T Cell Exhaustion and Immunotherapy

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Introduction

Cancer is one of the leading causes of death worldwide, affecting millions of patients and their families every year. The development of immunotherapies, such as chimeric antigen receptor T-cell (CAR-T cell) therapy, revolutionized cancer medicine. By programming the patient's own immune cells to target and destroy tumor cells, this personalized approach overcomes many issues associated with classical chemotherapeutics. Despite the promise of immunotherapies, their efficacy is limited by T cell exhaustion, a phenomenon where T cells lose their ability to fight tumors or viruses after prolonged exposure. This phenomenon is not easily reversible, which has led to many research efforts attempting to understand how to program T cells to become active fighters again. A thorough understanding of T cell exhaustion, therefore, is critical to developing novel strategies to overcome this issue and restore therapeutic efficacy.

In this article collection, we highlight publications that focus on T cell exhaustion in cancer. First, Yin et al. (2023) review T cell exhaustion in the context of CAR-T cell therapy. They explain how T cell exhaustion develops and analyze the factors that lead to the phenomenon. Additionally, they review strategies to regulate T cell exhaustion and improve CAR-T cell therapy. Next, Roe (2022) reviews the different types of lymphocyte exhaustion – namely, NK cell exhaustion, B cell exhaustion, and T cell exhaustion. The features, characteristics, and phenotypes of each type of lymphocyte exhaustion are described in detail, and their similarities and differences are highlighted.

Following these articles, Li et al. (2022) analyzes how T cell metabolism can regulate T cell exhaustion. They review metabolic factors that affect T cell exhaustion, such as antigen stimulation signal strength, cytokines, and epigenetics. Additionally, they discuss metabolic and epigenetic strategies to reverse T cell exhaustion. Next, Peng et al. (2023) studied the mechanisms behind T cell exhaustion in the pathogenesis of esophageal adenocarcinoma (EAC). They analyzed transcriptomic data and single-cell sequencing data from EAC patients to understand the regulation and impact of T cell exhaustion on the immune environment in EAC. T cell exhaustion risk scores were found to be significantly associated with EAC survival prognosis, in addition to weak responsiveness to immunotherapy. Together, these findings underscore the importance of T cell exhaustion as a potential biomarker in tumor microenvironment-related immune regulation of EAC.

Finally, the article collection is rounded out by an article from BD Biosciences (2023) that focuses on strategies and key considerations for the characterization and deep immunophenotyping of T cells. Differences between standalone panels and pre-optimized backbone panels are explained, followed by a discussion of important criteria for building backbone panels. The article also discusses the features and advantages of the BD Horizon™ Human T Cell Backbone Panel for T cell research.

Through the articles presented in this curation, we hope to provide researchers with a strong understanding of T cell exhaustion and its implications in cancer research. To gain a better understanding of tools available to study T cells, we encourage you to visit the BD Biosciences BD Horizon™ Human T Cell Backbone Panel page and explore the possibilities presented there for your research.

Emily E. Frieben, Ph.D.
Associate Editor, *Wiley Interdisciplinary Reviews*

References

Yin, X., He, L., Guo, Z. T-cell exhaustion in CAR-T-cell therapy and strategies to overcome it. *Immunology*. 2023; 169(4): 400–411. https://doi.org/10.1111/imm.13642

Roe, K. NK-cell exhaustion, B-cell exhaustion and T-cell exhaustion—the differences and similarities. *Immunology*. 2022; 166(2): 155–168. https://doi.org/10.1111/imm.13464

Li, F, Liu, H, Zhang, D, Ma, Y, Zhu, B. Metabolic plasticity and regulation of T cell exhaustion. *Immunology*. 2022; 167(4): 482–494. https://doi.org/10.1111/imm.13575

Peng, S., Han, X., Geng, W., Zhao, L. T-cell exhaustion: A potential target biomarker of the tumour microenvironment affecting oesophageal adenocarcinoma. *J Gene Med.* 2023; 25(7):e3496. https://doi.org/10.1002/jgm.3496

Strategies and considerations for the characterization and deeper immunophenotyping of T cells. (2023), [Blog Post], *BD Biosciences.* https://www.bdbiosciences.com/en-us/learn/science-thought-leadership/blogs/strategies-immunophenotyping-t-cells

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REVIEW

immunology
Insights into immunity in health and disease

T-cell exhaustion in CAR-T-cell therapy and strategies to overcome it

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Abstract

Tumour immunotherapy has achieved good therapeutic effects in clinical practice and has received increased attention. Cytotoxic T cells undoubtedly play an important role in tumour immunotherapy. As a revolutionary tumour immunotherapy approach, chimeric antigen receptor T-cell (CAR-T-cell) therapy has made breakthroughs in the treatment of haematological cancers. However, T cells are easily exhausted in vivo, especially after they enter solid tumours. The exhaustion of T cells can lead to poor results of CAR-T-cell therapy in the treatment of solid tumours. Here, we review the reasons for T-cell exhaustion and how T-cell exhaustion develops. We also review and discuss ways to improve CAR-T-cell therapy effects by regulating T-cell exhaustion.

KEYWORDS
CAR-T-cell therapy, immunotherapy, T-cell exhaustion

INTRODUCTION

Immunotherapy has achieved good therapeutic effects in tumour treatment, and more attention has been given to this method. As one of the most important immunotherapy methods, chimeric antigen receptor T-cell (CAR-T-cell) therapy has made breakthroughs in treating cancer. Normal T cells cannot directly recognize antigens in vivo. Their activation requires contact between T cells and antigen-presenting cells (APCs) followed by the interaction between the antigenic major histocompatibility complex (MHC)-peptide complex and T-cell receptor (TCR). Additionally, due to the low mutational burden of tumour cells, normal tumour-infiltrating T cells are inhibited by immune mechanisms that prevent the autoimmune attack of normal tissues [1]. Compared with normal T cells, CAR-T cells express genetically modified antigen-specific and non-MHC-restricted receptors, which contain a single-chain variable fragment (scFv), a transmembrane domain and an intracellular signalling domain [2]. The modified receptors endow CAR-T cells with the ability to directly recognize antigens and thus improve their therapeutic effect. Extensive studies have shown that although CAR-T-cell therapy has good effects in the treatment of haematological cancer, it is not as successful in treating solid tumours. One possible reason for this lack of efficacy is that the T cells infiltrating solid tumours are easily exhausted [3].

In acute infections, naïve T cells are activated and rapidly differentiate into effector T cells. This process of differentiation involves intense transcriptional and metabolic reprogramming, proliferation, and epigenetic changes. Upon activation, T cells seek to destroy the source of the cognate antigen, such as infected cells or tumour cells, by releasing cytokines and directly killing the target cells [4]. After the expansion of effector T cells and the removal of antigens, most T cells die, and a small fraction of T cells become memory T cells and remain for a long time. These memory T cells downregulate the activation signal and can differentiate into effector T cells

again after corresponding stimulation. Memory T cells rely on interleukin-7 (IL-7) and interleukin-15 (IL-15) for self-renewal in the absence of antigens. However, in chronic infection or cancer, T cells will differentiate according to a different path, leading to T-cell exhaustion [5]. The exhaustion of T cells involves hierarchical disappearance of the cell function, upregulation of a variety of coinhibitory receptors, changes in key transcription factors, metabolic changes, and loss of the ability to enter a quiescent state to form memory T cells [5–7].

T-cell exhaustion and its negative effects on therapy are major concerns for CAR-T-cell therapy in solid tumours. In this review, we focus on three aspects of the exhaustion of T cells: (i) the development of T-cell exhaustion; (ii) reasons for T-cell exhaustion; and (iii) ways to prolong the effective time of CAR-T-cell therapy by regulating the exhaustion of T cells in the treatment of solid tumours.

DEVELOPMENT OF T-CELL EXHAUSTION

T cells play a role in protecting the body and are responsible for clearing the antigen they recognize. However, in some special cases, T cells in the human body cannot perform their due duties due to T-cell anergy (a state of low reactivity due to the lack of a sufficient costimulatory or inflammatory response to acquire the effector function) [8] or T-cell exhaustion. The exhaustion phenomenon was first observed in CD8+ T cells in mice with chronic lymphocytic choriomeningitis virus (LCMV) infection [9]. Subsequently, it was also observed to occur in cancer and other models of infection [10]. In the initial stage of exhaustion, CD8+ T cells lose their interleukin-2 (IL-2)-producing functionality, high proliferation ability, and killing ability. In the mid-term stage, they lose other abilities, such as the ability to produce tumour necrosis factor. At this stage, some virus-specific cells may completely lose the ability to produce interferon-γ (INF-γ) or beta chemokines or undergo degranulation in some severe cases of exhaustion. In the final stage, antigen-specific T cells disappear, and CD8+ T cells become completely exhausted and dysfunctional [9, 11]. In general, the development of T-cell exhaustion is progressive and evolves gradually over a period of time. It takes weeks to observe a significant difference in the gene expression profiles between exhausted T cells and effector T cells or memory T cells [6]. In the early stage, the exhaustion state can be reversed. T cells isolated from mice in the first week of chronic LCMV infection could form memory T cells after they were transferred into noninfected mice; however, this ability disappeared if they were isolated from mice between weeks 2 and 4 of infection [9].

KEY REASONS FOR T-CELL EXHAUSTION

T-cell exhaustion may result from consistent exposure of T cells to antigens, sustained expression of inhibitory receptors, the microenvironment surrounding T cells, immunosuppressive cells, and alterations in the expression of transcription factors (Figure 1).

Consistent exposure of T cells to antigens

There is a close relationship between T-cell exhaustion and long-term exposure to antigens. Long-term exposure rather than transient or intermittent exposure to antigens is one of the most important reasons for T-cell exhaustion [12]. Early studies have found that the severity of T-cell exhaustion in the LCMV model is related to long-term antigen stimulation [9]. In human and mouse models, the degree of T-cell exhaustion is positively correlated with the duration of antigen stimulation [13, 14]. In a study on CD8+ T-cell exhaustion, after incubation of the T cells with antigen for a short period of time, some of the exhausted T cells could be functionally restored, but long-term incubation led to an inability to restore the functions of the exhausted T cells [13]. Consistent antigen exposure can lead to sustained expression of the programmed death-1 (PD-1) receptor in T cells, which is a hallmark of exhaustion [14]. Genes associated with TCR signalling (such as Batf, Egr2, Ezh2, Irf4, Nfatc1, Nfatc2, Nr4a1, Nr4a2, and Nr4a3) are significantly enriched in both tumour and chronic virus-specific CD8+ T cells [15–18]. This phenomenon is more pronounced when comparing the transcriptional profile between exhausted CD8+ T cells extracted from HIV-infected and melanoma patients [19] and can be replicated in vitro with repeated sustained antigen stimulation [20].

Sustained expression of inhibitory receptors

Exhausted T-cell cells have the significant feature of overexpression of multiple inhibitory receptors, such as PD-1, lymphocyte activation gene 3 protein (LAG3), 2B4 (CD244), CD160, T-cell immunoglobulin mucin-3 (TIM3), and CTLA4 [21]. These inhibitory receptors control autoreactivity and play an important role in the control of autoimmune diseases. Inhibitory receptors usually induce T-cell exhaustion through the following four different ways: inhibiting the intracellular signal of activating receptors, upregulating the genes related to T-cell exhaustion, influencing the metabolism of T cells, and preventing the

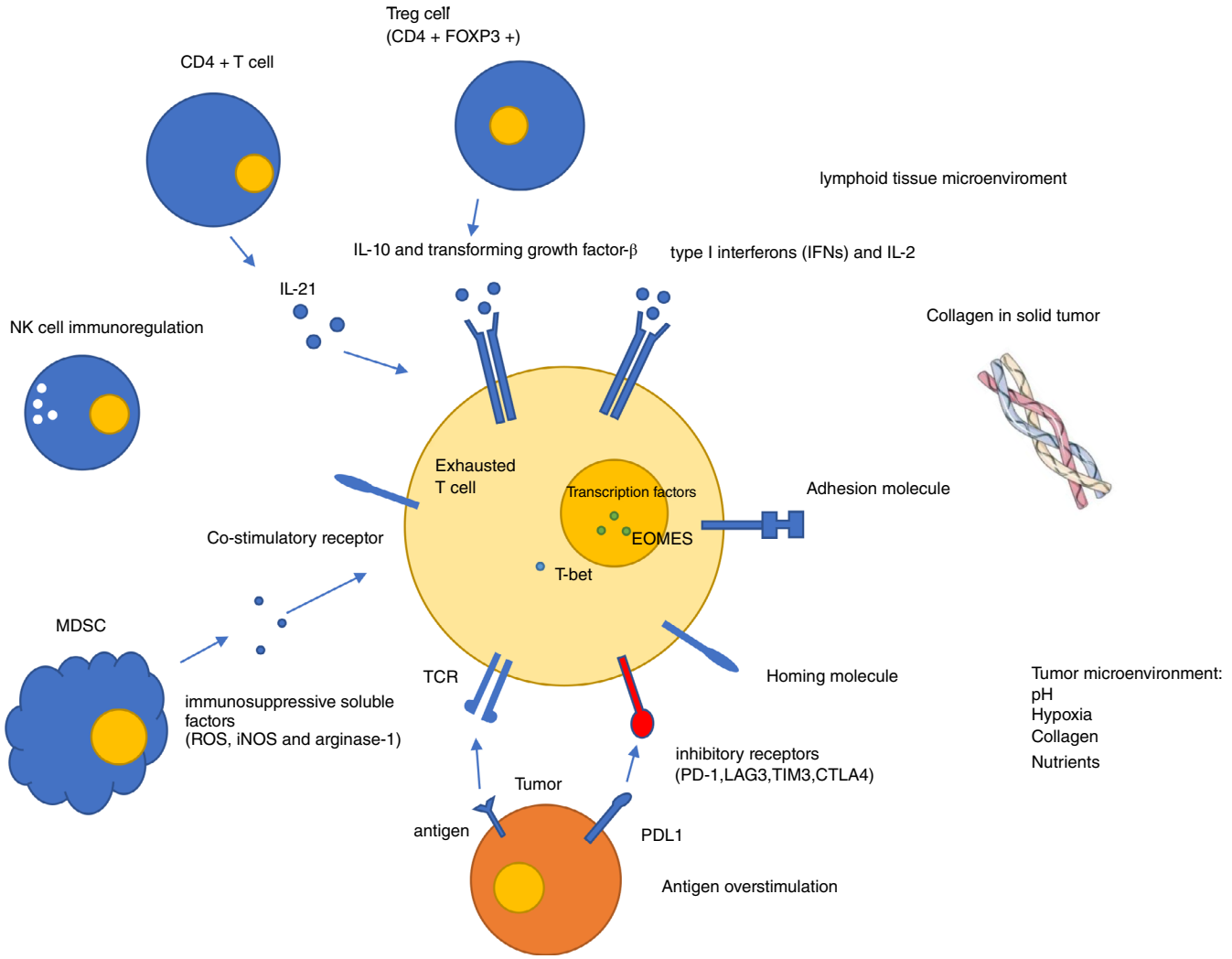


FIGURE 1 Factors that can cause T-cell exhaustion. There are five main factors that can cause T-cell exhaustion: (1) Consistent exposure of T cells to antigens; (2) inhibition of the expression of immune checkpoints and their ligands; (3) inhibition of T-cell function by IL-10, TGF-β and other inhibitory cytokines, production of metabolites (including adenosine, prostaglandins, and lactic acid), and physiological changes such as hypoxia, low pH and deprivation of nutrients (such as glucose or amino acids) in the microenvironment surrounding T cells; (4) recruitment of immunosuppressive cell populations such as Tregs and myeloid-derived suppressor cells; and (5) alteration of the expression of transcription factors in T cells such as T-bet and EOMES. Compared with memory T cells and effector T cells, exhausted T cells have different cell surface markers.

costimulatory signals [22, 23]. In general, the more inhibitory receptor expression there is, the more severe the exhaustion. T-cell exhaustion can be reversed by blocking these receptors [24]. For example, PD-1 can interact with PD-L1 [25], leading to immunosuppression and T-cell exhaustion. There are three mechanisms that may explain how PD-1 leads to T-cell exhaustion and suppresses the T-cell function. First, PD-1 may recruit phosphatases to inhibit TCR signalling [26]. Second, PD-1 may moderate the PI3K/AKT/mTOR pathway or Ras pathway and participate in the metabolism, survival, and growth of T cells [22]. Third, PD-1 may induce the expression of the basic leucine zipper ATF-like transcription factor (BATF) and inhibit the expression of effector genes [27].

The microenvironment surrounding T cells

Many soluble signalling molecules in the environment in which T cells are located regulate T-cell exhaustion, such as the immunosuppressive cytokines IL-10 and transforming growth factor-β (TGF-β) and the inflammatory cytokines type I IFNs and IL-2. In chronic infections, many types of cells can secrete IL-10, such as dendritic cells (DCs), monocytes, and CD4+ T cells [28–30]. Similar to PD-L1, IL-10 can also suppress T-cell activity during persistent viral infection, although these two function through distinct pathways [31]. Blocking IL-10 during chronic viral infection can help reverse T-cell exhaustion

and enhance virus control [32]. The cytokine TGF-β has potent immunosuppressive effects and is associated with immune checkpoint signalling pathways, which can lead to T-cell exhaustion [33, 34]. A bispecific protein containing anti-PD-1 fused with a TGF-β trap, which is secreted by T cells, improves the resistance to CAR-T-cell exhaustion [35]. Although type I IFNs such as IFN-α and IFN-β have antiviral functions in infection and thus promote the differentiation and activation of CD8+ T cells [36], blocking the IFN-α/β pathway reverses or prevents T-cell exhaustion during LCMV clone 13 infection [37]. In the absence of TCR signalling, exposure of CD8+ T cells to chronic inflammation alone, which gives rise to IFN-α/β signalling, impairs the differentiation of memory cells and thus leads to the exhaustion of T cells [38]. IL-2 can amplify CD8+ T-cell responses and optimize effector T-cell generation and differentiation into memory cells [39]. However, continuously high expression of IL-2 can activate the aryl hydrocarbon receptor and promote T-cell exhaustion [40]. In addition to the abovementioned cytokines, physiological changes in the solid tumour microenvironment (TME), including hypoxia, low pH, deprivation of nutrients (such as glucose or amino acids), and an increase in collagen, can also induce the exhaustion of CD8+ T cells.

