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REVIEW

Unlocking T cell exhaustion: Insights and implications for CAR-T cell therapy

Dian Xiong [a], **Haijun Yu** [b,*], **Zhi-Jun Sun** [a,*]

[a]*State Key Laboratory of Oral & Maxillofacial Reconstruction and Regeneration, Key Laboratory of Oral Biomedicine Ministry of Education, Hubei Key Laboratory of Stomatology, School & Hospital of Stomatology, Frontier Science Center for Immunology and Metabolism, Taikang Center for Life and Medical Science, Wuhan University, Wuhan 430079, China*
[b]*Department of Radiation and Medical Oncology, Hubei Province Cancer Clinical Study Center, Hubei Key Laboratory of Tumor Biological Behaviors, Zhongnan Hospital of Wuhan University, Wuhan 430071, China*

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Abstract Chimeric antigen receptor T (CAR-T) cell therapy as a form of adoptive cell therapy (ACT) has shown significant promise in cancer treatment, demonstrated by the FDA-approved CAR-T cell therapies targeting CD19 or B cell maturation antigen (BCMA) for hematological malignancies, albeit with moderate outcomes in solid tumors. However, despite these advancements, the efficacy of CAR-T therapy is often compromised by T cell exhaustion, a phenomenon that impedes the persistence and effector function of CAR-T cells, leading to a relapse rate of up to 75% in patients treated with CD19 or CD22 CAR-T cells for hematological malignancies. Strategies to overcome CAR-T exhaustion employ state-of-the-art genomic engineering tools and single-cell sequencing technologies. In this review, we provide a comprehensive understanding of the latest mechanistic insights into T cell exhaustion and their implications for the current efforts to optimize CAR-T cell therapy. These insights, combined with lessons learned from benchmarking CAR-T based products in recent clinical trials, aim to address the challenges posed by T cell exhaustion, potentially setting the stage for the development of tailored next-generation approaches to cancer treatment.

*Corresponding authors.
E-mail addresses: haijunyu@whu.edu.cn (Haijun Yu), sunzj@whu.edu.cn (Zhi-Jun Sun).

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1. Introduction

The advent of chimeric CAR-T cell therapy has marked a transformative period in cancer treatment options, as evidenced by the pivotal ZUMA-1, JULIET, and ELIANA trials[1]. These trials underscored the safety and efficacy of CAR-T therapies, such as axicabtagene and tisagenlecleucel, in patients with refractory or relapsed B-cell lymphomas, including diffuse large B-cell lymphoma (DLBCL) and primary mediastinal B-cell lymphoma (PMBCL). Further validation came from the TRANSCEND trial, which demonstrated the effectiveness of lisocabtagene maraleucel, another CAR-T product, in large B-cell lymphomas[2]. Building on these advancements, CAR-T cell therapy has been extended to various hematological malignancies, including acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL), with varying degrees of success[3]. However, despite showing promising efficacy against hematological malignancies, CAR-T cell therapies have mostly been unsuccessful in combating solid tumors up to this point[4]. On the one hand, CAR-T resistance can be attributed to antigen escape, where the selection pressure under CAR-T surveillance leads to the emergence of antigen-negative tumors. However, relapse also occurs with antigen-positive disease, suggesting that CAR-T cell-intrinsic factors such as T cell exhaustion could significantly contribute to poor anti-tumor response. Despite initial trafficking and reactivity, constant antigen exposure coupled with an immunosuppressive milieu provokes dysfunctional phenotypes. These exhausted CAR-T cells display a progressive decline in cytokine generation, proliferation, and ability to destroy target cells, hindering long-term disease management[4].

The resistance to CAR-T therapy caused by CAR-T cell exhaustion has prompted the dissection of CAR-T cell clonotype and functional state at high resolution by single-cell sequencing technologies[5]. In parallel, synergistic therapeutic combinations are being developed to potentiate CAR-T cell cytotoxicity and *in vivo* persistence[6]. Further, CRISPR-based genomic engineering tools are being employed to refine CAR-T cell specificity, persistence, and safety, while synthetic biology approaches are exploring optimized CAR expression *via* alternative promoter systems with tunable activity[7]. These strategies aim to elucidate the molecular underpinnings governing CAR-T cell function and inform the rational design of next-generation CAR-T cell therapies with improved potency and mitigated toxicity.

Long-standing evidence has identified T cell exhaustion as a pivotal element in the resistance to immunotherapy, manifested notably through the expression of PD-1 on tumor-infiltrating lymphocytes (TILs) which is associated with poor prognosis in various solid tumors[8,9]. As cytolytic T lymphocytes (CTLs) perform the primary role of tumor surveillance and clearance, the dysfunctional state observed with PD-1hi $CD8^+$ tumor-specific T cells (TSTs) is conveniently exploited by tumor cells as an immune evasion strategy to compromise T cell-based immunotherapies. Specifically, exhausted TSTs are characterized by TOX-dependent[10] sustained expression of inhibitory receptors (*e.g.*, PD-1 and TIM-3[11], altered transcriptional[12], metabolic[13] and epigenetic programs[14]), resulting in hierarchical loss of effector function and proliferative potential. Nonetheless, single-cell technologies such as spatially resolved transcriptional and epigenetic profiling are uncovering heterogeneity among T cell subsets distributed across developmental stages and the spatial immune contexture[15-17]. In light of this, T cell subsets early in development and away from the tumor immune microenvironment (TIME) exhibit minimal signs of exhaustion and are the principle contributor to durable immunotherapy response, *via* being memory-like and capable of becoming immune checkpoint blockade (ICB)-induced late-stage TILs, rather than being fully cytolytic themselves[17]. Understanding the cellular and molecular mechanisms behind T cell exhaustion would help the clinical implementation of CAR-T cell therapy by providing therapeutic targets for manipulating T cell fate in addition to diagnostic biomarkers for patient selection.

In this review, we explore the latest mechanistic insights into T cell exhaustion and discuss their clinical implications in the current landscape of CAR-T engineering and therapeutics. We contend that T cell exhaustion should be viewed not only as the cause but also the outcome of poor tumor control, thereby necessitating the use of adoptive CAR-T cells in recalcitrant ICI-resistant cases where terminal exhaustion programs are perpetuated in the immunosuppressive tumor microenvironment (TME). We further caution against direct manipulation of T cell exhaustion programs, such as PD-1 knockout, due to their role as protective measures against hyperstimulation. Together, these insights should inform the clinical implementation of safe and effective CAR-T based therapies for the next-generation of cancer therapy.

2. Mechanisms and clinical implications of tumor-specific T cell exhaustion

Ever since subsets of TILs were first observed with an "exhaustion profile", similar to their counterparts in chronic lymphocytic choriomeningitis virus (LCMV) infection models[18], T cell exhaustion has emerged as a clinically relevant topic in the field of onco-immunology. The lineage relationship between T cell populations developed in response to viral infection or cancer is being revealed by single-cell technologies pairing transcriptomics and epigenomics with TCR sequencing and clonotyping analysis[19-21]. A plethora of studies have identified several TST subtypes, each with a varying degree of resemblance to memory T (TMEM) cells, effector T (TEFF) cells, and terminally exhausted T (TEX) cells[15,22,23]. This section outlines studies that dissect the heterogeneity of $CD8^+$ T cell subsets, both in and out of the TIME. Drawing from these findings, we attempt to delineate the spatiotemporal dynamics of T cell exhaustion (Fig. 1), informing the design of ICI-based immunotherapies.

2.1. *T cell subsets inside the TIME*

Exhausted PD-1^+TSTs undergo differentiation into PD-1^{lo} or PD-1^{hi} subsets. Among them, the PD-1^{lo}TCF-1^+precursor exhausted T (TPEX) subset demonstrates self-renewal property and memory-like expansion into TEFF cells in response to ICB[17]. This ICB-permissive subset of TPEX cells actively expresses TMEM-associated genes (*e.g.*, *CCR7*, *LEF1*, and *SELL*) under the regulation of T cell factor 1 (TCF-1, encoded by *TCF-7*), which is the primary TF capable of inducing *de novo* accessible chromatin regions (ACRs) in lineage-determining genes and hence essential for the acquisition of TPEX identity[24]. In HPV-positive HNSCC setting, three distinct subsets of HPV-specific PD-1^+ TILs representing different stages of a shared differentiation trajectory are revealed by single-cell pseudotime analysis and TCR sequencing[20].

