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Title: In Situ Generation of Claudin18.2-Specific CAR-T Cells via Lipid Nanoparticle-Mediated mRNA Delivery

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

✓ Correct

2. Interview statements: Which interview statement filming option is the most appropriate for your group? **Please select one.**



Interview Statements are read by JoVE's voiceover talent.

Current Protocol Length

Number of Steps: 13

Number of Shots: 28

Interviews

NOTE to VO producer: Please generate VO for interviews

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. This research develops an in situ CAR-T strategy using Lipid Nanoparticle-delivered CAR mRNA targeting Claudin18.2.

1.1.1. B roll: 2.2.1

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. Conventional CAR-T cell production is costly, and its efficacy against solid tumors remains limited. This protocol enables transient, local CAR-T cell generation.

1.2.1. B roll: 2.5.1

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. This protocol allows researchers to evaluate the feasibility of using Lipid Nanoparticle-delivered mRNA to generate transient CAR-T cells for solid tumor therapy.

1.3.1. B roll: 3.2.1

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. A key experimental challenge is preventing mRNA degradation during in vitro transcription and nanoparticle preparation, as degradation can reduce encapsulation efficiency and CAR expression.

1.4.1. B roll: 4.6.1

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. Future studies will optimize lipid-nanoparticle targeting, enhance T-cell function, and combine immune checkpoint blockade to improve antitumor efficacy.

1.5.1. B roll: 5.2.1

Ethics Title Card

This research has been approved by the Ethics Committee for Laboratory Animals at Shanghai Tenth People's Hospital

Protocol

2. Lipid Nanoparticle Preparation by Microfluidic Mixing

Demonstrator: Jingyao Li

2.1. To begin, transcribe the CAR mRNA in vitro and purify the mRNA [1].

2.1.1. LAB MEDIA: JoVE71482_video_1 00:00-00:16. **TXT: See text protocol for in vitro transcription and purification of CAR mRNA**

Authors: Is it possible to film this wide shot covering the talent's face? If not, then no problem. I can use something from the existing footage

Response: Thank you for checking! Unfortunately, we don't have any additional video footage available, so please feel free to use the existing footage.

2.2. Calculate the required amount of mRNA using an 8-to-1 ratio of positively charged amino nitrogen groups to negatively charged phosphate groups. Dissolve the messenger RNA in sodium citrate solution to prepare the aqueous phase [1].

2.2.1. LAB MEDIA: JoVE71482_video_supplement——2.2.1 00:00-00:28

Please upload the new video: <https://review.jove.com/account/file-uploader?src=21802058>

Response: We have uploaded JoVE71482_video_supplement——2.2.1 to the website mentioned above.

2.3. Draw the organic and aqueous phases into separate 1-milliliter syringes, and remove any air bubbles [1].

2.3.1. LAB MEDIA: JoVE71482_video_1 00:09-00:16

Authors: I have merged and edited as suggested by you, please check if step 2.3 is correct now

Response: We checked and confirmed that there were no issues.

2.4. Load the 2 syringes into separate channels of the syringe pump [1]. Set the required flow rates for the organic and the aqueous phases. Add 5 milliliters of 50 millimolar sodium citrate buffer to the centrifuge tube connected to the outlet [2].

2.4.1. LAB MEDIA: JoVE71482_video_1 00:17-00:34

- 2.5. After flushing any residual washing solution out of the tubing, start the device, and collect the eluate [1].

2.5.1. LAB MEDIA: JoVE71482_video_1 00:35-00:38

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3. Lipid Nanoparticle Purification

- 3.1. Centrifuge the lipid nanoparticles dispersed in the sodium citrate solution in a 100 kilodalton ultrafiltration tube at 4,000 *g* for 5 to 10 minutes at room temperature [1].

3.1.1. LAB MEDIA: JoVE71482_video_1 00:39-00:45.

- 3.2. Redisperse the particles in 2.5 milliliters each of sodium citrate solution and 1x TBS, then repeat the centrifugation [1].

