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Review

Reversal of T-cell exhaustion: Mechanisms and synergistic approaches

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ABSTRACT

T cells suffer from long-term antigen stimulation and insufficient energy supply, leading to a decline in their effector functions, memory capabilities, and proliferative capacity, ultimately resulting in T cell exhaustion and an inability to perform normal immune functions in the tumor microenvironment. Therefore, exploring how to restore these exhausted T cells to a state with effector functions is of great significance. Exhausted T cells exhibit a spectrum of molecular alterations, such as heightened expression of inhibitory receptors, shifts in transcription factor profiles, and modifications across epigenetic, metabolic, and transcriptional landscapes. This review provides a comprehensive overview of various strategies to reverse T cell exhaustion, including immune checkpoint blockade, and explores the potential synergistic effects of combining multiple approaches to reverse T cell exhaustion. It offers new insights and methods for achieving more durable and effective reversal of T cell exhaustion.

1. Introduction

The concept of T-cell exhaustion was first proposed in a murine model of chronic lymphocytic choriomeningitis virus (LCMV) infection, where exhausted T cells were briefly described as activated T cells with no effector function [1]. With further research, T-cell exhaustion has been identified in the setting of viral infections, parasitic infections, tumors and other conditions. A comprehensive definition of T-cell exhaustion was proposed in 2019, with the main features including intrinsic dysfunction of the exhausted T cell, surface inhibitory receptor accumulation, effector function loss, dysregulation of various transcription factors, metabolic dysregulation leading to the disruption of the nutrient supply and accumulation of harmful metabolites, and epigenetic changes that induce abnormal transcriptional programs [2–4].

Various methods are used to reverse T-cell exhaustion. First, more studies have been conducted on the reversal of inhibitory receptor expression, mainly using immune checkpoint inhibitors (ICIs) to block inhibitory receptors, thus switching off their inhibitory signaling to block the exhaustion program. Second, exhaustion-related metabolic changes are currently targeted for reversal in humans mainly via intervention with transcription factors and metabolism-related enzymes to attenuate the exhaustion-related unfavorable metabolic state; ensure that the material and energy requirements supporting T-cell proliferation, differentiation and effector functions an be met; reduce the accumulation of harmful metabolites; and restore the normal effector function of cells [3]. In recent years, as research has progressed, the reversal of exhaustion at the epigenetic level has received increasing attention. The reversal of exhaustion at this level is achieved mainly through artificial interventions to modify chromatin; increase the chromatin accessibility of genes related to effector functions, memory and proliferation; reduce the chromatin accessibility of genes related to exhaustion; finally improving the cellular effector function at the genetic level [5]. There are various methods to reverse exhaustion, but clinical data prove that a single-pronged exhaustion reversal strategies have limitations, often only partially ameliorating the exhaustion-related changes, and that the reversal effects of these strategies are limited in persistence, drug resistance, and other aspects [6]. In contrast, multi-pronged synergistic reversal strategies can achieve more lasting and effective reversal effects with a wider scope and deeper level and thus have ideal research and clinical application prospects [7,8].

1.1. Molecular biological characteristics of exhausted T cells

Compared with normal T cells, exhausted T cells exhibit changes in number, effector function, surface inhibitory receptor expression status, transcriptional status, metabolic status, and epigenetic status [9]. The increased expression of various inhibitory receptors, such as

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programmed death 1 (PD-1), T-cell immunoglobulin domain and mucin domain-3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3), on the surface of exhausted T cells results in increased inhibitory signaling and subsequent decreases in the CD8 + T-cell number and effector function [10–13]. Moreover, the expression of various transcription factors is dysregulated in exhausted T cells, which promotes the loss of stemness in these cells and the disruption of their effector function. In addition, metabolic deregulation in exhausted T cells leads to a disruption of the material and energy supply required for cell proliferation, differentiation and physiological metabolic function, which further inhibits effector function and exacerbates the exhaustion of T cells [14,15]. Finally, exhausted T cells also undergo corresponding characteristic changes related to exhaustion at the gene level. DNA methylation, acetylation, and various histone modifications decrease the expression of genes controlling cellular effector functions, memory, and proliferation, and these changes genetically exacerbate the state of cell exhaustion [5]. Moreover, epigenetic changes also ensure the genetic stability of the exhausted state, enabling its maintenance in generations of progeny cells [16]. These characteristic changes related to exhaustion are markers of cell exhaustion and are also responsible for the abnormal effector function of exhausted T cells (Fig. 1). Therefore, targeting these characteristic changes related to exhaustion could reverse the overall exhausted state and improve the functional state of exhausted T cells.

2. Strategies to reverse T-cell exhaustion

2.1. Targeting cell surface inhibitory molecules to reverse T-cell exhaustion

Immune checkpoints are collective braking mechanisms that prevent the exhaustion of immune function during its execution. However, in tumors, the overexpression of programmed cell death 1 ligand 1 (PD-L1) on the surface of tumor cells induces the excessive upregulation of inhibitory receptors on the surface of T cells. This protective mechanism, which is initially beneficial for T cells and the immune system, eventually leads to uncontrolled tumor growth [17]. IIn response to the specific increase in the expression of multiple inhibitory receptors on the surface of exhausted T cells, a series of strategies for reversing T-cell exhaustion centered on blocking the actions of inhibitory receptors have been developed. The main strategies include 1) reducing the expression of inhibitory receptors and their ligands [17,18], 2) treating cells with ICIs to block the binding of ligands to immune checkpoints [19], and 3) modulating the form of inhibitory receptors [20] (Fig. 2). Although the three strategies involve different mechanisms, their ultimate goal is to block the immunosuppressive effects of checkpoint–ligand binding. Moreover, the specific mechanisms of action differ among immune checkpoint blockade strategies[21]. Currently, the blockade of various inhibitory receptors, such as PD-1, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and LAG-3, has been applied in clinical tumor treatment and has shown good therapeutic effects [22] (Table 1).

2.1.1. PD-1

PD-1 and PD-L1 are the most studied pair of immune checkpoint molecules; they are induced during the T-cell activation stage and are the main clinical targets for cancer immunotherapy due to their notable blocking effects [21,23]. Upon binding to its ligands, PD-1 aggregates near the TCR (T-cell receptor) and becomes phosphorylated on its immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM); this phosphorylation recruits protein tyrosine phosphatases such as SHP1 and SHP2. SHP1 and SHP2 can then dephosphorylate molecules within the TCR signaling complex, including Lck and ZAP-70, to counteract positive TCR signals. This process impairs the downstream PI3K-AKT pathway[24]. This inhibition eventually leads to an attenuation or loss of T-cell proliferation,

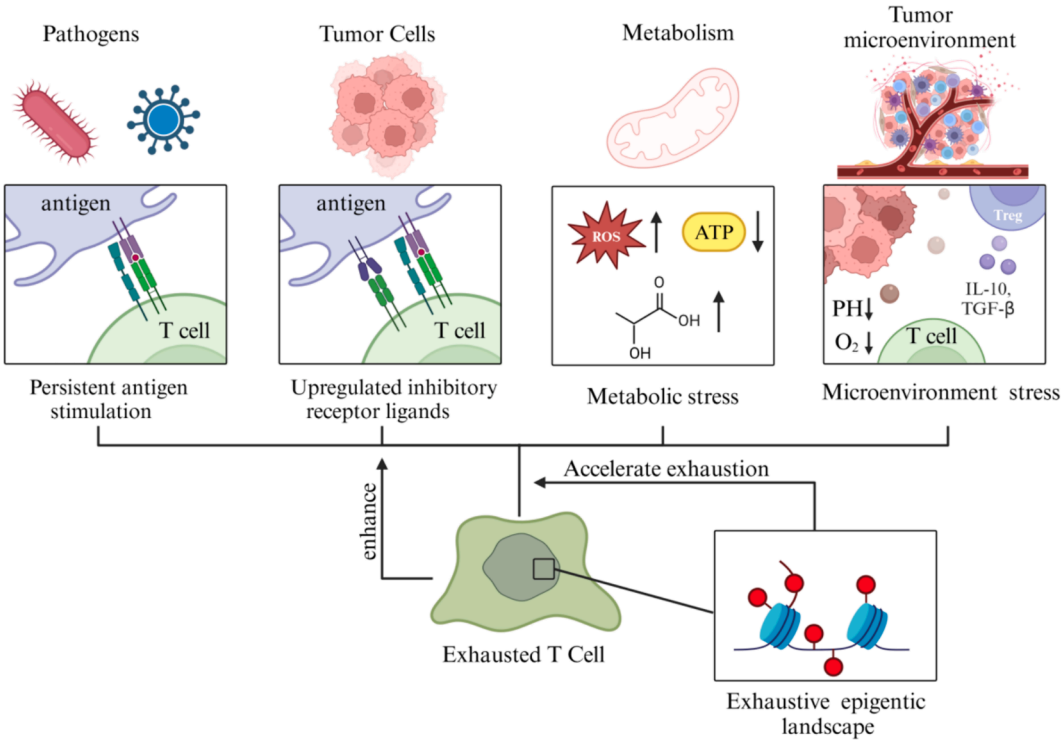

Fig. 1. Mechanism of T-cell exhaustion. The intricate process of T-cell exhaustion is characterized by multifaceted transformation. T cells in this state undergo a reduction in their numbers, a dampening of their effector functions, and a notable alteration in the expression of surface inhibitory receptors. Their transcriptional profiles are significantly altered, reflecting a shift in their genetic programming. Metabolically, these cells also exhibit changes that reflect their exhausted state, and their epigenetic landscape is reshaped. At the molecular level, T-cell exhaustion is characterized by a complex interplay of epigenetic modifications. DNA methylation, histone acetylation, and a variety of histone modifications that collectively contribute to the downregulation of genes pivotal for cells' effector capabilities, memory formation, and proliferative capacity.

