Chapter 13

Imaging Chimeric Antigen Receptor (CAR) Activation

Kendra A. Libby and Xiaolei Su

Abstract

The chimeric antigen receptor (CAR) has been extensively exploited in cancer immunotherapy. In spite of the success of CAR T cells in clinical applications, the molecular mechanism underlying CAR-T cell activation remains unclear. Key questions remain: how are CARs activated by tumor antigens? How do activated CARs transduce signaling to downstream pathways? Here we introduce a microscopy-based method for studying CAR signaling. We use an antigen-coated supported lipid bilayer to activate CARs and combine it with TIRF microscopy to visualize the initial activation process of CAR T cells. This enables monitoring CAR signaling at high spatial and temporal resolutions.

Key words Chimeric antigen receptor, T cell signaling, Microclusters, TIRF, CD19

1 Introduction

The development of chimeric antigen receptor (CAR)-armored T cells opened a new field in cancer immunotherapy [1]. T cells isolated from patients can be genetically engineered to express CARs for targeting specific cancer surface molecules. These engineered CAR-T cells are amplified in vitro before being sent back to patients to attack tumors. CAR-based therapy has been reported to successfully treat blood cancers including chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), and non-Hodgkin lymphoma [2–4], and new CARs are being designed and tested for treating solid tumors [5].

Although CAR T cells can effectively kill cancer cells, the molecular mechanisms underlying CAR-T cell activation are not well understood. CAR is usually designed as a single-pass transmembrane receptor (Fig. 1a). Its extracellular domain contains a single-chain antibody that recognizes antigens on the cancer cell surface. CD19, a B cell surface marker, is a commonly used target for CAR. A transmembrane domain, usually from CD8α or CD28, connects the extracellular ligand-binding domain to the intracellular signaling domains. The intracellular region of CAR contains a

Chaohong Liu (ed.), *T-Cell Receptor Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 2111,
https://doi.org/10.1007/978-1-0716-0266-9_13, © Springer Science+Business Media, LLC, part of Springer Nature 2020

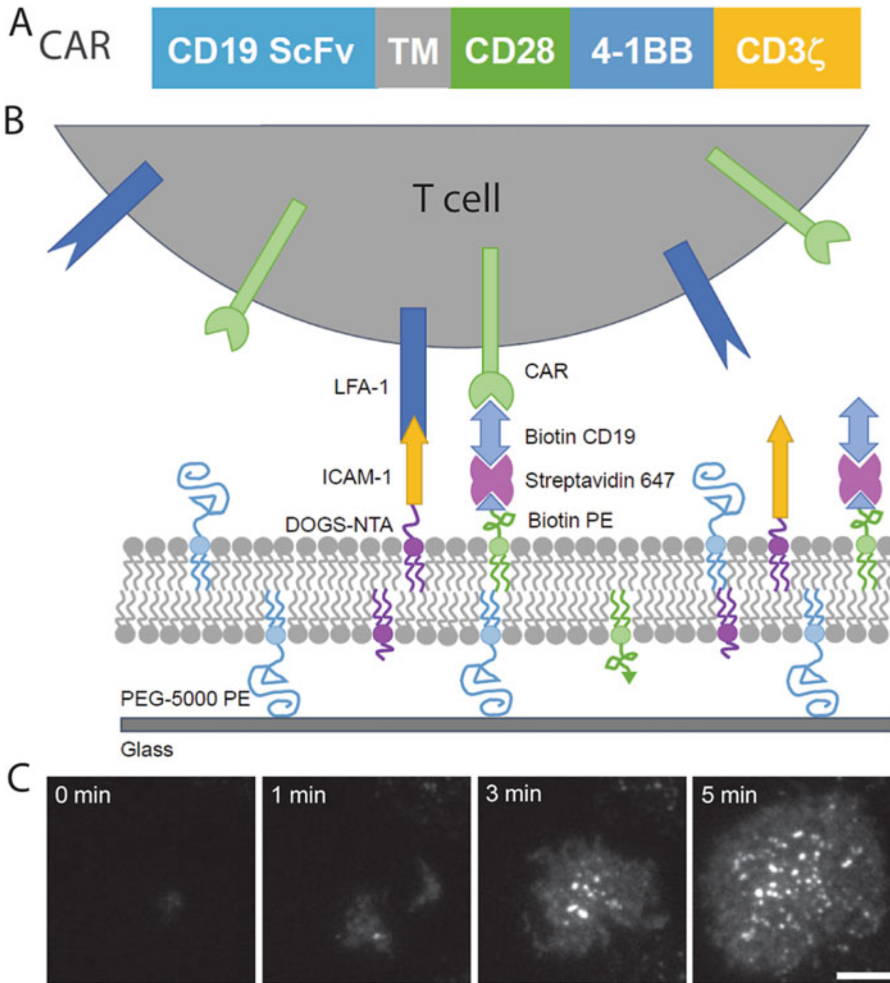

Fig. 1 A supported lipid bilayer system for CAR T cell activation. (**a**) The domain structure of CAR used in this study. (**b**) A functionalized lipid bilayer presents CD19 and ICAM-1 to CAR T cells. (**c**) TIRF microscopy reveals that CAR-GFP forms signaling microclusters as the T cell spreads on the CD19-coated bilayer. Scale bar: 5 μm

