frequencies of anti-CD19 CAR expression, at both 6 and 24 hr after electroporation, relative to the global T-cell population (Figures 5 and 6). Similar to Jurkat T cells, CAR expression was highest in primary T cells at 6 hr after electroporation. These results demonstrate the success of this workflow in generating CAR T cells expressing functional anti-CD19 CAR at the cell surface.

Viability Analysis of Anti-CD19 CAR T Cells

Cellular viability is a critical parameter in the execution of any transfection workflow. Therefore, in our experiments, we examined the impact of these anti-CD19 CAR mRNA electroporation–based transfections on primary human T-cell viability using two orthogonal and corroborative methods: automated cell counting of Trypan Blue–stained cells, and flow cytometric analysis of ReadiDrop 7-AAD–stained cells. Viability measurements from both methods were largely in agreement (Table 3). We found that untransfected cells had a resting viability of 70–80%, and that mock electroporations (without addition of mRNA) had a negligible impact (≤2% reduction). Electroporation of anti-CD19 CAR mRNA resulted in a viability of ~60%. We speculate that this further impact on viability would occur with the inclusion of any protein-coding mRNA, as the inclusion of GFP mRNA in place of anti-CD19 CAR mRNA also reduced viability (to 69%). Figure 7 and Table 3 summarize these data and illustrate the relatively low impact of using these specific electroporation conditions on primary T-cell viability in mRNA CAR T-cell workflows. These results also highlight the accuracy and reproducibility of viability measurements captured via these two distinct and orthogonal approaches.

