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REVIEW

Tonic signaling in CAR-T therapy: the lever long enough to move the planet

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Abstract **Chimeric antigen receptor (CAR) T-cell therapy has shown remarkable efficacy in treating hematological malignancies and is expanding into other indications such as autoimmune diseases, fibrosis, aging and viral infection. However, clinical challenges persist in treating solid tumors, including physical barriers, tumor heterogeneity, poor *in vivo* persistence, and T-cell exhaustion, all of which hinder therapeutic efficacy. This review focuses on the critical role of tonic signaling in CAR-T therapy. Tonic signaling is a low-level constitutive signaling occurring in both natural and engineered antigen receptors without antigen stimulation. It plays a pivotal role in regulating immune cell homeostasis, exhaustion, persistence, and effector functions. The "Peak Theory" suggests an optimal level of tonic signaling for CAR-T function: while weak tonic signaling may result in poor proliferation and persistence, excessively strong signaling can cause T cell exhaustion. This review also summarizes the recent progress in mechanisms underlying the tonic signaling and strategies to fine-tune the CAR tonic signaling. By understanding and precisely modulating tonic signaling, the efficacy of CAR-T therapies can be further optimized, offering new avenues for treatment across a broader spectrum of diseases. These findings have implications beyond CAR-T cells, potentially impacting other engineered immune cell therapies such as CAR-NK and CAR-M.**

Keywords CAR-T; tonic signal; CAR signaling; antigen receptor; CAR-NK; CAR-macrophage

Introduction to CAR-T cell therapy

Chimeric antigen receptor (CAR) T cell therapy, an engineered immune cellular immunotherapy, has demonstrated remarkable potential in the treatment of malignant tumors [1–4]. The CAR's extracellular domain typically comprises a single-chain variable fragment (scFv) that recognizes tumor antigens. Its intracellular portion usually consists of a co-stimulatory domain (CD28 or 41BB, etc.) and a CD3ζ domain, which collectively transmit downstream signals to activate T lymphocytes [5]. CAR-T cells possess high affinity and specificity for tumor antigens, rendering them highly effective in targeting and eliminating cancer cells. Furthermore, these engineered T cells can differentiate into memory phenotypes *in vivo*, providing long-term protection against tumor relapse [6,7]. Therefore, CAR-T therapy has shown revolutionary efficacy in treating hematological malignancies, leading to the Food and Drug Administration (FDA) approval of several CAR-T therapies [8,9].

The remarkable ability of CAR-T cells to efficiently eliminate specific cell populations within human bodies has significantly expanded the range of indications for CAR-T therapy beyond malignant tumors in recent years [10–12]. Notably, CAR-T therapy has proven clinically effective in treating autoimmune diseases, including systemic lupus erythematosus (SLE) [13–16], idiopathic inflammatory myopathies [17–19], systemic sclerosis [20,21] and myasthenia gravis [22–24]. Moreover, numerous clinical trials investigating the use of various CAR-T designs in different autoimmune conditions are currently underway. Furthermore, CAR-T cells targeting fibroblast activation protein (FAP) have also been successfully utilized to treat cardiac fibrosis, resulting in improved cardiac function [25,26]. In anti-aging research, CAR-T cells designed to target senoantigens such as urokinase-type plasminogen activator receptor (uPAR) and NKG2D ligands have shown promise in removing

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senescent cells, potentially delaying the aging process [27–30]. Although these applications are still in their nascent stages, the expanding scope of CAR-T cell therapy suggests it will play an increasingly multifaceted role in future medical advancements, offering tremendous potential and profound implications for a wide range of diseases.

As of August 2024, clinical trials for CAR-T therapies are demonstrating unprecedented global activity, with over 1700 registered trials on ClinicalTrials.gov, most of which are in early phases (including Phase I and Phase I/II trials). Notably, these trials exhibit a geographic concentration, predominantly in China and the United States (Fig. 1A). Similarly, the global distribution of users of the CAR-Toner website [31], an AI-based CAR optimizer, mirrors this trend, with a majority located in China and the United States (Fig. 1B).

The United States, having pioneered CAR-T research and application, has seen six CAR-T products approved by the FDA, offering advanced treatment options for patients [32,33]. Meanwhile, China, with its rapidly advancing biopharmaceutical industry, has achieved significant progress in the research, development, and commercialization of CAR-T therapies, with the National Medical Products Administration (NMPA) approving five CAR-T products [34–37]. Furthermore, Europe, Japan, and the Republic of Korea are actively conducting clinical trials to advance the availability of CAR-T therapies [38,39].

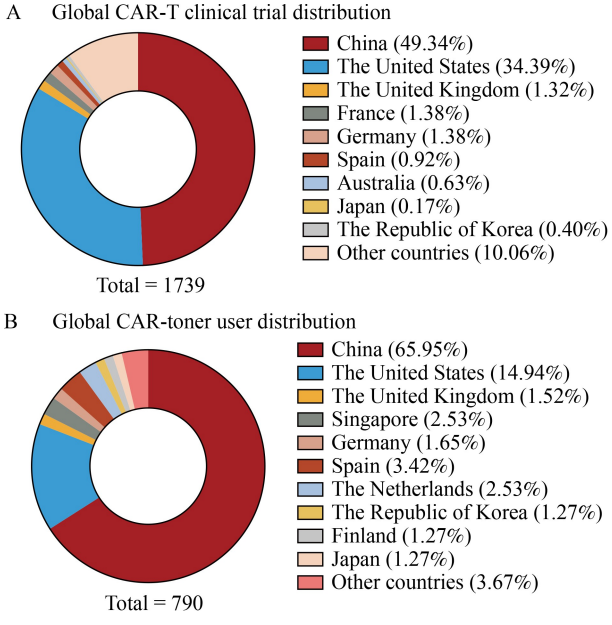

Fig. 1 Global distribution of CAR-T clinical trials and CAR-Toner users. (A) Geographical distribution of CAR-T clinical trials registered on ClinicalTrials.gov as of August 2024. (B) Worldwide distribution of CAR-Toner website users.