tandem fusion of the cytoplasmic domain of the T cell receptor (CD3ζ), and individual or combined co-receptors such as CD28 and 4-1BB. Intriguingly, the engagement of CAR with CD19 is sufficient to activate, at least qualitatively, all receptor signaling pathways [6], although the mechanism is not known.

To understand how CARs are activated by antigens and transduce signaling to downstream pathways, we developed a system for visualizing CAR signaling at high spatial and temporal resolutions. This system utilizes supported lipid bilayers to present antigens as well as other co-receptor ligands to CAR T cells. Supported lipid bilayers, mimicking the cell membranes of antigen-presenting cells, have been successfully used to present pMHC to activate the native

T cell receptor (TCR) [7]. It has also been utilized to study the mechanism of TCR signaling [8–10]. The supported lipid bilayer system has several features: (1) mobile antigens on membranes (in contrast to the immobile antigens attached to the glass surface) allow the reorganization of antigens and antigen-binding CARs to form higher order structures, e.g., signaling microclusters [11]; (2) a supported lipid bilayer that provides a planar surface, enabling high-quality imaging of CAR and other membrane-bound signaling protein dynamics by total internal reflection fluorescence (TIRF) microscopy; and (3) antigen density, co-receptor ligand density, and lipid composition that can be accurately controlled on the bilayer surface, enabling a quantitative understanding for CAR activation.

To prepare functionalized supported lipid bilayers, we attached the antigen CD19 to the supported lipid bilayer using a biotin-streptavidin method. To facilitate cell adhesion, we also attached ICAM-1, the ligand for integrin LFA-1, to the bilayer using a polyhistidine-NTA method (Fig. 1b). If desired, ligands for other (co)receptors (e.g., CD28, PD1) could be attached to bilayers as well. Once T cells expressing GFP-tagged CAR contact the CD19-functionalized membranes, they spread on the membranes. CARs, as visualized by TIRF microscopy, form microclusters to transduce signaling (Fig. 1c), reflecting an activation process which is similarly observed for the TCR [12]. The system could also be applied to study signaling molecules downstream CAR, including ZAP70, Gads, SLP76, Nck, or signaling reporters including calcium, MAPK, or NFAT. This would enable the monitoring of signaling kinetics at individual steps.

2 Materials

1. Lipid components: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (e.g., Avanti, 850457C), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-5000] (PEG-5000 PE) (e.g., Avanti, 880230C), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG (2000) Biotin) (e.g., Avanti, 880,129) and 1,2-dioleoyl-sn-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid) succinyl] (DOGS-NTA) (e.g., Avanti, 790404C) (*see* **Note 1**).
2. Proteins: His-tagged extracellular domain of ICAM-1 (e.g., Sino Biological, 10,346-H08H-50), Biotinylated extracellular domain of CD19 (e.g., Sino Biological, 11,880-H08H-B) (*see* **Note 2**) and Streptavidin Alexa Flour™ 647 (e.g., Thermo Fisher, S21374).

3. Tissue culture media: 10% heat-inactivated Fetal Bovine Serum (FBS) (e.g., Life Tech, 16140071) and 1% penicillin-streptomycin-glutamine (PSG) (e.g., Life Tech, MT30009CI) in RPMI 1640 (e.g., Thermo Fisher, 11875093).
4. 96-well glass bottom plate (requires flat glass, plastic wells with low fluorescence background, and resistance to treatment with acids, bases, and detergents, e.g., Matriplate MGB096-1-2-LG-L).
5. Hellmanex III (e.g., Sigma, Z805939).
6. Adhesive PCR Sealing Foil (e.g., Thermo Fisher, AB-0626).
7. Glass vials (e.g., National Scientific, B7800-2).
8. Gastight syringes 25 μL (e.g., Hamilton, 80,275) and 250 μL (e.g., Hamilton, 81,175).
9. Chloroform (e.g., Electron Microscopy Sciences, 12,550).
10. Argon.
11. NaOH.
12. PBS buffer: 155 mM NaCl, 3.0 mM Na_2HPO_4, 1.1 mM KH_2PO_4, pH 7.4.
13. High-speed centrifuge and polycarbonate centrifuge tubes.
14. Heat block.
15. Imaging media: 20 mM HEPES (e.g., Thermo Fisher, 15630-080) in RPMI 1640 no phenol red (e.g., Thermo Fisher, 11835-030).