The identity of stem-like TPEX is further maintained through a vital low expression of PD-1 that attenuates TCR and/or CD28 signaling to prevent T cell overstimulation and the consequent loss of TCF-1 expression[25]. In murine model PD-1-deficient $CD8^+$ T

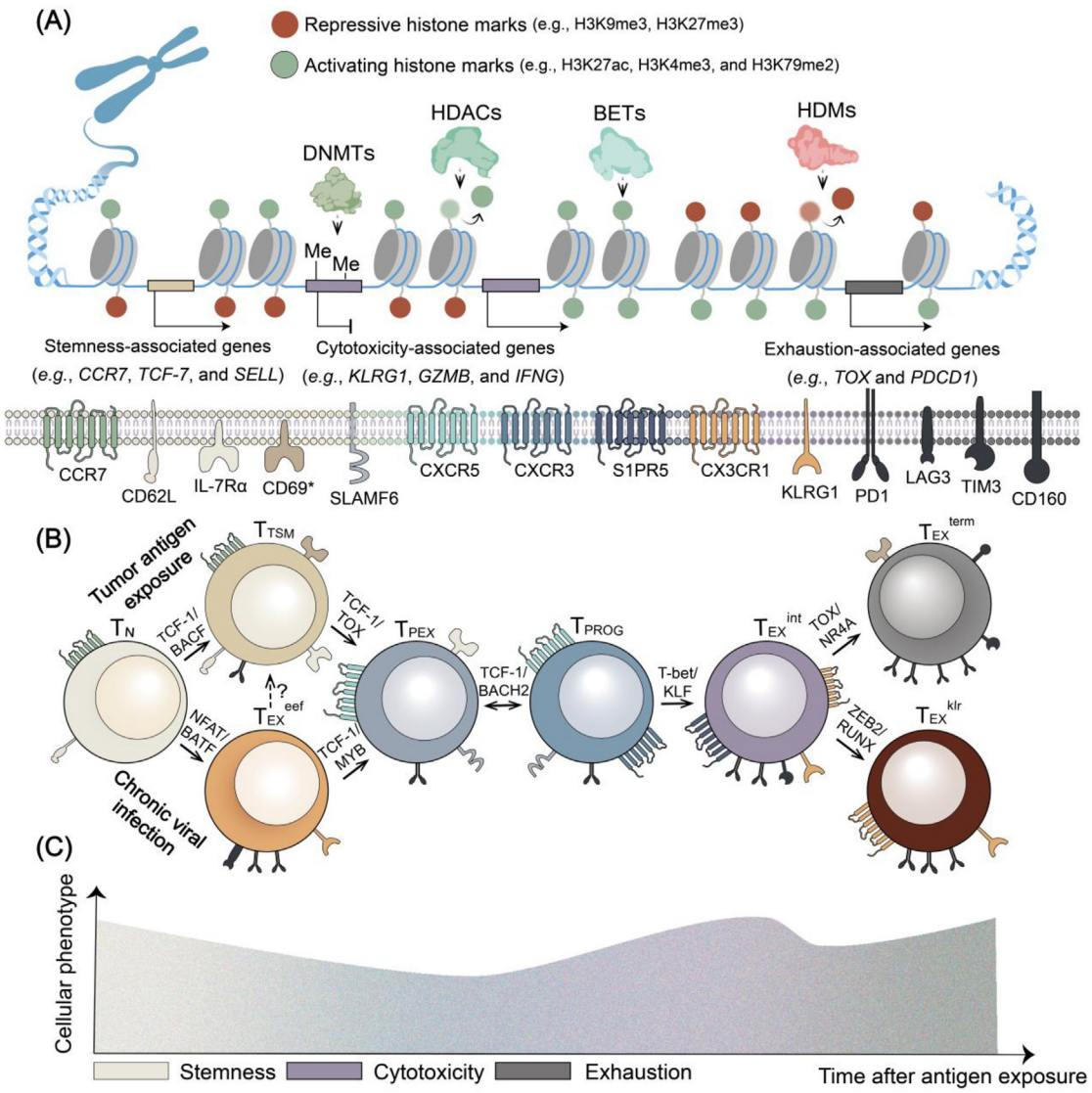

Figure 1 Genetic and phenotypic heterogeneity in the progression of T cell exhaustion. (A) Exhausted T cell subsets are characterized by differing epigenetic landscape. Naïve T cells (TN) are characterized with closed chromatin regions and repressive DNA methylation marks maintained by DNMT3a. Upon chronic exposure to tumor antigen, the chromatin regions near stemness-associated genes in TN cells are increased in accessibility, permitting the expression of transcription factors such as TCF-1. Epigenetic writers, removers and readers are also involved in the regulation of cytotoxicity-related ACRs in $S1PR5^+$ intermediate exhausted T cells (TEXint) and KLR^+ effector exhausted T cells (TEXklr), as well as exhaustion-related ACRs in TOX^+ TEX cells. (B) The progression of T cell exhaustion is governed by a temporal hierarchy of transcription factors. Lineage-determining TFs could influence the chromatin accessibility and induce transcription of a network of lineage-associated genes, driving the differentiation of T_N cells to become tumor-specific memory T cells (TTSM) in the TdLN (tumor-draning lymph nodes). Likewise, in chronic viral infection a subset of early effector T cells (TEXeff) is identified under the regulation of NFAT. This subset expressed high expression of exhaustion-related surface molecules, before differentiating into precursor $IL\text{-}7R^+SLAMF6^+$ exhausted T cells (TPEX) under the control of MYB. T_{PEX} could further differentiate into a progenitor exhausted T cell subset (TPROG), with upregulation of CXCR3 for trafficking into the TME. KLF, TOX and ZEB2 respectively dictates the differentiation of TEXint, TEX, and TEXklr cells. (C) The predominant phenotypes of T cells changes with prolonged exposure to antigen stimulation. *: CD69 expression is found on TdLN-TTSM and liver-derived T_{EX} cells, indicating tissue residency.

cells are observed with increased apoptosis and more profound defects in cytokine production, demonstrating a critical role for PD-1 in the maintenance of T cell functionality and persistence[26]. This pro-survival aspect of T cell exhaustion would have implications for engineered PD-1-deficient CAR-T products[27]. Studies suggest that when challenged by the same immunosuppressive TME, adoptive CAR-T cells display an increased propensity to exhaustion albeit similar epigenetic programs when compared with endogenous $CD8^+$ T cells[5]. This could be ascribed to the tonic signaling from the synthetic TCR in CAR-T cells even in the absence of antigen. In line, a transient cessation of such signaling could phenotypically revive exhausted CAR-T cells through EZH2-dependent epigenetic remodeling[28]. This reflects the need for applying a molecular "brake" in the design of CARs, in line

with how the low expression of PD-1 maintains TCF-1$^+$ TPEX identity[25] and how TOX promotes intratumoral persistence of TSTs by dampening TCR signaling[10].

Nevertheless, when compared with TMEM cells, TPEX is still characterized by an exhaustion-associated epigenome with upregulated ACR in genes associated with T cell dysfunction (*e.g.*, *TOX*, *TIGIT*, *PDCD1*, and *NFATC2*), as well as fewer associated with stemness (*e.g.*, *SELL* and *CCR7*)[22]. Moreover, despite its immunostimulatory effects on TPEX(*i.e.*, 4.4 fold-expansion), ICB paradoxically accelerates TPEX's differentiation into terminal exhausted PD-1^{hi} TEX cells, with a further loss of ACRs near genes related to progenitor biology (*e.g.*, *Il2*, *Lef1*, *Wnt2*, *Tcf7*, *Il7r*)[23,29]. In an adoptive TPEX transfer murine model treatment with anti-PD-1 inhibitors increased the proportion of TEX-like phenotype (from 52% to 67%)[30]. This result is in line with a recent study on the clinical use of bispecific T cell engagers (TCEs) where continued application of TCEs therapy caused the expansion of pre-existing T cells with exhausted phenotypes as a form of acquired resistance mechanism in non-responders[31]. Halting the use of TCEs at certain time points could potentially allow the restoration of early T cell subsets and rejuvenate their anticancer potential[32]. These results further underscore the necessity to extend our investigation beyond the tumor microenvironment and to identify early-stage T cell subsets as valuable diagnostic biomarkers[33].

2.2. *T cell subsets outside the TIME*

The migratory behavior of fresh TSTs from outside the TIME in response to ICB leads to the replacement of exhausted TME-TSTs. This process, known as "clonal replacement"[34], stems from the clinical observation that only a minimal overlap of TCR clonotypes exists between TILs derived from pre- and post-ICI treatment tumor samples: 84% TCRs in the post-treatment group are novel clonotypes, highlighting the limited proliferative ability of TIME populations including TPEX and TEX, and the expansion of systemic TSTs in responding to ICB[35]. The replenishment of the TIL cell pool from the periphery (*i.e.*, normal adjacent tissue[34], peripheral blood[36], and TdLN[37]) has been observed in multiple tumors[21,38] and credited with the successful prediction of immunotherapy response[36].