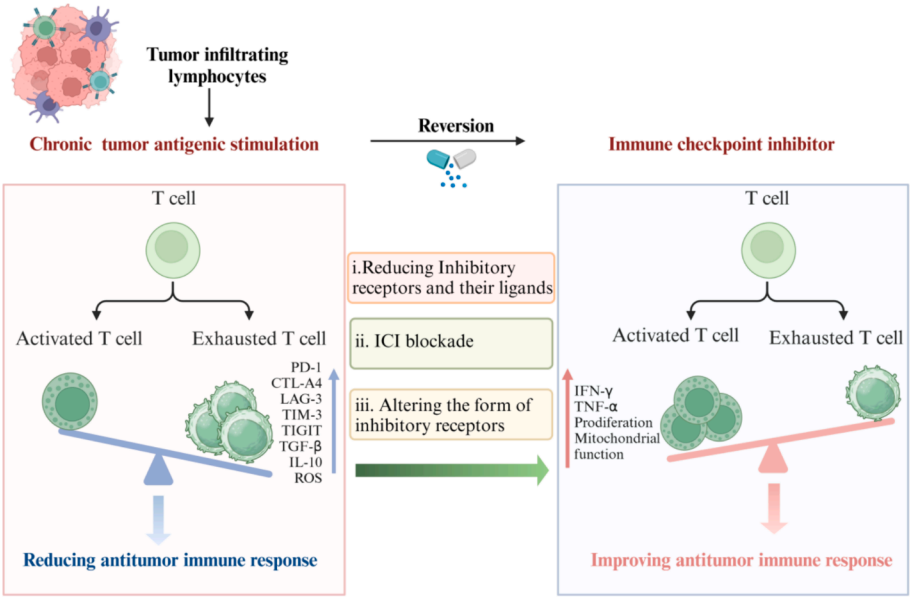

Fig. 2. Targeting of surface inhibitory molecules to reverse T-cell exhaustion. Under long-term stimulation with tumor antigens, the expression of multiple inhibitory receptors on the surface of exhausted T cells is increased. The main strategies to reverse the exhaustion of T cells focus on blocking inhibitory receptor activity and include the following three methods: 1) reducing the expression of inhibitory receptors and their ligands, 2) treating T cells with an immune checkpoint-blocking agent (immune checkpoint inhibitor, ICI) to block the ligand recognition and binding activities of the immune checkpoint, and 3) altering the form of the inhibitory receptor.

Table 1
Targeting inhibitory molecules to reverse T-cell exhaustion in tumors.

Target	Agent	generic name	Strategies	Clinical Outcomes	Reference
PD-1	Anti-PD-1 monoclonal antibody	Serplulimab	Combined with chemotherapy drugs	significantly improve PFS and OS	[36]
		Cemiplimab		improve PFS and OS The incidence of adverse events decreased	[29]
		Sintilimab		significantly improve OS	[30]
		Toripalimab		significantly improve EFS	[31]
		Camrelizumab		significantly improve OS	[32]
PD-1 /PD-L1	Anti-PD-1/PD-L1 monoclonal antibody	Pembrolizumab	Combined with chemotherapy drugs	significantly improve OS	[37]
PD-L1	Anti-PD-L1 monoclonal antibody	Nivolumab	Adjuvant immunotherapy	improve DFS	[38]
		Durvalumab	Combined with chemoradiotherapy	significantly improve EFS	[33]
		Avelumab	Avelumab plus axitinib	promising anti-tumour activity and acceptable toxicity	[34]
		Atezolizumab	Atezolizumab plus bevacizumab and chemotherapy	significantly improve PFS and OS	[35]
CTLA-4	Anti-CTLA-4 monoclonal antibody	Ipilimumab	Ipilimumab plus Nivolumab,	The effect is not obvious and induces adverse reactions	[49]
LAG-3	LAG-3-Ig fusion protein	/	Additional T-cell activation function	more significant effect	[64]
TIGIT	Anti-TIGIT monoclonal antibody	Ociperlimab (BGB-A1217)	Combined with tislelizumab	preliminary antitumor activity was observed	[69]
		Tiragolumab	Tiragolumab plus Atezolizumab	Antitumor activity was demonstrated	[70]
DNA methyltransferase	DNA methyltransferase inhibitors	Decitabine	Combined with PARP inhibitor	Reduce tumor growth and invasiveness of breast cancer	[148]
histone deacetylase	histone deacetylase inhibitors	Nothing	Combined with ICB	Improve ICB antitumor immune responses	[166]

activation, cytokine production and cytotoxicity [17]. Therefore, several clinical trials combining the use of SHP2 inhibitors with ICIs are underway (NCT04000529, NCT04418661). In addition to its effect on T cells, SHP2 also mediates signal transduction in a variety of cells, with the ultimate effects still awaiting the results of clinical trials [25]. Recently, researchers have shown that PD-1 signaling can downregulate Dynamin-related protein 1 (Drp 1) phosphorylation by regulating extracellular regulated protein kinases 1/2 and the mechanistic target of rapamycin (mTOR) pathway to block mTOR-mediated mitochondrial fission, decrease the mitochondrial number and attenuate mitochondrial function, rendering cells unable to adapt their biological activities. This process ultimately leads to the inhibition of T-cell activation, proliferation and infiltration [26]. Subsequently, Ma *et al.* successfully achieved a synergistic enhancement of PD-1 blockade in lung cancer mediated by a high level of Drp 1 expression and verified that PD-1 affects mitochondrial function by regulating the Drp 1 level [27,28]. Together, these results indicate that the PD-1/PD-L1 axis participates in the activation of multiple inhibitory signaling pathways and plays an important role in T-cell exhaustion.

Targeting PD-1 and PD-L1 with anti-PD-1 and anti-PD-L1 antibodies can block multiple immunosuppressive signals transduced via the PD-1/PD-L1 axis, restore the normal activation pathway, promote a favorable

mitochondrial functional state in T cells, and reverse exhaustion. Currently, a variety of commercial immune checkpoint antibodies, such as serplulimab, pembrolizumab, nivolumab, cemiplimab, sintilimab, toripalimab, camrelizumab, avelumab, atezolizumab and durvalumab, are used in the clinical treatment of various tumors, making treatments targeting immune checkpoints such as PD-1 more convenient, effective, safe and controllable [29–38]. Pembrolizumab was found to significantly prolong the survival of patients with melanoma [39], cervical cancer [40] and colorectal cancer [41], and the incidence of adverse events was also significantly lower than that associated with traditional chemotherapy. Nivolumab has shown better therapeutic effects on cancer types such as epithelial cell carcinoma [42]. According to the existing clinical data, the effects of commercial inhibitors targeting PD-1/PD-L1 on reversing T-cell exhaustion differ across tumor types.

Recently, clinical reports have indicated that ICB therapy may also lead to cytokine storms [43]. Regulators of immune inflammatory functions, such as Regnase-1, are believed to be crucial for modulating immune responses mediated by the interleukins IL-6 and IL-12 [44]. The combined use of these regulators with ICB may be beneficial for preventing cytokine storms. Therefore, accurately matching existing ICIs with the appropriate tumor types and establishing a mature reference system for matching to guide the clinical selection of ICIs is a future direction for increasing the therapeutic efficacy of ICB. In addition, the clinical application of multiple PD-1 and PD-L1 inhibitors and their combination with other ICIs or other therapies is rapidly increasing, and they are showing increasingly prominent therapeutic effects [45,46].

2.1.2. CTLA-4

The expression of CTLA-4, the earliest identified immune checkpoint molecule, is upregulated immediately after TCR binding [21]. Like PD-1, CTLA-4 also blocks T-cell activation by targeting the costimulatory receptor CD28 via a different mechanism of action. Since the binding of CTLA-4 to the costimulatory molecule ligand B7 is stronger than its binding to CD28, the activity of the positive costimulatory molecule CD28 is inhibited by competitive binding of B7 after TCR recognition, which then suppresses the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway to attenuate the activation of T cells. In addition, the activation of CTLA-4 itself induces a negative feedback coinhibitory signal mediated by SHP-2 and protein phosphatase 2A (PP2A) to inhibit T-cell activation [13,47].

An immune pathway analysis involving 33 cancer types revealed that CTLA-4 is strongly associated with T cells in almost all tumor types and is involved in multiple immune pathways [48]. This finding partially indicates the feasibility of targeting CTLA-4 in tumor immunotherapy. However, the specific mechanism of CTLA-4 blockade is not fully clear, and the clinical efficacy of the anti-CTLA-4 monoclonal antibody (mAb) ipilimumab is not ideal. In a randomized controlled trial involving patients with advanced melanoma, the progression-free survival (PFS) time of patients treated with ipilimumab alone was significantly shorter than that of patients treated with adoptive therapy [49]. Similarly, a series of clinical studies of ipilimumab combined with other ICIs (e.g., nivolumab) or chemotherapy showed that the addition of ipilimumab did not significantly improve the status of the tumor microenvironment and may result in more adverse effects [50–52]. Regarding the problem of CTLA-4 blockade inducing a series of adverse effects, some investigators have suggested that maintaining rather than inhibiting the expression of CTLA-4 may be a safer and more effective method for reversing exhaustion [53]. Given the complexity of targeting CTLA-4 in immunotherapy, whether this pathway is amplified or blocked needs further investigation. Moreover, the development of new anti-CTLA-4 antibodies and antibody–drug conjugates may solve the problems associated with the clinical use of CTLA-4 inhibitors.

2.1.3. LAG-3

LAG-3 is the third most commonly targeted immune checkpoint in human tumor immunotherapy after PD-1 and CTLA-4, and the third ICI target approved by the FDA. LAG-3 is expressed on the surface of stimulated CD4 + T cells and CD8 + T cells, and its upregulation triggers persistent suppression of T-cell effector functions in the context of tumor epigenetic regulation and continuous stimulation by tumor antigens. Clinical data on melanoma have shown that the median survival rate of patients who are positive for LAG-3 is less than one-third that of patients who are negative for LAG-3, suggesting that LAG-3 activation has a detrimental effect on the prognosis of cancer patients [54]. LAG-3 is structurally homologous to CD4 but has a much greater affinity for MHC; thus, LAG-3 blocks CD4–MHC-mediated T-cell activation through competitive inhibition [55]. Second, LAG-3 acts as an inhibitory molecule, and its intracellular segment motifs (FxxL, S484, KIEELE and EP) mediate a variety of inhibitory signal transduction pathways, further inhibiting the activation of T cells [56,57]. Recent studies have also shown that LAG-3 has noncanonical ligands in addition to MHC (e.g., galectin-3 and fibrinogen-like protein 1). The binding of LAG-3 to these noncanonical ligands mediates the MHC-independent T-cell effector inhibition function of LAG-3[58,59]. In addition to its direct effect on effector T cells, LAG-3 also suppresses the generation and reactivation of memory T-cell subsets, thus increasing its inhibitory effect on T-cell effector functions[60,61].