3.2.1. LAB MEDIA: JoVE71482_video_1 00:55-01:00.

- 3.3. Next, redisperse the particles in 1 milliliter of sodium citrate solution and 4 milliliters of 1x TBS, then centrifuge again [1]. Store the concentrated lipid nanoparticles in 1 milliliter of TBS solution at 4 degrees Celsius until use [2-TXT].

3.3.1. LAB MEDIA: JoVE71482_video_1 00:39-00:45

3.3.2. LAB MEDIA: JoVE71482_video_1 01:01-01:21 **TXT: See text protocol for LNP mRNA encapsulation efficiency**

4. T Cell Isolation and Activation

- 4.1. After thawing the PBMCs in a 37 degrees Celsius water bath, resuspend them in 1 milliliter of buffer containing PBS, 2% FBS and 1 millimolar EDTA [1].

4.1.1. LAB MEDIA: JoVE71482_video_2 00:00-00:12 **TXT: PBMCs: Peripheral Blood Mononuclear Cells**

Authors: We cannot state commercial names as per journal guidelines. Can you please provide the generic name (example, DPBS with supplements)

Response: EasySep™ Buffer consists of PBS, 2 % FBS and 1 mM EDTA.

- 4.2. Then, add 10 milliliters of immunomagnetic cell separation buffer, and mix thoroughly [1]. Centrifuge the cells at 500 *g* for 8 minutes, and count them [2].

4.2.1. LAB MEDIA: JoVE71482_video_2 00:13-00:26

4.2.2. LAB MEDIA: JoVE71482_video_2 00:30-00:40

- 4.3. Resuspend the cells in buffer and adjust the density to 5×10^7 cells per milliliter [1-TXT]. Then, add 50 microliters of isolation cocktail per milliliter of cell suspension, and incubate at room temperature for 5 minutes [2].

4.3.1. LAB MEDIA: JoVE71482_video_2 00:41-00:55 TXT: Buffer: PBS + 2% FBS + 1mM EDTA

Authors: Which buffer/reagent is used for resuspension here in 4.3.1?

Response: Resuspend using EasySep™ Buffer.

4.3.2. LAB MEDIA: JoVE71482_video_2 00:56-01:10

Authors:

Please check if the simplified narration is correct now in the steps 4.1 to 4.4:

Thaw PBMCs, resuspend in 1ml easysep buffer, wash with 10 ml separation buffer, centrifuge, count the cells. Resuspend in 1ml containing 5E7 cells and add 50ul isolation cocktail + 40 ul magnetic particles

Response: We have checked the steps and found no issues.

- 4.4. Next, add 40 microliters of the magnetic particles per milliliter of cell suspension and mix [1]. Place the tube on a magnetic stand for 10 minutes at room temperature [2].

4.4.1. LAB MEDIA: JoVE71482_video_2 01:11-01:18

4.4.2. LAB MEDIA: JoVE71482_video_2 01:19-01:26

- 4.5. Carefully transfer the enriched cell suspension to a new centrifuge tube [1]. Then, add 4 milliliters of fresh immunomagnetic cell separation buffer to the original tube containing the magnetic beads. Return the original tube to the magnet for a second 5-minute separation [2].

4.5.1. LAB MEDIA: JoVE71482_video_2 01:30-01:42

4.5.2. Reuse 4.4.2

Authors: Step 3.1.2.1 reads transfer the enriched suspension to a new tube, then remove the tube from the magnet and add buffer, which makes it unclear which tube is meant.

Response: The test tube into which the buffer was added was the one that originally contained the magnetic beads.

- 4.6. After washing the cells, centrifuge and count them [1]. Adjust the cell density to 1 million per milliliter with T cell culture medium [2].

4.6.1. LAB MEDIA: JoVE71482_video_2 02:00-02:11

4.6.2. LAB MEDIA: JoVE71482_video_2 02:12-02:22

4.7. Finally, add 12.5 microliters of human T cell activator CD3-CD28 (*C-D-3-C-D-28*) magnetic beads [1] and incubate the cells at 37 degrees Celsius in a 5 percent carbon dioxide incubator for 48 hours [2]. After lipid nanoparticle-mRNA transfection, assess the T-cell activation and CAR expression using flow cytometry [3-TXT].