Current designs of CAR

The CAR has undergone substantial evolution since its invention three decades ago. In 1989, Eshhar *et al*. proposed the first prototype of CAR, combining an antibody's variable region with a T cell receptor's (TCR) constant region [40,41]. A pivotal discovery followed in 1991 when Weiss *et al*. demonstrated that the cytoplasmic domain of CD3ζ is crucial for transducing signals to activate T cells [42]. Building on these foundations, the first generation of CAR-T cells was developed in 1993, in which a scFv derived from an antibody fused with a CD3ζ intracellular domain was ectopically expressed on T cells [43]. However, despite their ability to recognize tumor antigens, these first-generation CAR-T cells exhibited limited proliferative capacity and killing function, resulting in poor clinical efficacy [44–46].

To overcome these limitations, second-generation CARs incorporated additional co-stimulatory structural domains in the intracellular signaling tails. The inclusion of either CD28 or 41BB signaling domains significantly enhanced the tumor-killing ability and *in vivo* persistence of CAR-T cells [47–51]. This second-generation design is currently the most widely used CAR structure to date (Fig. 2).

Regarding the ectodomain of CAR, in addition to scFv, several other antibody and antibody alternatives have been used as antigen binding domains. These include camelid single-domain nanobody (VHH), shark single-domain antibody (VNAR), and variable lymphocyte receptor (VLR) from jawless vertebrates [52–58]. Recent studies suggested that different antibody forms and alternatives may result in varying CAR-T performance *in vivo* [31].

Distinct intracellular co-stimulatory domains have also

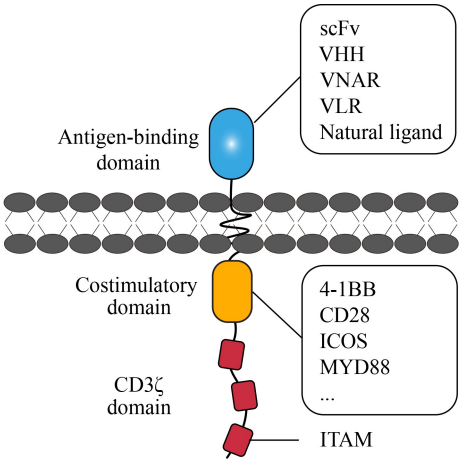

Fig. 2 Structure of a second-generation chimeric antigen receptor. The extracellular region of CAR is the antigen binding domain. The intracellular region consists of the co-stimulatory structural domain and the CD3ζ signaling structural domain.

been explored to optimize CAR-T function. While CD28 and 4-1BB remain the most commonly utilized, alternative domains, such as ICOS and MYD88, have also been investigated [59–63]. Building upon the commonly used second-generation CAR, scientists have continually refined the structure to enhance therapeutic efficacy. In 2009, June *et al*. developed the third-generation CAR, incorporating two co-stimulatory signaling domains (CD28 and 41BB) in its signaling tails, further amplifying the activation signals [64]. These third-generation CAR-T cells display increased cytokine secretion and proliferation capacity [65–68]. However, this boosted activation of CAR-T cells by secreting large amounts of cytokines may lead to safety risks. To optimize CAR-T cells to function within the tumor microenvironment, fourth-generation CAR-T cells have been developed. Based on the second-generation design, these CAR-T cells serve as delivery vehicles possessing the additional capability to secrete additional cytokines (e.g., IL15, IL12, IL18) to modulate the local immune milieu [69–75].

Limitations of current CAR-T therapy

Currently, CAR-T therapies exhibit significant efficacy in hematological malignancies [76–81]. A substantial proportion of patients with relapsed/refractory large B cell lymphoma (LBCL) and B cell acute lymphoblastic leukemia (B-ALL) achieve durable complete remissions following a single infusion of CAR-T cells [3,82–85]. Globally approved and marketed CAR-T therapies target CD19 in B cell lymphomas and BCMA in multiple myeloma. However, CAR-T therapy efficacy in solid tumors remains limited. As the application of CAR-T therapy expands, several clinical challenges persist in treating solid tumors with CAR-T therapy (Fig. 3).

Physical barriers Solid tumors are densely organized structures surrounded by tumor-associated fibroblasts (CAFs) and complex vascular networks, forming physical barriers that impede T cell infiltration [86–88]. Additionally, some tumor cells downregulate chemokine expression, hindering the effective recruitment of CAR-T cells to the tumor and thus limiting anti-tumor activities [89].