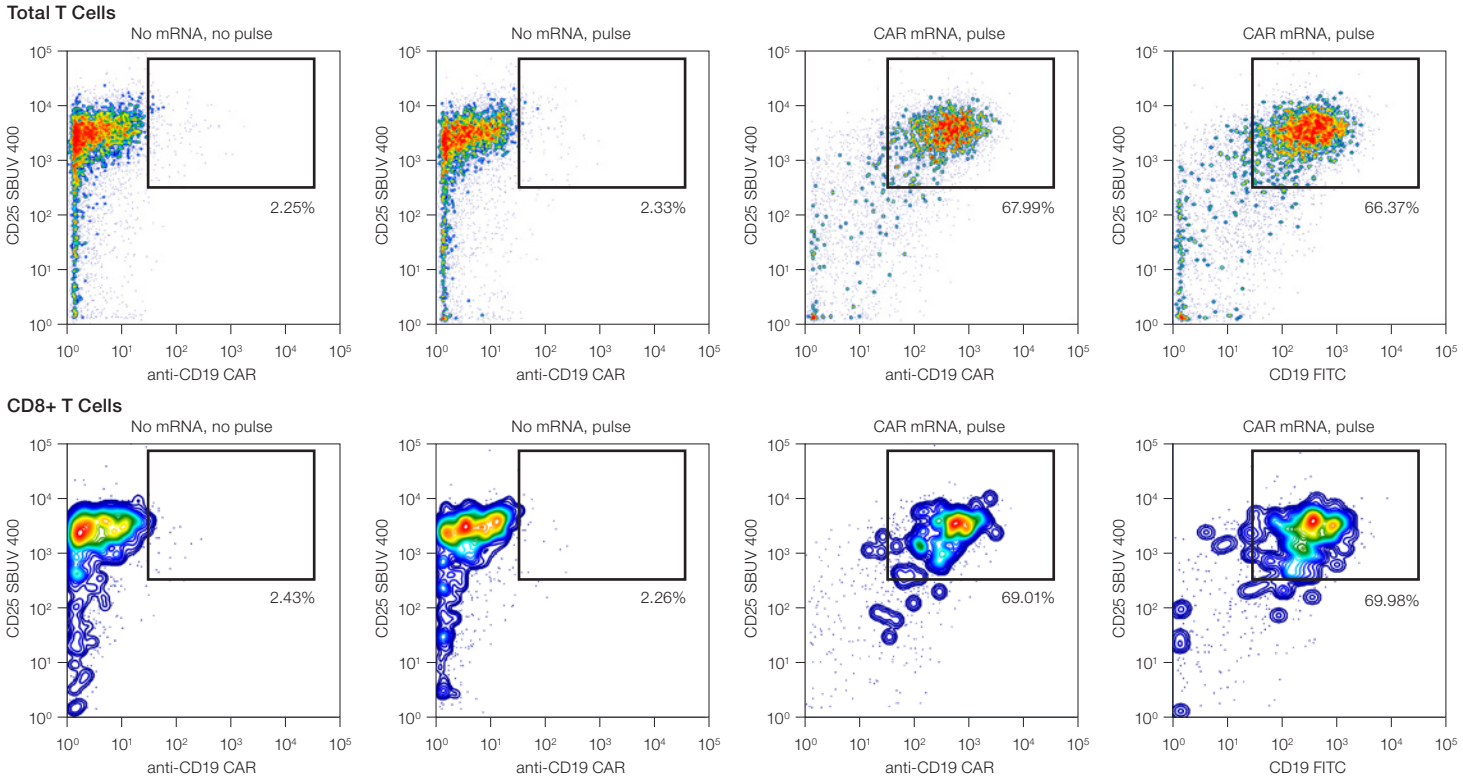

Fig. 5. Anti-CD19 CAR expression in total and CD8+ primary human T cells 6 hr after electroporation. Flow cytometry scatter plots (CD25+ vs. anti-CD19 CAR+ or CD19 FITC) measured from total or CD8+ primary human T cells. The gate shown in each scatter plot circumscribes CAR-positive cells. CAR, chimeric antigen receptor; FITC, fluorescein isothiocyanate; SBUV, StarBright UltraViolet.