The diversity of functional mechanisms underlying the LAG-3-mediated inhibition of T-cell effector functions also allows the targeting of LAG-3 to reverse T-cell exhaustion and increase the efficacy of tumor immunotherapy. Direct knockdown of LAG-3 or conventional blockade of its recognition using an anti-LAG-3 antibody can significantly improve T-cell effector functions. Moreover, due to its unique mechanism, LAG-3 signaling has little overlap with other immune checkpoint pathways, and LAG-3 thus has greater potential for synergistic immune checkpoint blockade and fewer toxic side effects [62,63]. Moreover, in addition to conventional anti-LAG-3 antibodies, LAG-3-Ig fusion protein agonists have been developed and used to reverse the immunosuppressive effects of LAG-3 [64]. Compared with conventional anti-LAG-3 antibodies, LAG-3-Ig has an additional T-cell activation function and thus has a more significant effect on improving T-cell effector functions.

In summary, LAG-3, a new-generation target for tumor immunotherapy, has diverse and unique mechanisms; thus, targeting LAG-3 either alone or in combination with other immune checkpoints or other exhaustion reversal strategies has considerable clinical potential [56]. The unique blocking mechanism and agonistic integrated fusion protein of LAG-3 provide new insights for the development of next-generation ICIs.

2.1.4. Other immune checkpoints

In addition to the three main immune checkpoints described above, an increasing number of novel immune checkpoint molecules, including TIM-3, T-cell immune receptor with Ig and ITIM domains (TIGIT) and cluster of differentiation 155 (CD155), have been identified and found to effectively reverse exhaustion. The expression level of TIM-3 was significantly increased in various exhausted T cells isolated from liver cancer, colorectal cancer and prostate cancer samples, and various ligands, including galectin 9 (Gal 9), phosphatidylserine (PtdSer), high mobility group box 1 protein (HMGB 1), and carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1), interact with TIM-3 to regulate T-cell effector function through the activation of different pathways [65]. Activation of the TIM 3/Gal 9 pathway leads to reduced production of cytokines such as interferon-γ (IFN-γ), interleukin 2 (IL-2) and tumor necrosis factor α (TNF α), while the TIM 3/PtdSer and TIM 3/CEACAM1 pathways are associated with antigen presentation and tolerance development, respectively [66].

Similarly, data for 33 tumor types suggest that TIGIT is upregulated in the vast majority of cancers and is strongly associated with prognostic parameters such as patient survival [67]. The main ligand of TIGIT, CD155, is highly expressed on the surface of tumor cells and significantly amplifies CD155/TIGIT axis signaling, leading to immune cell

dysfunction and facilitating tumor immune escape [68]. Therefore, a few commercial TIGIT antibodies have been developed and applied clinically, such as ociperlimab and tiragolumab, which have shown good therapeutic effects when used in combination with PD-1/PD-L1 inhibitors [69,70] (Table 1).

2.1.5. Limitations and research directions of ICB

ICB is a relatively mature means of reversing T-cell exhaustion, and with the continuous discovery of new immune checkpoints, its effects are becoming increasingly diverse [71]. However, the current clinical data suggest that many problems persist with ICB. The main problem is that the reversal effect of ICB is not durable, and the reversed T cells return to an exhausted state soon after continuous antigen stimulation; this phenomenon is related to the limitations of ICB therapy [72]. ICB only blocks the link during the exhaustion process and, to a certain extent, block the exhaustion effect; they do not achieve more durable reversal effects; for example, they do not eliminate chronic stimulation that causes CD8 + T-cell exhaustion [73], do not ameliorate the abnormal state of the energy supply [14], and do not reverse genetic causes of exhaustion [74]. Therefore, under continuous antigen stimulation, cell surface inhibitory receptor expression constantly increases, which eventually returns T cells to an exhausted state soon after reversal.

2.2. Targeting transcription factors to interfere with T-cell exhaustion

Transcription factors are messengers that regulate the biological activities of cells and participate in regulating the proliferation, differentiation and other biological activities of T cells. Various transcription factors (such as T-cell factor-1 (TCF-1), high-mobility group box protein of thymocyte selection (TOX), and eomesodermin (Eomes)) have been found to play key roles in the process of T-cell exhaustion. Moreover, different transcription factors mutually influence each other in the regulation of T-cell biological activities, and their regulatory effects occur throughout the life cycle of T cells and other immune cells. Targeting transcription factors has become an integral component of regulating T-cell effector functions to achieve durable and efficient reversal of T-cell exhaustion (Table 2).

2.2.1. TCF-1 and TOX

TCF-1 and TOX are involved in the differentiation of T-cell subsets and are differentially expressed in each subset. TCF-1 is highly expressed in naive T cells and progenitor-like T cells but is expressed at low levels in effector T cells and terminally exhausted T cells, and its high expression is eventually restored after the generation of memory T cells; this protein is responsible for the self-renewal of CD8 + T cells and the maintenance of memory and stemness [75–78]. In contrast, TOX is expressed mainly in mature effector T cells and is not only responsible for epigenetic remodeling related to effector functions but also closely associated with the occurrence of CD8 + T-cell exhaustion. The expression levels of TCF-1 and TOX show a dynamic balance in different periods, consistent with the differences in the effector functions of cells in different periods [79].

An analysis of the expression level of TCF-1 in different T-cell subsets and the corresponding functional status of the cells revealed that TCF-1 is closely related to the stemness, continuous proliferation and differentiation, and immune memory function of T cells [80]. The effector maintenance function of TCF-1 is achieved through multiple molecular mechanisms, including the TCF-1-mediated T-box expressed in T cells (T-bet)-to-Eomes transition, the TCF-1-driven c-Myb-mediated increase in B-cell lymphoma-2 (Bcl-2) expression, and the counterbalance of IFN via the TCF-1/B-cell lymphoma-6 axis [77,80–82]. An analysis of clinical data revealed that patients with high expression of TCF-1 in T-cell subsets had longer overall survival (OS) and progression-free survival

Table 2
Targeting transcription factors to reverse T-cell exhaustion.

transcription factors	function in T cell	signal pathway	Effects on T cell exhaustion	reference
TCF-1	mediates the bifurcation between divergent fates	i) TCF1/cMyb/Bcl2 ii) TCF1/Bcl6/IFN1	promote the fate shift of exhausted T cells to progenitor T cells	[8182]
TOX	involved in T cell development and differentiation	i) drive antigenic stimulation-mediated conversion into a unique epigenetic landscape of exhaustion ii) upregulation of exhaustion-related inhibitory receptors (such as PD-1, TIM-3, CD244, and TIGIT)	promote T cell exhaustion	[8586]
EOMES	involve in the regulation of T-cell effector functions, memory and proliferation	i) bind to transcriptional repressive regulatory regions in multiple genes (e.g., PD-1, CTLA-4, and CD39) and block their transcription ii) competes with T-bet for binding sites and impedes its positive effector function	facilitates the reversal of exhaustion promote T cell exhaustion	[9295]
T-bet	participate in T cell development ， differentiation and the initial priming of CD8 + effector T cells	−bet/lymphocyte function-associated antigen 1/IFN-γ	prevent the upregulation of exhaustion-related inhibitory receptors	[94]
IRF4	participate in the regulation of memory T cell differentiation	increase expression of inhibitory receptors; decrease cellular metabolic capacity;repressed the expression of TCF-1	promotes and sustains exhaustion	[97]
BATF	participate in the proliferation,the maintenance of cell number and phenotype of T cells; associated with effector T cell differentiation	remodel chromatin accessibility	BATF cKO T cells are more likely to remain naive and resistant to exhaustion	[104]
NFAT	participate in the proliferation and the maintenance of cell number and phenotype of T cells	i) inhibit the transcription of some exhaustion-related genes (under physiological conditions) ii) activator protein-1 as a switch negative feedback regulation	i) inhibit exhaustion ii) induce exhaustion	[102]
Blimp-1	coordinate T-cell proliferation and maturation	PRDM1 (Blimp-1coding genes) KO increased expression of TCF7 ，Myeloblastosis ，Bcl6 and Inhibitor of DNA-binding 3	drive T-cell exhaustion and terminal differentiation	[107]
NR4A3	coordinate T-cell proliferation and maturation.	PRDM1(KO)/NFAT2/NR4A3	promotes exhaustion	[107]
BCL6	promote stem-like programs in CD8 + T cells	antagonizing Blimp-1	reverses the T exhaustion	[108]
Stat5	fosters effector-like CD8 + T cell differentiation	antagonizing TOX IL-2/Stat5	directs Tex^{int} cell formation and re-instigates partial effector biology	[87]

(PFS) times than those with low TCF-1 expression [83]. This finding suggested that TCF-1 has positive effects on the maintenance of T-cell function and number and the attenuation of T-cell exhaustion in the context of chronic infections or tumors.

The effector function of TOX is a double-edged sword. On the one hand, TOX is a conventional transcription factor required for the effector functions of effector T cells. It plays an important role in T-cell differentiation and development and is highly expressed in a variety of effector memory T (TEM) cells [79,84]. On the other hand, TOX is considered a bridge between effector T cells and exhausted T cells, and sustained, robust TOX expression can drive antigen stimulation-mediated conversion into a unique epigenetic landscape and an exhaustion-associated transcriptional program that promotes the generation of exhausted T cells [85,86]. In addition, TOX promotes the endocytosis of PD-1 by binding to PD-1 in the cytoplasm, thereby maintaining high levels of PD-1 expression in cells and further accelerating the influx of exhaustion signals [84].

Based on the characteristics of TCF-1, Shan *et al.* expressed TCF-1 in exhausted T cells, enforced a preexisting stem cell-like transcriptional program, successfully upregulated T-cell effector function-related transcription factors (i.e., Eomes and Tox), suppressed the expression of key coinhibitory receptors (i.e., PD-1 and 2B4), shifted the T-cell fate toward a stem cell-like exhaustion state, and significantly increased the response capacity of exhausted T cells [80]. Scott *et al.* reported that under tumor microenvironmental stimulation, TOX-deficient CD8 + T cells exhibit low chromatin accessibility at the genetic locus of each inhibitory receptor, which successfully prevents the upregulation of exhaustion-related inhibitory receptors (such as PD-1, TIM-3, CD244, and TIGIT); however, unfortunately, the effector function of these seemingly nonexhausted CD8 + T cells is still dysregulated, indicating that blocking the TOX-mediated exhaustion pathway alone cannot completely restore the effector function of exhausted T cells [86]. However, treatments targeting TOX showed excellent synergy with other means of reversing exhaustion, as indicated by the use of the transcription factor signal transducer and activator of transcription 5 (Stat 5) to antagonize TOX, which successfully achieved partial reprogramming of the exhaustion-associated epigenetic landscape, restored the versatility of exhausted T cells, and significantly enhanced the reversal effect of ICB [87,88].