Tumor heterogeneity Solid tumors exhibit considerable heterogeneity, with cells within the same tumor potentially expressing different antigens [90]. This heterogeneity complicates the identification of universally effective tumor-specific antigens (TSAs). Consequently, CAR-T therapies targeting a single antigen may

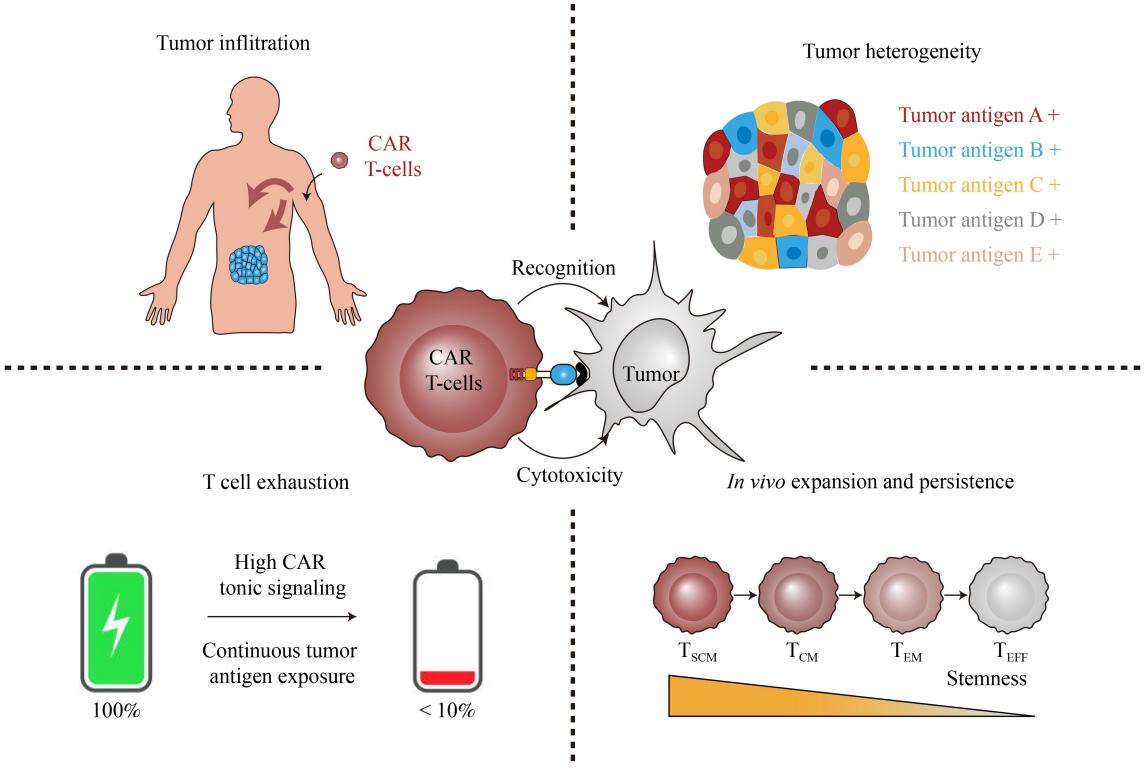

Fig. 3 Clinical challenges in CAR-T cell therapy for solid tumors. The compact and dense structure of solid tumors limits CAR-T cell infiltration, while high intratumoral heterogeneity facilitates immune escape. CAR-T cell exhaustion and functional impairment are induced by intrinsic tonic signaling and the tumor microenvironment. Furthermore, insufficient *in vivo* proliferation and differentiation of CAR-T cells into memory or stem cell-like phenotypes contribute to tumor relapse.

inadequately eliminate all tumor cells [91]. Current preclinical solid tumor targets include GD2 [44,92,93], mesothelin [94–98], HER2 [99–102], B7-H3 [103–106], EGFR [107–110], PSCA [111–113], GPC3 [114–118], Claudin18.2 [119,120], CEA [121–123] and CSPG4 [124–126]. However, compared to B cell malignancies, fewer clinical trials of CAR-T cell immunotherapy for solid tumors have been completed, highlighting the need for more effective tumor-antigen targets and novel strategies to target heterogeneous tumors.

CAR-T cell exhaustion CAR-T cells can reach a state of exhaustion in human bodies, which significantly inhibits their anti-tumor capacity [127,128]. Continuous exposure to high concentrations of tumor antigens or excessive tonic signaling can rapidly induce exhaustion, resulting in diminished cytokine secretion and proliferation [129]. Moreover, the tumor microenvironment contains immunosuppressive cells and cytokines, such as regulatory T cells (Tregs) [130–132], tumor-associated macrophages (TAMs) [133–136], and transforming growth factor β (TGFβ) [137–139], which inhibit CAR-T cell function through various mechanisms.

Limited *in vivo* persistence CAR-T cells may lose their capacity for self-renewal *in vivo* due to various reasons, compromising long-term tumor surveillance and increasing the risk of tumor relapse. The immunosuppressive and nutrient-deficient tumor microenvironment further impairs CAR-T cell proliferation and impedes their differentiation into memory or stem cell-like phenotypes [140]. Consequently, CAR-T cell numbers significantly decrease during later treatment stages, failing to maintain effective immune surveillance and increasing relapse risk.

To address these challenges, scientists are exploring diverse strategies. A comprehensive understanding of CAR downstream signaling mechanisms can significantly help the clinical application of CAR-T therapy in solid tumors.

Tonic signaling in natural immune antigen receptors

Tonic signaling is a low-level yet constitutive signal generated by immune antigen receptors in the absence of antigens. Tonic signaling is not unique to CAR-T cells, as it is also observed in unstimulated natural T and B lymphocytes. In the 1990s, scientists detected low-level phosphorylation of the CD3ζ chain in resting naive T lymphocytes, with the activity of Zap70, an essential kinase downstream of TCR, correlating with this phosphorylation [141,142]. Early studies suggested that tonic signaling was generated by T cells in peripheral lymphoid organs through transient contact with pMHC molecules on autologous cells [143]. Tonic signals play crucial roles in T cell biology as follows.