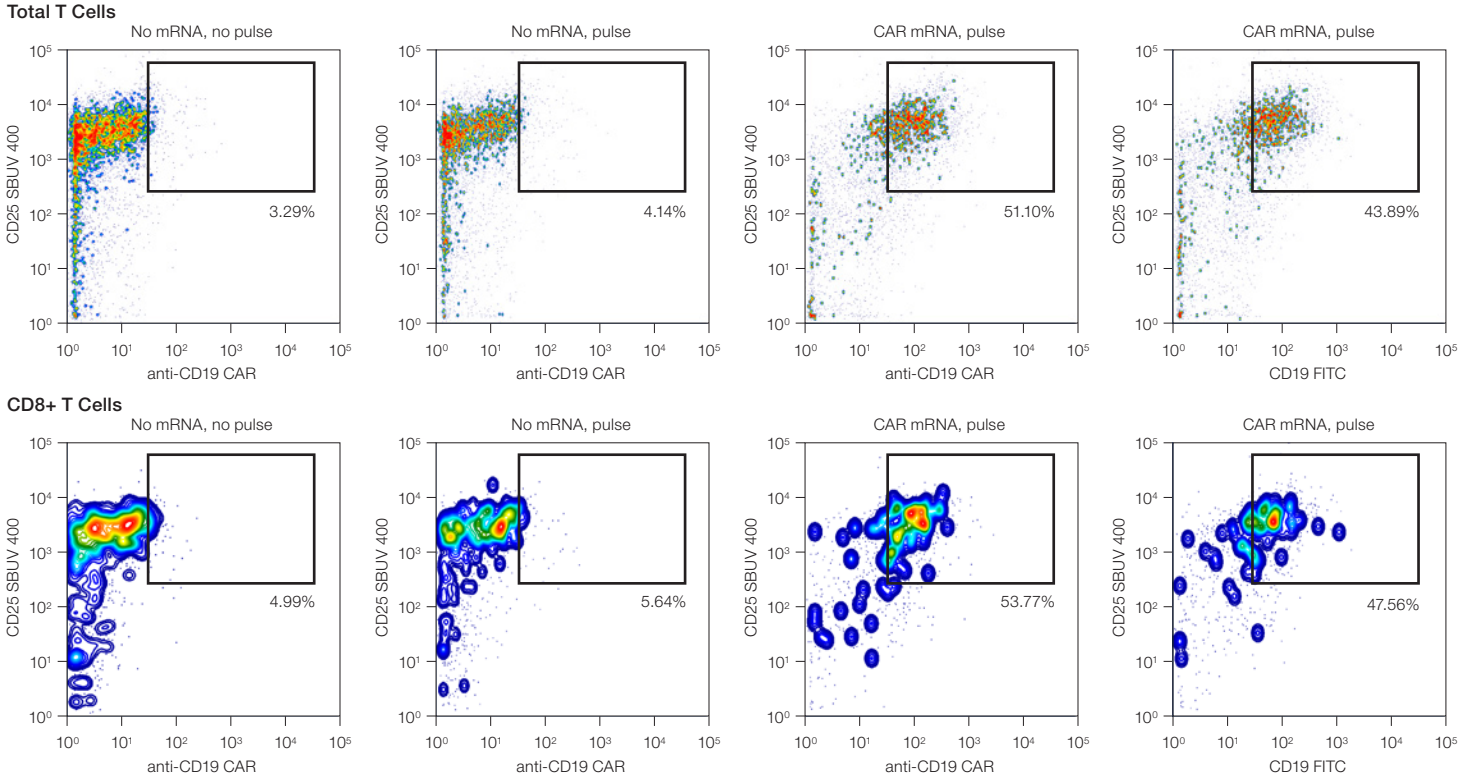

Fig. 6. Anti-CD19 CAR expression in total and CD8+ primary human T cells 24 hr after electroporation. Flow cytometry scatter plots (CD25+ vs. anti-CD19 CAR+ or CD19 FITC) measured from total or CD8+ primary human T cells. The gate shown in each scatter plot circumscribes CAR-positive cells. CAR, chimeric antigen receptor; FITC, fluorescein isothiocyanate; SBUV, StarBright UltraViolet.

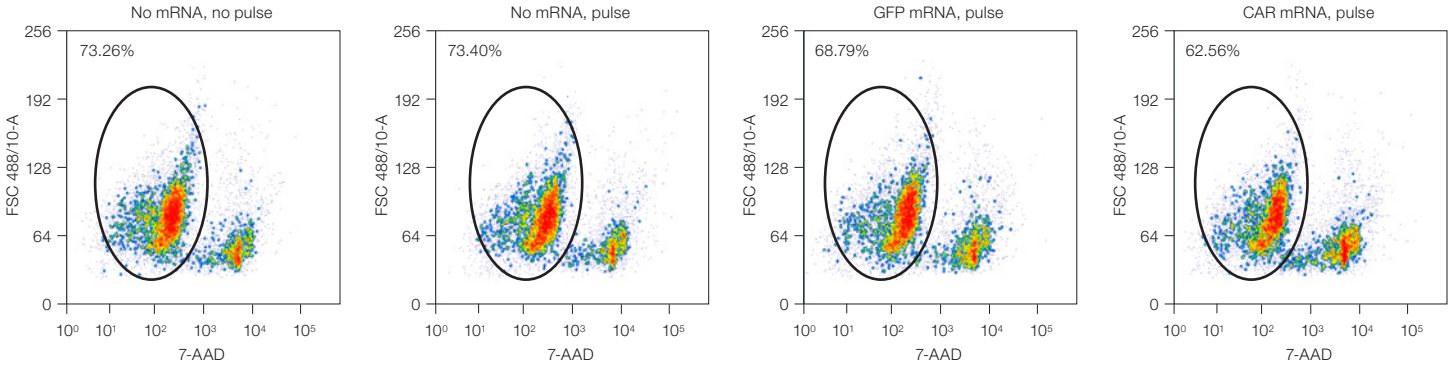

Fig. 7. Viability analysis of primary human T cells transfected with anti-CD19 CAR. Flow cytometry scatter plots (7-AAD+ vs. FSC) measured from primary human T cells. Cells were assayed 6 hr after electroporation (or mock-electroporation). Gates circumscribe live cells (cells negative for 7-AAD). Percentages shown in each panel refer to the percentage of viable cells. 7-AAD, 7-aminoactinomycin D; CAR, chimeric antigen receptor; FSC, forward scatter; GFP, green fluorescent protein.

Table 3. Viability.

	Viability, %	
Electroporation Condition	**Trypan Blue**	**ReadiDrop 7-AAD**
No mRNA, no pulse	81	73.26
No mRNA, pulse	79	73.40
GFP mRNA, pulse	69	68.79
CAR mRNA, pulse	58	62.56

7-AAD, 7-aminoactinomycin D; CAR, chimeric antigen receptor; GFP, green fluorescent protein.

Conclusions

While viral transduction is the most widely used method in CAR T-cell workflows, there are many established nonviral methods for introducing molecules into cells, and the choice will vary depending on application, cargo, and cell type, among other variables (Chong et al. 2021; Atsavapranee et al. 2021). We anticipate that the identification of a high-efficiency CAR mRNA transfection approach, as executed here via electroporation, will catalyze the wider adoption of mRNA transfection in CAR T-cell workflows.

In this study, we describe a successful nonviral method to produce CAR T cells using a high-efficiency and high-viability strategy for executing mRNA electroporations. These two transfection characteristics are vital in any CAR T-cell workflow to produce CAR T cells at a sufficient scale for clinical and therapeutic applications. More broadly, these attributes are critical to any experimental workflow involving transfections. We also note that this workflow is highly scalable and could easily be modified to produce a higher volume of CAR T cells for direct therapeutic applications, as previously described (Wiesinger et al. 2019).