Immunosuppressive cells and CD4+ helper T cells

Regulatory T cells (Tregs) are a subset of T cells that control autoimmunity in vivo. A high proportion of CAR-Treg cells can decrease the therapeutic effect of CAR-T cells. CAR-T cells with up to 5% CAR-Treg cells can lead to tumour recurrence [41]. Tregs can inhibit the response of T cells to infection. For example, when people are infected with HIV, the number of Tregs increases, which inhibits the antiviral ability of T cells. Tregs can produce IL-10, TGF-β, and other inhibitory cytokines [42]. Tregs are also correlated with the PD-1 pathway [42]. Simultaneous depletion of Tregs and blockade of PD-1 can induce synergistic effects on viral control and reverse the exhaustion of CD8+ T cells [43]. Therefore, Tregs are associated with the exhaustion of CD8+ T cells. In addition to Tregs, other immunoregulatory cells, including APCs, myeloid-derived suppressor cells (MDSCs) [44], and natural killer (NK) cells, can indirectly regulate the exhaustion of T cells [45]. Tumour-infiltrating APCs created a lymphoid-like microenvironment to support the survival of CD8+ T cells, and the occurrence of immune escape was accompanied by a loss of APC niche in solid tumour [46]. MDSCs produced soluble factors, such as reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and arginase-1, to inhibit T cells and increase T-cell exhaustion [44]. NK cells reduced T-cell exhaustion when they were used simultaneously with CAR-T cells [47].

CD4+ helper T cells play an important role in inhibiting the exhaustion of CD8+ T cells [48]. The loss of CD4+ T helper cells may lead to CD8+ T-cell exhaustion. During chronic infection, CD4+ helper T cells can produce IL-21, which, in turn, reduces CD8+ T-cell exhaustion [49, 50]. The depletion of CD4+ helper T cells leads to failed containment of chronic infection and more severe exhaustion of CD8+ T cells [51].

Alterations in the expression of transcription factors

The transcriptional profile of exhausted T cells is significantly different from that of effector and memory T cells. T-bet and EOMES are two important transcription factors that underlie the transcriptional profile and exhaustion of T cells. Subcellular localization of T-bet and Eomes dictates their regulatory effects on the exhaustion of T cells. Compared with memory T cells, exhausted T cells have a higher ratio of Eomes:T-bet in the nucleus during chronic LCMV infection in preclinical cancer models and human tumours [52]. Hypoglycaemia and hypoxia in the TME can downregulate T-bet expression and increase the number of exhausted T cells, thus inhibiting the function of tumour-infiltrating T cells [53]. The deletion of both alleles of Eomes in T cells prevents the production of antitumour cytotoxic T lymphocytes (CTLs), whereas the deletion of a single allele of Eomes decreases the exhaustion of CD8+ T cells [54].

T-bet and EOMES also affect exhausted T cells (Figure 2). Exhausted T cells can be classified into two subsets based on their expression of these two transcription factors: T-bet^{hi}PD1^{mid} exhausted T cells and EOMES^{hi}PD1^{hi} exhausted T cells. The former subset has relatively higher expression of T-bet than the latter subset, while the latter subset has relatively higher expression of EOMES. The number of T-bet^{hi}PD1^{mid} exhausted T cells is lower than the number of EOMES^{hi}PD1^{hi} exhausted T cells. T-bet^{hi}PD1^{mid} exhausted T cells retain partial proliferation potential, while EOMES^{hi}PD1^{hi} exhausted T cells have higher coinhibitory receptor expression and lower proliferative potential [55]. PD-1 blockade therapy has a certain effect on T-bet^{hi}PD1^{mid} exhausted T cells, while it has little effect on EOMES^{hi}PD1^{hi} exhausted T cells [55]. In CAR-T-cell therapy on large established pancreatic tumours and lymphoma, T-bet^{hi} CAR-T cells showed a better antitumour activity than normal CAR-T cells [56, 57]. In addition to the abovementioned T-bet and EOMES,

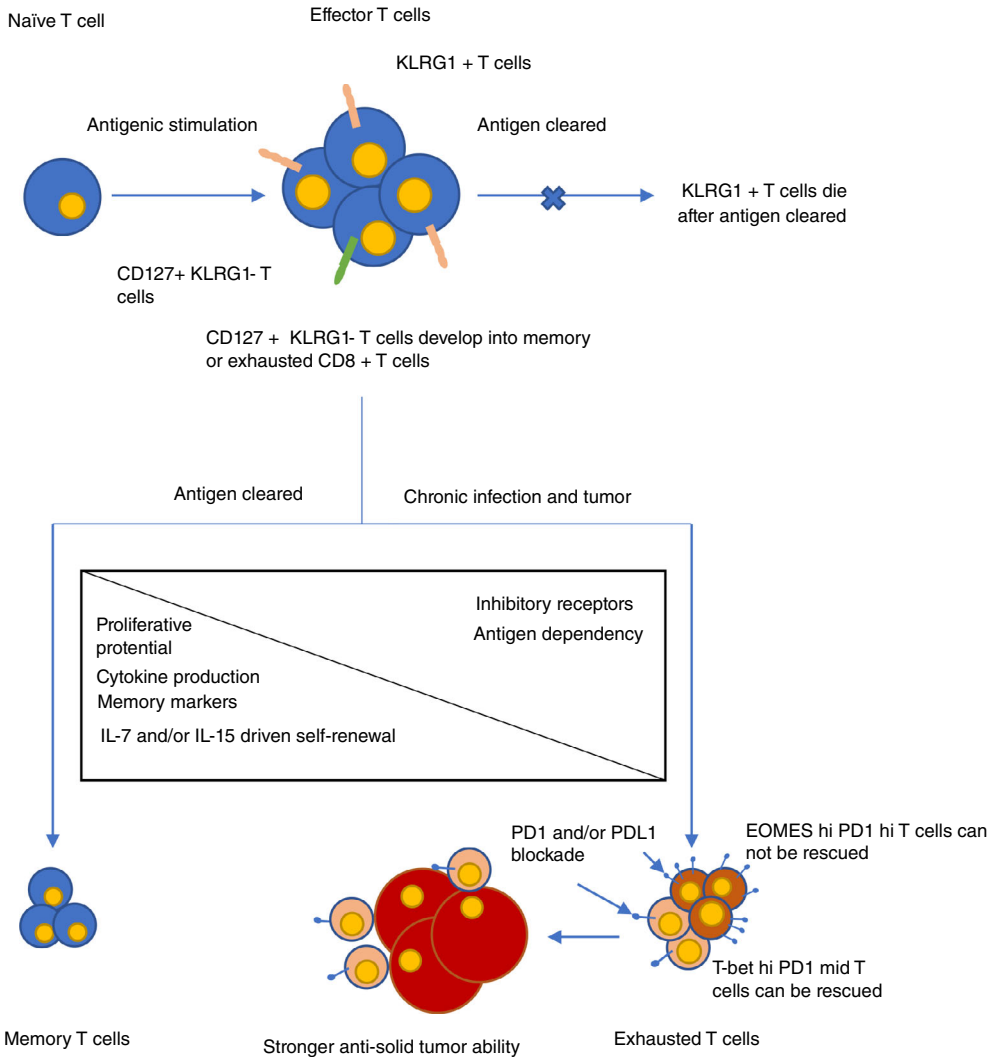


FIGURE 2 Development of T-cell exhaustion. Naïve T cells will proliferate to form an effector population upon activation by antigens. Most effector T cells, which express killer cell lectin-like receptor subfamily G member 1 (KLRG1), do not survive through the contraction phase, whereas a smaller number of effector T cells, which express CD127, go on to develop into memory or exhausted CD8+ T cells. When exposed to acute infection after the antigen is cleared, effector CD8+ T cells can differentiate into functional memory T cells. These memory T cells can survive by homeostatic self-renewal, which is driven by IL-7 and IL-15. However, in chronic infection and solid tumours with prolonged antigen stimulation, T cells will lose effector functions in a hierarchical manner and become exhausted. T-bet^{hi} T cells and CAR-T cells can be rescued by PD-1 or PD-L1 blockade and result in a stronger anti-solid tumour ability.

many other transcription factors are also involved in the exhaustion of CD8+ T cells, such as BLIMP1, NFAT, BATF, FOXO1, and FOXP1 [27, 58–62].

STRATEGIES TO PROLONG THE EFFECTIVE TIME OF CAR-T-CELL THERAPY IN THE TREATMENT OF SOLID TUMOURS BY INHIBITING T-CELL EXHAUSTION

Although CAR-T cells represent a breakthrough in the treatment of malignant haematological tumours, they do not achieve good therapeutic outcomes in the treatment of solid tumours. In the treatment of solid tumours compared with haematological tumours, CAR-T cells face three main challenges: recognizing tumours, entering tumours, and surviving in tumours. Prolonging the effective time of CAR-T cells in solid tumours can improve the therapeutic effect. Overcoming T-cell exhaustion can prolong the effective time of CAR-T cells in solid tumours. Here, we discuss ways to prolong the effective time of CAR-T cells in solid tumours by inhibiting T-cell exhaustion.

Utilization of immune checkpoint blockade

The immune checkpoint blockade plays an important role in preventing T-cell exhaustion in tumour immunotherapy. At present, drugs targeting PD-1, PD-L1, and CTLA-4 have been used in combination with CAR-T cells and have achieved good clinical results [63]. Anti-CTLA-4 therapy in combination with anti-VEGF antibodies was reported to have increased antitumour activity and prevent the immunosuppressive effects of galectin-1 [64]. Genetically engineered CAR-T cells can secrete antibodies to block their own immune checkpoints without adding artificial exogenous immune

checkpoint-directed antibodies. Researchers established CAR19 T cells that can constitutively secrete anti-PD-1 antibodies, and the modified CAR-T cells showed enhanced antitumour activity in a heterogeneous CD19+ lung cancer model [24]. Furthermore, the activity and proliferation of T cells were enhanced. Modified CAIX-CAR-T cells expressing anti-PD-L1 antibodies exhibited higher activity than ordinary CAR-T cells in clear cell renal cell carcinoma (ccRCC) mouse models, producing more cytokines, recruiting more immune cells, and promoting tumour regression [65]. Similarly, various types of CAR-T cells expressing antibodies against PD-1, PD-L1, CTLA-4, and other immune checkpoints have been developed [66].

In addition to generating T cells with expression of antibodies against immune checkpoint, directly reducing or blocking the expression of various inhibitory receptors at the genetic level is also an effective method. With the development of CRISPR/Cas9 technology, CAR-T cells with low expression of inhibitory receptors have been developed for solid tumour therapy. Compared with normal CAR-T cells, CRISPR/Cas9-edited CD133-specific CAR-T cells with the PD-1 knockout showed better tumour inhibition ability in mouse glioma models [67]. Currently, CRISPR/Cas9 technology is also used in CAR-T-cell therapy to induce the LAG-3 knockout [68].

Blocking immune checkpoints with CRISPR/Cas9 technology may be more advantageous than overexpressing antibodies against immune checkpoints. The repeated use of antibodies may lead to drug resistance. Although mesothelin-directed CAR-T cells, which overexpress a dominant-negative form of PD-1, have a high tumour-clearing capacity, they can only prevent tumour growth and do not clear the tumour due to the repeated use of anti-PD-1 antibodies in combination with mesothelin-28ζ or mesothelin-BBζ [69].

A tool called switch receptors can also be used to alleviate T-cell exhaustion. With this method, the extracellular ligand-binding domain of the inhibitory receptor is fused with the intracellular signalling domain of the activating molecule. In a breast cancer model, MUC1 CAR-T cells with a switch receptor containing an extracellular receptor for IL-4 fused with an intracellular signalling domain for IL-7 showed better proliferation and higher antitumour ability in the presence of IL-4 than MUC1 CAR-T cells without the switch receptor [70].

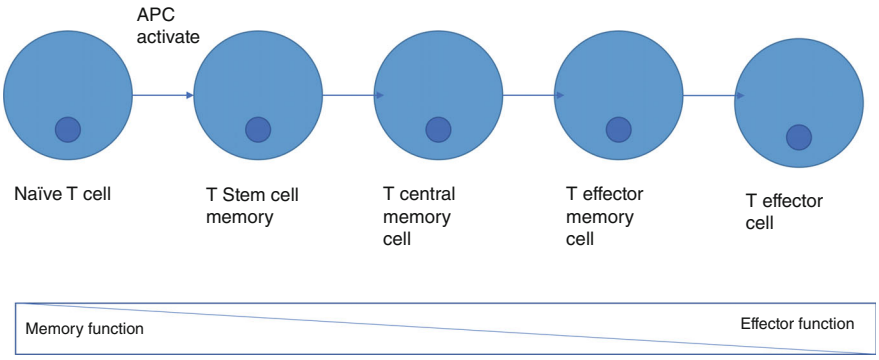
Modulation of transcription factors and gene expression profiles

The exhaustion of mesothelin-directed CAR-T cells is related to the transformation of CD8+ T cells into NK-like cells, and downregulation of the expression of ID3 and SOX4, two transcription factors responsible for the transformation, can improve the efficacy of CAR-T-cell therapy in solid tumour treatment by preventing or delaying CAR-T-cell dysfunction [13]. TCF-1 is a key transcription factor in pre-exhausted CD8 T cells. TCF-1 was reported to drive an effector versus exhausted CD8+ T-cell fate [71]. Therefore, modulation of this transcription factor can possibly promote the effective time of CAR-T cells. The upregulation of T-bet in CAR-T cells can promote anti-solid tumour ability in treating large established pancreatic tumours [57]. BATF and interferon regulatory factor 4 (IRF4) cooperate to counter T-cell exhaustion, and overexpression of BATF in CAR-T cells can promote the survival and expansion of tumour-infiltrating CAR-T cells [72].

Gene expression profiles play an important role in the transformation of effector T cells into exhausted T cells. The CAR-T cells isolated from complete responders were reported to have upregulation of genes associated with memory differentiation status, such as IL-6 and STAT3, and blockade of the expression of these genes can decrease the function of CAR-T cells; on the other hand, the CAR-T cells from nonresponders had the upregulation of genes more associated with an effector phenotype as well as glycolysis, exhaustion, and cell death by apoptosis [73]. Therefore, methods that can modulate gene expression profiles can possibly decrease T-cell exhaustion and prolong the effective time of CAR-T cells.

Overcoming inhibitory factors in the TME

In addition to the immune checkpoints mentioned above, many other factors in the TME affect T-cell exhaustion in solid tumours. Soluble immunosuppressive factors (such as adenosine, IDO1, VEGF, and TGF-β), immunosuppressive nontumour cells (such as MDSCs, tumour-associated macrophages (TAMs), and stromal cells), and collagen can promote the exhaustion of CD8+ T cells. Blocking the adenosine 2A receptor improved the effect of HER2 CAR-T cells in a syngeneic tumour model and enhanced the activation and cytokine production of the CAR-T cells [74]. Blocking IDO1 in a xenograft colon cancer model enhanced the effect of EGFRvIII CAR-T cells by significantly decreasing the growth rate of tumours [75]. Blocking VEGF increased the antitumour effect of CAR-T cells in multiple solid tumour models [64]. Clinical application of TGF-β-resistant CAR-T cells was proved to be feasible and generally safe in treating prostate cancer [76].



CD62L	+	+	+	-	-
CCR7	+	+	+	-	-
CD45RA	+	+	-	-	+
CD45RO	-	+	+	+	-
KLRG1	-	-	-	+	+

FIGURE 3 Markers of T-cell differentiation (modified after Chang et al.[83]). Upon encountering antigen, naïve T cells are activated and differentiate into Tscm, Tcm, Tem and effector T cells. T-cell differentiation can be classified according to the expression of cell surface markers and transcription factors and alterations in cellular metabolic pathways.

Inhibiting the anti-inflammatory cells in the TME or increasing the inflammatory phenotypes of other immune cells can inhibit T-cell exhaustion and increase the antitumour effect of CAR-T cells in solid tumours. Macrophages were found to be associated with resistance to VEGF blockade in murine ovarian cancer models, and in macrophage-deficient mice, there was no resistance to the VEGF blockade until macrophages were reintroduced into the tumours [77]. HER2 CAR-T cells needed M1 macrophages and IFNγ receptors on stromal cells to induce tumour rejection in a subcutaneous mouse model, indicating that functional T cells alone are not sufficient to clear tumours. Therapeutic methods that target stromal cells and recruit other types of inflammatory immune cells are necessary for CAR-T cells to clear tumours [78]. In a xenograft sarcoma model, GD2 CAR-T cells combined with all-trans retinoic acid, which can reduce MDSC numbers, showed significantly better antitumour effects than GD2 CAR-T cells alone [79]. Hypoxia in the TME can upregulate PDL-1 on MDSCs and increase the ability of MDSCs to suppress TILs [80].