T cell homeostasis Tonic signals produced by TCRs help to maintain the *in vivo* proliferation of T cells, a phenomenon referred to as T cell homeostasis, and T cells with deleted TCRs are gradually lost over time [144].

T cell development In the thymus, T cells undergo a developmental process from double negative (DN) to double positive (DP) to single positive (SP) [145]. Upon completion of the TCRβ chain rearrangement and expression in DN stage, it combines with pre-TCRα chain to form the pre-TCR complex. The pre-TCR complex is known to generate robust tonic signals in order to examine the functionality of the newly synthesized TCRβ chain (β-selection). In the absence of ligands, pre-TCR transmits downstream signals to prevent apoptosis and promote proliferation, enabling continued development and differentiation [146]. T cells that do not express the TCRβ chain and pre-Tα chain undergo apoptosis and failure of developmental processes in the thymus [147]. If newly synthesized TCRβ, generated by random VDJ recombination, fails to transmit the tonic signal, the thymocyte undergoes apoptosis. It has been reported that pre-TCR generates tonic signals through spontaneous dimerization via electrostatic interactions mediated by charged amino acids on the pre-Tα chain [148–150] (Fig. 4A). Four conserved charged residues (D22, R24, R102 and R117) of pre-Tα are essential for spontaneous dimerization and signaling of the pre-TCR [148]. When all four amino acids are mutated to alanine, pre-TCR signaling is weakened and β-selection is compromised.

After β-selection, T cells upregulate CD4 and CD8 expression, entering the DP phase. Subsequently, T cells expressing TCR will go through positive and negative selection, eliminating T cells unable to generate tonic signals in the thymic environment (neglect) as well as self-reactive T cells whose signals are so strong (negative selection) [151]. Consequently, T cells that complete the developmental process maintain low-intensity tonic signaling *in vivo*, sustaining homeostatic proliferation within human bodies, in the absence of foreign antigens.

For B lymphocytes, tonic signaling generated by the BCR is necessary to maintain their survival *in vivo*, since knockout of BCR leads to rapid B cell death [152]. BCR-mediated low-level activation signaling via the constitutive PI3K pathway has been shown to sustain the survival of mature B cells [153]. Similar to T cells, tonic signaling also plays a critical role in B cell development. During the early stages of B cell development, the μ heavy chain undergoes rearrangement to form the pre-B cell receptor (pre-BCR) together with the surrogate light chain (SLC) [154,155]. The SLC consists of VpreB and λ5. The non-Ig portion of the λ5 chain has seven positively charged arginine residues. When these seven arginines are mutated to serines, the spontaneous phosphorylation of the pre-BCR complex, followed by the transmission of downstream signals, is blocked [156].

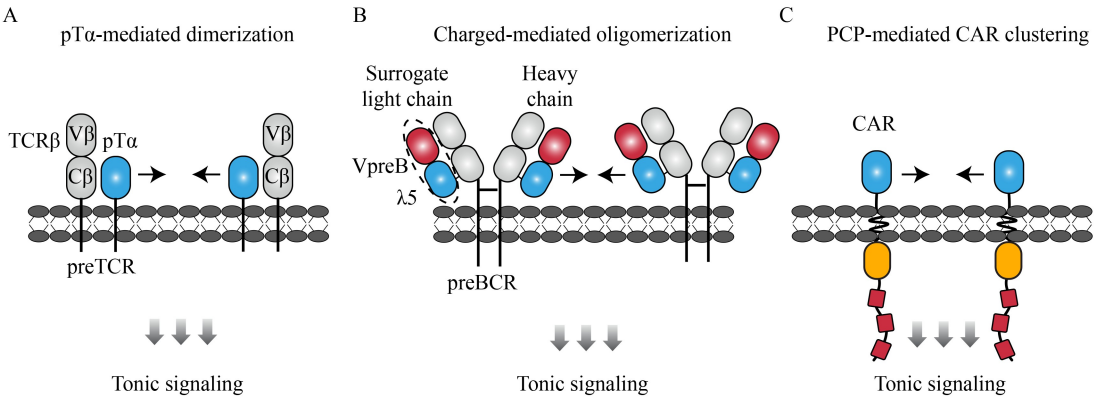

Fig. 4 Mechanisms of tonic signaling in pre-TCR, pre-BCR and CAR. Electrostatic interaction forces generated by charged amino acids on the surface of pre-TCR (A) and pre-BCR (B) drive spontaneous oligomerization of the receptor, resulting in a tonic signal. (C) Electrostatic interaction forces generated by positively charged patches (PCPs) on the CAR extracellular structure cause CAR oligomerization to generate tonic signal.

Therefore, the positively charged amino acid residues drive the oligomerization of the pre-BCR on the surface of the cell membrane, which is crucial for transmitting activation signals downstream and supporting early B cell development [156,157] (Fig. 4B).

Collectively, spontaneous aggregation of immune cell receptors or transient binding to self-antigens can trigger tonic signals in both T and B lymphocytes. Although this signal is not as potent as the strong signals triggered by foreign antigens, it is essential for lymphocyte function and survival.

CAR tonic signaling

CAR is a type of synthetic immune receptor comprising components derived from multiple natural immune receptors. Similar to TCR and BCR, CAR also generates tonic signals in the absence of target antigens, often displaying stronger tonic signal strength compared to TCR. In the absence of ligands, exogenous cytokines or feeder cells, some CAR-T cells still have the ability to proliferate autonomously over long time periods (constitutive growth phenotype) [158]. Recent studies have demonstrated that the tonic signaling of CAR-T has a significant impact on its anti-tumor efficacy and persistence *in vivo* [129,159]. By fine-tuning and engineering the molecular structure of CARs, the tonic signaling of CAR-T cells can be effectively modulated, thereby enhancing their overall efficacy in cancer treatment.

