RESEARCH HIGHLIGHT

Positively charged patches: tonic for CAR fitness

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Tonic signaling is a constitutive low-level activation of chimeric antigen receptors (CARs) in the absence of tumor antigen and plays an imperative role in the CAR-T efficacy. In a recent paper published in *Cell Research*, Chen et al. show that the positively charged patches on the surface of the CAR antigen-binding domain regulate CAR tonic signaling and open a door to further optimization of improvements of CAR.

CARs, or chimeric antigen receptors, are synthetic receptors that provide antibody-like recognition of target cells, typically cancer cells, and T cell-like signaling function to T lymphocytes. The CAR's design essentially consists of three major components: an extracellular domain consisting of a single-chain variable fragment (scFv) that recognizes the antigen, a transmembrane domain that anchors the receptor to the cell membrane, and an intracellular domain that activates downstream signaling pathways, such as cytokines, when the receptor binds to its antigen (Fig. 1a).[1]

CAR-T cell therapy has shown remarkable success in treating B-cell malignancies such as acute lymphoblastic leukemia and non-Hodgkin's lymphoma.[2] The success of CAR-T cell therapy has been driven by several factors, including the specificity of the CARs for cancer cells and the ability of CAR-T cells to persist and expand in the body. However, CAR-T cell therapy also faces several challenges that limit its effectiveness, mainly due to the limited in vivo persistency of CAR-T cells and impaired T cell function.[3] T-cell receptors and CARs exhibit tonic signaling, characterized by spontaneous but weak cell activation and low-level release of pro-inflammatory cytokines without tumor antigen stimulation.[4,5] While low-level tonic signaling may benefit CAR function, excessive tonic signaling has been associated with CAR-T cell dysfunction suggesting that this baseline CAR activation signaling should be fine-tuned for optimal antitumor function and improved clinical efficacy.[5,6] Interestingly, how CAR tonic signaling is initiated and its strength is regulated remains elusive. In a recent paper in *Cell Research*, Chen et al.[7] report that the strength of tonic signaling could be fine-tuned by optimizing positively charged patches (PCPs) on the surface of the antigen-binding domain of the CAR.

Chen et al. show the relationship between the strength of CAR tonic signaling, and CAR-T cell persistence and exhaustion. The authors developed an assay to quantify the strength of CAR tonic signaling and found that CARs which differed only in their antigen-binding domain exhibit different levels of tonic signaling strength, with GD2-CAR and CD19-CAR having the highest and lowest activities, respectively. Further, CAR-T cells with higher tonic signaling activity exhibited greater exhaustion, as evidenced by upregulation of exhaustion markers such as PD-1, TIM-3, and LAG-3, as well as impaired cytokine production and killing ability in vivo. Furthermore, the three-dimensional homology models of different CAR scFvs were created and analyzed using the SWISS homology modeler.[8] They found a statistically significant linear relationship between the PCP score and the CAR tonic signaling index, showing the key role of PCPs on the surface of the antigen-binding domain of CARs in regulating the tonic signaling strength of CARs.

An interesting finding of this study is adjusting the ionic strength of the ex vivo culture medium to optimize CAR tonic signaling and CAR-T fitness. Antigen-independent oligomerization of CARs with different charge properties shows that electrostatic interactions between CAR scFvs cause CAR clustering. CD19-CARs exhibit an even distribution of positively electrostatic fields and display a uniform distribution on the T cell; in contrast, CARs like GD2-CAR and CSPG4-CAR have primarily positively charged patches on their scFv surfaces and form aggregated puncta-like structures on the T cell membrane. T cells expressing ITAM-deficient GD2-CAR or CSPG4-CARs showed similar punctate phenotypes, demonstrating that CAR tonic signaling did not trigger this process. The study shows that the clustering of CARs can be reduced, and the function of highly tonic CAR-T cells can be improved by adjusting the ionic strength in the ex vivo culture medium, leading to better killing ability and more robust cytokine secretion against solid tumors.