Among the peripheral sites, the TdLN has been suggested as the main site of action for PD-(L)1 inhibitors[37]. A subset of TCF1$^+$TOX$^-$ TSTs in TdLNs (accounting for around 40% of total TdLN-TSTs at 8 weeks post-tumor induction) is identified with an epigenome distinct from their TCF1$^+$TOX$^+$ TPEX counterpart[17]. TCF1$^+$TOX$^-$ TST cells are featured with ACRs at binding motifs for several members of the ETS and Runx TF families, resembling canonical TMEM; hence this subset is termed TdLN-derived tumor-specific memory cells or TdLN-TTSM. This novel TdLN-TTSM cell population is phenotypically more memory-like (around 150-fold expansion versus 40-fold for TPEX cells upon antigen re-encounter) and more responsive to ICB with significant augmentation in quantity and cytokine production, hence being regarded as bona fide responders to ICB. Furthermore, TdLN-TTSM undergoes ICB-induced differentiation into TdLN-TPEX cells, before the latter clonally replace the terminally exhausted CD69$^+$Ly108$^-$ TEX cells in the TIME, overcoming spatial partitioning[23]. As for TPEX's presence in peripheral blood[39], scTCR-seq identified 51 out of 414 blood-derived TCRs that are both tumor-specific and tumor-infiltrating[40], however, a similar setup revealed that stem-like tumor neoantigen-specific TCRs are rarely detectable in the peripheral blood of melanoma patient[41], suggesting varying results due to tumor heterogeneity.

2.3. *Epigenetic and transcriptional underpinning of T cell exhaustion*

The phenomenal and functional diversity of T cell subsets is predetermined by epigenetic and transcriptional regulatory mechanisms. The epigenetic regulation of exhaustion-associated transcription factor (TF) is instrumental in orchestrating exhaustion programs[42]. Specifically, super-enhancers (SEs) upstream TOX gene locus are identified with increased chromatin accessibility leading to persisting TOX expression after the resolution of chronic viral infection. This process, where the epigenetic regulation of transcriptional programs persists beyond the initial signaling events and influences subsequent cellular development and responses, is commonly referred to as "epigenetic scars". As a result, TOX is able to alter the global landscape of chromatin accessibility, demonstrated by TOX$^{-/-}$CD8$^+$ T cells with ~4000 ACR changes with drastically reduced ability to become TEX cells[43]. These changes were characterized by increased ACRs in TEFF associated gene loci and decreased ACRs in TMEM and exhaustion gene loci, in line with the biology of precursor exhaustion as mentioned above. Immunoprecipitation followed by mass spectrometry (MS) identified that TOX binds to proteins involved in both activating and repressive chromatin remodeling events, such as the HBO1 complex and DNMT1. Similar epigenetic programs are observed in exhaustion-prone GD2-targeting HA-28z CAR-T cells by epigenome and enhancer connectome profiling[5].

The epigenetic heterogeneity in exhausted CD8$^+$ T cell subsets is feathered with key TFs. Before exhaustion programs set in, BACH2, a member of the BACH family of basic leucine zipper TFs, imposed stem-like transcriptional program in CD8$^+$ T cells and protect against exhaustion during early chronic viral infection. A key TF that dictates early commitment to exhaustion is TCF-1, as the TCF-1-centered transcriptional network affects the lineage specification and epigenetic landscape of TPEX cells[25]. Recently it has also been shown that MYB is also an indispensable TF for the development of an early CD62L$^+$ TPEX subset, which is responsible for the proliferative burst in response to PD-1 inhibitors in viral infection model[12].

At a later stage, following continual exposure to chronic infection or cancer antigens, the TPEX population undergoes *T-bet*[23]/*BATF*[44]-driven differentiation into transitory TEX cell states that are characterized by remnant effector functions. A CD101$^-$Tim3$^+$ subset of TEX^{int} resembles *bona fide* KLRG-1$^+$ TEFF cells with co-expression of Ki-67, granzyme B, S1PR1, and CX3CR1, demonstrating both proliferative and cytolytic functions[11]; TEX^{int} cells express NK-associated genes, as recently identified by combined longitudinal scRNA-seq and scATAC-seq. The differentiation of this TEX^{int} subset is dependent on TF Zeb2[19]. Finally, the re-engagement of TOX and its downstream TF network (*e.g.* Eomes, STAT1, and IRF1) would lead to the onset of terminal exhaustion[23]. Although TOX expression is shared by all stages of exhausted T cells, terminal exhausted TEX has the highest TOX expression, and can be further divided into two subsets, TEX^{term}, and TEX^{klr} as revealed by unbiased clustering in temporal scRNA-seq[19]. Taken together, a temporal hierarchy of TFs drives T cell exhaustion under epigenetic regulation, leading to a gradual loss of transcriptional plasticity in endogenous T cell subsets. This underscores the potential use of epigenetic drugs, such as the traditional DNA methylation

inhibitors, histone deacetylase (HDAC) inhibitors, and the newly established BET (bromodomain and extra-terminal proteins) inhibitors, as adjuvant agents in the process of CAR-T cell expansion and preparation.

2.4. *The spatiotemporal dynamics of T cell exhaustion programs*

Informed by prior studies on $CD8^+$ TST heterogeneity, we could characterize the spatiotemporal dynamics of tumor-specific T cell exhaustion programs in the context of immunotherapies (Fig. 1). First, in TdLNs, ICB-induced expansion of TTSM is coupled with an enhanced differentiation into TPEX; Second, with the help of an intact "cancer−immunity cycle", TdLN-TPEX traffic into the TIME, wherein ICB transcriptionally sustain the effector function of newly migrated TPEX and the TEX population they evolve into. Preclinical studies demonstrating disturbance in the cancer−immunity cycle provided supportive evidence for the proposed model of ICB therapeutic mechanism. Perturbation such as early blockade of LN egress[45] with S1PR1 agonist FTY720 or surgical removal of LN[17], abolishes the enhanced anticancer immunity conferred by PD-1/PD-L1 ICB; Conversely, increased frequency and maturation of tumor-associated high endothelial venules (TA-HEVs) could better mediate lymphocyte entry into TIME and improve ICI efficacy[46]. Further supporting evidence includes studies targeting PD-$L1^+CD103^+$ conventional type I dendritic cells in TdLNs (TdLN-cDC1)[47], which interact with PD-1^+ TdLN-TSTs and their interactions could predict early distant disease recurrence in melanoma patients[48]. Likewise, genetic ablation of PD-L1 in cDCs recapitulates the tumor control effects of PD-1 blockade[49], while in a cDC1-deficient *Batf*$3^{-/-}$ mouse model a limited number of TST is observed within TIME, neutering ICB effects[50].

Interestingly, akin to situations in auto-immune diseases where TEFF and TMEM cells could migrate back into the LNs for the maintenance or termination of inflammatory dendritic cells[51], the migration of TPEX from the peripheral to TIME niche seems to be bidirectional, recently demonstrated in an *in vivo* setup where labeling and tracking of TIME T cells are achieved through *in situ* photoactivation[52]. Contrary to common belief, more tumor-specific TPEX egress from TIME *via* lymphatic drainage and reenter the TdLNs than those retained within TIME[52], reflecting TPEX's preference for lymphoid environment[53]. Furthermore, as ICB acts on TdLN rather than exclusively on the TIME, adopting a strategy that modulates the systemic immune contexture and enhances the recruitment of viable TSTs from TdLN to the TIME may align well with the evolving trend in immunotherapy treatment.

2.5. *Clinical implications of tumor-specific T cell exhaustion*

Tumor-specific T cell exhaustion demonstrates clear clinical and prognostic relevance across both solid and hematological malignancies, correlating with outcomes and therapeutic responses in melanoma, non-small-cell lung cancer (NSCLC), breast cancer, pancreatic cancer, hepatocellular carcinoma, and acute B cell lymphoblastic leukemia[54,55]. For instance, high proportions of less-exhausted $TCF1^+PD\text{-}1^+CD8^+$ TPEX cell subsets associate specifically with improved progression-free survival (PFS) in melanoma and NSCLC patients receiving ICIs[30,56], while exhausted phenotypes marked by elevated PD-1 and TIM3 stratify patient outcomes in breast and lung squamous cell carcinomas[57]. These collective findings spotlight that combating T cell exhaustion represents a promising therapeutic avenue with broad applicability for enhancing outcomes across refractory cancers.