4.7.1. LAB MEDIA: JoVE71482_video_2 02:28-02:35

4.7.2. LAB MEDIA: JoVE71482_video_2 02:36-02:48

4.7.3. LAB MEDIA: JoVE71482_video_3 **TXT: See text protocol for functional validation of CAR T cells**

Results

5. Results

- 5.1. The CAR mRNA ran as a single sharp peak at the predicted size [1].
 - 5.1.1. LAB MEDIA: Figure 1B. *Video editor: Please emphasize the single peak labeled 1813 without deleting other parts*
- 5.2. The lipid nanoparticles showed over 90% mRNA encapsulation efficiency [1], a particle size of approximately 150 nanometers [2], a polydispersity index below 0.2 [3], and a slightly negative zeta potential [4].
 - 5.2.1. LAB MEDIA: Figure 2A. *Video editor: Please emphasize the encapsulation efficiency bar without deleting other parts*
 - 5.2.2. LAB MEDIA: Figure 2C. *Video editor: Please emphasize panel (1)*
 - 5.2.3. LAB MEDIA: Figure 2C. *Video editor: Please emphasize panel (2)*
 - 5.2.4. LAB MEDIA: Figure 2C. *Video editor: Please emphasize panel (3)*
- 5.3. The activated T cells showed a marked increase in CD69 and CD25 expression [1].
 - 5.3.1. LAB MEDIA: Figure 3A. *Video editor: Please use the updated figure. Please emphasize the blue/cyan "activated T" histogram in both the CD69 and the CD25*
- 5.4. CAR expression began 4 hours after transfection [1], peaked above 40 percent at 18 hours [2], and fell below 10 percent by 72 hours [3].
 - 5.4.1. LAB MEDIA: Figure 3B. *Video editor: Please emphasize the CAR-T value 11.8 at 4 h*
 - 5.4.2. LAB MEDIA: Figure 3B. *Video editor: Please emphasize the CAR-T row values 41.6 at 18 h*
 - 5.4.3. LAB MEDIA: Figure 3B. *Video editor: Please emphasize the CAR-T row values 3.67 at 72 h*
- 5.5. The obtained CAR-T cells showed greater than 50% killing of both Claudin18.2-positive gastric cancer cell lines [1]. In vivo experiments in the NUGC4 mouse model

demonstrated that the lipid-nanoparticle-CAR T cell group significantly inhibited tumor growth [2] compared to the PBS group [2].

5.5.1. LAB MEDIA: Figure 4A.

5.5.2. LAB MEDIA: Figure 4B. *Video editor: Please emphasize the PBS curve and the 3 treatment curves at day 14 without deleting other parts*

