#5184 From tLNP Screening to Functional Validation: *In Vitro* and *In Vivo* Efficacy of CAR-T Delivery

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Introduction

Targeted lipid nanoparticles (tLNPs) delivering chimeric antigen receptor (CAR)-encoded mRNA directly to T cells enable in situ generation of CAR-T effectors, offering a promising alternative to ex vivo CAR-T manufacturing. Here, we present a systematic evaluation of CAR mRNA formats and tLNP designs and demonstrate their functional performance *in vitro* and *in vivo*. After confirming the target antigen (CD19), we screened the single-chain variable fragments (scFvs) using a phage-display platform and designed the CAR architecture. We then optimized structural and sequence elements of the CAR mRNA, including the 5′ cap, untranslated regions, coding sequence, and poly(A) tail, and the designed CAR mRNAs were subsequently screening and identified by the *in vitro* assay toolbox. Following identification of the optimal CAR design and sequence, linear and circular CD19 CAR mRNAs were synthesized and compared in activated human T cells. Circular CAR mRNA exhibited higher transfection efficiency, greater expression magnitude, and more durable expression than the linear format, and T cells transfected with circular CAR mRNA displayed enhanced cytotoxicity against CD19+ Raji targets. Through formulation and process optimization, we identified a non-targeted LNP that efficiently transfects activated T cells and established a baseline platform for mRNA expression studies. Conjugation of anti-CD3 and anti-CD8 antibodies to the LNP surface produced CD3/CD8-tLNPs that selectively target T cells. Given the established pathogenic role of B cells in systemic lupus erythematosus (SLE)—including autoantibody production, immune-complex deposition, and downstream tissue inflammation—we evaluated the efficacy of CD19 CAR/tLNPs in a pristane-induced SLE model in CD34+ HSC-engrafted mice. Collectively, these results support a workflow that integrates CAR mRNA engineering, tLNP selection, and preclinical evaluation for the development of *in vivo* CAR-T therapeutics.

Rational Design and Sequence Optimization of CAR Enabled by Phage Display and mRNA Platforms

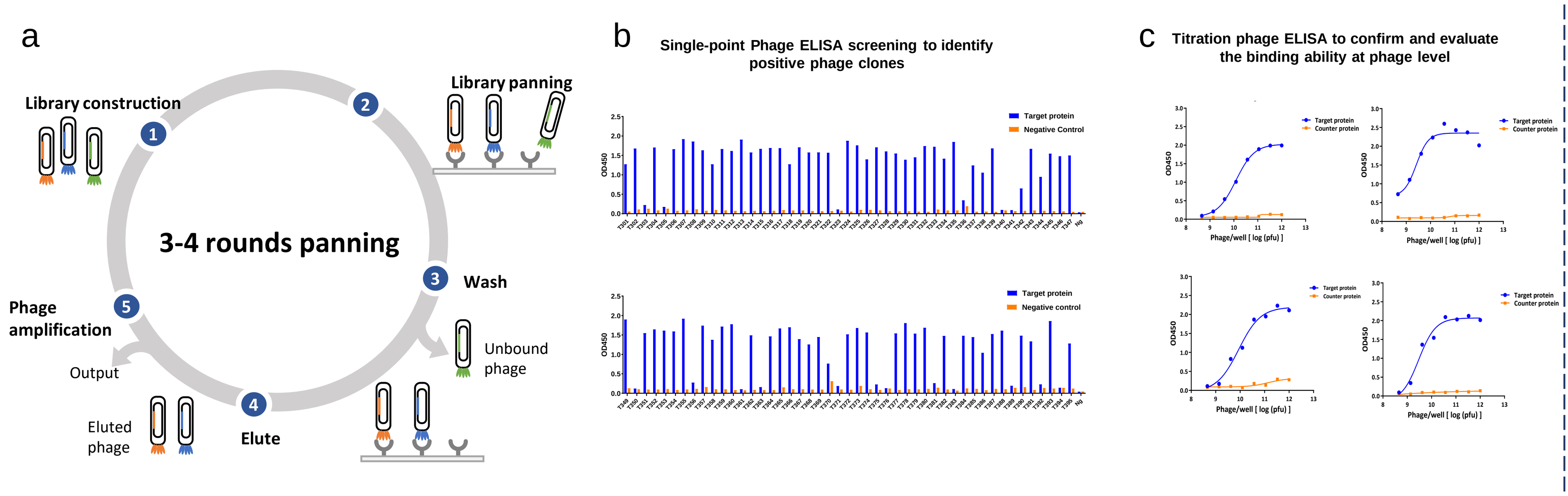
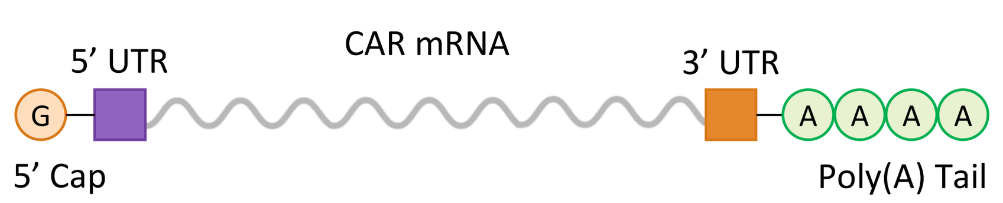