CAR-T cell immunotherapy has recently achieved remarkable clinical outcomes across hematological and solid malignancies[4,58-63] (Table 1). For example, disialoganglioside GD2-targeted CAR-T cells demonstrate early signs of efficacy in H3K27 M-mutated diffuse midline gliomas[64], while claudin18.2-specific CAR-T cells achieved promising response rates in advanced gastrointestinal cancers[65]. However, suboptimal therapeutic responses remain common, often attributable to exhaustion arising from immunosuppressive tumor microenvironments[4]. Indeed, determinants of response and resistance to CD19 CAR-T cell therapy in chronic lymphocytic leukemia implicate T cell exhaustion as a key driver of therapeutic failure by dampening cytotoxic capacity[66]. This is critical because CAR-T cell cytotoxicity underpins anti-tumor efficacy and relies on productive synapse formation and effector molecule release, which can be dysregulated by exhaustion[4]. For example, the resistance of solid tumors like glioblastoma to CAR-T cell killing stems from disrupted IFN-γ signaling that normally promotes cytotoxic CAR-T cell adhesion[67]. Additionally, the differentiation fate of CAR-T cells is controlled by transcriptional regulators like FOXP1 and KLF2, which respectively govern self-renewal *versus* terminal exhausted fates[68] (Fig. 1). Thus T cell exhaustion both directly and indirectly curtails CAR-T cell potency through multifaceted mechanisms compromising durable cytotoxicity. Strategies to mitigate exhaustion are therefore imperative to drive CAR-T cell efficacy across refractory cancers. In the following section, we will explore additional promising strategies to engineer CAR-T cells to combat exhaustion and improve therapeutic outcomes, discussing relevant preclinical studies and early-phase clinical trials implementing these innovative approaches.

3. Engineering strategies to counter CAR-T cell exhaustion

The development of CAR-T cells is a direct result of precise genetic engineering enabled by the CRISPR/Cas9 system. This powerful tool not only facilitates the engineering of T cells to express specific CARs but also significantly enhances the efficacy of the resulting CAR-T cells[7]. For example, CRISPR/Cas9 has proven to be integral to alloCAR-T cells, effectively circumventing challenges such as graft *versus* host (GvHD) or host *versus* graft disease (HvGD)[69]. CAR-T cell function can be further enhanced *via* CRISPR-mediated knockout of immune checkpoint molecules and intracellular negative regulators of TCR signaling. In addition, calibration of CAR antigen stimulation through rational incorporation of signaling domains has been explored to balance immediate cytotoxicity against risks of terminal exhaustion[6]. In the following sections, we will explore these strategies in greater detail, underscoring the opportunities for genomic engineering in preventing CAR-T cell exhaustion, thereby promoting their *in vivo* persistence and therapeutic efficacy.

3.1. *CRISPR-Cas9 editing for allogeneic CAR-T cell therapy*

Donor-derived allogeneic CAR-T cells (alloCAR-T cells) offer a compelling alternative when autologous (patient-derived) CAR-T cells are unavailable due to the costly and time-consuming preparation of primary cell-based treatments. The appeal of alloCAR-T cells therefore stems from their potential for "off-the-shelf" use[70], bypassing the necessity for patient-specific tissue samples and the intricate processes associated with autoCAR-T cell manufacturing and distributing. However, this requires genomic

Table 1 Clinical trials of commercial CD19-targeted CAR T product for the treatment of hematological malignancies.

CAR T product	Trial phase	Cancer type (number of patients)	Clinical outcome	Median follow-up	Side effect	Country/region	Company information	NCT number	Approval year
Kymriah	Single-center case series	DLBCL (24); FL (14)	ORR: 66%; CRR: 55%	61 months	CRS; other effects[b]	USA[c], EU, and other regions[a]	Novartis Pharma Inc.	NCT02030834	2017
Kymriah	Multi-center phase II	DLBCL, HGBCL or tFL (115)	ORR: 53%; CRR: 39%	40 months	Same with above	Same with above	Same with above	NCT02445248	2017
Yescarta	Multi-center phase I/II	DLBCL (77); PMBCL (8); tFL (16)	ORR: 74%; CRR: 54%	51 months	CRS; ICANS; other effects	USA[d], EU and China	Kite Pharma Inc.	NCT02348216	2017
Yescarta	Multi-center phase III	DLBCL (126); HGBCL (31); other (23)	ORR: 83%; CRR: 65%	25 months	Same with above	Same with above	Same with above	NCT03391466	2017
Tecartus	Multi-center phase II	MCL (68)	ORR: 91%; CRR: 68%	36 months	CRS; HLH/MAS; other effects	USA[e], EU	Kite Pharma Inc.	NCT02601313	2020
Breyanzi	Single-center phase I/II	tFL (13) and FL (8)	ORR: NR for FL and 46% for tFL; CRR: 88% for FL	38 months	CRS; ICANS; other effects	USA[f], EU	Juno Therapeutics	NCT01865617	2021
Breyanzi	Multi-center phase I	R/R CLL/SLL (23)	ORR: 82%; CRR: 45%	24 months	Same with above	Same with above	Same with above	NCT03331198	2021
Abecma	Multi-center phase III	MM (254)	ORR: 71%; CRR: 39%	19 months	CRS; HLH/MAS; other effects	USA[g], EU	Bristol Myers	NCT06045806	2023
Carvykti	Multi-center phase III	MM (165)	ORR: 99%; CRR: 86%	16 months	CRS; ICANS; Parkinsonism and Guillain-Barré syndrome; HLH/MAS; other effects	USA[g], EU, China and other regions	Legend Biotech	NCT04181827	2023
Relma-cel	Multi-center phase II	R/R LBCL(68)	ORR: 77%; CRR: 53%	18 months	CRS; ICANS; other effects	China[h]	JW Therapeutics	NCT04089215	2023
CNCT19	Single-center phase I	ALL(20)	CRR: 90%	10 months	CRS; ICANS; other effects	China[h]	Juventas Cell Therapy Ltd.	NCT02975687	2023

DLBCL, diffuse large B-cell lymphoma; HGBCL, high-grade B-cell lymphoma; tFL, transformed follicular lymphoma; FL, follicular lymphoma; PMBCL, primary mediastinal B-cell lymphoma; CLL/SLL, chronic lymphocytic leukemia/Small lymphocytic lymphoma; LBCL, large B-cell lymphoma; MM, multiple myeloma; ALL, acute lymphoblastic leukemia.

[a]Other regions including Japan, Australia.

[b]Other effects include possible hypersensitivity reactions, infections, prolonged cytopenia, hypogammaglobulinemia and secondary malignancies. Note that secondary T cell malignancies as adverse reactions have been updated for all six CAR T products listed here by FDA on Jan 19th, 2024.

[c]Approved by the FDA for R/R FL.

[d]For R/R DLBCL.

[e]For MCL and ALL.

[f]For R/R LBCL.

[g]For R/R MM.

[h]Approved by the Chinese NMPA for r/r B-ALL.

engineering to avert unintended immunological consequences of allogeneic mismatch, in the form of GvHD and HvGD.

The CRISPR/Cas9 system as a genomic editing tool has provided a safe and viable strategy to circumvent the risks of GvHD and HvGD. By disrupting endogenous TCRs in alloCAR-T cells, the CRISPR/Cas9 system attenuates the recognition of recipient cells as foreign. A seminal example involves the use of CRISPR/Cas9 to concurrently knock out the endogenous TCR alpha constant (TRAC) locus and knock in a CD19-targeted CAR construct[71]. The CAR-Transgene is positioned under transcriptional control of the native TRAC promoter. This enables reliable, high-level CAR expression in the engineered T cells, mirroring the constant expression pattern of the endogenous TCR alpha chain. The simultaneous CRISPR knockout of TRAC abrogates surface expression of the native TCR, preventing alloreactivity. The combined effect yields uniform CAR expression from the well-characterized TRAC promoter while eliminating the host alloreactive TCR. Further examples include combined CRISPR editing of the TRAC locus and beta-2-microglobulin (B2M)[72]. B2M is a requisite component of MHC class I and central to self-peptide presentation and immune surveillance. As MHC mismatches contribute substantially to the immunogenicity of allogeneic cell grafts, CRISPR-mediated deletion of B2M reduces direct allorecognition and mitigates the risk of an endogenous anti-CAR immune response. Such auto-immunity can further yield tonic signaling that instigates T cell exhaustion[73]. Collectively, CRISPR-based genetic engineering has progressively enhanced the *in vivo* persistence of alloCAR-T cells by mitigating tonic signaling that can precipitate exhaustion while preserving on-target antitumor activity.

Considerable clinical benefit has been achieved with CRISPR gene editing strategies for alloCAR-T cells[74]. For example, a phase I trial (NCT03690011) is investigating CRISPR-engineered alloCAR-T expressing a CAR-Targeting CD7 for the treatment of high-risk T cell malignancies. In parallel efforts, CRISPR-mediated multiplex editing has enabled generation of universal CD19/CD22 dual-targeted CAR-T cells from healthy donors[75]. Kagoya et al.[76] demonstrated the utility of multiplex editing to generate alloCAR-T cells lacking HLA class I, HLA class II, and TCR expression, enabling off-the-shelf use while controlling alloreactivity. Comparable CRISPR editing strategies have further been employed to generate universal alloCAR-T cells deficient in endogenous TCR and HLA class I expression for the treatment of refractory lymphoma[77]. By mitigating graft rejection, this approach yielded clinical responses in pilot cases.