The extracellular structural domains of CAR can significantly influence tonic signal intensity. Meanwhile, CARs with distinct antigen binding domains designed for different targets often confer unique tonic signaling characteristics to CAR-T cells [159]. For instance, CAR-T cells targeting c-Met and mesothelin exhibit a constitutive growth phenotype for ligand-independent expansion, whereas CAR-T cells targeting CD19 do not [158]. Furthermore, when the intracellular structures of the CARs remain identical, significant variations in tonic signal intensity are observed solely by replacing different scFvs. This finding strongly supports the role of antigen binding domains as a key determinant in tonic signal generation. Indeed, the mechanism of tonic signal formation is thought to involve scFv-driven CAR oligomerization on the cell membrane, thereby transmitting activation signals downstream [159]. Long *et al*. observed that GD2.CAR spontaneously formed clusters on the cell membrane, resulting in a stronger tonic signal, whereas CD19.CAR, which had a weaker tonic signal, was more evenly distributed on the cell membrane [129]. Chen *et al*. further demonstrated that oligomerization of scFv is mediated by electrostatic interaction forces generated by positively charged amino acid residues on its surface, providing a robust molecular mechanism for CAR tonic signaling [159]. Chen *et al*. calculated the surface charge distribution of scFv in CAR molecules targeting 10 different antigens (GD2, Her2, CSPG4, EGFR, mesothelin, GPC3, TRBC1, CD133, BCMA, and CD19) currently in the clinical use and determined their tonic signaling intensity. They found that CAR molecules with more positively charged patches (PCP) in scFv were more likely to spontaneously aggregate, generating stronger electrostatic fields and tonic signals (Fig. 4C). Consequently, quantifying the area of PCP (PCP scores) can serve as a predictor of the CAR tonic signaling, and modulating PCP scores through protein engineering can precisely control CAR-T cell tonic signals, thereby improving functionality (Fig. 5A, Table 1) [159]. Furthermore, in the process of manufacturing CAR-T, the ionic strength in the culture environment can affect the tonic signal of CAR-T cells. When the ionic strength in the culture medium is enhanced, it will weaken the electrostatic interaction force generated by the surface charge of CAR, preventing the spontaneous aggregation of CAR leading to the

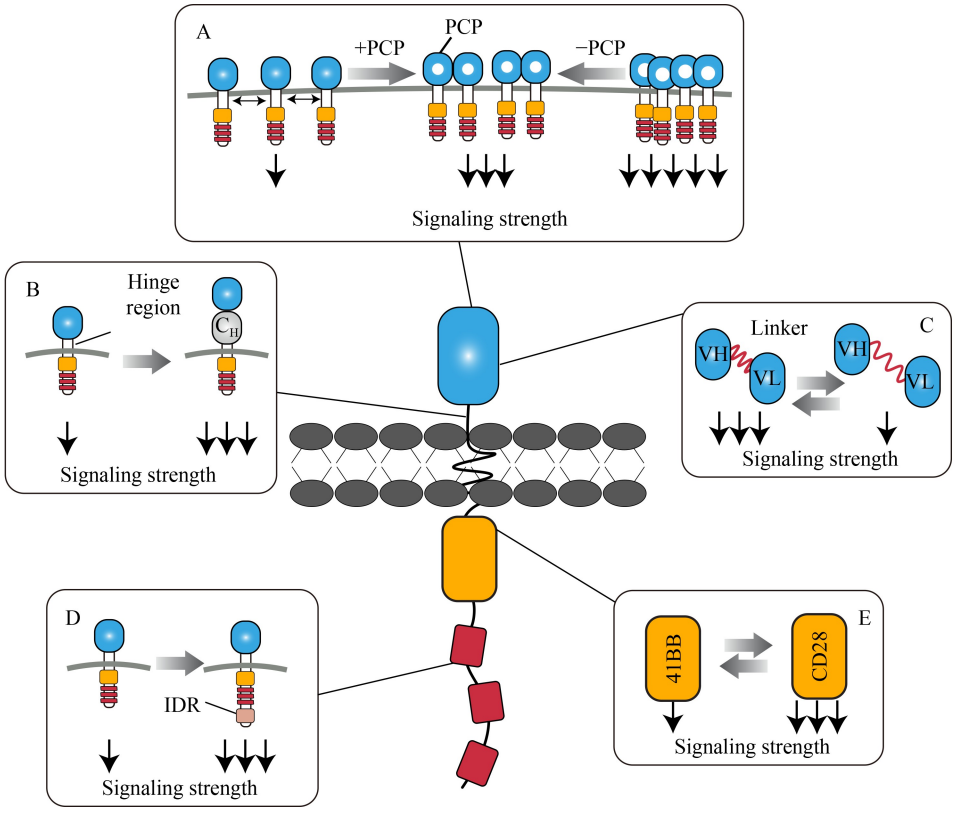

Fig. 5 Strategies for optimizing tonic signaling in CAR-T Cells. Tonic signal of CAR-T cells can be modulated by several strategies: (A) altering the size of positively charged patches (PCPs) on the single-chain variable fragment (scFv); (B) modifying the hinge region length between the scFv and transmembrane domain; (C) adjusting the length of the linker between variable heavy (VH) and variable light (VL) chains; (E) selecting different co-stimulatory domains; and (D) engineering the CD3ζ signaling domain. These modifications allow for fine-tuning of CAR-T cell activation and function.