Figure 1. A phage display platform for scFv discovery in CAR-T design. a, Workflow for scFv library panning. b, Single-point ELISA screening to identify positive phage binders. c, Titration phage ELISA to confirm and evaluate the binding ability at the phage level.



5′ Cap

A chemically modified cap that facilitates **efficient ribosome binding** during translation

Methods
- two-step multi-enzymatic reaction
- co-transcriptional method

UTR's

- 5′ UTR: facilitates the **initiation of translation**
- 3′ UTR: affects the **stability and half-life of mRNA**

Methods
- Optimize length, sequence and secondary structure
- Housekeeping gene UTRs boost target protein expression in specific cells/tissues.

Coding Sequence (CDS)

A critical determinant of the **immunogenicity** and **translational efficiency**

Methods
- Optimize codon adaptability and GC content
- Nucleotide modifications

Poly(A) Tail

Ensures **efficient translation** and mRNA **stability**, while **preventing degradation**

Methods
- Optimize length and type

Figure 2. After confirming the CAR construct, optimize the 5′ cap, UTRs, coding sequence, and poly(A) tail of the CAR mRNA to ensure efficient CAR expression in transfected T cells.

Six-assay Toolbox Enables High-throughput Screening and Identification of Optimized CAR

1. Short-term Cytotoxicity (Acute, 4–48 h)
Methods: IncuCyte, Luciferase, LDH |Quick Screen + Characterization

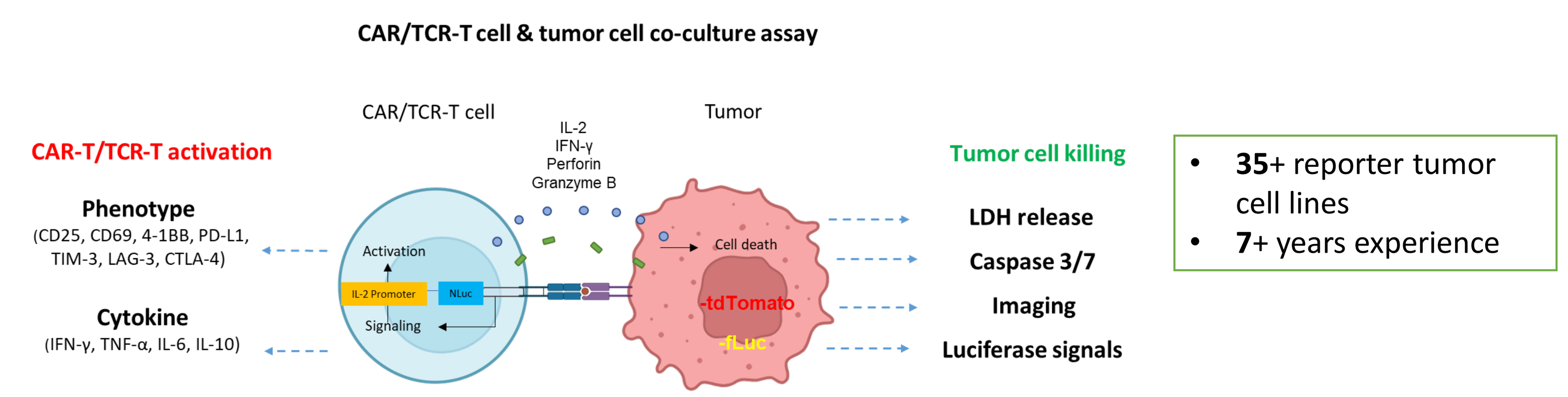
2. Long-term Killing / Persistence (5–14+ days)
Methods: Repeated challenge, real-time imaging, tumor Spheroids |Advanced Characterization