Recent new generation allogeneic CAR-T cell platforms have further demonstrated the ability to overcome two key limitations that have hindered the field−host immune rejection and poor persistence. For example, hypoimmune anti-CD19 CAR-T cells with disrupted MHC expression elicited potent antitumor activity and enhanced persistence compared to conventional allogeneic CAR-T cells in fully immunocompetent mouse models[78]. Similarly, Sleeping Beauty transposon-engineered allogeneic CAR-T cells with CRISPR-inactivated TCRs minimized GVHD while retaining robust CAR signaling and activity against $CD19^{+}$ tumors *in vivo*[79]; A BCMA-targeted allogeneic CAR-T cell product (ALLO-715) demonstrated high response rates and durable remissions with a favorable safety profile in relapsed/refractory multiple myeloma[80]. Additionally, studies of UCART19, an allogeneic anti-CD19 CAR-T cell generated by TALEN editing, have shown deep molecular remissions and bridging to transplant in pediatric and adult ALL[81,82]. Collectively, these pioneering trials underscore the potential of CRISPR-based engineering to overcome key limitations of alloCAR-T cell therapies, where precise modulation of T cell stimulation is key to balancing therapeutic efficacy (persistence) and safety (host rejection).

Future allogeneic CAR-T cell products will likely focus on multiplex editing to achieve multifunctional attributes in alloCAR-T cells while minimizing immunogenicity. This is now possible with the advent of streamlined CRISPR systems capable of generating up to four genomic alterations in a single step, underscoring the potential for alloCAR-T cell engineering in a therapeutic setting (Table 2)[72-83]. In a phase I trial, base editing was employed in alloCAR-T cells for the concurrent inactivation of CD52, which facilitates alloCAR-T survival from residual alemtuzumab-induced lymphodepletion (alemtuzumab is by protocol administered prior to CAR-T infusion to deplete native T cells in order to minimize HvGD), and CD7, so as to prevent fratricide by other CD7-targeted CAR-T cells[83]. The TCRβ chain was also deactivated to minimize GvHD. This leads to efficient preparation of universal CD7-targeted alloCAR-T cells, resulting in leukemic remission in a pediatric T-ALL patient 28 days after a single infusion[83]. The result demonstrates immense potential of multiplexed CRISPR editing in "off-the-shelf" use of CAR-T cell therapy.

3.2. *Revitalizing CAR-T cells via CRISPR targeting of immune regulators*

Transitioning from allogeneic to autologous CAR-T cells, CRISPR technology could be similarly applied to mitigate CAR-T cell exhaustion by editing immune regulators. The immunosuppressive tumor microenvironment, especially in the solid tumor setting, poses a significant challenge to the efficacy of CAR-T cell therapy. However, the advent of the CRISPR/Cas9 system has opened up new avenues for silencing immunosuppressive signaling, thereby enhancing the therapeutic potential of CAR-T cells. Novel strategies have thus emerged to engineer CAR-T cells by targeting key immunosuppressive mediators, including immune checkpoint pathways, transcriptional regulators, and other negative regulators of T cell function (Fig. 2).

3.2.1. *PD-1/PD-L1*

As mentioned, while blockade of the PD-1/PD-L1 axis holds promise to enhance CAR-T cell function, direct PD-1 ablation warrants a nuanced approach given its complex immunomodulatory roles. On the one hand, PD-1 has emerged as a key target for reinvigorating exhausted CAR-T cells due to its role as an intrinsic immunosuppressive receptor. PD-1 signaling inhibits TCR-mediated activation by recruiting SHP-1/2 phosphatases to block PI3K/AKT and Ras/MEK/ERK signaling. Additionally, PD-1 downregulates CDK and CK2 activity, inhibiting cell cycle progression and TCR expression[84]. Studies have shown that expression of a dominant-negative PD-1 receptor augmented the therapeutic efficacy of CD19-targeted CAR-T cells against refractory B cell lymphoma[85]. On the other hand, despite exhibiting exhaustion-associated impairments, $PD\text{-}1^{+}$ T cells represent the major intratumoral subset with residual effector function. In addition, PD-1 may act as a safeguard against hyperstimulation-induced cell death. Therefore, wholesale genetic deletion of PD-(L)1 risks aggravating exhaustion by removing crucial negative feedback controls. This underscores the need to better delineate the multifaceted immunobiology of PD-1 in order to strategically engineer CAR-T cells without precipitating unintended exhaustion.

Table 2 Overview of genetically modified alloCAR T cell therapies for hematologic and solid tumors.

CAR T variant	Target antigen	Cancer type	Engineering strategy	Ref.
Multiplex CRISPR-edited alloCAR T	CD19	B-cell leukemia/lymphoma	CRISPR multiplexing; TRAC, B2M, and PD-1 locus deletion	72
CRISPR-engineered alloCAR T	CD19	Pediatric BCP-ALL	Donor-derived, second-generation CAR; retroviral and lentiviral constructs with 4.1BB signaling	74
Multiplex CRISPR-edited alloCAR T	CD19/CD22	R/R B-ALL	CRISPR multiplexing; TRAC and CD52 locus deletion	75
tKOc alloCAR T	CD19	B-cell malignancies	HLA class I, II, and TCR triple knockout *via* Cas9/sgRNA ribonucleoprotein electroporation	76
Hypoimmune alloCAR T	CD19	B-cell malignancies[a]	CRISPR-Cas9-edited HIP T cells; B2M, CIITA, TRAC locus disruption; CD47 and anti-CD19 CAR *via* lentiviral transduction	78
SB transposon-engineered alloCAR T	CD19	Lymphoid malignancies[a]	Non-viral sleeping beauty transposons for CD19-28z.CAR transduction and CRISPR-based TCR inactivation	79
ALLO-715	BCMA	R/R multiple myeloma	Allogeneic anti-BCMA CAR	80
UCART19	CD19	Pediatric/adult B-ALL molecular remission	TALEN-based allogeneic CAR19 T; TCR α chain and CD52 locus deletion	81,82
Base-edited CAR7 T	CD7	T-ALL	Base editing to generate universal CAR T; CD7 inactivation; TCRβ chain inactivation	83

BCP-ALL, B-cell precursor acute lymphoblastic leukemia; R/R B-ALL, relapsed/refractory B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia.
[a]Using humanized mouse models.

Recent advances have demonstrated promising strategies to interfere with PD-1 signaling and augment CAR-T cell function. Chen et al.[86] engineered CAR-T cells targeting carcinoembryonic antigen to secrete a PD-1-TREM2 single-chain variable fragment (scFv). This bifunctional scFv abrogated PD-1/PD-L1 interactions while also blocking the myeloid inhibitory receptor TREM2, attenuating immunosuppression and enabling T cell infiltration and cytotoxicity. Additionally, Liu et al.[87] incorporated a chimeric PD1-CD28 switch receptor into CAR-T cells, where the PD1 extracellular domain provided inhibitory binding while the CD28 intracellular domain transduced costimulatory signals to enhance PI3K/Akt activation. This switch receptor configuration boosted the persistence and efficacy of CAR-T cells against solid tumors *in vivo*[87]. Moreover, CRISPR-Cas9-mediated genomic deletion of the endogenous *PDCD1* locus augmented the cytotoxic capacity of TCR and CAR-T cells in a clinical trial of multiplex-edited NY-ESO-1-targeted T cells[88]. Durable *PDCD1* deletion prevented exhaustion despite chronic antigen exposure. Together, these sophisticated engineering strategies demonstrate promising mechanisms to selectively modulate PD-1 signaling without fully abrogating this pathway's nuanced immunoregulatory roles. Additional investigation will further elucidate optimal modalities and therapeutic setups for strategic PD-1 inhibition to maximize CAR-T cell potency.