3 Methods

3.1 Construction of Jurkat CAR T Cells

1. Prepare tissue culture media by adding 10% heat-inactivated FBS and 1% PSG to RPMI 1640; filter media if necessary. Store media at 4 °C and warm to 37 °C before usage.
2. Maintain cells in tissue culture media. Cells double roughly every 24 h. Keep the cell density below 0.5 million/mL. Split cells when necessary.
3. Produce lentivirus of CAR-GFP using HEK293 cells.
4. Infect T cells with the CAR virus.
5. Keep the culture for 10 days until the CAR expression is stabilized.
6. Select CAR-GFP$^+$ T cells by fluorescence-activated cell sorting (FACS).

3.2 Preparation of Small Unilamellar Vesicles (SUV) for Making Membranes (We follow our previous protocol for making SUV and membranes [8] with slight modifications)

1. Clean glass vials with 5% Hellmanex III, rinse them with milli Q water, and dry in oven.
2. Warm lipid stocks to room temperature.
3. Rinse the glass vial with chloroform. Pour ~1 mL chloroform into each vial.
4. Use glass syringes to prepare a lipid mix, about 4 μmol each vial. Lipid composition: 98% POPC, 2% DOGS-NTA, 0.1% DSPE-PEG (2000) Biotin, and 0.1% PEG-5000 PE (*see* **Note 3**).
5. Dry the lipid mix with a steady argon flow (*see* **Note 4**). Use a ~45 °C water bath while drying to maintain lipid solubility as the chloroform evaporates. Multiple white layers will form on the walls of the vial after the lipids dry.
6. Dry lipids further in a desiccator for at least 2 h.
7. Resuspend the dried lipids in 1.5 mL PBS. Vortex to mix.
8. Transfer resuspended lipids into two 1.5 mL microcentrifuge tubes, adding 750 μL to each tube.
9. Freeze the resuspended lipids in liquid nitrogen and thaw in a room temperature water bath. Repeat this freeze-thaw cycle 30 times until the cloudy solution becomes clear (*see* **Note 5**). If desired, freeze the resuspension at −80 °C to store for future use.
10. Centrifuge the resuspended lipids at 48,000 × *g* for 45 min at 4 °C.
11. The supernatant now contains SUVs; transfer this to a clean tube. Avoid disrupting the white pellet at the bottom of the tube. Cover the SUV solution with argon and store at 4 °C. The SUV should be used within 2 weeks (*see* **Note 6**).

3.3 Preparation of Antigen-Functionalized Supported Lipid Bilayers (See Note 7)

1. In a 1 L beaker, immerse glass imaging plate into 1 L of 5% Hellmanex III. Microwave to 50 °C. Stir and incubate overnight at room temperature.
2. The next morning, rinse each well 10 times with ultrapure water. Blow-dry all wells and seal plate with adhesive foil.
3. Use a blade to open wells to be immediately used. Add 250 μL freshly made 5 M NaOH to each well. Incubate 1 h at 50 °C on a heat block. Remove NaOH and repeat cleaning (*see* **Note 8**).
4. Add 250 μL 5 M NaOH to each well and incubate overnight at room temperature.
5. The next morning, remove NaOH from wells and rinse twice with 500 μL ultrapure water. Rinse twice with 500 μL PBS.
6. Add 200 μL PBS and 20 μL SUV to each well (*see* **Note 9**). Tap the tube containing SUV solution gently to mix before adding. Incubate for 1 h at 37 °C to allow bilayer formation (*see* **Note 10**).

7. Remove 100 μL from each well and wash three times with 500 μL PBS.
8. Prepare a solution of 10 nM streptavidin Ax647 in PBS and add 100 μL to each well. Incubate for 30 min at 37 °C.
9. Remove 100 μL from each well and wash three times with 500 μL PBS.
10. Prepare a solution of 10 nM CD19-biotin and 10 nM ICAM-1-his in PBS. Add 100 μL to each well and incubate for 1.5 h at 37 °C.

3.4 Imaging CAR T Cell Activation

1. Spin down CAR T cells to have ~0.1 million per well and resuspend in imaging media.
2. Remove 100 μL of solution from the well. Wash once with 500 μL imaging media.
3. Assess the bilayer quality in each well. A good bilayer will be fluid (can be tested by FRAP of streptavidin 647) and cover the surface completely (*see* **Note 11**).
4. Add ~0.1 million cells to the well and begin time lapse imaging immediately afterwards (*see* **Note 12**). Following landing, the cells will spread and microclusters will form in ~1–2 min, indicating an activation of the proximal signaling.