3. Proliferation
Methods: CellTrace/CFSE, Ki-67, cumulative cell counts |Characterization

4. Activation & Exhaustion
Methods: Multicolor flow panel (CD69/PD-1), Ki-67 |Quick Screen + Characterization

5. Cytokine & Soluble Biomarkers
Methods: CBA/Luminex/MSD |Screen + Characterization

6. Specificity / Antigen Density
Methods: Graded antigen, Cross-reactivity |Safety Validation



- 35+ reporter tumor cell lines
- 7+ years experience

Figure 3. Six *in vitro* assay toolbox enabling systematic biological processes evaluation for CAR in WuXi Biology, WuXi AppTec. WuXi has established approximately 40 tumor single or dual- reporter cell lines to facilitate CAR T screening and functional assessment in last decades.

Preparation and Functional Validation of CAR mRNA Constructs

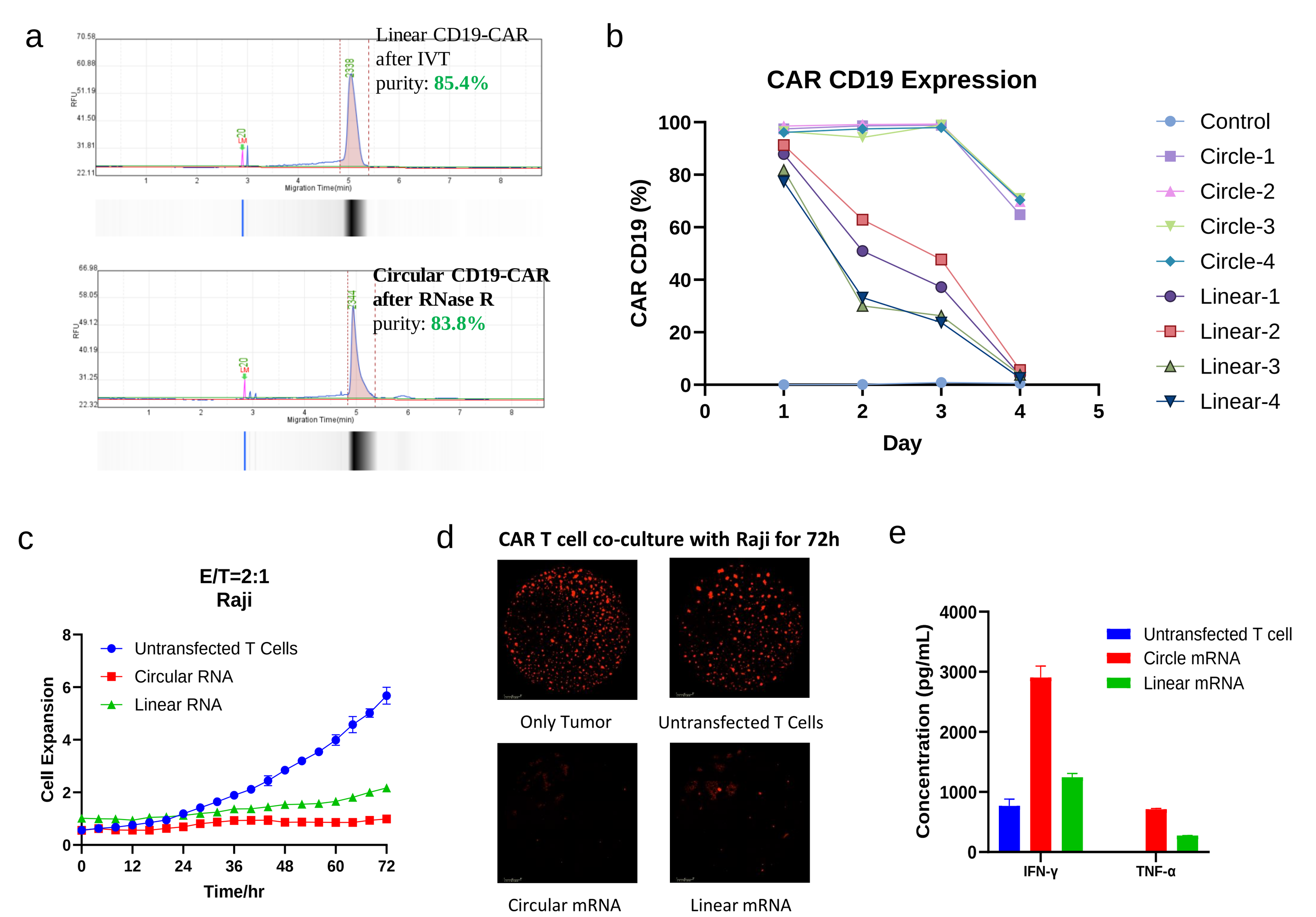


Figure 4. Circular mRNA sustains CD19 CAR expression and E:T-dependent cytotoxicity compared with rapidly decaying linear mRNA. a, Linear and circular mRNAs encoding a CD19 CAR were successfully prepared with high quality and integrity. b, CAR expression from linear mRNA declined rapidly after electroporation, whereas circular mRNA maintained a high CAR positivity rate during the first 3 days post-electroporation. c,d Both linear and circular CD19-CAR mRNAs mediated killing of CD19-positive target cells via incucyte. e, Circular CD19-CAR mRNAs triggers sever inflammation cytokine TNF-α and IFN-γ release than linear mRNA.