3.2.2. *TGF-β/SMAD*

The efficacy of CAR-T cell therapy against solid tumors is limited by the presence of soluble immunosuppressive factors in the tumor microenvironment such as transforming growth factor beta (TGF-β). Beyond pharmaceutical blockade[89], direct genetic disruption of TGF-β signaling pathways offers opportunities to enhance CAR-T cell function, as knockout or overexpression of TGF-β receptors (dnTGF-βRII) fine-tunes cellular responses to immunomodulatory cues[90]. For instance, CRISPR-mediated deletion of the TGF-β receptor TGFBR2 was found to reduce TREG induction and CAR-T cell exhaustion, enhancing CAR-T *in vivo* efficacy against xenograft solid tumor models. TGFBR2-deficient CAR-T cells effectively eliminated tumors when administered systemically or intratumorally, and controlled reinoculated contralateral tumors, due to increased central and effector CAR-TMEM subsets[91]. Similarly, expression of dnTGF-βRII and CCR8 in CAR-T cells countered the inhibitory effects of TGF-β and improved therapeutic efficacy in pancreatic cancer model[92]. In another study, a self-driving CD19-targeting CAR is designed, which could be induced to express dominant-negative TGFBRII upon CD19 engagement[93]. The self-driving circuit allows antigen-dependent upregulation of dnTGF-βRII and specifically countered the inhibitory effects of TGF-β on CAR-T cell activation within the immunosuppressive microenvironment. This selective expression enhanced CAR-T cell expansion and prevented exhaustion compared to constitutive or static dnTGF-βRII expression, as recent findings revealed TGF-β signaling is integral to core transcriptional programs that mediate tissue residency and stemness in CAR-TMEM cells[94]. Thus, programmable circuits that restrict dnTGF-βRII expression to tumor sites highlight an advantage over systemic approaches like pharmacologic TGF-β blockade, which risk abrogating TGF-β-mediated epigenetic and transcriptional remodeling requisite for optimal TMEM differentiation. Overall, conditional or constitutive genetic disruption of TGF-β signaling offers therapeutic potential to avert CAR-T cell exhaustion and improve therapeutic outcome against immunosuppressive solid tumors.

Beyond direct engineering of TGF-β/TGFBR, targeting downstream effectors of the TGF-β/SMAD signaling cascade also holds promise to enhance CAR-T cell function by disrupting SMAD-dependent programs that precipitate CAR-T exhaustion. For instance, a recent study found that lentiviral overexpression of the inhibitory SMAD, SMAD7, in EGFR-targeted CAR-T cells enhanced proliferative capacity, cytokine production, and tumor cytotoxicity to levels comparable to dnTGF-βRII expression[95]. Critically, SMAD7-overexpressing CAR-T cells mediated complete tumor regression *in vivo* by Day 20 post-infusion, whereas traditional

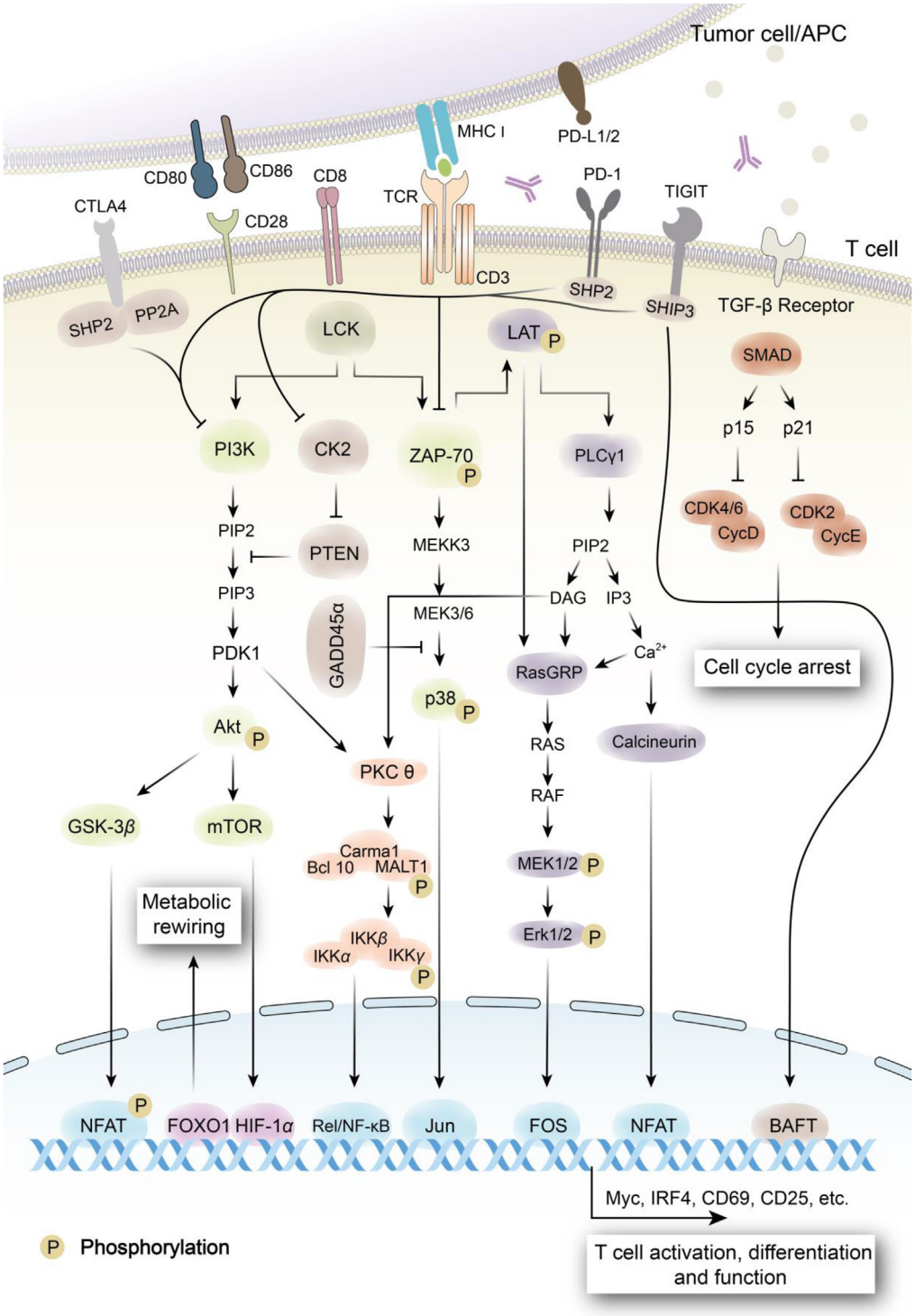

Figure 2 Immunosuppressive T cell signaling pathways. Upon receiving external stimuli, the inhibitory receptors on T cells are activated and modulate key molecules downstream of the TCR complex. These stimuli may include cognate ligand present on the surface of tumor cells or antigen-presenting cells, as well as paracrine cytokines in the TME. Activation of inhibitory receptors ultimately leads to alterations in gene expression programs and cellular biologies, leading to T cell dysfunction and immune evasion.

CAR-T cells only elicited partial responses. These data substantiate SMAD signaling modulation as a viable strategy to enhance CAR-T cell potency against immunosuppressive malignancies.

Multiple trials are investigating the therapeutic effects of TGFBR2 knockout or dnTGF-βRII expression in CAR-T cells, given preclinical data substantiating their potential to mitigate exhaustion. For instance, dnTGF-βRII expression in PSMA-targeted CAR-T cells enhanced antitumor functionality in preclinical prostate cancer models, leading to a phase I trial applying these cells in metastatic castration-resistant prostate cancer

(NCT03089203)[96]. Follow-up results from this seminal trial established feasibility and reasonable safety of TGF-β resistant PSMA-CAR-T cells[97]. However, while transient efficacy signals were observed, 5 of 13 patients experienced mild side effects as part of the cytokine release syndrome (CRS), alongside upregulation of additional inhibitory factors post-infusion, highlighting the need for combinational paradigms in treating immunosuppressive solid tumors. Together, these pioneering clinical setups help establish modulation of TGF-β signaling dynamics as a viable approach to potentiate CAR-T cell therapies.

3.2.3. *TOX/NR4A*

As a core transcriptional mediator of exhaustion, TOX cooperates with NR4A transcription factors to impose dysfunctional phenotypes in $CD8^+$ T cells. Studies on the transcriptional circuitry underpinning CAR-T cell exhaustion have revealed that coordinated upregulation of TOX and NR4A family transcription factors, the latter downstream of NFAT signaling, underlies the progressive dysfunction of TILs[98]. Specifically, TOX/NR4A act in part by suppressing synergistic immunostimulatory nodes like the BATF/IRF4 axis, which promotes effector function. Thus, ablation of TOX/NR4A mitigates exhaustion by relieving inhibition on BATF/IRF4-mediated CAR-T cell activation. Overexpression and mechanistic studies has validated the BATF/IRF4 counterbalances the immunosuppressive TOX/NR4A axis[99].

Multiple lines of investigation have explored targeting TOX/NR4A to enhance CAR-T cell activity against solid tumors. Knockout of *Tox* and *Tox2* was found to improve anti-tumor efficacy of CAR-T cells in murine models by relieving exhaustion[100]. Additionally, combined knockout of *Nr4a1*, *Nr4a2* and *Nr4a3* reprogrammed the epigenetic landscape of tumor-infiltrating CAR-T cells to promote cytotoxicity and tumor control[100,101]. However, recently it has been reported that TOX2 may play a more nuanced role by promoting central memory differentiation[102]. TET2 reduction led to enhanced TOX2 expression and central memory generation in CAR-T cells in a leukemia patient with complete remission. Thus, while TOX is a core exhaustion mediator, selective targeting and engineering of the TOX/NR4A axis is warranted to potentiate CAR-T cell therapy without compromising self-renewal ability of the adoptively transferred T cells.