Cytokines can polarize the tumour milieu and improve the recruitment and function of CAR-T cells. IL-12 can induce the recruitment of adoptively transferred VEGFR-2 CAR-T cells and prolong the survival of tumour-bearing mice [81]. Based on these findings, a new type of CAR-T cell called 'armoured' CAR-T cells, which can constitutively secrete cytokines, was developed and led to enhanced T-cell infiltration and function in solid tumours and showed less exhaustion [81].

Collagen in solid tumours plays a very important role in immunosuppression. Reduced collagen levels induced by the application of LOXL2 can diminish exhausted T cells and overcome resistance to anti-PD-L1 therapy [82].

Formation of memory T cells

The differentiation state of CAR-T cells plays an important role in determining the effect of tumour therapy. T cells can be divided into five categories based on the differentiation state: naïve T cells, T stem cell-like memory (Tscm) cells, central memory T (Tcm) cells, effector memory T (Tem) cells, and effector T (Teff) cells. Each category has its own biomarkers, as shown in Figure 3. In addition, cytokines such as IL-2, IL-12, IL-27, and IFN-γ are usually characteristic of Teff cells. Cytokines, such as IL-10, IL-21, IL-7, IL-15, and TGF-β, are associated with memory T cells, including Tscm, Tcm, and Tem cells. Genes such as T-bet, Id2, Blimp-1, Batf, and Stat4 are associated with Teff cells. The genes Id3, Bcl-6, Tcf-7, Stat3, Foxo1, and Eomes are proposed to be upregulated in memory T cells [57, 84].

The immunophenotypes of naïve, Tscm, Tcm, and Tem cells are superior to those of effector T cells in terms of survival, proliferation, antitumour effect, and survival in tumours when used in adoptive cell therapy (ACT), and these cells show less exhaustion [85]. The prognosis of ovarian cancer was observed to be associated with CD8+ Tem cell accumulation in ascites [86]. The induction of T-cell memory by CDK4/6 inhibition promoted antitumour immunity [87]. An increase in the CD8+ T-cell memory resulted in enhanced antitumour function in mice [88]. Better antitumour activity in vivo was obtained when the CAR-T-cell therapy had more CAR-T cells in the Tscm state [89].

At present, many methods have been developed to increase the number of memory T cells. Cytokines, metabolism, and cell cycle arrest play important roles in T-cell differentiation and T-cell exhaustion. Replacing the cytokine IL-2 with IL-15 in the culture environment

during the in vitro culture of CAR-T cells inhibited mTORC1 activity, glycolytic enzyme expression, and mitochondrial changes and, as a result, increased the number of CAR-T cells in the Tscm state [89]. High glycolytic activity in CD8+ T cells severely compromised the generation of long-lived memory cells by driving T cells towards a terminally differentiated state and, therefore, inhibiting glycolytic metabolism enhanced CD8+ T-cell memory [88]. Short-term priming with cell cycle CDK4/6 inhibitors promoted the functional acquisition of immunological memory T cells [87].

Modification of CAR intracellular signalling

CD28 and 4-1BB are the two most common costimulatory molecules and are linked to an antigen-recognition domain in CAR-T cells. CAR-T cells with the costimulatory molecule 4-1BB/CD3 have increased the survival time, decreased exhaustion, and a superior differentiated phenotype compared with those with CD28/CD3 [90, 91]. Though CD28/CD3 and 4-1BB/CD3 CAR-T cells phosphorylate the same protein in the same signalling pathway upon activation, the phosphorylation level induced by CD28/CD3 CAR-T cells were much higher than that induced by 4-1BB/CD3 CAR-T cells [90]. A higher level of phosphorylation can result in a stronger effector function; however, it can lead to increased expression of exhaustion markers. In line with this, CD28/CD3 CAR-T cells showed a shorter effective time and less viability than 4-1BB/CD3 CAR-T cells [90]. The 4-1BB/CD3 CAR-T cells had higher expression of memory-associated genes and performed better in vivo [90]. In a study treating PSCA+ metastatic prostate cancer with CAR-T cells, 4-1BB CAR-T cells showed less exhaustion and better antigen selectivity in vivo than CD28 CAR-T cells [91].

T cells sustain their effector function through the glycolytic metabolism, whereas naïve T cells primarily derive their energy from the oxidation of fatty acids. Compared with CD28 CAR-T cells, 4-1BB CAR-T cells proliferated better and had more central memory differentiation, a higher level of fatty acid oxidation, increased generation of mitochondria, and a lower level of glycolytic metabolism; therefore, the 4-1BB CAR-T cells demonstrated less exhaustion, longer effective time, and better in vivo antitumour ability [92].

The gene locus into which the CAR is inserted in developing CAR-T cells has a significant impact on the function of CAR-T cells. Insertion of a CD19-specific CAR into the T-cell receptor α constant (TRAC) locus using CRISPR/Cas9 technology resulted in not only uniform CAR expression in human peripheral blood T cells but also higher T-cell proliferation, more memory cells, less exhaustion, and better tumour rejection [93]. This strategy induced knockout of the TRAC locus, which possibly reduced the tonic signalling that would otherwise push T cells towards terminal differentiation and exhaustion.

Inducing a transient 'resting' state of CAR-T cells by inhibiting intracellular CAR signalling can restore functionality to exhausted CAR-T cells via epigenetic remodelling. The inhibition of intracellular CAR signalling can be achieved by treating CAR-T cells with the tyrosine kinase inhibitor dasatinib or using a drug-regulatable platform [94]. The inhibitor dasatinib can reversibly inhibit TCR and CAR signalling. With the drug-regulatable platform, a tonically signalling CAR is modified with a C-terminal destabilizing domain (DD), and this modification can enable drug-dependent control of CAR protein levels.

SUMMARY

As a revolutionary cancer therapy method, CAR-T-cell therapy has achieved great advances in the haematological tumour treatment. However, because tumour-infiltrating T cells can easily become exhausted and due to other reasons, such as T cell difficulty in penetrating tumours, CAR-T-cell therapy has not achieved a satisfactory effect in solid tumours. The process of T-cell exhaustion is progressive, and many mechanisms are involved. After CAR-T cells enter solid tumours, multiple factors can cause the exhaustion of tumour-infiltrating T cells, including (i) consistent exposure of the T cells to antigens; (ii) inhibition signal of the expression of immune checkpoints and their ligands; (iii) inhibition of T-cell function by IL-10, TGF-β, and other inhibitory cytokines, production of metabolites (including adenosine, prostaglandins, and lactic acid), and physiological changes such as hypoxia, low pH, and deprivation of nutrients (such as glucose or amino acids) in the microenvironment surrounding T cells; (iv) recruitment of immunosuppressive cell populations such as Tregs and MDSCs; and (v) alteration of the expression of transcription factors in T cells such as T-bet and EOMES. T-cell exhaustion is a very complicated process that involves a large number of factors that have not yet been identified. Only when the molecular mechanisms underlying the exhaustion of T cells are fully understood can effective methods to decrease the extent of T-cell exhaustion and improve CAR-T therapeutic effects in the treatment of solid tumours be devised. Therefore, the molecular mechanisms underlying T-cell exhaustion, such as molecular signalling network collaboration and transcriptional changes, still need to be further explored. The

discovery of more factors that induce T-cell exhaustion can help us find more efficient ways to improve the effect of CAR-T-cell therapy.

Since the emergence of CAR-T-cell technology, many methods have been proposed to improve the therapeutic effect of CAR-T cells in the treatment of solid tumours. Currently, there are five methods that can decrease T-cell exhaustion to improve the effect of CAR-T-cell therapy: (i) blocking immune checkpoints on the surface of T cells or tumour cells; (ii) modulating the transcription factor and gene expression profiles; (iii) overcoming inhibitory factors in the TME; (iv) inducing the formation and differentiation of memory T cells; and (v) genetically redesigning the structure of CARs to modulate CAR intracellular signalling. Among the above five methods, inducing the formation and differentiation of memory T cells is considered to have more advantages than the other strategies in terms of improving T-cell proliferation and resistance to exhaustion in vivo [83, 95]. Besides inhibiting T-cell exhaustion, other methods to improve the therapeutic effect of CAR-T cells on solid tumours have also been developed, such as developing CAR-T cells that can recognize more than one target on solid tumours [96] and promoting the infiltration of CAR-T cells [97]. With these methods, CAR-T-cell therapy will become a powerful tool and obtain a widespread application in treating solid tumours in the future.

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CONFLICT OF INTEREST STATEMENT

There are no potential conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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REFERENCES

1. Savage PA, Leventhal DS, Malchow S. Shaping the repertoire of tumor-infiltrating effector and regulatory T cells. Immunol Rev. 2014;259(1):245–58.
2. Rahbarizadeh F, Ahmadvand D, Moghimi SM. CAR T-cell bioengineering: single variable domain of heavy chain antibody targeted CARs. Adv Drug Deliv Rev. 2019;141:41–6.
3. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. Trends Immunol. 2015;36(4):265–76.
4. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol. 2009;27:591–619.
5. Wherry EJ. T cell exhaustion. Nat Immunol. 2011;12(6):492–9.
6. Doering TA, Crawford A, Angelosanto JM, Paley MA, Ziegler CG, Wherry EJ. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. Immunity. 2012;37(6):1130–44.
7. Schietinger A, Greenberg PD. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. Trends Immunol. 2014;35(2):51–60.
8. Schwartz RH. T cell Anergy. Annu Rev Immunol. 2003;21:305–34.
9. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol. 2003;77(8):4911–27.
10. Kim PS, Ahmed R. Features of responding T cells in cancer and chronic infection. Curr Opin Immunol. 2010;22(2):223–30.
11. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med. 1998;188(12):2205–13.
12. Bucks CM, Norton JA, Boesteanu AC, Mueller YM, Katsikis PD. Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion. J Immunol. 2009;182(11):6697–708.
13. Good CR, Aznar MA, Kuramitsu S, Samareh P, Agarwal S, Donahue G, et al. An NK-like CAR T cell transition in CAR T cell dysfunction. Cell. 2021;184(25):6081–100.
14. Oestreich KJ, Yoon H, Ahmed R, Boss JM. NFATc1 regulates PD-1 expression upon T cell activation. J Immunol. 2008;181(7):4832–9.
15. Ashouri JF, Weiss A. Endogenous Nur77 is a specific indicator of antigen receptor signaling in human T and B cells. J Immunol. 2017;198(2):657–68.
16. Mognol GP, Spreafico R, Wong V, Scott-Browne JP, Togher S, Hoffmann A, et al. Exhaustion-associated regulatory regions in CD8(+) tumor-infiltrating T cells. Proc Natl Acad Sci USA. 2017;114(13):E2776–85.
17. Chen J, Lopez-Moyado IF, Seo H, Lio C-WJ, Hempleman LJ, Sekiya T, et al. NR4A transcription factors limit CAR T cell function in solid tumours. Nature. 2019;567(7749):530–4.
18. Man K, Gabriel SS, Liao Y, Gloury R, Preston S, Henstridge DC, et al. Transcription factor IRF4 promotes CD8(+) T cell exhaustion and limits the development of memory-like T cells during chronic infection. Immunity. 2017;47(6):1129–41.
19. Streeck H, Brumme ZL, Anastario M, Cohen KW, Jolin JS, Meier A, et al. Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells. PLoS Med. 2008;5(5):e100.
20. Zhao M, Kiernan CH, Stairiker CJ, Hope JL, Leon LG, van Meurs M, et al. Rapid in vitro generation of bona fide exhausted CD8+ T cells is accompanied by Tcf7 promotor methylation. PLoS Pathog. 2020;16(6):e1008555.
21. Hashimoto M, Kamphorst AO, Im SJ, Kissick HT, Pillai RN, Ramalingam SS, et al. CD8 T cell exhaustion in chronic infection and cancer: opportunities for interventions. Annu Rev Med. 2018;69:301–18.
22. Patsoukis N, Brown J, Petkova V, Liu F, Li L, Boussiotis VA. Selective effects of PD-1 on Akt and Ras pathways regulate molecular components of the cell cycle and inhibit T cell proliferation. Sci Signal. 2012;5(230):a46.

23. Odorizzi PM, Wherry EJ. Inhibitory receptors on lymphocytes: insights from infections. J Immunol. 2012;188(7):2957–65.
24. Li S, Siriwon N, Zhang X, Yang S, Jin T, He F, et al. Enhanced cancer immunotherapy by chimeric antigen receptor-modified T cells engineered to secrete checkpoint inhibitors. Clin Cancer Res. 2017;23(22):6982–92.
25. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008; 26:677–704.
26. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. J Exp Med. 2012;209(6):1201–17.
27. Quigley M, Pereyra F, Nilsson B, Porichis F, Fonseca C, Eichbaum Q, et al. Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. Nat Med. 2010;16(10):1147–51.
28. Ng CT, Oldstone MB. Infected CD8α$^-$ dendritic cells are the predominant source of IL-10 during establishment of persistent viral infection. Proc Natl Acad Sci USA. 2012;109(35): 14116–21.
29. Said EA, Dupuy FP, Trautmann L, Zhang Y, Shi Y, el-Far M, et al. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. Nat Med. 2010;16(4):452–9.
30. Richter K, Perriard G, Behrendt R, Schwendener RA, Sexl V, Dunn R, et al. Macrophage and T cell produced IL-10 promotes viral chronicity. PLoS Pathog. 2013;9(11):e1003735.
31. Brooks DG, Ha SJ, Elsaesser H, Sharpe AH, Freeman GJ, Oldstone MB. IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. Proc Natl Acad Sci USA. 2008;105(51):20428–33.
32. Ejrnaes M, Filippi CM, Martinic MM, Ling EM, Togher LM, Crotty S, et al. Resolution of a chronic viral infection after interleukin-10 receptor blockade. J Exp Med. 2006;203(11): 2461–72.
33. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity. 2009;30(5):636–45.
34. Oida T, Xu L, Weiner HL, Kitani A, Strober W. TGF-β-mediated suppression by CD4+CD25+ T cells is facilitated by CTLA-4 signaling. J Immunol. 2006;177(4):2331–9.
35. Chen X, Yang S, Li S, Qu Y, Wang HY, Liu J, et al. Secretion of bispecific protein of anti-PD-1 fused with TGF-β trap enhances antitumor efficacy of CAR-T cell therapy. Mol Ther Oncolytics. 2021;21:144–57.
36. Curtsinger JM, Mescher MF. Inflammatory cytokines as a third signal for T cell activation. Curr Opin Immunol. 2010; 22(3):333–40.
37. Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, et al. Blockade of chronic type I interferon signaling to control persistent LCMV infection. Science. 2013;340(6129):202–7.
38. Stelekati E, Shin H, Doering TA, Dolfi DV, Ziegler CG, Beiting DP, et al. Bystander chronic infection negatively impacts development of CD8(+) T cell memory. Immunity. 2014;40(5):801–13.
39. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol. 2012;12(3):180–90.
40. Liu Y, Zhou N, Zhou L, Wang J, Zhou Y, Zhang T, et al. IL-2 regulates tumor-reactive CD8(+) T cell exhaustion by activating the aryl hydrocarbon receptor. Nat Immunol. 2021;22(3): 358–69.
41. Haradhvala NJ, Leick MB, Maurer K, Gohil SH, Larson RC, Yao N, et al. Distinct cellular dynamics associated with response to CAR-T therapy for refractory B cell lymphoma. Nat Med. 2022;28(9):1848–59.
42. Veiga-Parga T, Sehrawat S, Rouse BT. Role of regulatory T cells during virus infection. Immunol Rev. 2013;255(1):182–96.
43. Penaloza-MacMaster P, Kamphorst AO, Wieland A, Araki K, Iyer SS, West EE, et al. Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. J Exp Med. 2014;211(9):1905–18.
44. Goh C, Narayanan S, Hahn YS. Myeloid-derived suppressor cells: the dark knight or the joker in viral infections? Immunol Rev. 2013;255(1):210–21.
45. Waggoner SN, Cornberg M, Selin LK, Welsh RM. Natural killer cells act as rheostats modulating antiviral T cells. Nature. 2011;481(7381):394–8.
46. Jansen CS, Prokhnevska N, Master VA, Sanda MG, Carlisle JW, Bilen MA, et al. An intra-tumoral niche maintains and differentiates stem-like CD8 T cells. Nature. 2019; 576(7787):465–70.
47. Bachiller M, Perez-Amill L, Battram AM, Carné SC, Najjar A, Verhoeyen E, et al. NK cells enhance CAR-T cell antitumor efficacy by enhancing immune/tumor cells cluster formation and improving CAR-T cell fitness. J Immunother Cancer. 2021;9(8):e2866.
48. Lu YJ, Barreira-Silva P, Boyce S, Powers J, Cavallo K, Behar SM. CD4 T cell help prevents CD8 T cell exhaustion and promotes control of mycobacterium tuberculosis infection. Cell Rep. 2021;36(11):109696.
49. Williams LD, Bansal A, Sabbaj S, Heath SL, Song W, Tang J, et al. Interleukin-21-producing HIV-1-specific CD8 T cells are preferentially seen in elite controllers. J Virol. 2011;85(5): 2316–24.
50. Chevalier MF, Julg B, Pyo A, Flanders M, Ranasinghe S, Soghoian DZ, et al. HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function. J Virol. 2011;85(2):733–41.
51. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J Virol. 1994;68(12):8056–63.
52. McLane LM, Ngiow SF, Chen Z, Attanasio J, Manne S, Ruthel G, et al. Role of nuclear localization in the regulation and function of T-bet and Eomes in exhausted CD8 T cells. Cell Rep. 2021;35(6):109120.
53. Zhang Y, Kurupati R, Liu L, Zhou XY, Zhang G, Hudaihed A, et al. Enhancing CD8(+) T cell fatty acid catabolism within a metabolically challenging tumor microenvironment increases the efficacy of melanoma immunotherapy. Cancer Cell. 2017; 32(3):377–91.
54. Li J, He Y, Hao J, Ni L, Dong C. High levels of Eomes promote exhaustion of anti-tumor CD8+ T cells. Front Immunol. 2018; 9:2981.
55. Paley MA, Kroy DC, Odorizzi PM, Johnnidis JB, Dolfi DV, Barnett BE, et al. Progenitor and terminal subsets of CD8+ T