5.5.3. LAB MEDIA: Figure 4B. *Video editor: Please emphasize the PBS curve*

1. Lipid

Pronunciation link: <https://www.merriam-webster.com/dictionary/lipid>

IPA: /'lɪpɪd/

Phonetic Spelling: lih·pid

2. Nanoparticle

Pronunciation link: <https://www.merriam-webster.com/dictionary/nanoparticle>

IPA: /'nænəʊ,pɑːrtɪkəl/

Phonetic Spelling: nan·oh·par·tih·kuhl

3. Microfluidic

Pronunciation link: <https://www.merriam-webster.com/dictionary/microfluidic>

IPA: /,maɪkroʊfluˈɪdɪk/

Phonetic Spelling: my·kroh·floo·id·ik

4. Transcribe

Pronunciation link: <https://www.merriam-webster.com/dictionary/transcribe>

IPA: /trænˈskraɪb/

Phonetic Spelling: tran·skrybe

5. In vitro

Pronunciation link: <https://www.merriam-webster.com/dictionary/in%20vitro>

IPA: /ɪnˈviːtroʊ/

Phonetic Spelling: in·vee·troh

6. Aqueous

Pronunciation link: <https://www.merriam-webster.com/dictionary/aqueous>

IPA: /'eɪkwɪəs/

Phonetic Spelling: ay·kwee·uhs

7. Citrate

Pronunciation link: <https://www.merriam-webster.com/dictionary/citrate>

IPA: /'sɪ,treɪt/

Phonetic Spelling: sih·trayt

8. Syringes

Pronunciation link: <https://www.merriam-webster.com/dictionary/syringe>

IPA: /səˈrɪndʒɪz/

Phonetic Spelling: suh·rin·jiz

9. Eluate

Pronunciation link: <https://www.merriam-webster.com/dictionary/eluate>

- IPA: /'elju,ert/
Phonetic Spelling: el·yoo·ayt
10. Kilodalton
Pronunciation link: No confirmed link found
IPA: /,kɪloo'də:ltən/
Phonetic Spelling: kil·oh·dawl·tuhn
11. Ultrafiltration
Pronunciation link: <https://www.merriam-webster.com/dictionary/ultrafiltration>
IPA: /,ʌltrəfɪl'treɪʃən/
Phonetic Spelling: uhl·truh·fil·tray·shuhn
12. Centrifugation
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifugation>
IPA: /,sentrɪfjə'geɪʃən/
Phonetic Spelling: sen·trih·fyuh·gay·shuhn
13. Encapsulation
Pronunciation link: <https://www.merriam-webster.com/dictionary/encapsulation>
IPA: /ɪn,kæpsə'leɪʃən/
Phonetic Spelling: in·kap·suh·lay·shuhn
14. PBMCs
Pronunciation link: No confirmed link found
IPA: /,pi:,bi:,em'si:z/
Phonetic Spelling: pee·bee·em·seez
15. Mononuclear
Pronunciation link: <https://www.merriam-webster.com/dictionary/mononuclear>
IPA: /,mɑ:noo'nʊ:kliər/
Phonetic Spelling: mon·oh·noo·klee·ur
16. Resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /,ri:sə'spend/
Phonetic Spelling: ree·suh·spend
17. EDTA
Pronunciation link: <https://www.merriam-webster.com/dictionary/EDTA>
IPA: /,i:,di:,ti:'eɪ/
Phonetic Spelling: ee·dee·tee·ay
18. Immunomagnetic
Pronunciation link: No confirmed link found
IPA: /,ɪmjənoʊmæg'netɪk/
Phonetic Spelling: im·yuh·noh·mag·net·ik
19. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /'maɪkroʊ,li:tərz/
Phonetic Spelling: my·kroh·lee·terz
20. Transfection
Pronunciation link: <https://www.merriam-webster.com/dictionary/transfection>

IPA: /træns'fekʃən/

Phonetic Spelling: trans·fek·shuhn

21. Cytometry

Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>

IPA: /saɪ'tɑ:mətri/

Phonetic Spelling: sy·tom·uh·tree

22. Polydispersity

Pronunciation link: No confirmed link found

IPA: /ˌpɑːlɪdi'spɜːsəti/

Phonetic Spelling: pol·ee·dih·spur·suh·tee

23. Zeta

Pronunciation link: <https://www.merriam-webster.com/dictionary/zeta>

IPA: /'zertə/

Phonetic Spelling: zay·tuh

24. Claudin18.2

Pronunciation link: No confirmed link found

IPA: /'klɔːdɪn eɪ'tiːn pɔɪnt tuː/

Phonetic Spelling: claw·din eighteen point two

25. Gastric

Pronunciation link: <https://www.merriam-webster.com/dictionary/gastric>

IPA: /'gæstrɪk/

Phonetic Spelling: gas·trik

26. In vivo

Pronunciation link: <https://www.merriam-webster.com/dictionary/in%20vivo>

IPA: /ɪn'viːvoo/

Phonetic Spelling: in·vee·voh

27. NUGC4

Pronunciation link: No confirmed link found

IPA: /ˌenˌjuːˌdʒiː'siː fɔːr/

Phonetic Spelling: en·yoo·jee·see four