4 Notes

1. To prevent oxidation of lipids after opening, store the unused lipids in a glass vial, and fill the space above the lipids with argon. Store at −20 °C. Alternatively, we recommend using the bottling service provided by Avanti to order small aliquots of lipids.
2. ICAM-1 and CD19 used here are only the extracellular domain. They are purified from the Human cells, containing post-translational modifications.
3. POPC provides the structural component of the membrane. DOGS-NTA binds polyhistidine-tagged proteins. DSPE-PEG (2000) Biotin binds streptavidin, which can further recruit biotinylated molecules. PEG-5000 PE provides a cushion layer between the membrane and glass (Fig. 1b). A direct contact of membranes to the glass might reduce membrane fluidity. For any new composition of lipids, the membrane fluidity needs to be determined by FRAP (fluorescence recovery after photobleaching).
4. Dry the lipids using a gentle argon flow. Chloroform evaporation will take heat away and drop the temperature of the lipid mix. A water bath is necessary to maintain a stable temperature.

5. The exact cycle number may vary depending on lipid composition. Water bath-based sonication may help to reduce the freeze-thaw cycle number.
6. The SUV storage period may vary. To test how long one is able to store a specific lipid mixture, use FRAP to assess bilayer quality over time.
7. The key to making high quality bilayers is a sufficient cleaning of glass. Any dirt or dust on the glass will result in membrane defects (such as holes and tears) and reduce the mobility of lipids and membrane-associated proteins. We found a sequential cleaning using Hellmanex and NaOH is effective in removing dirt.
8. Ensure that the heat block remains around 50 °C, as high temperatures may melt the glue of the imaging plate, potentially leading to leaky wells. Similarly, avoid reheating the same plate too many times.
9. Depending upon how many lipids are lost during centrifugation, the lipid concentration after SUV preparation may vary. If the bilayer only covers part of the glass surface, increase the amount of SUV added.
10. Once a bilayer has been established, it must not dry out. Ensure that there is always at least 50 μL of solutions to cover the bottom of each well.
11. A fluid bilayer will look like television static as the individual streptavidin 647 molecules move around. There should be no dark holes or bright clumps. Any bilayer deficiencies are likely due to poor cleaning of the glass.
12. One can seed a few cells first to find the right TIRF angle and focal plane before adding the majority of cells for imaging. The number of cells one needs to add may vary depending upon how many actually interact with the bilayer; adjust the number of cells added as needed to get the desired density.

Acknowledgments

This work was supported by a Yale College First-Year Summer Research Fellowship in the Sciences and Engineering awarded to Kendra Libby. Xiaolei Su was supported by Grant#IRG 17-172-57 from the American Cancer Society, Hood Foundation Child Health Research Award, Gilead Sciences Research Scholars Award in Hematology/Oncology, The Andrew McDonough B+ Foundation, and The Rally Foundation.

References

1. June CH, Sadelain M (2018) Chimeric antigen receptor therapy. N Engl J Med 379(1):64–73
2. Ghobadi A (2018) Chimeric antigen receptor T cell therapy for non-Hodgkin lymphoma. Curr Res Transl Med 66(2):43–49
3. Lee DW et al (2015) T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet 385 (9967):517–528
4. Porter DL et al (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365(8):725–733
5. Martinez M, Moon EK (2019) CAR T cells for solid tumors: new strategies for finding, infiltrating, and surviving in the tumor microenvironment. Front Immunol 10:128
6. Karlsson H et al (2015) Evaluation of intracellular signaling downstream chimeric antigen receptors. PLoS One 10(12):e0144787
7. Dustin ML et al (2007) Supported planar bilayers for study of the immunological synapse. Curr Protoc Immunol. Chapter 18: p. Unit 18 13
8. Su X et al (2017) Reconstitution of TCR signaling using supported lipid bilayers. Methods Mol Biol 1584:65–76
9. Su X et al (2016) Phase separation of signaling molecules promotes T cell receptor signal transduction. Science 352(6285):595–599
10. Huang WYC et al (2019) A molecular assembly phase transition and kinetic proofreading modulate Ras activation by SOS. Science 363 (6431):1098–1103
11. Dustin ML, Groves JT (2012) Receptor signaling clusters in the immune synapse. Annu Rev Biophys 41:543–556
12. Grakoui A et al (1999) The immunological synapse: a molecular machine controlling T cell activation. Science 285(5425):221–227