cells cooperate to contain chronic viral infection. Science. 2012;338(6111):1220–5.
56. Gacerez AT, Sentman CL. T-bet promotes potent antitumor activity of CD4(+) CAR T cells. Cancer Gene Ther. 2018;25(5–6):117–28.
57. Chmielewski M, Abken H. CAR T cells releasing IL-18 convert to T-bet(high) FoxO1(low) effectors that exhibit augmented activity against advanced solid tumors. Cell Rep. 2017;21(11):3205–19.
58. Agnellini P, Wolint P, Rehr M, Cahenzli J, Karrer U, Oxenius A. Impaired NFAT nuclear translocation results in split exhaustion of virus-specific CD8+ T cell functions during chronic viral infection. Proc Natl Acad Sci USA. 2007;104(11):4565–70.
59. Shin H, Blackburn SD, Intlekofer AM, Kao C, Angelosanto JM, Reiner SL, et al. A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. Immunity. 2009;31(2):309–20.
60. Liikanen I, Lauhan C, Quon S, Omilusik K, Phan AT, Bartrolí LB, et al. Hypoxia-inducible factor activity promotes antitumor effector function and tissue residency by CD8+ T cells. J Clin Invest. 2021;131(7):e143729.
61. Rao RR, Li Q, Bupp MRG, Shrikant PA. Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8+ T cell differentiation. Immunity. 2012;36(3):374–87.
62. Stephen TL, Rutkowski MR, Allegrezza MJ, Perales-Puchalt A, Tesone AJ, Svoronos N, et al. Transforming growth factor β-mediated suppression of antitumor T cells requires FoxP1 transcription factor expression. Immunity. 2014;41(3):427–39.
63. Moon EK, Wang LC, Dolfi DV, Wilson CB, Ranganathan R, Sun J, et al. Multifactorial T-cell hypofunction that is reversible can limit the efficacy of chimeric antigen receptor-transduced human T cells in solid tumors. Clin Cancer Res. 2014;20(16):4262–73.
64. Wu X, Li J, Connolly EM, Liao X, Ouyang J, Giobbie-Hurder A, et al. Combined anti-VEGF and anti-CTLA-4 therapy elicits humoral immunity to Galectin-1 which is associated with favorable clinical outcomes. Cancer Immunol Res. 2017;5(6):446–54.
65. Suarez ER, Chang DK, Sun J, Sui J, Freeman GJ, Signoretti S, et al. Chimeric antigen receptor T cells secreting anti-PD-L1 antibodies more effectively regress renal cell carcinoma in a humanized mouse model. Oncotarget. 2016;7(23):34341–55.
66. Yoon D, Osborn M, Tolar J, Kim C. Incorporation of immune checkpoint blockade into chimeric antigen receptor T cells (CAR-Ts): combination or built-In CAR-T. Int J Mol Sci. 2018;19(2):340.
67. Hu B, Zou Y, Zhang L, Tang J, Niedermann G, Firat E, et al. Nucleofection with plasmid DNA for CRISPR/Cas9-mediated inactivation of programmed cell death protein 1 in CD133-specific CAR T cells. Hum Gene Ther. 2019;30(4):446–58.
68. Zhang Y, Zhang X, Cheng C, Mu W, Liu X, Li N, et al. CRISPR-Cas9 mediated LAG-3 disruption in CAR-T cells. Front Med. 2017;11(4):554–62.
69. Chen N, Morello A, Tano Z, Adusumilli PS. CAR T-cell intrinsic PD-1 checkpoint blockade: a two-in-one approach for solid tumor immunotherapy. Onco Targets Ther. 2017;6(2):e1273302.
70. Bajgain P, Tawinwung S, D'Elia L, Sukumaran S, Watanabe N, Hoyos V, et al. CAR T cell therapy for breast cancer: harnessing the tumor milieu to drive T cell activation. J Immunother Cancer. 2018;6(1):34.
71. Chen Z, Ji Z, Ngiow SF, Manne S, Cai Z, Huang AC, et al. TCF-1-centered transcriptional network drives an effector versus exhausted CD8 T cell-fate decision. Immunity. 2019;51(5):840–55.
72. Seo H, Gonzalez-Avalos E, Zhang W, Ramchandani P, Yang C, Lio CJ, et al. BATF and IRF4 cooperate to counter exhaustion in tumor-infiltrating CAR T cells. Nat Immunol. 2021;22(8):983–95.
73. Fraietta JA, Lacey SF, Orlando EJ, Pruteanu-Malinici I, Gohil M, Lundh S, et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. Nat Med. 2018;24(5):563–71.
74. Beavis PA, Henderson MA, Giuffrida L, Mills JK, Sek K, Cross RS, et al. Targeting the adenosine 2A receptor enhances chimeric antigen receptor T cell efficacy. J Clin Invest. 2017;127(3):929–41.
75. Huang Q, Xia J, Wang L, Wang X, Ma X, Deng Q, et al. miR-153 suppresses IDO1 expression and enhances CAR T cell immunotherapy. J Hematol Oncol. 2018;11(1):58.
76. Narayan V, Barber-Rotenberg JS, Jung IY, Lacey SF, Rech AJ, Davis MM, et al. PSMA-targeting TGFβ-insensitive armored CAR T cells in metastatic castration-resistant prostate cancer: a phase 1 trial. Nat Med. 2022;28(4):724–34.
77. Dalton HJ, Pradeep S, McGuire M, Hailemichael Y, Ma S, Lyons Y, et al. Macrophages facilitate resistance to anti-VEGF therapy by altered VEGFR expression. Clin Cancer Res. 2017;23(22):7034–46.
78. Textor A, Listopad JJ, Wuhrmann LL, Perez C, Kruschinski A, Chmielewski M, et al. Efficacy of CAR T-cell therapy in large tumors relies upon stromal targeting by IFNγ. Cancer Res. 2014;74(23):6796–805.
79. Long AH, Highfill SL, Cui Y, Smith JP, Walker AJ, Ramakrishna S, et al. Reduction of MDSCs with all-trans retinoic acid improves CAR therapy efficacy for sarcomas. Cancer Immunol Res. 2016;4(10):869–80.
80. Noman MZ, Desantis G, Janji B, Hasmim M, Karray S, Dessen P, et al. PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. J Exp Med. 2014;211(5):781–90.
81. Chinnasamy D, Yu Z, Kerkar SP, Zhang L, Morgan RA, Restifo NP, et al. Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. Clin Cancer Res. 2012;18(6):1672–83.
82. Peng DH, Rodriguez BL, Diao L, Chen L, Wang J, Byers LA, et al. Collagen promotes anti-PD-1/PD-L1 resistance in cancer through LAIR1-dependent CD8(+) T cell exhaustion. Nat Commun. 2020;11(1):4520.
83. Kishton RJ, Sukumar M, Restifo NP. Metabolic regulation of T cell longevity and function in tumor immunotherapy. Cell Metab. 2017;26(1):94–109.
84. Chang JT, Wherry EJ, Goldrath AW. Molecular regulation of effector and memory T cell differentiation. Nat Immunol. 2014;15(12):1104–15.
85. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell subset with stem cell-like properties. Nat Med. 2011;17(10):1290–7.

86. Lieber S, Reinartz S, Raifer H, Finkernagel F, Dreyer T, Bronger H, et al. Prognosis of ovarian cancer is associated with effector memory CD8(+) T cell accumulation in ascites, CXCL9 levels and activation-triggered signal transduction in T cells. Onco Targets Ther. 2018;7(5):e1424672.
87. Lelliott EJ, Kong IY, Zethoven M, Ramsbottom KM, Martelotto LG, Meyran D, et al. CDK4/6 inhibition promotes antitumor immunity through the induction of T-cell memory. Cancer Discov. 2021;11(10):2582–601.
88. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. J Clin Invest. 2013;123(10): 4479–88.
89. Alizadeh D, Wong RA, Yang X, Wang D, Pecoraro JR, Kuo CF, et al. IL15 enhances CAR-T cell antitumor activity by reducing mTORC1 activity and preserving their stem cell memory phenotype. Cancer Immunol Res. 2019;7(5):759–72.
90. Salter AI, Ivey RG, Kennedy JJ, Voillet V, Rajan A, Alderman EJ, et al. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. Sci Signal. 2018;11(544): t6753.
91. Priceman SJ, Gerdts EA, Tilakawardane D, Kennewick KT, Murad JP, Park AK, et al. Co-stimulatory signaling determines tumor antigen sensitivity and persistence of CAR T cells targeting PSCA+ metastatic prostate cancer. Onco Targets Ther. 2018;7(2):e1380764.
92. Kawalekar OU, O'Connor RS, Fraietta JA, Guo L, McGettigan SE, Posey AD Jr, et al. Distinct signaling of coreceptors regulates specific metabolism pathways and impacts memory development in CAR T cells. Immunity. 2016;44(2):380–90.
93. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJC, Hamieh M, Cunanan KM, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017;543(7643):113–7.
94. Weber EW, Parker KR, Sotillo E, Lynn RC, Anbunathan H, Lattin J, et al. Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. Science. 2021;372(6537):a1786.
95. Liu Q, Sun Z, Chen L. Memory T cells: strategies for optimizing tumor immunotherapy. Protein Cell. 2020;11(8):549–64.
96. Liang Z, Dong J, Yang N, Li SD, Yang ZY, Huang R, et al. Tandem CAR-T cells targeting FOLR1 and MSLN enhance the antitumor effects in ovarian cancer. Int J Biol Sci. 2021;17(15): 4365–76.
97. Fang J, Ding N, Guo X, Sun Y, Zhang Z, Xie B, et al. αPD-1-mesoCAR-T cells partially inhibit the growth of advanced/refractory ovarian cancer in a patient along with daily apatinib. J Immunother Cancer. 2021;9(2):e1162.

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REVIEW

NK-cell exhaustion, B-cell exhaustion and T-cell exhaustion—the differences and similarities

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Abstract

T-cell exhaustion has been extensively researched, compared with B-cell exhaustion and NK-cell exhaustion, which have received considerably less attention; and there is less of a consensus on the precise definitions of NK-cell and B-cell exhaustion. NK-cell exhaustion, B-cell exhaustion and T-cell exhaustion are examples of lymphocyte exhaustion, and they have several differences and similarities. Lymphocyte exhaustion is also frequently confused with anergy, cellular senescence and suppression, because these conditions can have significant overlapping similarities with exhaustion. An additional source of confusion is due to the fact that lymphocyte exhaustion is not a binary state, but instead has a spectrum of severity induced by different levels and duration of continuous antigenic stimulation. Concurrent multiple types of lymphocyte exhaustion are possible, and this situation is herein called poly-lymphocyte exhaustion. Poly-lymphocyte exhaustion for the same cancer or pathogen would be especially dangerous. As there are significant advantages for a pathogen by inducing poly-lymphocyte exhaustion in an immune system, there are pathogens with an evolved capability to induce poly-lymphocyte exhaustion. These pathogens may include certain manipulative viruses, bacteria, fungi and protozoan parasites.

KEYWORDS

B-cell exhaustion, latent Infections, NK-cell exhaustion, T-Cell Exhaustion, viral Infections

Abbreviations: ABCs, age-associated B cells; APO-1, apoptosis antigen-1; B cell, bursa of Fabricius, bone marrow-derived cell; BAFF, B-cell activating factor; BAFF-R, B-cell activating factor receptor; BCR, B-cell receptor; BTLA, B- and T-lymphocyte attenuator; CD, cluster of differentiation; CMV, cytomegalovirus; CTLA-4, cytotoxic T-lymphocyte antigen-4; DC, dendritic cell; DNA, deoxyribonucleic acid; DNAM, DNAX accessory molecule; DNAX, deoxyribonucleic acid X; EGR2, early growth response gene 2; EOMES, eomesodermin; FCRL4, Fc receptor-like-4; HIV, human immunodeficiency virus; IgG, immunoglobulin-G; Ig, immunoglobulin; IL-10, interleukin-10; IL-6, interleukin-6; ILT2, Ig-like transcript 2; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell immunoglobulin-like receptor; KLRG1, killer cell lectin-like receptor G1; LAG-3, lymphocyte activation gene-3; LAIR-1, leucocyte-associated Ig-like receptor-1; LAMP-1, lysosomal-associated membrane glycoprotein-1; LCMV, lymphocytic choriomeningitis virus; LILR, leucocyte immunoglobulin-like receptor; LIR, leucocyte immunoglobulin-like receptor; MDSC, myeloid-derived suppressor cell; NFAT, nuclear factor of activated T-cells; NK-cell, natural killer cell; p38 MAPK, p38 mitogen-activated protein kinase; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype; SIGLEC, sialic acid-binding Ig-like lectin; T-cell, thymus-derived cell; TACTILE, T-cell activation, increased late expression; TCR, T-cell receptor; TGF, transforming growth factor; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TIM-3, T-cell immunoglobulin and mucin domain-3; TLM, tissue-like memory; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α; TRAIL-R, TNF-related apoptosis-inducing ligand receptor.

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INTRODUCTION

T-cell exhaustion has been extensively investigated and discussed, but NK-cell exhaustion and B-cell exhaustion have received considerably less attention. There are significant differences and similarities between these various types of lymphocyte exhaustion. There can also be substantial and severe consequences. Some pathogens are capable of inducing various types of lymphocyte exhaustion during chronic and/or latent infections from which they can benefit. These pathogens include certain especially manipulative viruses, bacteria, fungi and protozoan parasites. What if two or more types of lymphocyte exhaustion occur? Actually, multiple lymphocyte exhaustion is possible and can result in a category of lymphocyte exhaustion, which is herein called 'poly-lymphocyte exhaustion'. Before discussing this further, lymphocyte exhaustion, anergy, cellular senescence and suppression will be distinguished, and then, NK-cell exhaustion, B-cell exhaustion and T-cell exhaustion characteristics will be discussed in more detail.

DISCUSSION

Lymphocyte exhaustion, anergy, cellular senescence and suppression

Lymphocyte exhaustion can be defined as the progressive loss of functionalities and a reduced proliferative capability triggered by chronic antigen stimulation due to chronic infections or cancers [1]. Anergy in lymphocytes is a functionally defined state of hyporesponsiveness in which lymphocytes do not proliferate and have defective production of major growth factors and/or inflammatory cytokines following an antigen encounter and subsequent receptor–ligand ligation [1]. Cellular senescence is a permanent G1 cell cycle arrest that restricts the life span of cells to control cell replication, as a result of oncogene activation, deoxyribonucleic acid (DNA) telomere degradation, epigenomic changes, or stressful stimuli including reactive oxygen species, ionizing radiation or certain chemicals [1]. Senescent cells remain viable, and most of them secrete several pro-inflammatory cytokines, chemokines, growth factors and proteases (proteinases), a state called senescence-associated secretory phenotype (SASP), which can have detrimental consequences [1]. Suppression of lymphocytes such as NK-cells is a cellular dysfunction variously caused by tissue hypoxia (reduced oxygen levels); neuroendocrine activation (e.g., cortisol and other glucocorticoids); a hypercoagulable blood state (e.g. increased tissue factor, fibrin, thrombin and platelet activation); pro-inflammatory prostaglandins (e.g. prostaglandin E2); anti-inflammatory phase factors interleukin-6 (IL-6), interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1); regulatory T-cells; and myeloid-derived suppressor cells (MDSCs). [2]

In summary, for NK-cell, B-cell and T-cell lymphocytes generally, exhaustion could be defined as an impaired state resulting from antigenic overstimulation, anergy could be defined as an impaired state resulting from inadequate stimulation of the activating receptors, senescence could be defined as an impaired state resulting from the ageing of the lymphocyte, and suppression could be defined as an impaired state resulting from the inhibiting actions of external non-antigenic factors.