Table 1 CAR modification strategies for regulating tonic signal strength in CAR-T cells

Modification of CAR	Targets of CAR	Tonic signal	Functional consequences
Changing the size of the PCP	CD19.CAR	Enhance	Enhanced proliferative capacity and *in vivo* anti-tumor efficacy in mice
	GD2.CAR	Weaken	Reduced exhaustion and enhanced *in vivo* anti-tumor efficacy in mice
Changing the linker's length between VH and VL	CD22.CAR	Enhance	Increased cytokine secretion, enhanced anti-tumor efficacy in mice *in vivo* and enhanced efficacy in clinical trials
Changing the length of hinge region	PSCA.CAR	Weaken	Enhanced persistence *in vivo* in mice, enhanced anti-tumor efficacy *in vivo* in mice
Replacement of co-stimulatory domain	CD19.CAR, GD2.CAR	Weaken	Reduced exhaustion, enhanced *in vivo* persistence in mice, enhanced *in vivo* anti-tumor efficacy in mice
Addition of CD3e structural domain	CD19.CAR	Weaken	Reduced exhaustion and enhanced *in vivo* anti-tumor efficacy in mice
Addition of IDR structural domain	CD19.CAR, Her2.CAR	N/A	Increased cytokine secretion and enhanced *in vivo* anti-tumor efficacy in mice

reduction of the tonic signal [159].

Based on this principle, Qiu *et al*. developed an artificial intelligence (AI)-based PCP prediction program (CAR-Toner) that rapidly and accurately predicts PCP scores based on CAR amino acid sequences and provides protein engineering optimization strategies [31]. This tool significantly improves the speed of CAR-T cell therapy product development and optimization, leading to its widespread adoption by CAR-T scientists globally (Fig. 1B).

Modulation of CAR-T tonic signaling can also be achieved by changing other extracellular structural components. For example, shortening the connecting sequence between VH and VL in scFv can promote the

spontaneous aggregation of CAR, leading to the enhancement of tonic signal intensity [160] (Fig. 5C, Table 1). Similarly, adjusting the length of the spacer between scFv and the transmembrane region (hinge region) can also regulate tonic signals. For example, removing the CH2CH3 structural domains in the hinge region will result in a significant reduction of tonic signals in CAR-T cells [161] (Fig. 5B, Table 1).

In addition to modifying extracellular domains, altering the intracellular structural domains of CAR can effectively regulate tonic signaling in CAR-T cells. First, replacing different co-stimulatory structural domains can significantly modulate tonic signaling. Compared to using CD28 as the co-stimulatory structural domain, incorporating 4-1BB or ICOS as the co-stimulatory structural domain often results in a reduction in the tonic signal intensity of CAR-T cells [129] (Fig. 5E). Furthermore, OX40 has been added to CAR-T cells to enhance the proliferation and cytotoxicity of CAR-T cells [162,163]. OX40 signaling reduces apoptosis of CAR-T cells mainly through the upregulation of genes encoding members of the Bcl-2 family and enhances the proliferation of CAR-T cells through activating the NF-κB, MAPK and PI3K-AKT pathways [162]. The effect of OX40 and its downstream signals on tonic signals deserves further exploration. Secondly, engineering the CD3ζ signaling transduction domains provides another avenue for tonic signaling optimization. Mutating ITAMs within the CD3 region to modulate its signaling capacity or blocking CD3 ubiquitination to prevent CAR degradation both likely affect CAR tonic signaling [164,165]. One study optimized signaling in CAR-T cells by adding a CD3ε signaling domain to the intracellular end of the CD28ζ CAR molecule. This modification significantly reduced the production of cytokines (e.g. IL2, IFN-γ, TNF-α) in response to antigenic stimulation, while enhancing cellular persistence and anti-tumor effects in a murine tumor model [166]. Similarly, the insertion of the CD3ε cytoplasmic structural domain between 41BB and CD3ζ in the 41BBζ CAR structure also improved CAR-T cell performance. The RK motif of CD3ε facilitated LCK recruitment, enhancing signaling and cytotoxic capabilities [167]. Reduced basal CAR phosphorylation level and weaker tonic signaling were also observed. Moreover, adding intrinsically disordered regions (IDRs) after the CD3ζ domain has been shown to induce CAR condensation at the cell membrane, enhancing antigen sensitivity and tumor-killing function [168]. This result may be due to that IDR-induced CAR aggregation generates a stronger tonic signal and therefore enhances the cytotoxicity of the CAR-T (Fig. 5D).

Peak Theory

While CAR-T tonic signals are known to have a profound impact on the efficacy of CAR-T therapies, it is worth noting that the relationship between tonic signal intensity and anti-tumor function is not linearly correlated. We propose that the relationship between CAR-T cell efficacy and tonic signal intensity follows a bell-shaped distribution curve (Peak Theory) (Fig. 6). Excessively weak signals can impair the proliferative capacity of CAR-T cells, leading to poor *in vivo* persistence and increased risk of tumor relapse. Conversely, overly strong signals can lead to rapid exhaustion of CAR-T cells, compromising their ability to kill tumors. Therefore, moderate tonic signal strength is the key to ensuring optimal CAR-T therapy efficacy.