It is generally well accepted that healthy cells are required for transfection workflows. Additionally, appropriate handling of primary T cells in this workflow requires treatment with activating reagents, routine examination of cell health and viability during cell culture, and determination of the optimal time point to perform electroporation.

The parallel use of automated cell counting of Trypan Blue–stained cells and flow cytometric analysis of 7-AAD–stained cells allowed us to determine the relatively low impact of our mRNA transfections on cell health and viability. The concordance between these assays indicates that either approach is sufficient to assay cell health and viability in this CAR T-cell workflow. Our immunostaining and flow cytometry experiments revealed robust cell surface expression of anti-CD19 CAR in immortalized and primary human T cells, including CD8+ primary human T cells. Importantly, in our experiments, the binding of the anti-CD19 CAR to FITC-conjugated recombinant CD19 protein most likely indicates that the anti-CD19 CAR is properly folded and able to bind its ligand.

The optimal electroporation conditions determined for primary human T cells in this study, including electrical parameters, expression time point, and mRNA concentration, should serve as a useful guide for CAR T-cell development (Table 4). However, these parameters may not be optimal for all experimental conditions (that is, for all cell types, CAR constructs, mRNA preparations, etc.). We suggest conducting a set of pilot experiments to empirically determine the optimal parameters for your experimental system that will lead to the best efficiency and viability results, as we did here and in our previous study (Wong et al. 2020). The Bio-Rad Gene Pulser Xcell Electroporation System is uniquely suited to this optimization process, as it enables flexible modification of the key parameters of electroporation.

Taken together, our study highlights the utility of the Gene Pulser Xcell Electroporation System in executing high-efficiency mRNA transfections of primary human T cells with low impact on cellular health and viability.

Table 4. Optimized electroporation conditions used in this study for Jurkat and primary human T cells.

Parameter	Item/Condition
Electroporation system	Gene Pulser Xcell Electroporation System
mRNA concentration	150 µg/ml
Pulse voltage	200 V
Wave type	Square
Pulse length	2 ms
Pulse number	1 pulse
Cuvette size	0.2 cm
Electroporation buffer	Gene Pulser Electroporation Buffer
Electroporation volume	0.2 ml
Cell number	$2x10^6$ cells
Expression time (following electroporation)	6 hr

References

Atsavapranee ES et al. (2021). Delivery technologies for T cell gene editing: Applications in cancer immunotherapy. EBioMedicine 67, 103354.

Chong ZX et al. (2021). Transfection types, methods and strategies: a technical review. PeerJ 9, e11165.

Foster JB et al. (2019). Purification of mRNA encoding chimeric antigen receptor Is critical for generation of a robust T-cell response. Hum Gene Ther 30, 168–178.

Krug C et al. (2014). A GMP-compliant protocol to expand and transfect cancer patient T cells with mRNA encoding a tumor-specific chimeric antigen receptor. Cancer Immunol Immunother 63, 999–1008.

National Cancer Institute (2022). CAR T cells: Engineering patient's immune cells to treat their cancers. https://www.cancer.gov/about-cancer/treatment/research/car-t-cells, accessed August 25, 2022.

Sterner RC and Sterner RM (2021). CAR-T cell therapy: Current limitations and potential strategies. Blood Cancer J 11, 69.

Stock S et al. (2019). Optimizing manufacturing protocols of chimeric antigen receptor T cells for improved anticancer immunotherapy. Int J Mol Sci 20, 6223.

Vannucci L et al. (2013). Viral vectors: a look back and ahead on gene transfer technology. New Microbiol 36, 1–22.

Wiesinger M et al. (2019). Clinical-scale production of CAR-T cells for the treatment of melanoma patients by mRNA transfection of a CSPG4-specific CAR under full GMP compliance. Cancers (Basel) 11, 1198.

Wong L et al. (2020). Optimizing electroporation conditions for high-efficiency mRNA transfections of CD8+ T cells with the Gene Pulser Xcell Electroporation System. Bio-Rad Bulletin 7304.

Zhang C et al. (2017). Engineering CAR-T cells. Biomark Res 5, 22.

Zhang Z et al. (2018). Optimized DNA electroporation for primary human T cell engineering. BMC Biotechnol 18, 4.

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