Summary of NK-cell exhaustion

Natural killer (NK) cells are essential innate immune cells and share several characteristics and functions similar to the adaptive immune system's cytotoxic T-cells [3–5]. NK-cells, like T-cells, are lymphocytes that play a role in the recognition and elimination of cells infected by intracellular pathogens and the elimination of cancerous cells [6]. A variety of activating and inhibitory receptors are expressed on the NK-cell surface, and the balance and spatial–temporal integration of signals from these receptors determine the NK-cell effector functions [5]. As NK-cell functions are regulated by the integration of activating and inhibitory signals from cell surface receptors, reduced or weakened signals from activating receptors will lead to domination by inhibitory signals and a decreased NK-cell functionality. [6]

Functionally impaired NK-cells exhibit a decreased expression of the transient degranulation marker lysosomal-associated membrane glycoprotein-1 (LAMP-1) also called CD107a, a decreased production of the cytokines interferon-γ and tumour necrosis factor-α (TNF-α), and a decreased production of the cytotoxic secretions of perforin and granzymes [5]. Reduced effector functions and phenotypic changes have been observed in both NK-cells and T-cells, but in contrast to T-cell exhaustion, which is more clearly defined, no consensus has been reached on the definition of NK-cell exhaustion. [5]

Furthermore, conventional NK-cells lack antigen specificity and typically live less than 10 days in humans, and 7–10 days in mice [6]. Given this short life span and a lack of antigen specificity, conventional NK-cell exhaustion may be less important than other more common lymphocyte dysfunctions, such as anergy, cellular senescence or suppression. [6]

Adaptive long-lived NK-cells and their exhaustion

In addition to conventional NK-cells, there are also long-lived NK-cells, and it is known that long-lived NK-cells

can develop traits of adaptive immunity in certain circumstances including cytomegalovirus (CMV) infections [5]. In a remarkable contrast to conventional NK-cells, adaptive NK-cells are long-lived, possess memory recall and exhibit some degree of antigen specificity [6]. Furthermore, adaptive NK-cells can live for several months without detectable CMV and are stably maintained at elevated levels for years in some individuals. [6]

Phenotypic characteristics

Many, but not all, adaptive NK-cells express the lectin-like heterodimeric activating receptor CD94/NKG2C [6]. Their expansion can be driven through CD94/NKG2C interactions with CMV-encoded peptides, although adaptive NK-cells also arise in individuals who lack NKG2C [7,8]. Adaptive NK-cells have a distinct metabolism and exhibit an epigenetic signature closer to effector CD8+ T-cells than conventional NK-cells [8]. Because they are also long-lived and contribute to the control of pathogens such as CMV, adaptive NK-cells may become exhausted with chronic activation [6]. CMV exposure increases the number of NKG2C+ NK-cells that exhibit a skewed killer cell immunoglobulin-like receptor (KIR) profile having expressions of both inhibitory KIRs and activating KIRs. [9]

One group studied the occurrence of NK-cell exhaustion after chronic stimulation and proposed a paradigm of NK-cell exhaustion similar to T-cell exhaustion [4]. It was shown that prolonged cytokine exposures with IL-15 for more than five days, or following murine CMV infections in mice, stimulated sustained NK-cell proliferation and decreased expressions of the NK-cell proliferation marker Ki67, and decreased production of the cytokine interferon-γ and granzyme B. [4]

Master regulators of epigenetic reprogramming for exhaustion

It was also reported that tumour-induced NK-cell dysfunctions were caused by exhaustion due to substantial proliferation of NK-cells, in addition to a significant decrease in the activating T-box transcription factors eomesodermin (Eomes) and T-bet [10]. The expression of transcription factors Eomes and T-bet are important for T-cell and NK-cell maturation, differentiation, and functionality [11]. Low expression of these transcription factors has been found during both NK-cell exhaustion and senescence [4]. Decreased expression of Eomes and several activating receptors including NKG2D, and increased expressions of the inhibitory receptors NKG2A and killer cell lectin-like receptor G1 (KLRG1) were also observed during NK-cell exhaustion and impaired NK-cell functionality. [4]

Adaptive NK-cell exhaustion inhibitory receptors

In addition to NK-cell functional impairment, numerous studies have also shown that an imbalance in the expressions of NK-cell surface activating and inhibitory receptors indicates NK-cell exhaustion [12]. Inhibitory receptors include certain killer cell immunoglobulin receptors (KIRs), CD94/NKG2A and leucocyte immunoglobulin-like receptors (LIRs), which in turn inhibit NK-cell activation and functionalities [3]. Major activating receptors include the cytotoxic receptors NKG2D and DNAX accessory molecule (DNAM-1), whose activating signals are transmitted through receptors binding to immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor proteins [13]. The binding of the appropriate matching ligands with these NK surface receptors results in the transduction of either activating or inhibitory signals through various signalling pathways, which eventually converge on processes regulating NK-cell cytotoxicity, including degranulation and cytokine release. [13]

Increased expression of inhibitory receptors and decreased expression of activating receptors usually correspond to reduced NK-cell functionalities [5]. However, NK-cell suppression and NK-cell exhaustion overlap with different types of stimuli; therefore, it is unlikely that these states can be distinguished entirely by receptor expression levels [6]. Furthermore, because of the large number of receptors expressed, and as several receptors recognize the same ligands, there is no consensus on which of these receptors are exhaustion markers [5]. However, it is clear that the altered expression of multiple receptors is essential for NK-cell exhaustion, because single activating receptors, with the exception of CD16, are not able to individually activate NK-cells entirely by themselves. [13]

Inhibitory receptors that have been implicated in NK-cell exhaustion include programmed cell death protein 1 (PD-1), lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin and mucin domain-3 (TIM-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), and T-cell activation, increased late expression (TACTILE) also called CD96 [6]. PD-1 expression is a major receptor indicating T-cell exhaustion, and several researchers have also associated PD-1 expression with NK-cell exhaustion [3]. However, the expression of PD-1 by exhausted NK-cells is highly disputed, and other researchers have shown that the receptor TIGIT and to some degree the receptors TIM-3 and LAG-3 are more important indicators of NK-cell exhaustion. [3]

Furthermore, some studies of NK-cell dysfunction have shown little importance for the expression of PD-1 [3]. For example, a study in melanoma patients observed a correlation between peripheral NK-cell expressions of TIM-3 and the stage of melanoma, suggesting increased TIM-3 expression from cancer progression and an increased burden of disease-induced NK-cell exhaustion [14]. However, there was no observed difference in PD-1 or CTLA-4 expressions between melanoma patients and healthy controls [14]. Another study analysed NK-cells in 20 non-small-cell lung cancer patients and detected virtually no PD-1 expressions on the NK-cells [15]. In summary, some studies contradict any link between NK-cell exhaustion and PD-1 receptor expressions.

In the case of mice, studies of NK-cells from mouse tumours indicated that co-inhibitory receptor TIGIT is the critical marker for dysfunctional NK-cells [16]. In fact, TIGIT+NK-cells had decreased expressions of interferon-γ, tumour necrosis factor (TNF), LAMP-1 also called CD107a, and TNF-related apoptosis-inducing ligand receptor (TRAIL-R) consistent with reduced NK-cell functions, although research has also shown TIGIT expression on healthy NK-cells [17,18]. Ligand binding to TIGIT was associated with reduced NK-cell cytotoxicity, and blockade of the TIGIT receptor reversed NK-cell dysfunction with major antitumour effects in murine tumour experiments [16]. TIGIT+NK-cells may also be inhibited by TIGIT ligands expressed by cancer cells and myeloid-derived suppressor cells (MDSCs), thus indicating that TIGIT is a significant inhibitory receptor in multiple pathways [18]. However, it is yet to be proven whether TIGIT expression itself indicates intrinsically dysfunctional NK-cells, or whether ligand binding to TIGIT is required for inhibitory effects. [3]

Furthermore, some studies indicate that TIGIT expression levels on NK-cell surfaces are variable among healthy human individuals [17]. Although increased TIGIT expression on healthy human NK-cells is proportional to reduced cytotoxic capability, these NK-cells still mostly maintain their functionality [17]. Therefore, the increased expression of an inhibitory receptor, or decreased expression of an activating receptor, does not necessarily correspond to a severely dysfunctional NK-cell in the case of chronic infections and cancers [5]. In other words, the altered expression of a single receptor is unlikely to significantly alter NK-cell activation status, because multiple activating receptors and/or inhibitory receptors have to be reduced or increased in expression, respectively, to correspond to NK-cell exhaustion. [5]

In addition to decreased cytotoxicity and cytokine secretion, NK-cells from human individuals with malignancies or chronic viral infections often show a loss of activating receptors [6]. For example, NKG2D is an activating receptor that binds ligands produced as a result of cellular stress, and NKG2D has a reduced expression on NK-cells from patients with digestive cancers, chronic

TABLE 1 Features of NK-Cell Exhaustion

Feature	Function	Expression	Comments
TIGIT	Receptor	Increased [5,6]	Inhibitory receptor
LAG-3	Receptor	Increased [6]	Inhibitory receptor
TIM-3	Receptor	Increased [5]	Inhibitory receptor
PD-1	Receptor	Increased [5,6]	Inhibitory receptor, highly disputed re: NK-cells
NKG2A	Receptor	Increased [4–6]	Inhibitory receptor
KIRs	Receptor	Increased [12]	Inhibitory receptors, but other KIRS are activating
CD96	Receptor	Increased [6]	Inhibit/activate receptor, also called TACTILE
NKG2D	Receptor	Decreased [4,5,10]	Activating receptor
NKp30	Receptor	Decreased [5]	Activating receptor
NKp44	Receptor	Decreased [5]	Activating receptor, inhibitory with inhibit. ligand
NKp46	Receptor	Decreased [5,10]	Activating receptor
DNAM-1	Receptor	Decreased [5,12]	Activating receptor, also called CD226
CD16	Receptor	Decreased [6]	Activating receptor
CD49b	Receptor	Decreased [10]	Activating receptor
CD107a	Receptor	Decreased [5,17]	Degranulation marker also called LAMP−1
Interferon-γ	Cytokine	Decreased [5,6]	Important NK-cell secretion
TNF-α	Cytokine	Decreased [5]	Important NK-cell secretion
Perforin	Cytotoxin	Decreased [5]	Important NK-cell secretion
Granzymes	Cytotoxins	Decreased [5]	Important NK-cell secretions

lymphocytic leukaemia, breast cancer, chronic hepatitis B virus and hepatitis C virus [6]. CD16, another activating receptor that binds the Fc portion of antibodies, had reduced expressions on NK-cells from breast cancer patients. [6]

Table 1 lists various increased or decreased features of NK-cell exhaustion, but the list is not comprehensive.

Summary of B-cell exhaustion

B-cell exhaustion essentially refers to the inhibition of normal B-cell functions as a consequence of chronic diseases or chronic and/or latent pathogen infections [19–21]. This inhibition can be long-term, as in the case of viral infections, such as hepatitis C infections, even after the virus has departed, hypoproliferation and other aspects of B-cell exhaustion appeared to be durably genetically programmed into B-cells specific for hepatitis C antigens [22]. B-cell exhaustion from chronic infections has also sometimes been called B-cell anergy. [22]

B-cell exhaustion includes increased expression of multiple inhibitory receptors, impaired or unusual expressions of chemokine and adhesion ligands and receptors, and poor proliferative responses to a variety of antigen and lymphocyte stimuli [19,20]. Inhibitory receptors having immunoreceptor tyrosine-based inhibitory motifs (ITIMs) are variously expressed during B-cell and other lymphocyte activation and differentiation [21]. These receptors bind with distinct ligands and activate specific intracellular signalling pathways, and are essential for regulation of B-cells and other lymphocytes [21]. Although inhibitory receptors are critical for controlling the function of B-cells and other lymphocytes, their continuous and high expression can result in decreased cell function, anergy or exhaustion, which has been observed in chronic and/or latent pathogen infections and autoimmune diseases. [21]

Tissue-like memory B-cell exhaustion

B-cell exhaustion is linked to a weaker antibody response against pathogens [19,20]. Much of the research on B-cell exhaustion has been concerned with human immunodeficiency virus (HIV) infections, because of B-cell exhaustion observed in several HIV-infected patients. [19]

Phenotypic characteristics

A pathogenic hallmark for B-cells from HIV-1-infected individuals is the loss of CD21 expression on mature B-cells [23]. CD21, also known as complement receptor type 2 or the complement C3d receptor, has been shown to play an important role in activation/interaction of B-cells with HIV-1 [23]. HIV-1 particles interact directly with B-cells both in peripheral (circulating) blood and in lymph nodes of HIV-1-infected patients, and the HIV virus is bound to B-cells through the CD21 receptor with a fragment of complement C3 [23]. A population of CD27-/$CD21^{low}$ tissue-like memory (TLM) B-cells has been observed in the blood of HIV-infected individuals enriched with HIV-specific B-cells [19]. TLM B- cells, sometimes also called $CD19^{+}CD10^{-}CD27^{-}CD21^{low}$ B-cells, are numerically expanded during HIV-1 infections [23]. In addition, an accumulation of a functionally impaired subpopulation of $CD20^{high}CD27^{-}CD21^{low}$ TLM B-cells in the peripheral blood of HIV-infected patients is also likely induced by chronic B-cell activation. [21]

TLM B-cells display a lower proliferative capability compared with naive and classical memory B-cells, and exhibit a lower epitope diversity and a lower number of cell divisions [23]. B-cell exhaustion during HIV-1 infection was confirmed by blockade of inhibitory receptors, such as inhibitory receptor Fc receptor-like-4 (FCRL4) and sialic acid-binding Ig-like lectin 6 (Siglec-6), which led to higher B-cell proliferation and cytokine releases [21]. This result suggests chronic B-cell activation induces B-cell exhaustion [23]. In addition, the fortunate patients who could control HIV infections also had a high frequency of exhausted, TLM B cells during undetectable or low viral loads [24]. This suggests that the loss of CD21 expression is not caused exclusively by a high viral load and that the accumulation of dysfunctional $CD21^{low}$ B-cells may be linked to intrinsic infection-induced B-cell alterations. [24]

Furthermore, HIV-induced B-cell exhaustion includes unusual expression levels of chemokine and inhibitory receptors, and higher expressions of specific receptors, including the leucocyte immunoglobulin-like receptors (LILRs), such as the inhibitory receptor variously known as LIR-1/Ig-like transcript 2 (ILT2)/LILRB1/subfamily B (CD85j) [19,20]. In addition, there are significantly high expressions of the integrin alpha X adhesion receptor (CD11c), and certain chemokine receptors, including the CXCR3 and CCR6 receptors; and low expressions of the L-selectin receptor (CD62L), and certain chemokine receptors, including the CXCR4, CXCR5 and CCR7 receptors. [19]

Metabolic rewiring

HIV infections are linked to numerous phenotypic and functional B-cell abnormalities, including a decreased frequency of $CD27^{+}$ memory B-cells; an increase in immature/transitional B-cells; the increased expression of CD38, CD70, CD86 and CD95, which is also called Fas,

FasR, or apoptosis antigen-1 (APO-1); and decreased expressions of CD22 (Siglec-2), a sialic acid-binding Ig-like lectin; CD25; B-cell activating factor receptor (BAFF-R) and leucocyte-associated Ig-like receptor-1 (LAIR-1) also known as CD305 [24]. In addition, the overexpression of CD95 (Fas) has been repeatedly identified as a marker of B-cell activation that can lead to Fas/FasL-mediated apoptosis, which is also associated with a decrease in antigen-specific B-cell responses. [24]

In contrast to CD27+ classical memory B-cells or CD27-/CD21^{high} naive B-cells, a feature of these peripheral blood TLM B-cells is the increased expression of the inhibitory receptor Fc receptor-like-4 (FCRL4) [21]. Functional analyses of the ITIM-containing intracellular domain of FCRL4 indicates that FCRL4 has a profoundly negative regulatory effect on B-cell receptor (BCR) signalling by inhibiting BCR-mediated calcium mobilization, tyrosine phosphorylation of several intracellular proteins, and the activation of protein kinase pathways [21]. Increased expression of inhibitory receptor FCRL4 on TLM B-cells and, to a lesser degree, on classical memory B-cells in the blood is unique to HIV infections, which suggests that FCRL4 may be essential to B-cell inhibition [19]. Given its extensive inhibitory role, FCRL4 may be an important inhibitory receptor in B-cell exhaustion associated with chronic HIV infections. [21]

Besides higher expressions of FCRL4, TLM B-cells also have increased expressions of other ITIM-bearing receptors that serve as negative regulators of BCR-mediated activation [21]. These include FcγRIIB (CD32b), a low-affinity receptor for the antibody immunoglobulin G (IgG); CD22; CD85d of the leucocyte Ig-like receptor (LILR) family; and other B-cell inhibitory receptors, such as CD72, LAIR-1 and the programmed cell death 1 (PD-1) receptor [21]. The increased expression on TLM B-cells of these inhibitory receptors for ligand binding may also contribute to impaired B-cell proliferation and functionality, including inhibited HIV-specific antibody responses [21]. Furthermore, additional research has shown that chronic antigenic stimulation or T-cell-independent B-cell activation concurrent with elevated B-cell activating factor (BAFF) levels can also contribute to B-cell exhaustion [19,25].

In summary, HIV-induced B-cell exhaustion includes increased expressions of multiple inhibitory receptors, reduced proliferative potential, stunted replication history and reduced immunoglobulin diversity [19].

B-cell exhaustion can also be caused by non-viral pathogens

B-cell exhaustion is not limited only to chronic viral infections. B-cell exhaustion characteristics have also been found in the blood of individuals living in mosquito-infected areas having the protozoan parasite *Plasmodium falciparum* (which causes malaria) [26]. FCRL4, the cell surface marker that defines TLM B- cells and is characteristic of exhausted memory B-cells, was expressed at significantly higher levels on atypical memory B-cells compared with classic memory B-cells and naive B-cells [26]. The expressions of inhibitory and homing receptors on atypical memory B-cells, with exceptions, were comparable to those on the TLM B-cells observed in HIV-infected individuals [26]. Atypical memory B-cells showed increased expressions of the receptors CD85j, CD22, CD11c and CXCR3; CXCR4, CD62L, CXCR5 and CCR7 expressions were decreased, and little difference was observed in the expressions of CCR6 or CD72 [26].