2.2.2. Eomes and T-bet

Eomes and T-Bet are members of the T-box transcription factor family and are involved in regulating T-cell effector functions, memory and proliferation [89,90]. Eomes is differentially expressed in T cells at all stages of their normal life cycle and in the setting of chronic infections or tumors. Some researchers have shown that the expression level of Eomes is lowest in naive T cells, relatively high in effector and memory T cells with a normal activation status, and highest in exhausted T cells. Throughout the development of chronic infections and tumors, the level of Eomes in T cells increases, and this increase corresponds to the activation of cellular functions [91]. Interestingly, these transcription factors play synergistic and seemingly antagonistic roles in the development of T-cell effector functions and exhaustion, respectively. On the one hand, Eomes cooperates with T-bet during the early cell development stage to drive the maturation of effector T cells. On the other hand, under continuous antigen stimulation, a high level of Eomes can inhibit T-cell effector functions and instead promote exhaustion.

Eomes has two functions: it promotes the differentiation and maturation of T cells at early developmental stages, and it subsequently cooperates with T-bet. He *et al.* showed that Eomes is required for the full development of antitumor cytotoxic T lymphocytes (CTLs) and that Eomes promotes CD8 + T-cell effector functions by inhibiting the expression of inhibitory receptors on the surface of CD8 + T cells [92,93]. A reduction in Eomes expression results in decreased production of effector-related cytokines (IFN-γ and TNF-α) in T cells and an attenuation of cytotoxic effects. In severe cases, complete loss of Eomes results in impaired proliferation and abnormal effector functions of CD8 + T cells, a phenomenon associated with increased TCF-1 expression and decreased TOX expression in CD8 + T cells due to Eomes deficiency [91]. Although a certain level of Eomes is necessary for the development of effector functions in CD8 + T cells, an excessive level of Eomes induces T-cell exhaustion, which antagonizes the effect of the transcription factor T-bet on effector functions. The mechanism involves downregulation of the expression of maturation- and memory-related genes (such as C-C motif chemokine receptor 7 (Ccr 7), Tcf-1 and C-X-C motif chemokine receptor 3 (Cxcr 3)); the production of proliferation-, effector-, and chemotaxis-related cytokines; an increase in the expression of inhibitory receptors (e.g., PD-1); and the upregulation of genes involved in apoptosis [91]. T-bet is necessary for the early proliferation and differentiation of T cells, and the α chain of LFA1 is targeted to participate in the initiation of the initial effector function of T cells [94]. Some researchers believe that the dual effect of Eomes may be caused by competition between Eomes and T-bet for the same active site and the difference in the strength of the interaction between the two proteins. Compared with that of T-bet, the intensity of action of Eomes is much weaker; therefore, when Eomes expression is low, the number of active sites is relatively sufficient, and the effects of Eomes and T-bet are synergistic. However, when the level of Eomes is too high, cell proliferation and effector function are reduced due to competition with T-bet for binding sites, which results in an exhausted state [95].

Normally, Eomes can directly bind to transcriptional repressive regulatory regions in multiple genes (e.g., PD-1, CTAL-4, and CD39) to block their transcription, thus maintaining inhibitory receptor expression at a low level. However, Eomes is downregulated in tumor cells, and its transcriptional blockade of inhibitory receptors is weakened; therefore, an appropriate increase in the insufficient level of Eomes in the tumor environment facilitates the reversal of exhaustion [92]. However, due to the competitive relationship between Eomes and T-bet for receptor gene binding and the positive correlation between Eomes and immune checkpoint expression identified during viral infections, the upregulation of Eomes in the tumor environment should be reasonably controlled; otherwise, the uncontrolled upregulation of Eomes promotes the restoration of exhaustion [91,95,96]. In conclusion, an excessive or inadequate Eomes level can lead to the exhaustion of CD8 + T cells. Therefore, regulating the level of Eomes in CD8 + T cells and maintaining it within the appropriate range are key to reducing the occurrence of CD8 + T-cell exhaustion and reversing the existing exhausted state through the Eomes pathway.

2.2.3. IRF4, BATF, and NFAT

Interferon regulatory factor 4 (IRF4), basic leucine zipper ATF-like transcription factor (BATF) and nuclear factor of activated T cells (NFAT) are three TCR-responsive transcription factors with highly similar or even overlapping gene binding sites. They work synergistically to regulate transcription in exhausted T cells and are highly expressed in exhausted T cells in the setting of chronic infections and tumors [97,98].

IRF4, BATF and NFAT not only participate in the initial proliferation of normal antigen-specific T cells and the maintenance of the cell number and phenotype during infections but also promote the occurrence of exhaustion under certain conditions [97,99,100]. For example, IRF4 exerts distinct effector functions in cooperation with different transcription factors [101]. In the absence of activator protein-1 (AP-1), the positive effect of NFAT on the number and function of CD8 + T cells is switched to a proexhaustion effect [102].

In exhausted T cells, IRF4 promotes exhaustion via multiple mechanisms, including increased expression of inhibitory receptors (e.g., PD-1, TIM-3, and LAG3), impaired secretory function of effector-related cytokines, and a decreased cellular metabolic capacity [97]. Recently, several researchers have shown that downregulating IRF4 can reduce the expression of inhibitory receptors and that the anabolic capacity associated with efficient function can be partially restored,

demonstrating the feasibility of reversing the state of cell exhaustion by reducing the excessive level of IRF4 in exhausted T cells [103]. By analyzing gene expression and chromatin accessibility during the differentiation of BATF-deficient CD8 + T cells, Tsao *et al.* confirmed that BATF is involved in regulating chromatin accessibility and is closely related to effector T-cell differentiation [104]. Sun and colleagues reported that BATF functions in exhausted T cells through various pathways, such as the PD-1/BATF, IL-21/BATF, and BRD4/BATF axes. Additionally, BATF can enhance the durability of CAR-T cells and reverse the exhaustion of tumor-infiltrating T cells, indicating its potential as a valuable target for cancer immunotherapy [105]. Under physiological conditions, by regulating its downstream targets, NFAT can inhibit the transcription of some exhaustion-related genes, thus preserving some of the effects of tumor-infiltrating lymphocyte (TIL) depletion. However, in the absence of its companion transcription factor AP-1, NFAT exerts a proexhaustion effect on CD8 + T cells, and negative feedback regulation during proliferation is predominant, which in turn induces exhaustion [102].

IRF4, BATF and NFAT can all enhance T-cell effector functions. However, this enhancing effect is often achieved at the expense of accelerating cellular exhaustion, and the memory function and proliferation of cells are affected when the effector function is enhanced. Therefore, the expression levels of IRF4, BATF, and NFAT must be precisely regulated and balanced to enhance T-cell effector functions while ensuring the adequate memory function and proliferation of antigen-specific T cells to avoid accelerating the exhaustion process due to dysregulated cell proliferation[106].

2.2.4. Other transcription factors

Many other transcription factors are directly or indirectly involved in the regulation of T-cell exhaustion, including B lymphocyte-induced maturation protein-1 (Blimp-1) and neuron-derived orphan receptor 1 (NR4A3), which work together to coordinate T-cell proliferation and maturation [107]; BCL6 [94], which antagonizes Blimp-1 to promote stem-like programs in CD8 + T cells in the context of cancer; TOX [85], which promotes cell exhaustion by modifying the CD8 T-cell epigenetic landscape and transcriptional programs; and Stat5, which restores exhausted T cells to a durable effector state by antagonizing TOX [87]. According to current research, the changes in the expression and effects of transcription factors during exhaustion are undoubtedly very complex, and the same transcription factor may have completely different functions when expressed at different levels or in cooperation with other factors. The mechanism underlying the occurrence and development of T-cell exhaustion cannot be explained through the action of a single transcription factor. The overall perspective must be considered to explore the synergistic, agonistic or antagonistic interactions between transcription factors, to truly understand each step of the mechanism underlying exhaustion, and to determine how to prevent or reverse the occurrence of adverse exhaustion processes.

2.3. Targeting metabolic reprogramming to reverse T-cell exhaustion

Cells exhibit different metabolic states at different stages to adapt to the changing requirements for effector functions and proliferation [109,110]. The metabolism of sugars and lipids, which are responsible for the energy supply, and the metabolism of various proteins, amino acids, reaction byproducts, and waste products all indicate and influence the functional state of cells [3]. Currently, an increasing number of scholars believe that metabolic dysfunction is closely related to the occurrence and progression of T-cell exhaustion [111–113]. T cells can be roughly divided into four subtypes, namely, naive T cells, memory T cells, effector T cells, and exhausted T cells (including progenitor T cells and terminally exhausted T cells), according to their effector functions and metabolic states. In terms of energy metabolism, naive T cells and memory T cells are relatively static, and they satisfy their low demand for energy mainly through oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO). Effector T cells are more active and have a greater demand for energy. Under energy supply stress, metabolic reprogramming occurs to allow the supplementation of OXPHOS with aerobic glycolysis to meet the high energy demand [6,114,115]. Due to mitochondrial damage, exhausted T cells cannot rely on OXPHOS to meet their energy needs and instead compensate for the deficient mitochondrial energy supply by increasing aerobic glycolysis [113] (Fig. 3).

2.3.1. Reversal strategies centered on the regulation of glucose metabolism

Glucose uptake is decreased in exhausted T cells, consistent with an insufficient energy supply. Second, in exhausted T cells, the mitochondrial number is decreased, the mitochondrial volume is abnormally increased, the mitochondrial membrane potential is abnormal, the mitochondrial structure is disrupted, the structure of cristae—closely related to effector function—is abnormal, and many depolarized mitochondria accumulate, which exacerbates the dysregulation of glucose metabolism disorders from another aspect [116,117]. Mechanistically, hypoxia in the tumor microenvironment induces Blimp-1-mediated inhibition of peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α-dependent mitochondrial reprogramming. Eventually, the cells become dysfunctional. On the other hand, the loss of mitochondrial function leads to an increase in the intracellular level of reactive oxygen species (ROS), which suppresses the proliferation and self-renewal of T cells and further promotes exhaustion.