3.2.4. *Other immune regulators*

Similar to the NR4A factors, the E3 ubiquitin ligase CBL-B has emerged as a downstream effector of NFAT-mediated TCR signaling that acts as a critical checkpoint governing CAR-T cell exhaustion. CBL-B is also similarly upregulated on dysfunctional $PD\text{-}1^+/TIM\text{-}3^+$ CAR-T cells infiltrating solid tumors[103]. Genetic ablation of CBL-B *via* CRISPR-Cas9 effectively reinvigorates CAR-T cell function by restoring cytokine production, cytotoxicity, and resistance to PD-1/PD-L1 mediated suppression[104]. For instance, CRISPR-mediated CBL-B deletion in a CEA-targeted CAR-T cells inhibited exhaustion against MC38 colon tumors, as evidenced by reduced PD-1/TIM-3 expression and increased IFN-γ and TNF-α levels that promoted tumor clearance[103]. Additionally, viral integration of dominant-negative CBLB mutations presents another emerging paradigm to manipulate CAR-T cell signaling dynamics for better therapeutic efficacy, akin to dnTGF-βRII approaches[105]. Ongoing clinical trials are exploring the therapeutic potential of transient CBL-B silencing to enhance solid tumor CAR-T cell therapies (NCT03087591, NCT02315612). However, CBL-B targeting requires careful calibration, as systemic knockout causes autoimmunity[106]. Further research on the nuanced immunomodulatory functions of CBL-B will inform engineering strategies to mitigate CAR-T cell exhaustion without compromising immunological balance.

Recent analyses have revealed the integral cooperative interplay between Regnase-1 and Roquin-1 in governing T cell activation and self-tolerance[107]. These mRNA regulatory proteins interact physically to form a checkpoint hub that post-transcriptionally suppresses shared inflammatory gene targets. Structural studies defined the binding interface on Roquin-1 that interacts with Regnase-1. Analyses of knockout T cells reveal both distinct and overlapping immunomodulatory phenotypes resulting from single *versus* dual protein ablation. While heightened activation and persistence changes elicit eventual systemic autoimmunity, mutations specifically disrupting the Regnase-1/Roquin-1 interface conversely enabled superior tumor-specific T cell functionality. Despite provoking autoantibody production, impaired cooperative regulation improved T cell exhaustion resistance and intratumoral accumulation.

Furthermore, an additional recent study explored modulated disruption of this axis to enhance engineered human T cell function without provoking overt autoimmunity[108]. Individual knockout of Regnase-1 or Roquin-1 alone improved CAR-T cell cytokine production, proliferation, and tumor infiltration. Moreover, combined dual knockout further synergistically augmented antitumor potency beyond single deletion, albeit with accompanying toxicity risks in murine models at maximal levels. This exemplifies the delicate signaling balance of this checkpoint pathway requiring careful calibration. Further mapping of precise regulatory complex dynamics will inform rational engineering approaches to precisely tune Regnase-1/Roquin-1 activity to resist T cell exhaustion without eliciting systemic autoreactivity.

3.3. *Engineering of CAR activation dynamics to prevent exhaustion*

Beside the exclusion of immune negative regulators, advanced CAR engineering involves carefully calibrated, conditional activation dynamics, aiming to prevent overstimulation and balancing efficacy with safety (Fig. 3). CAR engineering requires a delicate balance as augmenting early activation signals promotes immediate cytotoxicity but risks driving terminal exhaustion and relapse. This dichotomy is evidenced by a CD19-CAR construct harboring dual CD28 and CD3ζ signaling domains, which have shown improved efficacy against low-antigen tumors[109]. This is a direct result of lowered threshold for CAR activation through increased expression of immunoreceptor tyrosine-based activation motif (ITAM) which benefits immune recognition of poorly immunogenic tumors. However, in other settings similar CD19-CAR constructs are engineered with stepwise mutation of ITAM multiplicity, in an attempt to temper the signaling strength of CARs and analyze its effect on CAR-T cell differentiation[110]. Strikingly, the result showed that the single ITAM-containing CAR outperformed the double- and triple-ITAM-containing CARs *in vivo*, due to a higher percentage of $CD62L^+CD45RA$ TCM population. In contrast, tonic CAR signaling from redundant ITAMs dose-dependently skewed differentiation towards short-lived effector phenotypes, at the expense of memory formation and long-term tumor control.

Beyond driving terminal differentiation, CAR overstimulation and prolonged CAR-antigen interaction can also precipitate antigen escape and CAR-T fratricide killing. Hamieh et al.[111] reports

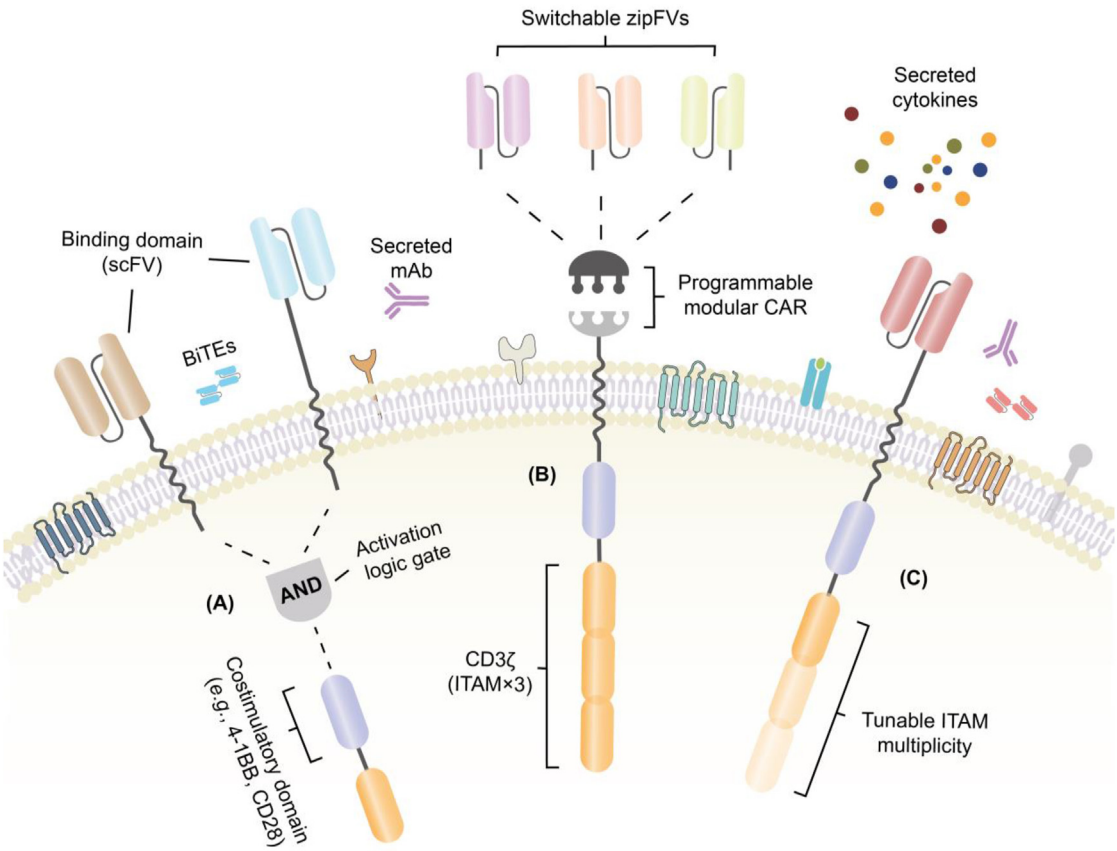

Figure 3 Engineering CAR activation dynamics to prevent T cell exhaustion. To mitigate overstimulation and prevent T cell exhaustion, a suite of engineering approaches has been developed to finely tune CAR T cell activation. These strategies aim to balance potent anti-tumor responses with longevity and safety. (A) Logic-gated activation where a dual antigen-recognition system is in place for CAR T cells engineered with multiple scFvs that recognize distinct antigens. This "AND" logic gate requires simultaneous binding of both antigens to trigger downstream signaling, reducing the risk of accidental activation and preventing exhaustion by ensuring that CAR T cells are activated only in the specific context of tumor presence. (B) Modular programmable CAR describes advanced systems like SUPRA CAR and switchable receptors, which allow for the temporal modulation of CAR activation. These include designs that enable transient cessation of signaling or inducible activation, thereby reducing the risk of exhaustion by allowing periods of rest and recovery for the T cells. (C) Intracellular signaling modulation can be achieved by design of single or multiple ITAMs for CAR intracellular domains, so as to adjust signal strength. Careful calibration of co-stimulatory signals from domains such as CD28 or 4-1BB ensures robust initial activation while preserving T cell memory potential. ITAM, immunoreceptor tyrosine-based activation motif; scFV, single-chain variable fragment; zipFV, leucine zipper-fused variable fragment; BiTEs, bispecific T-cell engager.