Atypical memory B-cells have been observed in both mice and humans

Atypical memory B-cells can be classified by the expression of T-bet, although heterogeneity exists even within this B-cell subset [27]. CD11c is a reliable marker for this subset as nearly all CD11c$^+$ B-cells express T-bet; however, a large percentage of CD11c$^-$T-bet$^+$ B-cells can also arise during infections and increased cellular age [27]. These observations about CD11c also extend to human T-bet$^+$ B-cells. [27]

The atypical memory B-cells were once considered abnormal, but recent studies found that T-bet$^+$ B-cells are normal components of the human immune system, and increased populations can be induced by certain intracellular pathogens [27]. CD21$^-$T-bet$^+$ B-cells are a small subset present in the circulating blood of healthy individuals, and vaccinations with live influenza virus vaccines cause an expansion of these cells in circulating blood [27]. T-bet$^+$ B-cells are generated by and essential to respond to intracellular infections, and their populations are transiently increased during acute HIV, influenza and Ebola virus infections; and elevated populations are maintained during HIV, hepatitis C virus infections, malaria and tuberculosis infections, implying that T-bet$^+$ B-cells are fundamentally involved in the Th1-type immune response. [27]

T-bet$^+$ B-cells are also characterized by reduced expressions of CD21, a receptor expressed by pre-immune and resting memory B-cells, and variable expressions of CD27, a receptor normally associated with antigen experience; CD27 expression on T-bet$^+$ B-cells is particularly low during chronic human infections [27]. Atypical B-cells have low to undetectable levels of the surface markers CD23 and CD21, but it is now understood that this phenotype includes some additional subsets that do not express T-bet and which are unrelated to the T-bet$^+$ subset [27].

Recent studies suggest that B-cells expressing T-bet are the specific subset expanded in intracellular infections, autoimmune diseases and cellular ageing. [27]

T-bet$^+$ B-cell subsets have been seen in both mice and humans in primary and secondary humoral immune responses, and T-bet$^+$ B-cells observed in cases of murine ageing were sometimes called 'age-associated B-cells' (ABCs) [28,29]. ABCs do not proliferate from B-cell receptor activation, but instead proliferate in response to endosomal Toll-like receptor (TLR) signals, particularly from TLR7 and TLR9, and with increased T-bet expression, when activated they usually release antibodies of the immunoglobulin G-2 (IgG_2) isotype. [29]

Table 2 lists various increased or decreased surface features of B-cell exhaustion, but the list is not comprehensive.

Summary of T-cell exhaustion

T-cell exhaustion, anergy, senescence and suppression can be very difficult to distinguish, and a summary of their differences would clarify the discussion of T-cell exhaustion.

T-cell anergy, senescence, suppression and exhaustion

T-cell anergy is a functional state of hyporesponsiveness in which T-cells do not proliferate or produce the T-cell growth factor IL-2 after appropriate ligation of an antigen and T-cell receptor (TCR) [1]. Another characteristic of anergic CD4$^+$ and CD8$^+$ T-cells is an impaired production of inflammatory cytokines, including interferon-γ and TNF-α [1]. Although there are no definitive surface markers indicating anergic T-cells, epigenetic factors such as histone modifications, and the transcription factor early growth response gene 2 (Egr2) help establish and maintain T-cell anergy [1].

Decreased expressions of receptors CD27 and CD28, and higher expressions of receptors CD57 and killer cell lectin-like receptor subfamily G (KLRG1), usually indicate

TABLE 2 - Features of B-cell exhaustion

Feature	Function	Expression	Comments
PD-1	Receptor	Increased [21]	Inhibitory receptor
FCRL4	Receptor	Increased [21]	Inhibitory receptor
Siglec-6	Receptor	Increased [21]	Inhibitory receptor
LAIR-1	Receptor	Disputed [21,24][a]	Inhibitory receptor, called CD305
LILR	Receptor	Increased [19,20]	Inhibitory receptor
CD32b	Receptor	Increased [21]	Inhibitory receptor for IgG, called FcγRIIB
CD38	Receptor	Increased [24]	Inhibitory receptor
CD70	Receptor	Increased [24]	Inhibitory receptor
CD72	Receptor	Increased [21][a]	Inhibitory receptor
CD85d	Receptor	Increased [21]	Also called ILT4 or LILRB2
CD85j	Receptor	Increased [19,20]	Also called LIR−1, ILT2 or LILRB1
CD86	Receptor	Increased [24]	Inhibitory receptor
CD95	Receptor	Increased [24]	Receptor, also called Fas, FasR or APO−1
CD11c	Receptor	Increased [19]	Adhesion receptor, also called integrin alpha X
CXCR3	Receptor	Increased [19]	Chemokine receptor
CXCR4	Receptor	Decreased [19]	Chemokine receptor
CXCR5	Receptor	Decreased [19]	Chemokine receptor
BAFF-R	Receptor	Decreased [24]	BAFF means B-cell activating factor
CCR6	Receptor	Decreased [19][a]	Chemokine receptor
CCR7	Receptor	Decreased [26]	Chemokine receptor
CD21	Receptor	Decreased [21,23]	Activating receptor
CD22	Receptor	Decreased [24] [a]	Activating, also called Siglec−2
CD25	Receptor	Decreased [24]	Activating receptor
CD62L	Receptor	Decreased [19]	Cell adhesion receptor, also called L-selectin

[a]No change or a decrease or increase may be seen in atypical memory B-cells.

CD4 and CD8 T-cell senescence, but there are no definitive surface markers [30]. T-cell senescence has been defined as a state that is maintained by internal cell stress signalling by increased p38 mitogen-activated protein kinase (p38 MAPK), resulting from DNA damage, reactive oxygen species (ROS) activation or other stresses [30]. Senescent T-cells secrete a variety of generally pro-inflammatory factors known as the 'senescence-associated secretory phenotype' (SASP), which negatively impact multiple organs and contribute to age-associated inflammation, a state called 'inflamm-ageing' [1,30].

There are arguments that replicative cell senescence from telomere loss does not apply to lymphocytes, because unlike most somatic cells, lymphocytes during activation can reactivate telomerase expression, and this was demonstrated in human and murine T-cells and B-cells after specific (antigen) or non-specific stimulation (e.g. mitosis inducing anti-CD3 antibodies or lectins) [31,32]. Elevated telomerase activity may support the survival, proliferation and expansion of antigen responses in vivo, but lymphocytes still have a limited life span because their telomerase activity levels are insufficient to totally stop telomere loss [31]. Furthermore, resting human T-cells have little or no telomerase activity [31].

T-cell suppression has frequently been used interchangeably with T-cell exhaustion, causing considerable confusion. T-cell suppression with decreased T-cell functionality can be caused by multiple factors, including the influence of cytokines including IL-10 from dendritic cells and exhausted CD4 T-cells, interferon-α and interferon-β, and TGF-β secreted by regulatory T-cells or myeloid-derived suppressor cells [33,34]. Furthermore, because CD8 T-cells share several lymphocyte characteristics with NK-cells, T-cell suppression is very probable when there is tissue hypoxia (reduced oxygen); neuroendocrine activation; a hypercoagulable state (e.g. increased tissue factor, fibrin, thrombin); pro-inflammatory prostaglandins (e.g. prostaglandin E2); and anti-inflammatory phase factors and interleukin-6 (IL-6), just as in the case of NK-cells [15].

T-cell exhaustion characteristics

T-cell exhaustion is defined by reduced functionality, but is recoverable by manipulating extrinsic regulatory pathways, such as by checkpoint receptor blockades [30]. Exhausted T-cells are physiologically intact and result from chronic infections or cancer where chronic antigenic stimulation from a source that cannot be cleared prevents many of the responding T-cells from reverting to quiescent memory cells [30]. A heightened inflammatory status (i.e. a certain cytokine/chemokine environment together with chronic antigen exposure) may render T-cells exhausted, especially in the absence of CD4+ T-cell help [30].

Master regulators of epigenetic reprogramming for T-cell exhaustion

T-cell exhaustion, driven by chronic T-cell receptor stimulation and the nuclear factor of activated T-cells (NFAT), also affects T-cells transcriptionally and epigenetically, because the nuclear factor TOX also seems to play a central role in priming CD8 T-cells for exhaustion at the transcriptional and epigenetic levels [1].

Phenotypic characteristics of T-cell exhaustion

T-cell exhaustion exhibits a decreased production of effector cytokines, including IL-2, interferon-γ or TNF, abnormal expressions of certain chemokines, and a continuous and increased expression of multiple inhibitory receptors, which will be discussed in more detail [1]. T-cell exhaustion results from long-duration antigen exposures and continuous inflammation, and these long-duration conditions can result from several diseases and infections by chronic and/or latent pathogens [35–39].

If antigen exposures are chronic and continuous for over two to four weeks, T-cell exhaustion is possible [40,41]. T-cell exhaustion is induced even with continuous low levels of antigens, and T-cell exhaustion can include metabolic exhaustion and mitochondrial dysfunctions in the T-cells, which can reduce their proliferation and functionalities and possibly reduce these T-cells numerically [40,41]. T-cell exhaustion severity is worsened by increased antigen abundance (titre) and/or by longer duration of antigen levels [35–39]. T-cell exhaustion can be induced by latent infections of several protozoan, fungal, viral or bacterial pathogens, including *Toxoplasma gondii*, [37] and hepatitis B or C virus [42–44]. Inflammation is also possible during any protozoan, fungal, viral and bacterial pathogen infection from the production of several inflammatory cytokines [45].

T-cell exhaustion is induced by cancers or chronic and/or latent pathogen infections that cause long-duration inflammation and antigen stimulation of T-cells with significant antigen titres [35–37]. However, if any of these conditions are not met, as for short or very recent pathogen infections, or when pathogen infections produce very little inflammation or a very low antigen titre, then T-cell exhaustion will be unlikely [37]. Furthermore, if a mild genetic strain of a pathogen, or a prompt and effective

drug treatment of an active pathogen infection can avoid T-cell exhaustion, then sometimes a latent pathogen infection can induce CD4 T-cells and CD8 T-cells to produce increased amounts of interferon-γ that stimulates an improved immune response to later infections [37,46]. In summary, T-cell exhaustion may or may not happen depending on the genetic strain of a pathogen, the duration of infection and timely drug treatments, for infections involving viral, bacterial, fungal or protozoan parasite pathogens [37].

T-cell exhaustion affects CD4 T-cells and CD8 T-cells

T-cell exhaustion has major impacts on pathogen suppression involving CD4 T-cells and CD8 T-cells [36,41,47]. Several T-cell receptors participate in T-cell exhaustion, including costimulatory receptors and inhibitory receptors [36]. An increased number of multiple inhibitory receptors expressed correspond to an increased level of T-cell exhaustion [41]. The increased T-cell inhibitory receptors include the programmed cell death protein 1 (PD-1), lymphocyte activation gene-3 protein (LAG-3), B- and T-lymphocyte attenuator (BTLA), CD244, CD160, T-cell immunoglobulin domain and mucin domain-containing protein 3 (TIM-3), and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) [39,41,48]. The expression levels of the various inhibitory receptors increase with the increase in T-cell exhaustion; for example, severe T-cell exhaustion from chronic viral infections often corresponds to high levels of expression of the inhibitory TIM-3 receptor on T-cells. [48]

It should be noted that several inhibitory receptors are also expressed to some degree during the differentiation or activation of T-cells and that expression during active infections does not necessarily correspond to T-cell exhaustion caused by chronic infections [49]. It is known that during acute infections, cytokines affect T-cells and can significantly increase T-cell expression of inhibitory receptors in as little as 24 h, and certainly increase their expression within 48 to 72 h [49]. A primary role for the cytokine IL-27 has been proposed, at least in mice, because it is a major driver of IL-10 production and was observed to act as a master controller of several negative regulatory surface receptors, such as PD-1, TIM-3, LAG-3 and TIGIT. [30]

T-cell exhaustion can be reversed and/or prevented by blockades of various costimulatory and inhibitory T-cell receptors, such as a blockade of the interleukin-10 receptor, a blockade of the PD-1 receptor on CD8 T-cells, and a blockade of type I interferon (interferon-α and interferon-β) signalling to CD4 T-cells [41,46]. While the type I interferon-α and interferon-β are critical antiviral cytokines in the early phase of infection for CD8 T-cell activation and differentiation, their long-duration presence can induce CD4 T-cell exhaustion [36,37,47]. CD4 T-cell exhaustion also increases CD8 T-cell exhaustion, because the absence of an interleukin-21 signal that is normally secreted by CD4 T-cells will directly increase CD8 T-cell exhaustion [36,37,47]. In addition, the loss of CD4 T-cell functions from CD4 T-cell exhaustion will decrease the levels of interferon-γ, which is an important cytokine that controls acute and chronic intracellular infections by both viral and protozoan parasite infections. [37,50]

Particularly manipulative pathogens have evolved considerable skills for inducing T-cell exhaustion. Chronic infections with *T. gondii* have induced T-cells to have higher expressions of inhibitory PD-1 receptors and increased expressions of programmed cell death ligand 1 (PD-L1) by *T. gondii*-infected cells. [51]

T_{REG} cells also appear to have a role in T-cell exhaustion and T-cell suppression, including the maintenance of CD8 T-cell exhaustion [41,48]. T_{REG} cells can inhibit antigen-specific T-cells by the secretion of inhibitory cytokines including IL-10, interleukin-35 (IL-35) and TGF-β, and by the use of their IL-2 receptors (CD25) to act as a IL-2 sink to reduce extracellular IL-2 and thereby reduce T-cell proliferation [48,52]. Studies of lymphocytic choriomeningitis virus (LCMV) infections in mice and HIV infections in humans have proved that several immune cells can produce IL-10 during chronic infections, such as dendritic cells (DCs), monocytes and/or CD4 T-cells [41]. Concurrent blockade of IL-10 receptors and inhibitory PD-1 receptors in mice reverses CD8 T-cell exhaustion and improves control of viral infections in mice, which suggests IL-10 has a role in controlling CD8 T-cell exhaustion. [41]

Table 3 lists some features that have been linked to T-cell exhaustion, but the list is not comprehensive.

Table 4 compares causes, features and secretions that have been linked to B-cell, NK-cell, and T-cell exhaustion, but the list is not comprehensive.

Poly-lymphocyte exhaustion

B-cell exhaustion, NK-cell exhaustion and T-cell exhaustion have previously been discussed in isolation, where each type of lymphocyte exhaustion cripples each lymphocyte's essential roles in immune system defence. Can exhaustion of two or three of these lymphocyte types occur concurrently? As sufficient chronic and continuous antigen stimulation can cause any of the three types of lymphocyte exhaustion, multiple concurrent lymphocyte exhaustion is possible, and this novel category

TABLE 3 Features of T-cell exhaustion

Feature	Function	Expression	Comments
TIGIT	Receptor	Increased [39]	Inhibitory receptor
LAG-3	Receptor	Increased [41,48]	Inhibitory receptor
TIM-3	Receptor	Increased [41,48]	Inhibitory receptor
PD-1	Receptor	Increased [41,48]	Inhibitory receptor
BTLA	Receptor	Increased [39,48]	Inhibitory receptor, B- and T-lymphocyte attenuator
CD160	Receptor	Increased [41,48]	Inhibitory receptor
CD244/2B4	Receptor	Increased [41,48]	Inhibitory receptor, also called 2B4
CTLA-4	Receptor	Increased [41,48]	Inhibitory receptor
Interferon-α	Receptor	Increased? [41,46]	Inhibitory receptor role after extended time[a]
Interferon-β	Receptor	Increased? [41,46]	Inhibitory receptor role after extended time[a]
IL-2	Cytokine	Decreased [1,38]	Important T-cell secretion
Interferon-γ	Cytokine	Decreased [39,41]	Important T-cell secretion
TNF-α	Cytokine	Decreased [39,41]	Important T-cell secretion
Perforin	Cytotoxin	Decreased [35,38]	Important T-cell secretion
Granzyme	Cytotoxin	Decreased [35,38]	Important T-cell secretion

[a]The interferon-α and interferon-β receptors after long stimulation can cause T-cell exhaustion. It is not certain whether these receptors also have an increased expression on exhausted T-cells.

of lymphocyte exhaustion will herein be called 'poly-lymphocyte exhaustion'. The simplest and most common example of poly-lymphocyte exhaustion would be caused by two different cancers or pathogens, where each type of cancer or pathogen induces one type of lymphocyte exhaustion.

The more interesting type of poly-lymphocyte exhaustion occurs when only one type of cancer or pathogen induces poly-lymphocyte exhaustion. As B-cells, NK- cells and T-cells play complementary roles for an integrated and effective immune defence, poly-lymphocyte exhaustion affecting two or even three of these lymphocytes is particularly important. For example, NK-cell exhaustion and T-cell exhaustion have been separately linked to cytomegalovirus (CMV) infections, so the components of this type of poly-lymphocyte exhaustion have already been observed [11,13,37]. In addition, concurrent B-cell exhaustion and T-cell exhaustion have been reported in cases of the dangerous protozoan parasite *Plasmodium falciparum*. [53] Poly-lymphocyte exhaustion may also be possible for *Toxoplasma gondii*, [37] hepatitis B or C virus, and other manipulative pathogens [42–44]. The implications of poly-lymphocyte exhaustion regarding the same cancer or pathogen are significant, enabling cancers or pathogen infections by viruses, protozoan parasites, bacteria or fungi to overwhelm most or all lymphocyte defences. As poly-lymphocyte exhaustion would be very helpful to a pathogen's survival, it is likely that several pathogens have already evolved such a capability against mammalian immune systems.