Additionally, the authors tuned down the PCPs on the CAR surface. The positively charged amino acids, such as lysines in the framework region of scFv, were mutated to uncharged residues. This resulted in CARs with reduced electrostatic interactions and established a direct association between PCPs, CAR tonic signaling, and exhaustion (Fig. 1b, c). They furthermore showed that reducing PCPs could also be achieved by grafting a complementarity determining region into less charged framework regions of the antigen-binding domain of the CAR. Furthermore, Chen et al. convincingly showed that CARs with inefficient tonic signaling can be rescued by tuning up PCPs on the surface of a CAR by introducing extra PCPs. This eventually restores inefficient CAR tonic signaling, leading to enhanced T-cell persistence and antitumor activity of a CAR-T cell. They further investigated the effect of mutations within the scFv framework region of the CD19-CAR and GD2-CAR and reported that they did not significantly affect a CAR's antigen affinity, antigen specificity, or safety profile.

These findings help us understand that optimal CAR-T cell fitness could be achieved by fine-tuning tonic signaling by altering PCPs in CAR design. More importantly, it provides a new strategy to improve by engineering out exhaustion-inducing domains in a CAR. However, further studies are needed to validate these findings and investigate the long-term effects of PCP-modified CARs in clinical settings. A key question arising from this study is

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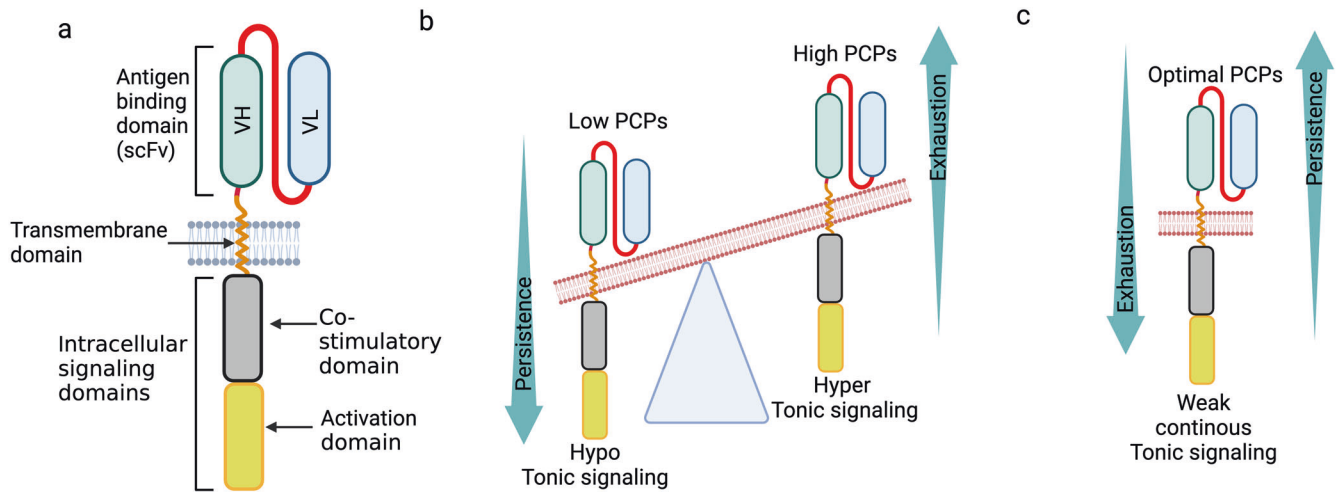

Fig. 1 Schematic illustration depicting CAR design and the effect of PCPs on the fitness of CARs. a The three main domains of a CAR, antigen-binding domain, transmembrane domain and intracellular signaling domain. **b** The effect of low and high PCPs on tonic signaling, CAR persistence and exhaustion. **c** An optimal PCPs for spontaneous weak tonic signaling and stable CAR functions. Created with BioRender.com

whether these findings will apply to all CARs? Can we have a universal CAR construct or structure where we only need to graft our scFv of interest, and the CAR would be ready for use? These open-ended questions are raised after the Chen et al. findings, and their answers lie in future studies.

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ADDITIONAL INFORMATION

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