Development and Therapeutic Efficacy of mRNA-encoded CAR Delivered via tLNP

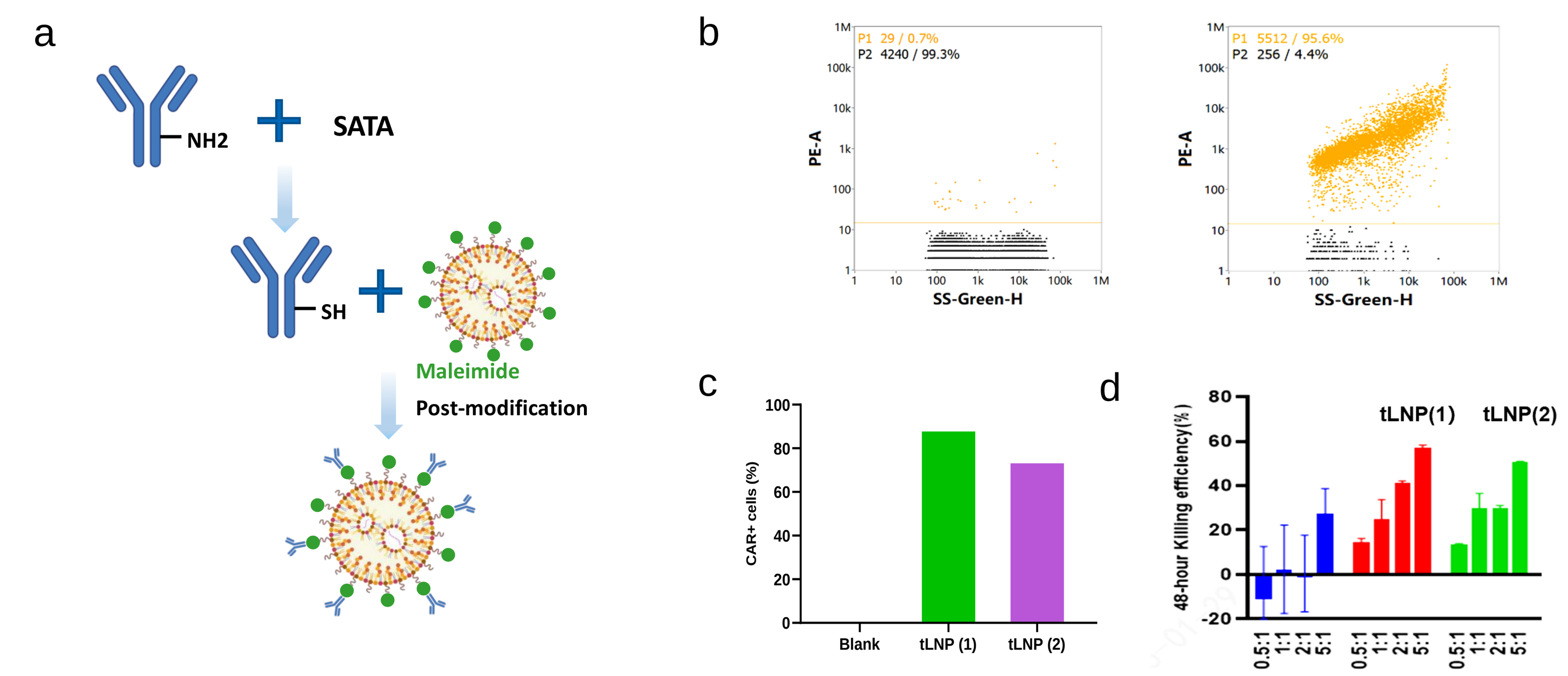


Figure 5. CD3-Targeted LNPs Enable Efficient CAR mRNA Delivery and Generation of Potent CD19 CAR T Cells. a, Preparation of antibody-targeted LNPs using a post-modification method. b, Greater than 95% conjugation efficiency of CD3 antibody to LNPs. c, Delivery of CAR mRNA to CD3+ T cells via CD3-targeted LNPs achieved CAR expression rates of 70–85%. d, CD19 CAR T cells generated with targeted LNPs exhibited potent *in vitro* cytotoxicity against Raji tumor cells.