a mechanism of trogocytosis where high-affinity CAR-antigen binding actively strips target antigens from tumor cells. This facilitates tumor immune escape by reducing surface antigen density and hence CAR-T cell recognition and killing. Additionally, trogocytosis promotes fratricide of CAR-T cells which now display the scavenged antigens, exacerbating dysfunction even in mainstream CD28 and 4-1BB-containing CAR-T cells[111]. Thus, intensified CAR-antigen stimulation compromises durable therapeutic efficacy, highlighting the need to allow for transient cessation of CAR signaling in engineering designs, which has been shown to restore exhausted cells by enabling epigenetic remodeling[28]. Using a drug-inducible CAR down-regulation system, temporary withdrawal of stimulation prevented exhaustion and redirected cells towards memory-like fates, even restoring anti-tumor functionality in CAR-T cells that had already acquired phenotypic and transcriptional signs of terminal exhaustion[28]. Collectively, these findings challenge the paradigm of exhaustion as an irreversible state, and suggest that optimal anti-tumor CAR-T cells may require calibrated stimulation, rather than maximal activation. A dynamic modulation of CAR activation incorporating periodic rest schedules could potentiate cytotoxicity against poorly immunogenic tumors, while sustaining long-term tumor control through memory formation.

Such temporal dynamic modulation of CAR activation is enabled by transcriptional control over receptor expression. Split, universal, and programmable (SUPRA) CAR systems have been developed to implement multi-input logic operations functional in diverse cell types[112]. In a similar vein, systems like VIPER CARs utilize a viral protease domain to render CAR expression dependent on FDA-approved protease inhibitors[113]. Both ON and OFF switch designs were engineered, enabling orthogonal control over multiple co-expressed CAR constructs through different inhibitor drugs. Further conditional activation has been achieved using camelid VHH antibodies as CAR scaffolds that are directly modulated by the small molecule drug methotrexate[114]. This approach enables rapid, transient control

over CAR activity without increasing viral payload size. A similar lenalidomide-responsive degron motifs have been utilized to construct reversible ON and OFF switch CAR circuits[115]. Recently a CD19-targeted CAR-T construct co-expressing a safety switch based on an inducible caspase 9 domain along with a ΔNGFR selection marker was recently examined in a clinical trial where, despite anti-seizure prophylaxis, a patient developed grade 3−4 neuropathic toxicity after receiving CAR-T infusion[116]. However, administration of the small molecule rimiducid induced rapid apoptosis of the engineered CAR-T cells, leading to subsequent neurologic improvement. This example underscores the potential of integrating inducible safety switches to mitigate severe on-target, off-tumor effects in novel CAR-T cell therapies.

Additionally, endogenous gene promoters can be harnessed to couple CAR-Transcription to native modes of physiologic T cell regulation. As mentioned, CRISPR-mediated knock-in of the CD19-specific CAR construct into the TRAC locus places the receptor under control of the TCRα promoter[71]. CD19-CAR expression is therefore silenced upon T cell maturation absent cognate antigen stimulation. Furthermore, synthetic promoters have been engineered to restrict CAR-Transcription specifically to the immunosuppressive tumor niche, responding to cues like hypoxia, IFN-γ, and NF-κB. This targeted expression limits tonic signaling in normal tissues. An alternative approach utilizes composite response elements like AP1, NFAT, and STAT5 to yield "self-driving" CAR circuits[93]. The CAR promoter activates upon initial antigen encounter, potentiating cytotoxicity. But self-limiting negative feedback subsequently dampens transcription, preventing chronic stimulation. Thus, diverse transcriptional regulatory designs enable tight control over CAR expression, allowing tailored transition between stimulated and resting states to sustain anti-tumor function.

Other approaches to temporally control CAR signaling include synthetic promoters responsive to the tumor microenvironment which restrict expression to the local site. Beyond transcriptional regulation, CAR activation can be uncoupled from antigen recognition using switchable or split receptor architectures, where soluble switch molecules or exogenous dimerization factors are required to activate signaling[117]. Split designs also enable geometric control over the immunological synapse between CAR-T cells and targets. By tuning the stoichiometry of split CAR components, signal strength can be precisely modulated to limit toxicity while still eliciting cytotoxicity. Adaptor molecules further mediate specific spatial interactions between CAR-T cell and target cell to control activation[118]. Collectively, these diverse strategies for engineering dynamic CAR circuits enable calibrated stimulation in response to tumor cues or external regulation. Such intelligent receptor designs hold promise to enhance the safety and efficacy of CAR immunotherapies.

4. Conclusions and perspectives

The functional decline in $CD8^+$ TSTs undermines the efficacy of cancer immunotherapies, prompting the mechanistic understanding of T cell exhaustion for therapeutic targeting. Ongoing efforts are classifying TSTs into distinct categories based on their individual transcriptional and epigenetic features. These categories span a spectrum of cell states, from those resembling stem or progenitor cells (*e.g.*, TPEX) to those that exhibit signs of exhaustion (*e.g.*, TEX^{term} and TEX^{klr})[16,23,33]. Along this spectrum, decline in cellular plasticity is evident, with terminal exhausted TST subsets showing minimal response to ICIs[15,23,30]. Further, both local and systemic environment cues have been recognized as mediators in reviving T cell function post-ICB treatment, where responsive TST subsets (*e.g.*, TdLN-TTSM) are identified in both peripheral and intratumoral niches[17]. This highlights the potential use of cutting-edge spatially resolved single cell technology (*e.g.* spatial-CUT&Tag[119]), combined with data-rich resources such as the Cancer Genome Atlas (TCGA), in dissecting the transcriptional and epigenetic heterogeneity of both immune and non-immune elements and their interplay (*e.g.*, tertiary lymphoid structures, TLS) within the TME.

While CAR-T cell immunotherapy has achieved remarkable clinical success, prevailing reliance on viral vectors for delivery of CRISPR-Cas9 raises significant safety concerns, risking immunogenicity-mediated rejection and insertional mutagenesis. Current CRISPR-based engineering still depends on viral machinery due to substantial challenges with non-viral approaches, where electroporation of plasmids or mRNA directly into primary T cells can cause toxicity and unacceptable loss of viability. Further challenges exist in achieving homogeneous targeting, as low transfection efficiency coupled with random integration precipitates heterogeneous editing outcomes. However, in a recently completed phase I trial, delivery of CRISPR elements (*i.e.*, ribonucleoproteins) *via* optimized electroporation enables nonviral, site-specific integration and genomic engineering[120]. Despite lower CAR^+ cell percentages, this approach nonetheless mediated a high complete remission rate (87.5%) with durable responses in 8 lymphoma patients. Single cell profiling further revealed a desirable TMEM-enriched CAR-T profile, and notably, with PD-1 expression that balance enhanced cytotoxicity and protection against hyperstimulation[120], consistent with prior discussions on the multifaceted role of PD-1 signaling in CAR-T cell-therapy. Looking ahead, precision electroporation strategies that enable viral-free targeted engineering hold promise to achieve safe and customizable CAR-T cell-therapies for broader clinical implementation.

However, on-target off-tumor toxicities (OTOT) poses additional challenge to such effort[53]. The distribution of target antigens in healthy tissues can lead to systemic toxicity, highlighting the need to balance cytotoxicity with safety through nuanced calibration of immune synapse signaling, as previously discussed. Various strategies now seek to enhance the tumor-specificity of CAR-T cells. For instance, affinity tuning of antigen recognition domains to fine-tune CAR activation threshold could improve discrimination between tumor and healthy cells[121]. Multi-antigen logic gating is also being tested with multiplex CRISPR to improve specificity[112,123], while dynamic control switches and locoregional delivery offer real-time modulation of CAR-T cell cytotoxicity, potentially averting off-target effects[122,123]. Achieving further specificity to tumor tissues may require equipping CAR-T cells with nuanced sensors capable of discrimination beyond antigen signature (*e.g.*, oncogenic metabolites, dysbiotic microbes, aberrant physiological traits, etc.)[124]. Ongoing elucidation of the complex immunobiology governing T cell reactivity and exhaustion will inform next-generation designs to overcome current limitations. Still, the guiding principles should remain anchored in balanced signaling dynamics that potentiate CAR-T cell therapy without exhausting therapeutic efficacy or inflicting toxicity.

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Author contributions

Dian Xiong: Conceptualization, Data curation, Investigation, Methodology, Writing − original draft, Writing − review & editing, Resources. Haijun Yu: Conceptualization, Funding acquisition, Supervision. Zhi-Jun Sun: Resources, Supervision, Writing − review & editing, Conceptualization, Data curation.

Conflicts of interest

The authors declare no conflicts of interest.

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