Exhaustion-related changes in mitochondria can result from exhaustion, but they are also the factors that drive or further exacerbate exhaustion [118,119]. Due to mitochondrial damage, the OXPHOS capacity is reduced and replaced by an increased capacity for aerobic glycolysis. Through gene enrichment analysis of metabolic pathways such as glycolysis, mitochondrial respiration, lipid metabolism, and the pentose phosphate pathway, Hao *et al.* found that mitochondrial gene expression was decreased in terminally exhausted T cells, revealing a relationship between mitochondrial function and T-cell failure, and speculated that this change could drive HIF-1α-mediated glycolytic reprogramming [113]. As such, alleviating oxidative stress and repairing mitochondria can help reverse T-cell exhaustion. Recently, evidence of the noncanonical functions of TERT has shown that oxidative stress induces the translocation of TERT to mitochondria, providing protection against oxidative stress and restoring mitochondrial function [120]. TERT is expected to become a new target that helps T cells regain their functionality.

Notably, ICB, the most commonly used method for reversing exhaustion at present, cannot reverse mitochondrial damage. Therefore, if ICB could promote mitochondrial biogenesis or reverse mitochondrial damage and thus restore the normal energy metabolism of cells, the effectiveness and durability of its exhaustion-reversing effect could be expected to increase [121]. Unlike mitochondrial damage, the changes in T-cell energy metabolism during exhaustion reflect the energy requirements of different cellular functional states. Simply forcing the reversal of energy metabolism to its initial mode cannot reverse the exhaustion state. Strategies to meet cellular energy requirements are more likely to restore the effector functions of CD8 + T cells [122]. Fisicaro *et al.* showed that CD8 + T-cell exhaustion can be reversed to some extent by targeted metabolism in the context of viral infections [123].

Various methods have been developed to restore the metabolic state of exhausted T cells. For example, ICB cannot directly increase the metabolic capacity of cells, but can reduce the adverse effects of inhibitory receptors on metabolic function. PD-1 downregulates mTOR activity by blocking the PI3K-AKT-mTOR axis, thus inhibiting glucose transport and utilization, and inhibiting PD-1 signaling can attenuate this inhibition, thereby increasing the ability of T cells to utilize glucose [124–126]. Chondroitin sulfate synthase 1 (CHSY1) can activate the succinic acid metabolism pathway and promote CD8 + T-cell failure, which causes liver metastasis of CRC. Artemisinin, a CHSY1 inhibitor,

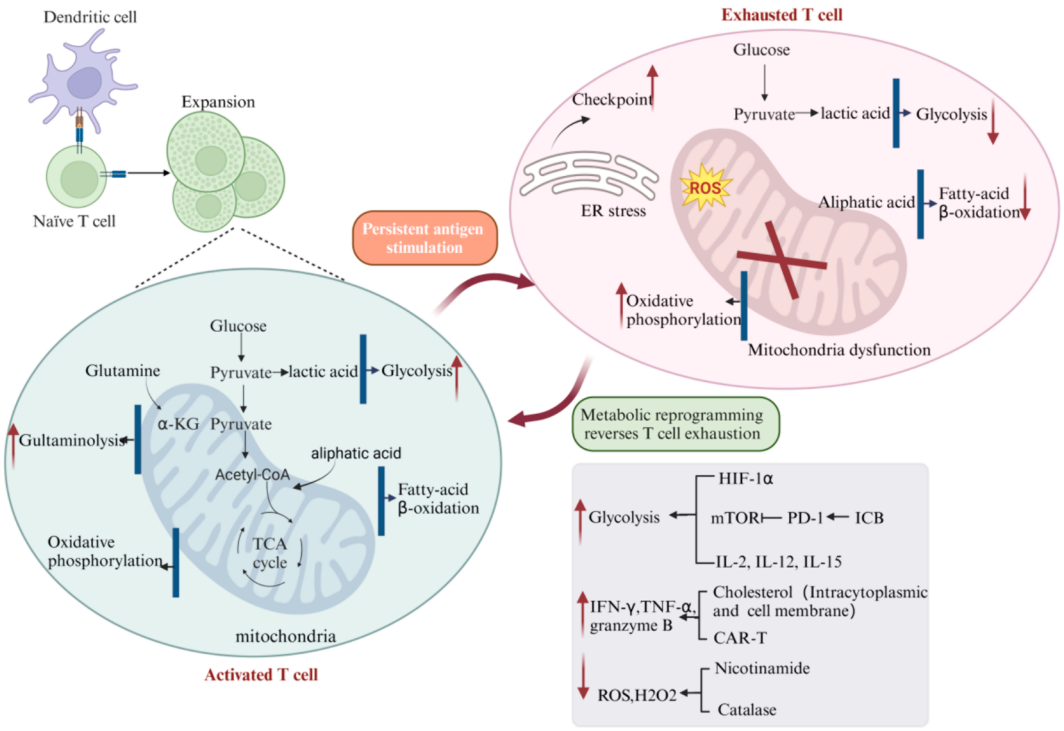

Fig. 3. Metabolic reprogramming reverses T-cell exhaustion. When T cells are activated and differentiated into effector T cells under antigen stimulation, anaerobic glycolysis and glutamate metabolism increase. In T cells under continuous antigen stimulation, anaerobic glycolysis and fatty acid β-oxidation are decreased, mitochondrial oxidative phosphorylation is increased, and the ROS level in these cells with mitochondrial dysfunction is increased, leading to exhaustion. The cytokine HIF-1α can drive an increase in glycolysis, and immune checkpoint blockade can increase glycolysis through the mTOR pathway to reverse T-cell exhaustion. Intracellular and cell membrane cholesterol and CAR-T cells can promote the production of effector cytokines by T cells. Nicotinamide and catalase can decrease ROS and H_2O_2 levels to promote T-cell activation and memory differentiation, attenuate the exhaustion phenotype and increase the expression of activation and memory markers.

can reverse T-cell exhaustion [127]. Some cytokines (e.g., IL-2, IL-10, IL-12, and IL-15) have been found to improve the energy metabolic state in exhausted T cells. Recently, Xia *et al.* reported that T-cell exhaustion, which occurs in the tumor environment, can also regulate the intensity of the response to immunotherapy by regulating energy metabolism. These authors noted that reference effector T cells primarily use glycolytic metabolism and maintain an efficient energy supply for glycolysis by increasing mitochondrial biogenesis, which can promote metabolic adaptation and maintain the immune function of these cells. The predominance of OXPHOS and FAO, the main metabolic programs, can increase mitochondrial biogenesis in reference memory T cells. This phenomenon can effectively improve cellular immune memory and metabolic adaptability [121]. In addition, regarding severely damaged or dysfunctional mitochondria, although mitochondrial autophagy leads to a short-term reduction in the number of CD8 + T cells, it is also highly important for the regulation of the long-term cell state, long-term cell survival, and metabolic state required for memory T-cell development [128].

2.3.2. *Reversal strategies centered on modulating lipid distribution*

Lipids play an important role in exhausted T cells by functioning as raw materials for acetyl-CoA synthesis and regulating gene expression and are involved in regulating T-cell differentiation [3]. Notably, lipids both induce exhaustion and promote the effector functions of T cells, depending on their subcellular localization. In the tumor microenvironment, exogenous lipids amplify the stimulus signal by inducing TCR aggregation on cells, leading to endoplasmic reticulum (ER) stress in CD8 + T cells and increased immune checkpoint expression, and ultimately promoting the occurrence of T-cell exhaustion [3,129]. Unlike extracellular lipids, intracellular lipids generally compensate for insufficient glucose metabolism in exhausted T cells, providing a new route of energy acquisition for exhausted T cells, which is beneficial for achieving T-cell effector functions and the continuation of biological activities [125,130]. An intracellular cholesterol deficiency inhibits proliferation, induces apoptosis, and drives the exhaustion of T cells [131].

The subcellular localization of lipids controls their differing or even completely opposite effects on cells, a phenomenon fully represented by the opposite effects of extracellular cholesterol and plasma membrane cholesterol on CD8 + T cells. After abundant extracellular cholesterol in the tumor microenvironment is internalized by TILs, the ER stress sensor X-box binding protein 1 is activated, inducing the expression of inhibitory receptors such as PD-1 and 2B4, after which the levels of various effector function- and proliferation-related cytokines decrease, promoting the occurrence of T-cell exhaustion. This process eventually leads to an attenuation of the function of CD8 + T cells in antitumor immunity [132]. In contrast, increases in the abundance of cholesterol in the cytoplasm and on the cell membrane have a positive effect on the proliferation of CD8 + T cells. Studies have shown that cholesterol is necessary for TCR aggregation and immune synapse formation. After TCR aggregation, the expression of granzyme B, IFN-γ and TNF-α is increased. Increases in the cytotoxicity and proliferation of CD8 + T cells ensue [132–134]. Subsequent findings reported by Bazioti and Chaves-Filho *et al.* verified the effect of intracellular cholesterol on promoting the proliferation of CD8 + T cells from both positive and negative aspects. Their report noted that deficiencies in Abca1 and Abcg1 (two transporters mediating intracellular lipid efflux) lead to the accumulation of cholesterol in the T-cell membrane and induce cell proliferation. A decrease in the membrane cholesterol level induces T-cell exhaustion [131,135]. Based on these observations, the cellular localization of lipids is the key factor influencing the effector function of CD8 + T cells. Recently, the team of Jessica E. Thaxton reported that metabolic stress in

the tumor microenvironment leads to an increase in the activity of acetyl-CoA carboxylase (ACC) in T cells. This activity causes T cells to preferentially store lipids rather than breaking them down, leading to the accumulation of lipid droplets within T cells. The metabolic pathway for energy production through fatty acid oxidation is suppressed, hindering the effector function of T cells. The use of ACC inhibitors can rescue the metabolism of T cells, increasing the preference of lipid processing for fatty acid oxidation over lipid storage, enhancing the antitumor effects of T cells and improving the mitochondrial structure in T cells [136].

To date, the regulation of lipid distribution and lipid metabolism combined with other traditional means of reversing exhaustion have been shown to reverse exhaustion. For example, in CAR-T-cell therapy, the antitumor function of CAR-T cells is significantly improved by increasing cholesterol levels in CAR-T cells and reducing cholesterol levels in the tumor microenvironment [130,131,137]. Compared with those treated with ICB alone, CD8 + T cells treated with a modality combining ICB with the correction of the abnormal lipid metabolic status showed a more complete reversal effect [130].

2.3.3. Reversal strategies centered on the clearance of accumulated ROS

ROS promote the transformation of innate immune cells into adaptive immune cells [138]. Low concentrations of ROS are beneficial for cell survival and activation, but high concentrations of ROS induce cell damage, resulting in a loss of the ability to synthesize anti-inflammatory cytokines in T cells (e.g., IL-10 and transforming growth factor β can result in a weak immune response and inhibited immune function) [115,139,140].