Figure 1 is a conceptual diagram of the simplest poly-lymphocyte exhaustion induced when the lymphocyte receptors and inhibitory receptors of two different types of lymphocytes are activated by two separate host cells, one host cell infected by a first intracellular pathogen and a second host cell infected by a second intracellular pathogen. The second type of lymphocyte is drawn in dotted lines to indicate it would normally not be concurrently present with the first type of lymphocyte. This is a basic example of poly-lymphocyte exhaustion induced by a combination of two different types of pathogens.

Figure 2 is a simplified conceptual diagram of poly-lymphocyte exhaustion induced when the lymphocyte receptors and inhibitory receptors of two different types of lymphocytes are activated by a host cell infected by an intracellular pathogen. The first type of lymphocyte is drawn in dotted lines to indicate it would not typically be concurrently present with the second type of lymphocyte.

CONCLUSION

B-cell exhaustion, NK-cell exhaustion and T-cell exhaustion are examples of lymphocyte exhaustion, and they have several differences and similarities. Lymphocyte exhaustion is also frequently confused with anergy, cellular senescence and suppression, because these lymphocyte states can have significant overlapping similarities with exhaustion. An additional source of confusion is also caused by the fact that lymphocyte

TABLE 4 Comparing B-cell, NK-cell and T-cell exhaustion causes, features and secretions

Causes	B-cell exhaustion	NK-cell exhaustion	T-cell exhaustion
Chronic antigen initiation	Induced	Induced	Induced
Longer duration effects	Increased	Increased	Increased
Higher antigen level effects	Increased	Increased	Increased
Features	**B-cell exhaustion**	**NK-cell exhaustion**	**T-cell exhaustion**
TIGIT inhibitory receptor		Increased	Increased
LAG-3 inhibitory receptor		Increased	Increased
TIM-3 inhibitory receptor		Increased	Increased
PD-1 inhibitory receptor	Increased	Increased/Disputed	Increased
BTLA inhibitory receptor			Increased
CD160 inhibitory receptor			Increased
CD244 inhibitory receptor			Increased
CTLA4 inhibitory receptor			Increased
Interferon-α inhibitory receptor[a]			Increased?
Interferon-β inhibitory receptor[a]			Increased?
NKG2A inhibitory receptor		Increased	
KIRs inhibitory receptors		Increased	
CD96 inhibit/activate receptor		Increased	
NKG2D activating receptor		Decreased	
DNAM-1 activating receptor		Decreased	
CD16 activating receptor		Decreased	
CD107a degranulation marker		Decreased	
FCRL4 inhibitory receptor	Increased		
Siglec-6 inhibitory receptor	Increased		
LAIR-1 inhibitory receptor	Increased[b]		
LILR inhibitory receptor	Increased		
CD32b inhibitory receptor	Increased		
CD38 inhibitory receptor	Increased		
CD70 inhibitory receptor	Increased		
CD72 inhibitory receptor	Increased[b]		
CD85d inhibitory receptor	Increased		
CD85j inhibitory receptor	Increased		
CD86 inhibitory receptor	Increased		
CD95 inhibitory/death receptor	Increased		
CD11c adhesion receptor	Increased		
CXCR3 chemokine receptor	Increased		
CXCR4 chemokine receptor	Decreased		
CXCR5 chemokine receptor	Decreased		
BAFF-R receptor	Decreased		
CCR6 chemokine receptor	Decreased[b]		
CCR7 chemokine receptor	Decreased		
CD21 activating receptor	Decreased		
CD22 activating receptor	Decreased[b]		
CD25 activating receptor	Decreased		

(Continues)

TABLE 4 (Continued)

Features	B-cell exhaustion	NK-cell exhaustion	T-cell exhaustion
CD62L adhesion receptor	Decreased		
Secretions	**B-cell exhaustion**	**NK-cell exhaustion**	**T-cell exhaustion**
Interferon-γ (cytokine)		Decreased	Decreased
TNF-α (cytokine)		Decreased	Decreased
Perforin (cytotoxin)		Decreased	Decreased
Granzymes (cytotoxins)		Decreased	Decreased

[a]The interferon α and β receptors after long stimulation can cause T-cell exhaustion. It is not certain if these receptors also have an increased expression on exhausted T-cells.

[b]No change or a decrease or increase may be seen in atypical memory B-cells.

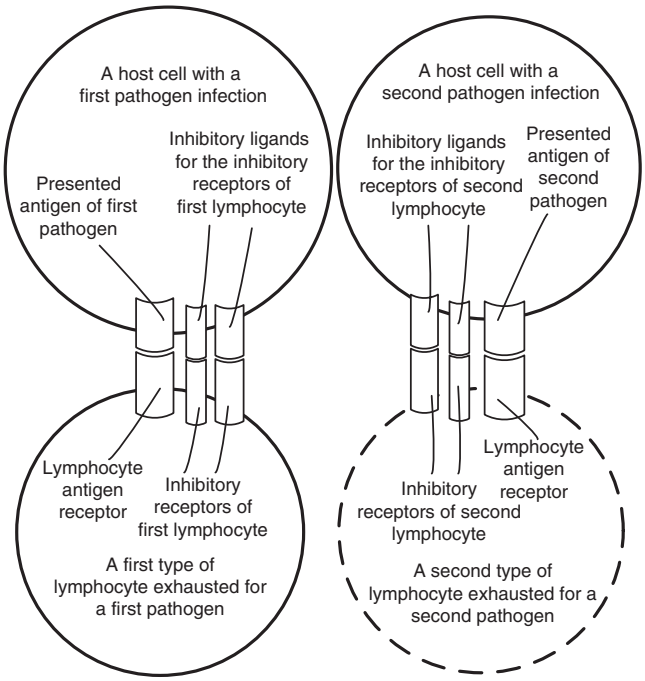

FIGURE 1 Conceptual diagram of the simplest poly-lymphocyte exhaustion induced when the lymphocyte receptors and inhibitory receptors of two different types of lymphocytes are activated by two separate host cells, one host cell infected by a first intracellular pathogen and a second host cell infected by a second intracellular pathogen

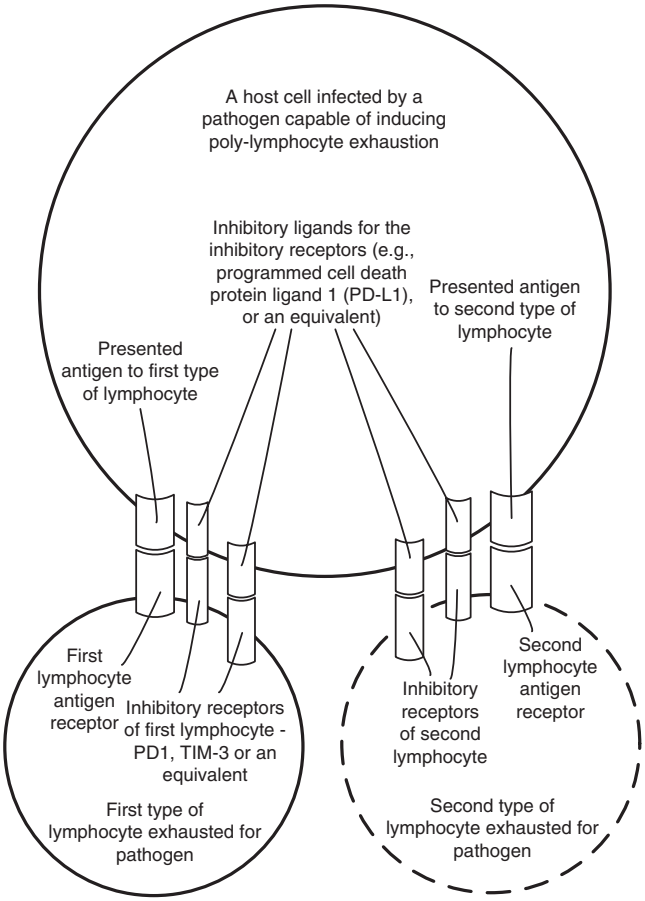

FIGURE 2 Simplified conceptual diagram of poly-lymphocyte exhaustion induced when the lymphocyte receptors and inhibitory receptors of two different types of lymphocytes are activated by a host cell infected by an intracellular pathogen

exhaustion is not a binary state, but instead has a spectrum of severity induced by various levels of continuous antigenic stimulation and duration. T-cell exhaustion has been researched more than B-cell exhaustion and NK-cell exhaustion, and there is less of a consensus on the precise definitions of B-cell and NK-cell exhaustion. In summary, for the B-cell, NK-cell and T-cell lymphocytes generally, exhaustion could be defined as an impaired state resulting from antigenic overstimulation, anergy could be defined as an impaired state resulting from inadequate stimulation of the activating receptors, senescence could be defined as an impaired state resulting from the ageing of the lymphocyte, and suppression could be defined as an impaired state resulting from the inhibiting actions of external non-antigenic factors. Concurrent multiple types of lymphocyte exhaustion are possible, and this situation is herein called poly-lymphocyte exhaustion. As there would be several advantages for a pathogen against an immune system

impaired by poly-lymphocyte exhaustion, there are probably several pathogens with an evolved capability for inducing poly-lymphocyte exhaustion.

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REFERENCES

1. Kaiser M, Semeraro MD, Herrmann M, Absenger G, Gerger A, Renner W. Immune aging and immunotherapy in cancer. Int J Mol Sci. 2021;22:7016. https://doi.org/10.3390/ijms22137016
2. Market M, Tennakoon G, Auer RC. Postoperative natural killer cell dysfunction: The prime suspect in the case of metastasis following curative cancer surgery. Int J Mol Sci. 2021;22:11378. https://doi.org/10.3390/ijms222111378
3. Judge SJ, Murphy WJ, Canter RJ. Characterizing the dysfunctional NK cell: Assessing the clinical relevance of exhaustion, anergy, and senescence. Front Cell Infect Microbiol. 2020;10:49. https://doi.org/10.3389/fcimb.2020.00049
4. Alvarez M, Simonetta F, Baker J, Pierini A, Wenokur AS, Morrison AR, et al. Regulation of murine NK cell exhaustion through the activation of the DNA damage repair pathway. JCI Insight. 2019;5:e127729. https://doi.org/10.1172/jci.insight.127729
5. Lee EHC, Wong DCP, Ding JL. NK cells in a *Tug-of-War* with cancer: The roles of transcription factors and cytoskeleton. Front Immunol. 2021;12: 734551. https://doi.org/10.3389/fimmu.2021.734551
6. Merino AM, Kim H, Miller JS, Cichocki F. Unraveling exhaustion in adaptive and conventional NK cells. J Leukoc Biol. 2020;108:1361–8. https://doi.org/10.1002/JLB.4MR0620-091R
7. Hammer Q, Rückert T, Borst EM, Dunst J, Haubner A, Durek P, et al. Peptide-specific recognition of human cytomegalovirus strains controls adaptive natural killer cells. Nat Immunol. 2018;19:453–63. https://doi.org/10.1038/s41590-018-0082-6
8. Liu LL, Landskron J, Ask EH, Enqvist M, Sohlberg E, Traherne JA, et al. Critical role of CD2 co-stimulation in adaptive natural killer cell responses revealed in NKG2C-deficient humans. Cell Rep. 2016;15:1088–99. https://doi.org/10.1016/j.celrep.2016.04.005
9. Béziat V, Liu LL, Malmberg JA, Ivarsson MA, Sohlberg E, Björklund AT, et al. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. Blood. 2013;121:2678–88. https://doi.org/10.1182/blood-2012-10-459545
10. Gill S, Vasey AE, De Souza A, Baker J, Smith AT, Kohrt HE, et al. Rapid development of exhaustion and down-regulation of eomesodermin limit the antitumor activity of adoptively transferred murine natural killer cells. Blood. 2012;119:5758–68. https://doi.org/10.1182/blood-2012-03-415364
11. Simonetta F, Pradier A, Roosnek E. T-bet and eomesodermin in NK cell development, maturation, and function. Front Immunol. 2016;7:241.
12. Bi J, Tian Z. NK cell dysfunction and checkpoint immunotherapy. Front Immunol. 2019;10:1999. https://doi.org/10.3389/fimmu.2019.01999
13. Chen Y, Lu D, Churov A, Fu R. Research progress on NK cell receptors and their signaling pathways. Mediators Inflamm. 2020;2020:6437057. https://doi.org/10.1155/2020/6437057
14. da Silva IP, Gallois A, Jimenez-Baranda S, Khan S, Anderson AC, Kuchroo VK, et al. Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. Cancer Immunol Res. 2014;2:410–22. https://doi.org/10.1158/2326-6066.CIR-13-0171
15. Datar I, Sanmamed MF, Wang J, Henick BS, Choi J, Badri T, et al. Expression analysis and significance of PD-1, LAG-3, and TIM-3 in human non-small cell lung cancer using spatially resolved and multiparametric single-cell analysis. Clin Cancer Res. 2019;25:4663–73. https://doi.org/10.1158/1078-0432.CCR-18-4142
16. Zhang Q, Bi J, Zheng X, Chen Y, Wang H, Wu W, et al. Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity. Nat Immunol. 2018;19:723–32. https://doi.org/10.1038/s41590-018-0132-0
17. Wang F, Hou H, Wu S, Tang Q, Liu W, Huang M, et al. TIGIT expression levels on human NK cells correlate with functional heterogeneity among healthy individuals. Eur J Immunol. 2015;45:2886–97. https://doi.org/10.1002/eji.201545480
18. Sarhan D, Cichocki F, Zhang B, Yingst A, Spellman SR, Cooley S, et al. Adaptive NK cells with low TIGIT expression are inherently resistant to myeloid-derived suppressor cells. Cancer Res. 2016;76:5696–706. https://doi.org/10.1158/0008-5472.CAN-16-0839
19. Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. J Exp Med. 2008;205:1797–805. https://doi.org/10.1084/jem.20072683
20. Khoder A, Alsuliman A, Basar R, Sobieski C, Kondo K, Alousi AM, et al. Evidence for B cell exhaustion in chronic graft-versus-host disease. Front Immunol. 2018;8:1937. https://doi.org/10.3389/fimmu.2017.01937
21. Kardava L, Moir S, Wang W, Ho J, Buckner CM, Posada JG, et al. Attenuation of HIV-associated human B cell exhaustion by siRNA downregulation of inhibitory receptors. J Clin Invest. 2011;121:2614–24. https://doi.org/10.1172/JCI45685
22. Kaplan DE. Persistence of exhaustion in cured hep C. Blood. 2017;130:4. https://doi.org/10.1182/blood-2017-05-786368
23. Amu S, Ruffin N, Rethi B, Chiodi F. Impairment of B-cell functions during HIV-1 infection. AIDS. 2013;27:2323–34. https://doi.org/10.1097/QAD.0b013e328361a427
24. Sciaranghella G, Tong N, Mahan AE, Suscovich TJ, Alter G. Decoupling activation and exhaustion of B-cells in spontaneous controllers of HIV infection. AIDS. 2013;27:175–80. https://doi.org/10.1097/QAD.0b013e32835bd1f0
25. He B, Qiao X, Klasse PJ, Chiu A, Chadburn A, Knowles DM, et al. HIV-1 envelope triggers polyclonal Ig class switch recombination through a CD40-independent mechanism involving BAFF and C-type lectin receptors. J Immunol. 2006;176:3931–41. https://doi.org/10.4049/jimmunol.176.7.3931
26. Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, Traore B, et al. Atypical memory B-cells are greatly expanded in individuals living in a malaria-endemic area. J Immunol. 2009;183:2176–82. https://doi.org/10.4049/jimmunol.0901297
27. Knox JJ, Myles A, Cancro MP. T-bet^{+} memory B cells: Generation, function, and fate. Immunol Rev. 2019;288:149–60. https://doi.org/10.1111/imr.12736