Several studies provide supporting evidence for the Peak Theory. For instance, GD2.CAR and CSPG4.CAR, which exhibit higher tonic signal intensity, also display more severe exhaustion phenotypes, whereas CD19.CAR, with weaker tonic signaling, do not show a exhaustion phenotype [159]. Thus, for CARs with strong tonic signaling (GD2.CAR and CSPG4.CAR), reducing positively charged residues on their surfaces through point mutations resulted in reduced tonic signaling and exhaustion, resulting in enhanced anti-tumor efficacy. Conversely, for CARs with weak tonic signaling (CD19.CAR), augmenting tonic signaling by increasing the PCP area improved *in vivo* persistence and anti-tumor function [159].

Similarly, studies have showed that shortening the sequence between the heavy and light chains of the CD22.CAR scFv can promote spontaneous aggregation of CAR molecules on the cell membrane, which enhances CAR signaling and clinical efficiency [160]. Watanabe *et al*. obtained results consistent with the Peak Theory by adjusting the length of the spacer between the scFv and the transmembrane region [161]. They found that PSCA.CAR with a full-length spacer region (CH2CH3) had strong CD3ζ phosphorylation signals in the absence of antigenic stimulation, and the expression of CD25 was also maintained at a high level, which led to faster

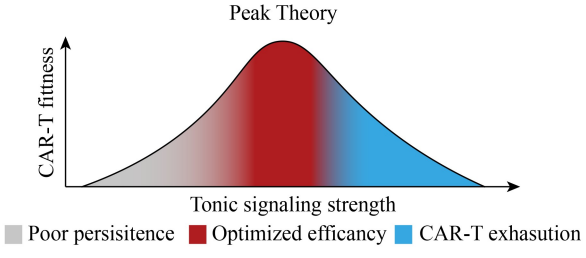

Fig. 6 Peak Theory: optimizing tonic signaling intensity in CAR-T Cells. A conceptual model for the relationship between tonic signaling intensity and CAR-T cell fitness posits an optimal range of tonic signal strength that maximizes CAR-T cell efficacy. Insufficient tonic signaling may compromise CAR-T cell persistence, while excessive signaling can lead to T cell exhaustion.

senescence of PSCA.CAR-T cells. Complete removal of the CH2CH3 region substantially attenuated PSCA.CAR-T cell tonic signals but also impaired T cell recognition and killing ability. In contrast, when the spacer region was tuned to a medium length (only one CH3 region), the tonic signal of PSCA.CAR-T cells was moderate, which was able to restore the killing ability while avoiding the accelerated senescence and death of cells [161]. The phenomenon aligns well with the Peak Theory and provides strong supporting evidence. Interestingly, a study by a University of California, Los Angeles (UCLA) research team independently verified the basal signals of CARs should be weak but not zero [169]. Other studies also discussed the impact of the hinge and transmembrane (TM) regions of CAR on CAR-T cell function. Altering the length or origin of the hinge and TM region enables CAR-T cells to proliferate more slowly and secrete fewer cytokines, but still maintain anti-tumor functions and mitigate toxic side effects such as neurotoxicity and CRS [170,171]. The above results are likely also due to the effects of the hinge and TM region on the tonic signaling of CAR-T cells, which also suggests that excessive tonic signaling may not lead to better efficacy, but rather enhance toxic side effects.

It is worth mentioning that, in addition to the tonic signal intensity, the duration of signal transmission is also a crucial factor. Li *et al*. elucidated that the transport and degradation mechanism of CAR receptors is dependent on CAR ubiquitination modification [165]. In resting T cells, CAR receptors are continuously internalized on the surface of resting CAR-T cells, and the internalized CAR receptors return to the cell surface to maintain dynamic balance. In activated T cells, CAR undergoes fast ubiquitination followed by lysosomal degradation, which results in CAR downmodulation [165]. Based on this principle, the mutations at the intracellular domain of CAR that block CAR ubiquitination produce a recyclable CAR, which greatly extends the half-life of CAR on the cell membrane and prolongs the duration of CAR signaling [165]. When applied to CD19.CAR, known for weak tonic signaling, the recyclable CAR design significantly increased CAR-T *in vivo* persistence, consistent with the Peak Theory.

Physiologic significance of tonic signaling

The intensity of the signals received by T cells, as a key regulatory factor, profoundly affects the formation of their functional properties. Specifically, T cell antigen sensitivity demonstrates a significant positive correlation with signal strength. During T cell development, surface expression of protein CD5 gradually increases, with high expression of CD5 on mature T cells. Some studies have reported that the surface expression level of CD5 directly correlates with the intensity of TCR signaling [172]. Furthermore, when naïve CD4 T cells are divided by CD5 expression, it can be observed that the higher CD5 expression correlates with elevated basal CD3 phosphorylation levels, indicating high tonic signaling [173]. It has also been reported that CD5 expression also correlates with T cell sensitivity to foreign antigens. Naïve CD8 T cells with high CD5 expression can be induced to exhibit stronger activation and proliferation phenotypes by foreign antigens [174].

Given CD5's positive correlation with both TCR tonic signal intensity and T cell antigen recognition sensitivity, it is reasonable to infer an intrinsic link between T cell tonic signal intensity and antigen recognition sensitivity. Higher tonic signal intensity may reflect a more active and/or sensitive state of intracellular signal transduction pathway within the T cell, which allows the T cell to respond faster and generate stronger signals when exposed to antigens, thereby enhancing recognition of the antigen. Similarly, Stefanová *et al*. pointed out in their study that the tonic signaling generated by interaction with autologous pMHC lowers the activation threshold of T cells, allowing the TCR to become more sensitive when it actually encounters a foreign antigen [175]. Consequently, increasing the basal TCR signaling intensity enables T cells to recognize previously unresponsive low-abundance antigens [176]. This principle can also be extended to CAR-T cell therapy, where CAR replaces TCR as the signaling sensor on CAR-T cell surfaces. Similarly, the sensitivity of CAR-T cells to tumor antigens can also be enhanced by improving CAR tonic signaling, which helps to kill tumor cells with low antigen density.