Abundant ROS accumulate in the tumor microenvironment and in exhausted T cells. This accumulation results mainly from dysfunction caused by mitochondrial hyperpolarization, an increase in basal metabolism in tumor cells, and an increase in the activity of enzymes related to ROS production [139,141]. In such an oxidizing tumor microenvironment with high ROS levels, tumor cells are only slightly affected by the overexpression of antioxidant system components, while the effector functions of immune cells, such as T cells and NK cells, are severely impaired, a phenomenon related to the oxidation of various proteins that regulate T-cell function [139]. Scharping *et al.* reported that ROS can drive T-cell exhaustion by acting as phosphatase inhibitors [118]. In addition, ROS can induce or amplify the exhaustion of T cells by altering the epigenetic landscape of T cells. For example, nitric oxide (NO) can influence histone methylation to promote exhaustion by inhibiting the catalytic activity of the histone demethylase lysine demethylase 3A (KDM3A) [142]. Abnormally elevated superoxide concentrations within mitochondria can influence exhaustion by decreasing DNA methylation levels [143].

Notably, targeting the anoxic characteristics of the tumor microenvironment, correcting its anoxic state, and reducing ROS accumulation induced by abnormal metabolic patterns associated with anoxia can inhibit the terminal exhaustion of CAR-T cells to a certain extent, maintain their responsiveness to ICB, and significantly increase the sustainability of CAR-T-cell therapy [104]. Alavi *et al.* reported that niacinamide, an oxidation inhibitor capable of clearing ROS, has positive effects on increasing proliferation and preventing the development of exhausted T cells [144]. SSubsequent studies have shown that the appropriate removal of excess hydrogen peroxide (H_2O_2) by exogenous catalase can promote T-cell activation and memory differentiation and reduce the development of exhaustion phenotypes [145]. In summary, targeted clearance of both intracellular and extracellular ROS facilitates the reversal of exhaustion and improves cellular effector functions.

2.4. Targeting epigenetic remodeling to reverse T-cell exhaustion

Genetic information is the central controller of cell proliferation and differentiation to support effector functions and plays a leading role in various biological activities, such as cell metabolism, transcription and translation. Although the nucleotide sequence does not change with the differentiation stage, unique epigenetic characteristics are acquired in each stage, and epigenetic changes occur throughout the cellular life cycle [146]. Indeed, epigenetic changes, mainly DNA modifications, histone modifications, alterations in chromatin accessibility, and long-range chromatin interactions, also occur in exhausted T cells. Epigenetic alterations determine exhaustion at the genetic level and profoundly and persistently affect the functional status of T cells [15] (Fig. 4).

2.4.1. DNA and histone modifications

During T-cell exhaustion, DNA and histones undergo modifications with multiple chemical groups, which affect chromatin accessibility at modified sites. When chemical group modifications occur on key transcriptional elements such as promoters and enhancers, the expression of the corresponding gene changes. Transcription factor genes with high accessibility are easily accessible to transcriptases, the expression of the corresponding transcription factors is relatively high, and their effects are obvious. However, genes with low accessibility are repressed via transcription factor inaccessibility, and transcriptases cannot function.

The most representative epigenetic modification of DNA is methylation. In general, DNA methylation has a negative effect on chromatin accessibility; thus, during exhaustion, it occurs mainly in the promoter or enhancer regions of proliferation-, effector function- and memory-related genes, which reduces the transcription of the gene by decreasing chromatin accessibility, and the cellular effector function and proliferation ability are subsequently attenuated [147]. Demethylation mainly occurs at the promoters and enhancers of immunosuppressive genes, driving exhaustion via another mechanism. For example, PD-1 promoter demethylation occurs in exhausted T cells, which upregulates the expression of the inhibitory receptor PD-1 and subsequently increases the probability of PD-1 binding to PD-L1, which increases the transmembrane transduction of inhibitory signals, eventually leading to the inhibition of cellular effector functions [148]. Common types of histone modifications include histone methylation and histone acetylation. Interestingly, the effects of histone methylation on gene expression can be either inhibitory, such as suppressor of variegation 3–9 homolog 1 (SUV39H1)-mediated methylation of histone H3 lysine 9 (H3K9me3), or activating, such as H3K4 methylation [147]. However, the effect of histone acetylation on gene expression is often stimulatory. In addition, both nonhistone proteins and histones undergo phosphorylation and ubiquitination. Regardless of the form of modification, the core mechanism remains the same, i.e., regulating the transcription of genes by altering chromatin conformation and accessibility.

2.4.2. Chromatin accessibility

Chromatin accessibility is the core controller of gene expression levels. The above principles of DNA modification and histone modification involve the regulation of gene transcription by changing chromatin accessibility and ultimately regulating cellular effector functions [147,149]. Ge *et al.* noted that the effects of cellular epigenetic changes and changes in cellular metabolism during cancer progression are reciprocal [149]. OOn the one hand, epigenetic changes in exhausted T cells determine the functional characteristics of the cells, and the related mechanism of action involves the control of transcription factor synthesis, the regulation of cell surface marker expression, and the regulation of metabolic processes. On the other hand, transcription factors and metabolic conditions drive the epigenetic landscape in cells [122]. Sun *et al.* noted that some cellular metabolites can be used as substrates or cofactors of chromatin-modifying enzymes and subsequently drive epigenetic changes [150], consistent with the findings of Dan *et al.*, who proposed that phosphatase of activated cells 1 recruits the Mi-2 β nucleosome-remodeling and histone deacetylase complex, eventually leading to chromatin remodeling in effector T cells [151].

Notably, the epigenetic hierarchy is more stable than the other hierarchies [74,152]. After the cessation of antigen stimulation, the

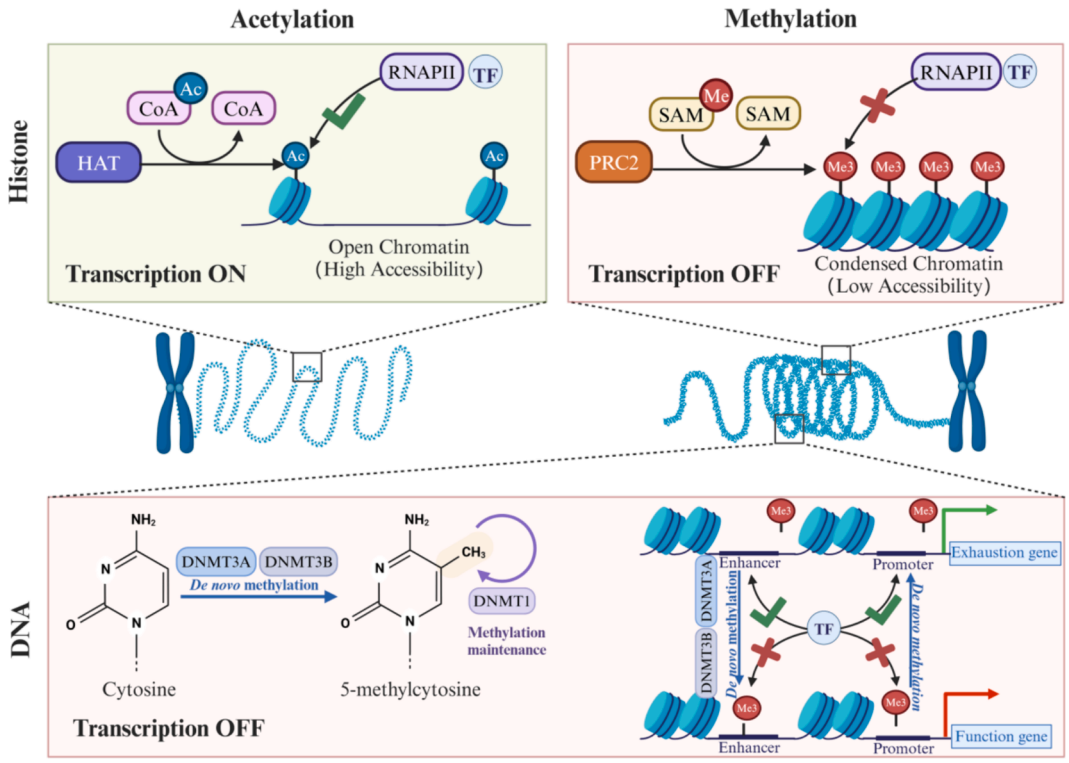

Fig. 4. Epigenetic remodeling reverses T-cell exhaustion. Changes in the epigenetic landscape during T-cell exhaustion are driven predominantly by histone and DNA modifications. 1) Histone modification: The spatial charge distribution of histone proteins in the regulatory regions of exhaustion-related genes is changed through acetylation such that the originally compacted chromatin is loosened and its accessibility is increased, which is conducive to the binding of transcription factors and transcriptases to initiate the exhaustion program. The methylation of histones in the regulatory regions of effector function-related genes increases the compaction and decreases the accessibility of chromatin, and transcription factors and transcriptases cannot readily bind to these regions, impairing the effector functions of these cells via transcriptional repression. 2) DNA modification: A three-dimensional conformational change is caused by DNA methylation of promoters and enhancers and the binding of transcription factors, and the original conformation can be restored after demethylation.

reversal of epigenetic changes is extremely limited, and the progeny cells still retain an exhaustion-associated epigenetic signature. Even after ICB, only approximately 10 % of the epigenetic changes are reversed, and the expression of only a few of the genes related to effector functions, memory, and proliferation is restored. The vast majority of exhaustion-associated features remain stable and play a role in triggering the exhaustion of T cells [74]. This feature partially explains the inability to maintain the effects of ICB in the long term. Thus, reversal of the epigenetic hierarchy is important for achieving complete and durable reversal of T-cell exhaustion. However, reversal of the epigenetic hierarchy is different from other reversal modes, and it is generally not a single-pronged approach to reverse exhaustion but rather is meant to enhance the reversal effect of other modes through mutual cooperation with these modes. Existing research reports and clinical data indicate that the reversal of ICB, the reversal of transcription factor blockade, and the reversal of unfavorable metabolic states show more durable and potent effects in combination with the reversal of epigenetic changes [153].

3. Multipronged strategies for the reversal of T-cell exhaustion

Single-pronged strategies for the reversal of T-cell exhaustion are very limited and cannot achieve an efficient and durable reversal effect [72,154,155]. As the understanding of T-cell exhaustion has increased, the discovery of multipronged synergistic reversal strategies has made it possible to achieve more durable and effective reversal of exhaustion [154,156,157].