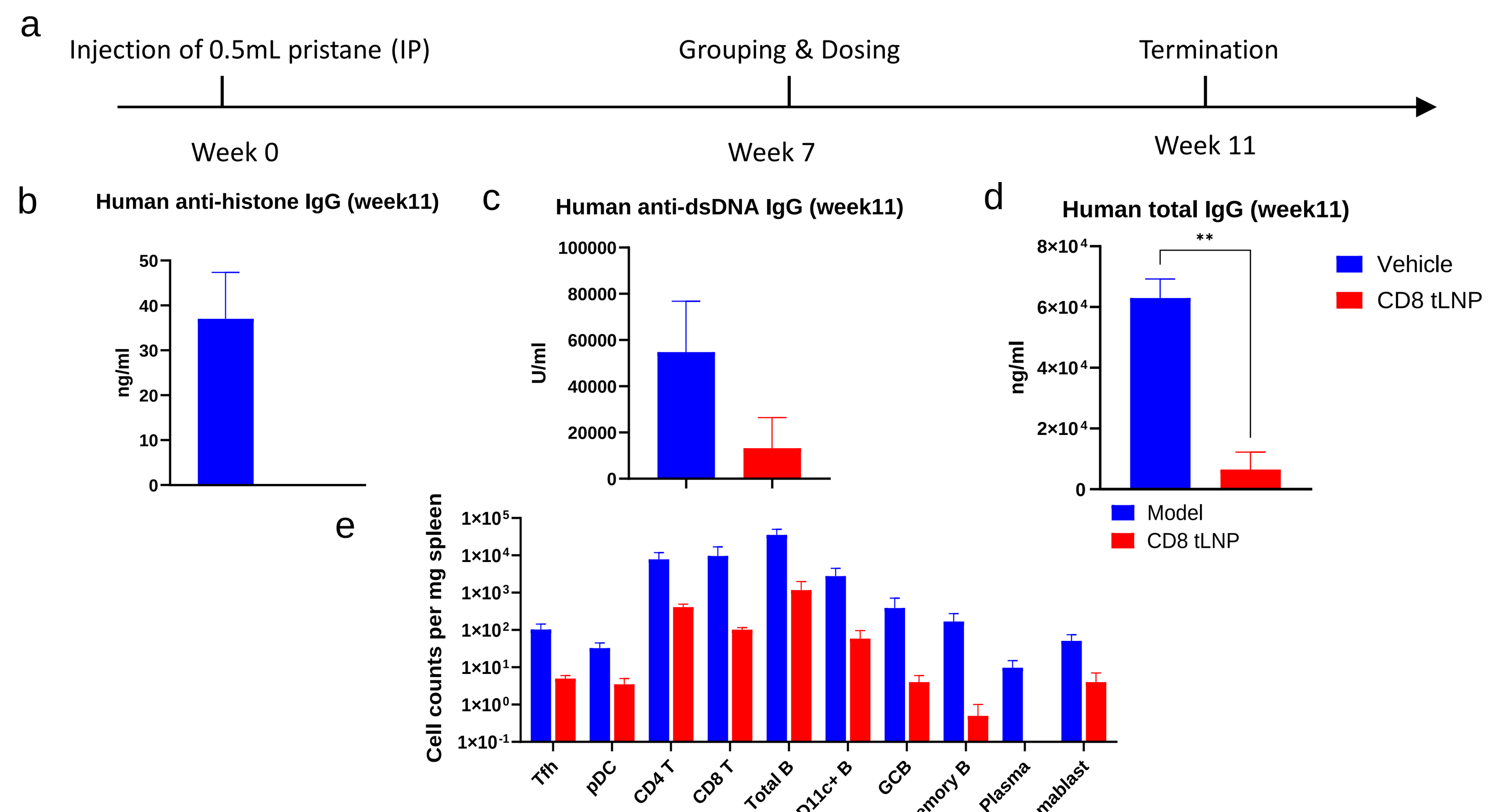


Figure 6. a, Schematic of the pristane-induced SLE model. b–d, Plasma levels of autoantibodies at endpoint: anti-histone IgG, anti-dsDNA IgG, and total IgG. e, Immune cell populations in the spleen.

Conclusion

- Phage display enables high-throughput identification of high-affinity, antigen-specific scFvs, greatly facilitating the rational design of *in vivo* CAR-T constructs.
- Following scFv screening and optimization of mRNA structural elements (5′ cap, UTRs, coding sequence, and poly(A) tail), the resulting mRNA constructs can be functionally evaluated using reporter systems such as Jurkat76 IL-2-NLuc.
- After selection of the optimized CAR construct, CD19 CAR mRNA was produced by *in vitro* transcription (IVT). Compared with conventional linear CAR mRNA, circular CAR mRNA demonstrated more sustained expression and superior cytotoxic activity.
- Encapsulation of CD19 CAR mRNA in CD3/CD8-targeted LNPs enabled efficient transfection of T cells and robust killing of CD19-positive target cells.
- The pristane-induced lupus model is a well-established murine model of systemic lupus erythematosus (SLE) that reproduces key features of the human disease, including autoantibody production and dysregulated immune responses. Treatment with targeted LNPs produced potent therapeutic effects in this model, including reduced autoantibody levels and decreased pathogenic immune cell populations.

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