In addition to enhancing antigen sensitivity, the intensity of tonic signals can alter CAR-T cell metabolic states and promote their metabolic reprogramming. Lakhani *et al*. reported that basal phosphorylation of CD3ζ correlates with the metabolic activity of CAR-T cells [177]. They observed varied metabolic reprogramming in CAR-T cells with different scFv structural domains, e.g. high metabolic activity of GD2.CAR-T cells with strong tonic signaling and low metabolic activity of CD19.CAR-T cells with weak tonic signaling. Basal CD3ζ phosphorylation triggers a series of downstream signaling events that upregulate the expression of key proteins in metabolic pathways such as glycolysis, nucleotide synthesis and glutaminolysis, which ultimately leads to an increase in basal metabolic activity of CAR-T cells [177]. CAR-T cell differentiation was also related to tonic signal, high tonic signal resulted in the downregulation of many genes associated with T cell stemness and quiescence, such as TCF7, IL7R, CCR7, and KLF2, while the expression of genes associated with effector cell differentiation and exhaustion, including PDCD1, TIGIT, GZMB and FASL, were upregulated [159]. Similarly, a study by Watanabe

et al. demonstrated that a lower tonic signal resulted in more CAR-T cells being maintained in an undifferentiated phenotype, whereas a higher tonic signal resulted in an increased percentage of effector memory cells [161].

In summary, tonic signaling holds unique and crucial physiologic significance for CAR-T cells, playing a vital role in regulating antigen sensitivity, metabolic activity and differentiation phenotype. Moderate enhancement of tonic signals can improve the recognition sensitivity of CAR-T cells to antigens, enabling CAR-T cells to be activated by low-abundance tumor antigens to initiate effective immune responses. This strategy is important in addressing tumor heterogeneity and improving therapeutic targeting. Meanwhile, tonic signaling, as a trigger for metabolic reprogramming, can guide CAR-T cells to transition from a low metabolically active state to a highly metabolically active state. By regulating tonic signaling, CAR-T cells can be guided to adopt more efficient metabolic pathways to meet the energy required for their rapid proliferation and immune response, thereby improving therapeutic efficacy.

Conclusion and CAR tonic signaling in other cell therapies

CAR tonic signaling provides a crucial perspective for the field of immune cell therapy. However, investigations into CAR tonic signaling in alternative cell therapies, such as CAR-NK, CAR-Treg, and CAR-Macrophage, remain limited. These studies necessitate consideration of the distinct biological properties, functional mechanisms, and clinical applications of these diverse cell types.

CAR-Treg Expressing CAR on regulatory T cells harnesses the immunosuppressive functions of regulatory T cells (Treg cells) with the aim of modifying Treg cells to treat GvHD [178–180], organ transplant rejection [181–184], asthma [185,186] and other autoimmune diseases [187–191]. Unlike conventional CAR-T cells, the primary function of Treg cells is to suppress the immune response rather than exert cytotoxic effects. Consequently, applying the Peak Theory to CAR-Treg cell therapy presents additional complexities. Optimizing the tonic signaling of CAR-Treg cells requires careful balancing of their immunomodulatory activity, stable regulatory T phenotype, and Treg cell exhaustion.

CAR-NK CAR-NK cells exhibit lower *in vivo* persistence compared to their CAR-T counterparts, presenting a significant challenge in maintaining therapeutic efficacy. This limitations is typically addressed through two primary strategies: (1) multiple administrations to sustain CAR-NK cell numbers [192], or (2) supplementation or expression with cytokines such as IL-12, IL-15, and IL-18 to induce memory-like phenotypes [193–197]. Notably, appropriate modulation of CAR-NK cell tonic signaling may offer an alternative approach to enhance proliferative capacity and overcome the issue of low *in vivo* persistence, potentially improving the overall therapeutic potential of these cells.

CAR-macrophage Macrophage-based cell therapy represents a distinctive approach in the field of immunotherapy, leveraging both the phagocytic capacity of macrophages to eliminate tumor cells and their regulatory role in the immune microenvironment [198–201]. Within the tumor microenvironment, macrophages exhibit diverse polarization states, primarily categorized as anti-tumor M1 type and tumor growth-promoting M2 type [202–204]. Notably, the progression of malignant tumors can induce macrophage polarization toward the M2 phenotype [205]. Thus, in the context of CAR-macrophage therapy, careful consideration of tonic signaling is crucial, with particular emphasis on the polarization state of CAR-macrophages and their interactions within the tumor microenvironment. Optimizing the tonic signaling of CAR-macrophage cells can aim to enhance M1 polarization state while inhibiting the M2 polarization state, thereby potentiating their anti-tumor effects.

Despite the promising potential of CAR tonic signaling in advancing CAR-T cell therapies, research on tonic signaling in CAR-Treg, CAR-NK, and CAR-macrophage cells remains limited. A deeper understanding of tonic signaling in these cell types is crucial, necessitating comprehensive studies to define clear detection indicators and elucidate the underlying mechanisms specific to each specific cell type. These advancements hold the potential to significantly enhance treatment outcomes across a broad spectrum of diseases, ultimately enabling these innovative therapies to revolutionize various clinical applications.

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Compliance with ethics guidelines

Conflicts of interest Yuwei Huang and Haopeng Wang declare that they have no conflict of interest.

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