3.1. Combined reversal strategies centered on ICB

ICB is a relatively mature exhaustion reversal strategy that has been used in clinical practice and has achieved good results, but it still has some limitations [72,158]. The core problem is that the reversal effect is not durable and is prone to tolerance development over time [158,159]. Considering the above factors leading to the limitations of ICB, researchers have proposed multiple combination reversal strategies incorporating ICB. For example, various combination strategies, including ICB combined with epigenetic reversal, ICB combined with an improvement of the tumor microenvironment, ICB combined with transcription factor regulation, and ICB combined with metabolic regulation, all showed better efficacy than ICB alone [160–163] (Fig. 5).

3.1.1. ICB can be combined with epigenetic remodeling to achieve more effective reversal

The epigenetic landscape of exhausted cells is not completely improved after ICB treatment, the cell exhaustion program remains active, and T cells treated with ICB gradually become exhausted again [74,152]. In addition, epigenetic information is heritable; thus, after ICB treatment, the progeny cells of exhausted T cells still carry exhaustion-related genetic information, which can easily induce exhaustion in progeny cells [16,164]. Therefore, in the long term, the combination of epigenetic remodeling with ICB is highly important.

In 2013, a study by Wrangle *et al.* showed that among patients first treated with epigenetic regulatory drugs (DNA methyltransferase inhibitors (DNMTis) and histone deacetylase inhibitors (HDACis)) and then treated with an anti-PD-1 antibody, more than 80 % did not experience further tumor progression within the following 6 months, initially indicating the effectiveness of combining ICB with epigenetic

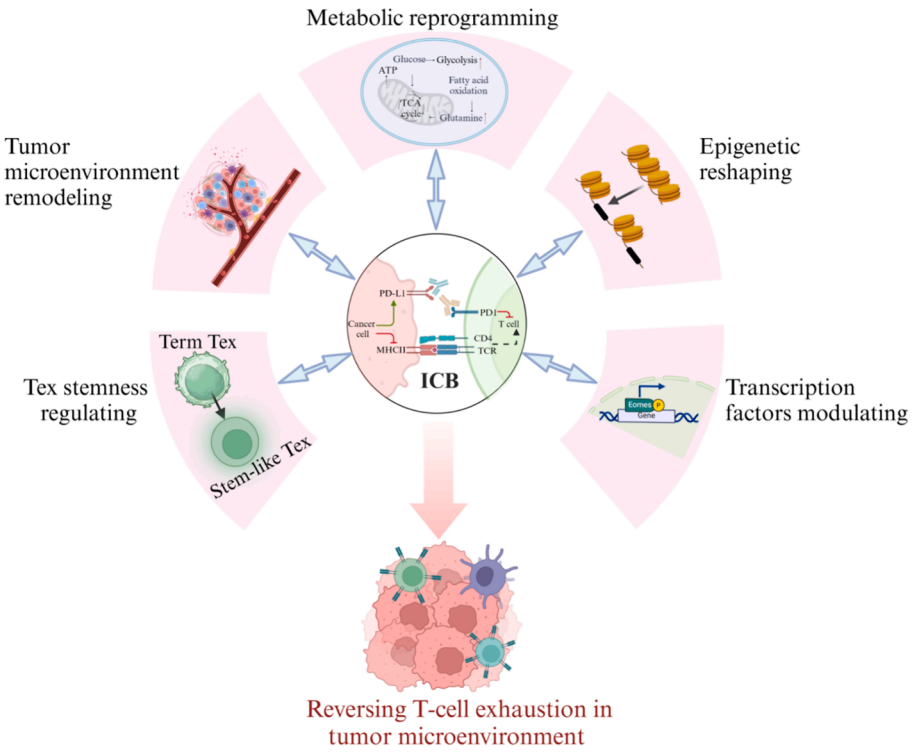

Fig. 5. Combination strategies for reversing T-cell exhaustion centered on ICB. ICB can be used alone or as a part of a multipronged strategy in combination with epigenetic remodeling, improvement of the metabolic status, alteration of the tumor microenvironment, regulation of exhausted T-cell stemness, and increasing the ICB response to achieve more effective reversal.

remodeling [165]. Subsequent mechanistic studies revealed that HDAC6i treatment enhanced the antitumor effect of ICB by down-regulating the inhibitory receptor PD-L1, reducing the infiltration of Treg cells and regulating the level of effector molecules in the tumor microenvironment [166,167]. As research has continuously progressed, an increasing number of epigenetic modulators are being applied in combination with ICB. In Hodgkin's lymphoma patients, the objective response rate to the combination of PD-1 blockade and decitabine increased by 16 %, and the PFS time of patients receiving the combination treatment was significantly longer than that of patients in the control group treated with PD-1 blockade alone, again revealing the effectiveness of ICB therapy combined with epigenetic remodeling [168]. More encouragingly, in a tumor-bearing mouse model, ICB in combination with epigenetic regulatory drugs had unexpected therapeutic effects on some tumor types without conventional PD-1 or CTLA-4 blockade, and more than 80 % of the tumor-bearing mice were cured [169]. This result suggests that by combining treatment with epigenetic regulation, we can not only enhance the therapeutic effect of the original therapeutic strategy but also have the opportunity to cure some once incurable tumor types, indicating the infinite possibilities of synergistic ICB and epigenetic therapy.

3.1.2. ICB can be combined with improvement of the metabolic status to eliminate internal proexhaustion factors

ICB fails to change the adverse metabolic pattern in exhausted T cells, and the disruption of material and energy metabolism in these cells is not significantly reversed, thus continuing to exert proexhaustion effects. Therefore, the coordinated restoration of cellular metabolic function after ICB treatment is anticipated to enhance the ability of ICB to reverse exhaustion [170].

First, reversed T cells remain in a proexhausted state with abnormal mitochondrial function and an insufficient energy supply. Zhai *et al.* successfully reconstructed the local cryoablation function of exhausted T cells by attenuating mitochondrial depolarization in T cells to address this limitation [163]. Chowdhury *et al.* reported that peroxisome proliferator-activated receptors could activate mitochondria in CTLs, increase OXPHOS and glycolytic activity, promote the proliferation of naive T cells, and synergistically enhance the effect of ICB treatment by increasing the number of functional CTLs [171]. Second, accumulated lactate in the tumor environment suppresses effector T-cell functions not only directly but also indirectly by supporting a subset of immunosuppressive cells. Thus, limiting glycolysis to reduce lactate production could also help to restore T-cell effector functions and enhance the therapeutic effect of ICB [172].

In addition to the several known metabolic regulatory mechanisms described above, many relatively unknown metabolic regulatory pathways have been found to enhance ICB efficacy; statins are representative effectors of these mechanisms, and statin use has been found to be associated with increased survival rates for individuals with a variety of cancers (e.g., head and neck cancer and non-small cell lung cancer) [173,174]. An analysis of 253 patients treated with PD-1 inhibitors by Cantini *et al.* revealed that statin use was strongly associated with longer PFS and OS for patients with malignant pleural mesothelioma and with longer PFS for patients with non-small cell lung carcinoma [175]. Many other similar metabolic regulators (such as peroxide regulators and cholesterol regulators) have been identified. An increasing number of analyses have shown that these metabolic regulators are clearly correlated with the efficacy of ICB, but the specific mechanism underlying their synergy with ICB needs further investigation [173,174,176,177].

3.1.3. ICB can be combined with improvement of the extracellular environment to eliminate external proexhaustion factors

The tumor microenvironment is complex and includes various cellular components (Treg cells, B cells, dendritic cells, NK cells, etc.), as well as various noncellular factors, such as the oxygen content, pH, cellular metabolites, and other secreted cellular products (antibodies, cytokines, etc.). These factors jointly participate in the growth, metabolism and functional status of immune cells and tumor cells [178].

Tumor cells generate a unique extracellular environment that is suitable for their own survival and drives the exhaustion and death of antitumor immune cells [179,180]. Therefore, when the T-cell exhaustion that occurs in the tumor environment is reversed through ICB treatment, synergistic regulation of the tumor microenvironment is indispensable for overcoming drug resistance and achieving a more effective and durable reversal effect [181–183].

Currently, synergistic effects with ICB therapy have been achieved for treatments targeting several aspects of the tumor microenvironment [161]. At the cellular level, Loeuillard *et al.* targeted tumor-associated macrophages (TAMs) and granulocytic myeloid-derived suppressor cells (G-MDSCs), enhancing the effects of ICB through the dual inhibition of these cells [184]. In addition, the application of fibroblast growth factor receptor (FGFR) inhibitors to attenuate the immunosuppressive tumor microenvironment also showed synergistic therapeutic effects with ICB, as FGF/FGFR signaling regulates immune cells, angiogenesis, and the epithelial–mesenchymal transition in the tumor microenvironment [185]. At the metabolic level, statins significantly enhanced the efficacy of ICB by regulating lipid metabolism in the tumor microenvironment and shaping inflammatory immune phenotypes, and these findings were further supported by subsequent data [186]. At the cytokine level, IFN has been shown to act directly on tumor cells and immune cells to inhibit tumor growth and to have a synergistic effect with ICB treatment [162,187]. Zhu *et al.* reported that IFN induced the production of chemokines to recruit cytotoxic T cells and that compared with anti-PD-1 antibody treatment alone, IFN treatment combined with PD-1 blockade significantly increased T-cell infiltration [188]. Generally, the tumor microenvironment results in the upregulation of PD-1 expression in T cells through the IFN/IFNAR1/JAK 1/STAT 3 signaling pathway in response to the recruitment of immune cells. However, after synergistic PD-1 blockade, this coping strategy of tumor cells fails, thus resulting in significant synergistic effects. Therefore, the core mechanism underlying the synergistic effects of combined ICB and IFN treatment is believed to be mediated through ICB as an auxiliary strategy to synergistically enhance the IFN-induced recruitment of chemokines to cytotoxic T cells rather than through ICB as the primary strategy, with IFN exerting a synergistic effect with ICB. Furthermore, hypoxia in the tumor microenvironment was found to be associated with tolerance to PD-1 blockade in head and neck squamous cell carcinoma (HNSCC), and the amelioration of hypoxia is often accompanied by increased effectiveness of anti-PD-1 therapy [189]. The mechanism of action linking hypoxia to ICB resistance was reported in a study of breast cancer, which showed that hypoxia induced the production of a series of cytokines (including HIF-1α) to cause chromatin remodeling in CD8 + T cells and effector changes in the epigenetic hierarchy, eventually leading to poor immune cell effector function and resistance to ICB therapy [190]. Subsequently, several studies have documented that simultaneous targeting of PD-L1 and microenvironmental hypoxia can reactivate the tumor-killing effect of T cells to some extent and enhance the efficacy of ICB [191–193]. The accumulation of acidic metabolic waste products is another major feature of the tumor microenvironment. The metabolic reprogramming induced by this acidic environment is closely related to the malignant progression of tumor cells and stemness maintenance. Previously, this external environment-induced acidosis was considered completely detrimental to tumor elimination, but Cheng *et al.* recently reported that this stemness maintenance is equally effective in T cells [194,195]. Therefore, approaches for enhancing the efficacy of tumor immunotherapy by regulating the pH of the tumor microenvironment remain to be further studied.

3.1.4. ICB can be combined with cell stemness regulation to increase the number of ICB-responsive cells

The poor efficacy of ICB is also associated with an insufficient number of responsive T cells [196]. The reversal of T-cell exhaustion is the basis of the restoration of effector functions in T cells. Studies have shown that only progenitor exhausted T cells (stem cell-like exhausted T cells) respond to ICIs, while terminally exhausted T cells with a more extensive exhaustion phenotype are not responsive to ICIs [197–199].

Sun *et al.* induced a stem cell-like exhausted T-cell-transcriptional program while inhibiting the transcription of terminally exhausted T-cell-related genes, skewing the conversion of exhausted CD8 + T cells into progenitor exhausted T cells, and successfully enhanced the reversal effect of ICB [80,108]. In addition to converting terminally exhausted T cells into a progenitor exhausted state, Li *et al.* reported that decitabine directly increased progenitor T-cell proliferation, decreased T-cell proliferation, and increased ICB efficacy by increasing the number of exhausted T cells that can respond to ICB [200]. Regardless of whether terminally exhausted T cells are converted into progenitor exhausted T cells through cytokine-mediated regulation or through a direct enhancement of progenitor exhausted T-cell proliferation, the core mechanism through which ICB efficacy is increased is an increase in the number of progenitor exhausted T cells that can respond to ICB treatment. Therefore, more attention should be given to the composition and cell count of the exhausted T-cell subset during ICB treatment to ensure that a sufficient number of responsive cells is present to facilitate a stronger ICB reversal effect.

3.1.5. Combinations of ICB for different targets

In addition to combinations of ICB with other exhaustion reversal strategies, targeting several different immune checkpoints via the ICB reversal approach showed a far greater reversal effect than the blockade of a single immune checkpoint. A case study of basal cell carcinoma showed a subsequent increase in the expression level of LAG-3, another surface inhibitory receptor on CD8 + T cells, in patients receiving anti-PD-1 treatment. This increase may be related to the compensation of the immune system for the downregulation of an inhibitory signaling pathway, providing a rationale for the prominent synergistic effect of combined blockade of PD-1 and LAG-3 [201]. In addition, due to its unique mechanism of action, LAG-3 has great potential for targeting in ICB combination therapies, providing a theoretical foundation for the subsequent combination of anti-PD-1 and anti-LAG-3 antibodies in clinical patients with various tumor types [202–204]. Interestingly, Meyiah *et al.* analyzed different ICI combinations and found that simultaneously targeting both PD-1 and TIGIT or PD-1 alone had a more significant synergistic effect with treatments targeting TIM-3 than other combinations. The above findings were tested in subsequent studies, where tumor-infiltrating CD8 + T cells from patients receiving these two ICB combinations were associated with longer disease-free survival (DFS) times than those from patients receiving other ICB combinations [205]. The mechanism underlying the synergism between PD-1 and TIGIT was revealed by Lee *et al.*, who reported that PD-1 inhibition mainly enhanced the short-term effector function of T cells, while TIGIT inhibition was responsible for the long-term maintenance of low differentiation and exhaustion of T cells; these effects were complementary at the temporal level, which explained the noticeable effect of combination treatments targeting both PD-1 and TIGIT [206]. However, the mechanism underlying the strong synergy between PD-1 and TIM-3 is unclear and needs further study.

With the advancement of nanotechnology, nanoparticle (NP)-based delivery systems have also been used in combination with inhibitory molecules to reverse T-cell exhaustion [207]. By modifying antibodies and other ligands on the surface of NPs, the specific and efficient uptake of NPs can be induced. For instance, PD-1 siRNA is transported into T lymphocytes by lipid-coated calcium phosphate, enhancing the cellular uptake of the siRNA and reducing the expression of PD-1 [208].

In addition to targeting only immune checkpoints expressed on CD8 + T cells, simultaneous targeting of immune checkpoints on CD8 + T cells and surface ligands on the corresponding tumor cells has also shown excellent synergistic effects. For example, simultaneous blockade of the PD-1 and PD-L1 immune checkpoints showed superior efficacy to single blockade of PD-1 or PD-L1 in the treatment of serous ovarian cancer, an effect related to the ability of this combination to uniquely

induce the activation of naive CD8 + T cells into a more active, cytotoxic, and proliferative progenitor exhaustion phenotype [209].

In conclusion, different immune checkpoints activate different inhibitory signaling pathways, and the duration and mechanism of inhibition of each signaling pathway are also different. Therefore, targeting multiple immune checkpoints simultaneously allows a more sustained and comprehensive blockade of inhibitory signals, resulting in more durable and effective reversal of exhaustion.

3.2. Combinations of other reversal strategies

Most of the combination strategies for the reversal of CD8 + T-cell exhaustion described above have ICB as the core. However, combinations of other strategies, such as transcription factor regulation, metabolic reprogramming, attenuation of the immunosuppressive microenvironment, epigenetic reprogramming, and adoptive immunotherapy, have also shown stronger reversal effects than a single strategy.

The regulation of cytokines occurs throughout multiple stages of cell proliferation, differentiation and effector function execution, and various types of cytokines and diverse functions are involved. Cytokines interact to form a complex and precise regulatory network. Therefore, synergy between cytokines and combinations of cytokines and other exhaustion reversal strategies are feasible approaches for achieving the more durable and effective reversal of T-cell exhaustion. Guo *et al.* reported that stimulation with the anti-inflammatory cytokine IL-10 synergized with adoptive immunotherapy to achieve a durable cure and even eradication of tumors in a solid tumor mouse model due to the metabolic reprogramming of IL-10-induced exhaustion of T cells via pyruvate carrier-dependent OXPHOS in mitochondria [210]. SSimilarly, IL-15 stimulation during the in vitro expansion of CAR-T cells allows progeny cells to maintain a stem cell-like phenotype, with a higher mitochondrial oxygen consumption rate (OCR) and spare respiratory capacity (SRC) than those of general CAR-T cells, resulting in attenuated exhaustion characteristics, greater proliferative capacity, and greater activity against tumor cells [211]. The combination of cytokine modulation with CAR-T-cell therapy is thus an effective strategy for increasing CAR-T-cell efficacy. Moreover, intercytokine synergy also showed efficacy in terms of reversing exhaustion. For example, dual knockdown of PRDM1 and NR4A3 skewed CAR-T cells toward a TCF-1 stem cell-like phenotype, achieved regulatory effects exceeding those of PRDM1 and NR4A3 alone, and greatly increased the anti-exhaustion ability and antitumor activity of CAR-T cells [107].

As a factor that induces exhaustion, the tumor microenvironment often plays a negative role during and after the reversal of T-cell exhaustion, attenuating the reversal of exhaustion and even reinducing exhaustion. For example, the poor efficacy of CAR-T-cell therapy is partially due to the persistent effects of the immunosuppressive microenvironment. Lactate accumulation caused by uncontrolled glycolysis in tumor cells has an inhibitory effect on CAR-T-cell function. In response to this limitation, Sun *et al.* used the lactate generation inhibitor oxamate to not only reprogram glucose metabolism in some tumor cells but also alleviate the immunosuppressive effect of lactate accumulation in the tumor microenvironment, which ultimately significantly increased the therapeutic effect of CAR-T cells [212].

As genetic information is the central controller of cellular biological activities, the combination of other exhaustion reversal strategies with epigenetic reversal undoubtedly leads to a greater reversal of exhaustion. Akbari *et al.* summarized strategies for synergistic epigenetic and CAR-T-cell therapy from multiple perspectives, including promoting the CAR-T-cell stem cell phenotype through epigenetic reprogramming, increasing the infiltration of CAR-T cells, reducing inhibitory receptor expression, and increasing the stability of metabolic patterns in CAR-T cells [213]. Although some ideas remain only theoretical and no specific applications have been explored, these ideas still provide insights for the more durable and effective reversal of exhaustion.

4. Perspectives

T-cell exhaustion promotes tumor immune escape; thus, reversing this exhaustion and restoring the antitumor activity of exhausted T cells are crucial. As the mainstream means of reversing exhaustion, ICB can increase the objective remission rate in some cancers; however, most patients still do not achieve complete remission, and ICB is ineffective in some patients. The main problem is that the reversal effect of ICB alone is insufficient. Therefore, under sustained antigen stimulation, the reversal effect of ICB is not durable, and after exhaustion reversal, T cells soon become exhausted again. With the development of various new technologies, the unique transcriptional landscape, metabolic state, and epigenetic landscape of exhausted T cells, as well as the tumor microenvironment in which they are located, have been clearly delineated, proving that exhaustion results from the combined regulation of multiple factors. Various factors are related to each other; for example, metabolic signals and immune signals are closely related, while epigenetic modifications often participate in regulating the exhausted state through the regulation of transcription factors and the expression of metabolism-related genes. Therefore, the identification of multipronged combination strategies for reversing exhaustion is an important direction for future clinical research.

Moreover, the exhaustion-reversing effect shows clear interindividual variability in clinical practice. The effect of exhaustion reversal varies across tumor types treated with the same reversal method and even across patients with the same tumor type. In addition to studying the developmental stage and degree of complementation of efficient combined reversal strategies, we need to further study the characteristics of different tumor types, identify the factors underlying each specific mechanism of exhaustion reversal, and explore their applicability for different tumor development stages and different degrees of T-cell exhaustion to maximize the efficacy of immunotherapy in tumor patients. Moreover, further research is needed to better understand the specific state of T-cell exhaustion in different individuals, develop personalized reversal strategies, and thus support individualized treatment.

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CRediT authorship contribution statement

Yang Hu: Writing – original draft. **Yaqi Zhang:** Investigation. **Fenfen Shi:** Supervision. **Ruihan Yang:** Investigation. **Jiayu Yan:** Investigation. **Tao Han:** Writing – review & editing, Funding acquisition. **Liping Guan:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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