28. Johnson JL, Rosenthal RL, Knox JJ, Myles A, Naradikian MS, Madej J, et al. The Transcription factor T-bet resolves memory B cell subsets with distinct tissue distributions and antibody specificities in mice and humans. Immunity. 2020;52:842–855. e6. https://doi.org/10.1016/j.immuni.2020.03.020
29. Myles A, Gearhart PJ, Cancro MP. Signals that drive T-bet expression in B cells. Cell Immunol. 2017;321:3–7. https://doi.org/10.1016/j.cellimm.2017.09.004
30. Pawelec G. Is there a positive side to T cell exhaustion? Front Immunol. 2019;10:111. https://doi.org/10.3389/fimmu.2019.00111
31. Plyasova AA, Zhdanov DD. Alternative splicing of human telomerase reverse transcriptase (hTERT) and its implications in physiological and pathological processes. Biomedicines. 2021;9:526. https://doi.org/10.3390/biomedicines9050526
32. Patrick M, Weng NP. Expression and regulation of telomerase in human T cell differentiation, activation, aging and diseases. Cell Immunol. 2019;345:103989. https://doi.org/10.1016/j.cellimm.2019.103989
33. Bourhis J, Stein A, Paul de Boer J, Van Den Eynde M, Gold KA, Stintzing S, et al. Avelumab and cetuximab as a therapeutic combination: An overview of scientific rationale and current clinical trials in cancer. Cancer Treat Rev. 2021;97:102172. https://doi.org/10.1016/j.ctrv.2021.102172
34. Studstill CJ, Hahm B. Chronic LCMV infection is fortified with versatile tactics to suppress host T cell immunity and establish viral persistence. Viruses. 2021;13:1951. https://doi.org/10.3390/v13101951
35. McLane LM, Abdel-Hakeem MS, Wherry EJ. CD8 T cell exhaustion during chronic viral infection and cancer. Annu Rev Immunol. 2019;37:457–95. https://doi.org/10.1146/annurev-immunol-041015-055318
36. Kurachi M. CD8+ T cell exhaustion. Sem in Immunopath. 2019;41:327–37.
37. Roe K. A role for T-cell exhaustion in long COVID-19 and severe outcomes for several categories of COVID-19 patients. J Neurosci Res. 2021;. https://doi.org/10.1002/jnr.24917
38. Dyck L, Mills KHG. Immune checkpoints and their inhibition in cancer and infectious diseases. Eur J Immunol. 2017;47:765–79.
39. Kong Y, Zhu L, Schell TD, Zhang J, Claxton DF, Ehmann WC, et al. T-cell immunoglobulin and ITIM domain (TIGIT) associates with CD8+ T-cell exhaustion and poor clinical outcome in AML. Clin Cancer Res. 2016;22:3057–66.
40. Pallett LJ, Schmidt N, Schurich A. T cell metabolism in chronic viral infection. Clin Exp Immunol. 2019;197:143–52.
41. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol. 2015;15:486–99.
42. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. Cell. 2009;138:30–50.
43. McHugh D, Caduff N, Murer A, Engelmann C, Deng Y, Zdimerova H, et al. Infection and immune control of human oncogenic γ-herpesviruses in humanized mice. Philo Tran R Soc Lond B Biol Sci. 2019;374:20180296. https://doi.org/10.1098/rstb.2018.0296
44. Schildermans J, De Vlieger G. Cytomegalovirus: A troll in the ICU? overview of the literature and perspectives for the future. Front Med. 2020;7:188. https://doi.org/10.3389/fmed.2020.00188
45. Roe K. An inflammation classification system using cytokine parameters. Scand J Immunol. 2021;93:e12970. https://doi.org/10.1111/sji.12970
46. Abdel-Hamed EF, Ibrahim MN, Mostafa NE, Moawad H, Elgammal NE, Darwiesh EM, et al. Role of interferon gamma in SARS-CoV-2-positive patients with parasitic infections. Gut Pathog. 2021;13:29. https://doi.org/10.1186/s13099-021-00427-3
47. Osokine I, Snell LM, Cunningham CR, Yamada DH, Wilson EB, Elsaesser HJ, et al. Type I interferon suppresses de novo virus-specific CD4 Th1 immunity during an established persistent viral infection. Proc Natl Acad Sci USA. 2014;111:7409–14.
48. Gunasinghe SD, Peres NG, Goyette J, Gaus K. Biomechanics of T cell dysfunctions in chronic diseases. Front Immunol. 2021;12:600829. https://doi.org/10.3389/fimmu.2021.600829
49. Legat A, Speiser DE, Pircher H, Zehn D, Fuertes Marraco SA. Inhibitory receptor expression depends more dominantly on differentiation and activation than "exhaustion" of human CD8 T cells. Front Immunol. 2013;4:455. https://doi.org/10.3389/fimmu.2013.00455
50. Halonen SK, Weiss LM. Toxoplasmosis. In: Handbook of Clinical Neurology, Vol. 114. Elsevier B.V., Amsterdam, Netherlands; 2013, pp. 125–45. https://doi.org/10.1016/B978-0-444-53490-3.00008-X
51. Bhadra R, Gigley JP, Weiss LM, Khan IA. Control of *Toxoplasma* reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1-PDL-1 blockade. Proc Natl Acad Sci USA. 2011;108:9196–201.
52. Eggenhuizen PJ, Ng BH, Ooi JD. Treg enhancing therapies to treat autoimmune diseases. Int J Mol Sci. 2020;21:7015.
53. Illingworth J, Butler NS, Roetynck S, Mwacharo J, Pierce SK, Bejon P, et al. Chronic exposure to Plasmodium falciparum is associated with phenotypic evidence of B and T cell exhaustion. J Immunol. 2013;190:1038–47. https://doi.org/10.4049/jimmunol.1202438

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REVIEW

immunology
Insights into immunity in health and disease

Metabolic plasticity and regulation of T cell exhaustion

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Abstract

The metabolic reprogramming during T cell activation and differentiation affects T cell fate and immune responses. Cell metabolism may serve as the driving force that induces epigenetic modifications, contributing to regulating T cell differentiation. Persistent pathogen infection leads to T cell exhaustion, which is composed of two main subsets and with distinct metabolic characteristics. The progenitor exhausted T cells utilize mitochondrial fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) for energy, while terminally exhausted T cells mainly rely on glycolytic metabolism with impaired glycolysis and OXPHOS. Here, we compiled the latest research on how T cell metabolism defines differentiation, focusing on T cell exhaustion during chronic infections. In addition, metabolic-related factors including antigen stimulation signals strength, cytokines and epigenetics affecting T cell exhaustion were also reviewed. Furthermore, the intervention strategies on metabolism and epigenetics to reverse T cell exhaustion were discussed in detail, which may contribute to achieving the goal of prevention and treatment of T cell exhaustion.

KEYWORDS
chronic infection, epigenetics, glycolysis, T cell exhaustion, T cell metabolism

INTRODUCTION

During chronic pathogen infection, antigen-specific T cells become exhausted, displaying poor effector function and losing memory potential.[1, 2] Targeting immune checkpoint molecules like programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG-3) and T-cell immunoglobulin and mucin domain 3 (TIM-3) have been widely described in reinvigorating T cell exhaustion.[3] Multi-antibody combination therapy has shown a great ability to rejuvenate exhausted T cells, but the reinvigoration is not durable, and still has many limitations.[4, 5] Thus, it is necessary to investigate the novel mechanisms of T cell exhaustion including factors involved in metabolism or metabolic-related epigenetic regulation of T cell exhaustion to explore new therapeutic targets against T cell exhaustion.

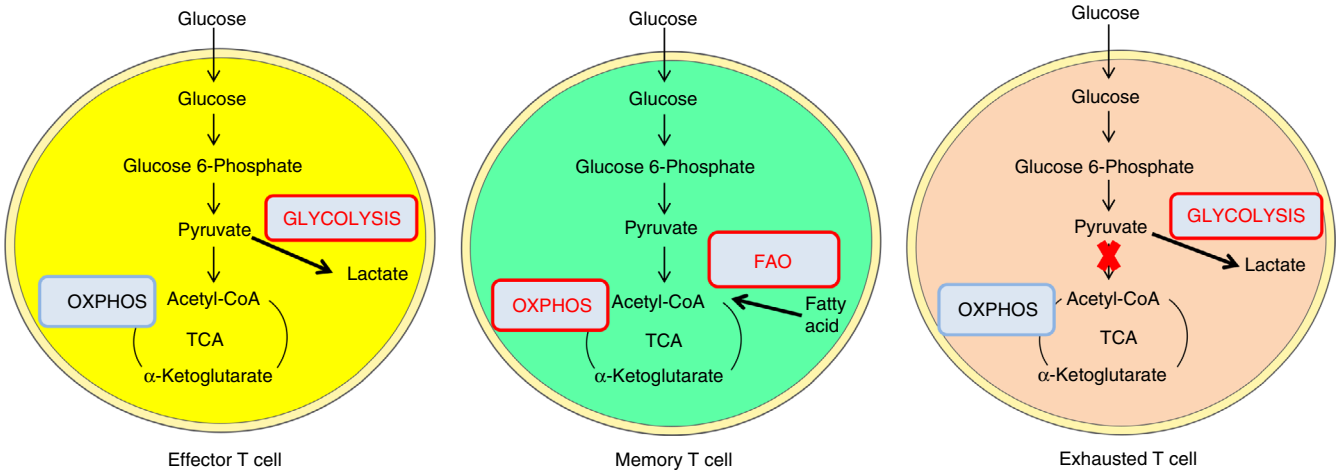

FIGURE 1 Metabolic programming at different T cell subsets. Different T cell subpopulations use specific metabolic programs to support their functions. Effector T cells need a large amount of energy for rapid supply due to their proliferation, differentiation and effector functions, and use glycolysis for rapid energy supply. Memory T cells mainly utilize FAO and OXPHOS for energy. Exhausted T cells mainly rely on glycolysis for energy. FAO, fatty acid oxidation; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid cycle.

THE VARIABLE METABOLIC DEMANDS THROUGHOUT T CELL DIFFERENTIATION

Following antigen stimulation, naïve T cells are activated and differentiated into effector T cells and memory T cells, which could be divided into effector memory T cells (T_{EM}), central memory T cells (T_{CM}), tissue-resident memory T cells (T_{RM}) and stem cell memory T cells (T_{SCM}).[6–8] Traditionally, it is believed that following antigen stimulation, the majority of T cells differentiate into short-lived effector cells, which would die by apoptosis following antigen clearance.[9] Only a small fraction of T cells (about 5%–10%) survive, known as memory precursor T cells, which further differentiate into memory T cell subsets.[10] Recently, a novel model on asymmetric division suggests that T cell fates are determined by the asymmetric division, the daughter cells adjacent to the immune synapse differentiating into effector T cells, while those from the distal pole giving rise to long-lived memory cells.[7, 11]

Memory T cell subsets show different phenotypes and functions.[10, 12] T_{CM} exhibits high level of IL-7 receptor α (CD127), lymph-node homing selectin CD62L and chemokine receptor CCR7,[9] which mainly exists in the lymph nodes, and can survive for more than 10 years. T_{CM} has robust proliferative capacity upon re-activation.[13] Compared with T_{CM}, T_{EM} is defined by decreased expression of CD62L and CCR7, high expression of killer cell lectin-like receptor G1 (KLRG1), and decreased proliferative potential.[13, 14] T_{EM} is enriched in spleen and peripheral nonlymphoid organs, such as lung, liver and gut, and survives for about 1 month.[15] Specially, tissue-resident memory T cell (T_{RM}) resides in peripheral tissues without recirculating, and displays tissue-residency markers including integrin CD103 and CD69.[16, 17] Additionally, T_{SCM} is the precursor of memory cells and presents naïve-like biomarkers (CCR7, CD27, CD127 and CD62L). T_{SCM} has enhanced self-renewal and proliferation capabilities, and has a multipotent developmental potential to generate multiple subsets of memory cells.[18, 19] The memory T cell mentioned in this article mainly refers to T_{CM}.

The metabolic requirements for different kinds of T cells are variable[20–22] (Figure 1). Naïve T cells maintain a quiescent state with relatively low energetic requirements, and mainly derive adenosine triphosphate (ATP) from oxidative phosphorylation (OXPHOS) in the mitochondrion. Additionally, fatty acids and amino acids can be degraded into pyruvate, acetyl coenzyme A (acetyl-CoA) or other intermediates to provide energy.[23] Upon activation, the metabolism of effector T cells is reprogrammed into aerobic glycolysis and OXPHOS to meet the increased demand for energy and intermediate products for rapid cell growth and division.[24, 25] In this process, since the available amino acids are shunted away from the tricarboxylic acid (TCA) cycle into protein synthesis and available fatty acids are shunted into membrane synthesis, most ATPs are generated by glycolysis.[23]

Compared with naïve and effector T cells, memory T cells increase mitochondrial mass, mainly utilize fatty acid oxidation (FAO) and mitochondrial OXPHOS for their long-term survival, and sustain a recall response upon secondary antigenic stimulation.[21, 26] Compared with T_{EM} cells, T_{CM} has a more elevated mitochondrial spare respiratory capacity (SRC), which indicates that T_{CM} may be more dependent on mitochondrial respiration for energy.[27] During glucose starvation, T_{EM} has a limited ability to

upregulate fatty acid synthesis and OXPHOS, which indicates that T_{EM} is less dependent on FAO than T_{CM}.[28]

Inhibiting glycolysis or augmenting OXPHOS can alter their phenotype and favour memory T cell formation.[29–31] Accumulated data show that T cells taking up limited glucose are metabolically suited for long-lived memory cell formation. Metformin or rapamycin treatment promotes memory T cell formation by enhancing FAO and OXPHOS at the expense of glucose utilization.[31, 32] Lactate dehydrogenase A (LDHA) inhibitor combined with IL-21 promotes the formation of T_{SCM} and improves antitumor response.[33] Conversely, increased glycolysis may favour the formation of T_{EM}.[27] Moreover, augmenting glycolytic metabolism by overexpressing the glycolytic enzyme phosphoglycerate mutase-1 (PGAM-1) drives T cells toward a terminally differentiated state, and limits the formation of long-lived memory T cells.[29]

In short, metabolic reprogramming orchestrates T cell differentiation from naïve T cells to effector and memory T cell subsets. The metabolic characteristics and interventions of different memory T cells remain to be explored.

THE METABOLIC REQUIREMENTS OF EXHAUSTED T CELLS

T cell exhaustion happens in chronic infections with lymphocytic choriomeningitis virus (LCMV), hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (*M. tuberculosis*).[1, 3, 34] Continuous antigen stimulation, high levels of reactive oxygen species (ROS) and hypoxia drive T cell exhaustion.[35] Considering exhausted $CD4^+$ T cells are heterogeneous,[36] the exhausted T cells mentioned in this article mainly refer to exhausted $CD8^+$ T cells. T cell exhaustion is a process in which T cells gradually lose their function: first, loss of interleukin (IL)-2 production, proliferation potential and cytolytic activity, followed by decreased expression of interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), at last impairing the ability to confer protection.[2, 3] Exhausted T cells overexpress various inhibitory receptors such as PD-1, TIM-3 and LAG-3, et al.[37] In addition, T cell exhaustion is accompanied by changes in the expression of transcription factors, such as upregulated expression of B lymphocyte-induced maturation protein-1 (Blimp-1)[38] and basic leucine zipper transcription factor ATF-like gene (BATF),[39] and downregulated expression of T-box–containing protein expressed in T cells (T-bet).[40] Traditionally, it is believed that exhausted T cells are derived from terminally differentiated effector T cells. However, emerging insights reveal that exhausted T cells are heterogeneous and can be derived from memory precursor effector cells or memory T cells at different differentiation stages.[41]

Exhausted T cells are phenotypically and functionally heterogeneous,[42, 43] which could be divided into two main subsets: progenitor exhausted T cells and terminally exhausted T cells. Progenitor exhausted $CD8^+$ T cells are a kind of 'stem-like' exhausted T cell population with an intermediate expression of PD-1, expression of CD127, chemokine receptor CXCR5 and T-cell factor 1 (TCF-1), which is also critical for memory formation.[1, 41] This subset has the capabilities of self-renewal and proliferation, and can respond well to PD-1/PD-ligand 1 (PD-L1)

BOX 1 Key players in cell metabolism

Anaerobic glycolysis: Glycolysis is a process of metabolizing glucose to pyruvate. Anaerobic glycolysis refers to the process in which glucose is degraded into lactate in the cytoplasm under anaerobic conditions along with the production of two ATPs per molecule of glucose.

Tricarboxylic acid (TCA) cycle: It is the central hub of mitochondrial metabolism that plays a pivotal role in coordinating the metabolism of carbohydrates, fats and proteins into ATP and carbon dioxide (CO_2).

Oxidative phosphorylation (OXPHOS): In the presence of sufficient oxygen, most glycolysis-derived pyruvate will be oxidized to acetyl coenzyme A (acetyl-CoA) by pyruvate dehydrogenase (PDH), then enter the TCA cycle and completely oxidize to CO_2 in the mitochondrion with up to 36 ATPs per molecule of glucose generated. During this process, oxygen is essential and acts as the ultimate electron acceptor to completely oxidize glucose.

Aerobic glycolysis or Warburg effect: A phenomenon of predominantly converting most glucose to lactate regardless of the availability of oxygen. It was first described in cancer cells and then in normal proliferating T cells.

Fatty acid oxidation (FAO): The glycerol and fatty acids produced by hydrolysis of fats and oils could be fully oxidized and decomposed to produce CO_2, water, and acetyl-CoA, and release some ATP under sufficient oxygen supply. FAO-generated acetyl-CoA then enters the TCA cycle, which couples with OXPHOS to generate ATP.

Metabolic reprogramming: It refers to alternations in metabolic of immune cells induced by infection and inflammation to match shift in metabolic requirements.

blockade.[44, 45] In contrast with progenitor exhausted T cells, terminally exhausted T cells show impaired proliferative capacity and do not respond to PD-1 pathway blockade,[46] which are characterized by high levels of PD-1 and TIM-3 and loss of TCF-1 expression.[43, 44, 47, 48] The exhausted T cells mentioned in this article mainly refer to terminally exhausted T cells (Box 1).

The two kinds of exhausted T cells also show different metabolic characteristics. The progenitor exhausted T cells have a catabolic metabolism characterized with mitochondrial FAO and OXPHOS,[49] while terminally exhausted T cells mainly rely on glycolytic metabolism with impaired glycolysis and OXPHOS.[50–53] Terminally exhausted T cells have reduced glucose uptake and low expression of transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which engages in mitochondrial biogenesis.[51, 52] In terminally exhausted T cells, increased mitochondrial mass and decreased mitochondrial membrane potential result in the inability of exhausted T cells to effectively utilize OXPHOS to provide energy.[50, 54] Impaired mitochondrial OXPHOS limits the proliferation and effector function of T cells by limiting ATP synthesis.[55]

Mitochondria in different T cell subsets have distinct morphological changes,[56, 57] and the shape of mitochondria determines T cell fate.[58] Effector T cells have fissed mitochondria with loose cristae, which become fused in memory T cells and have tighter cristae and expanded space. Altering mitochondrial fission/fusion induces the changes in cristae morphology, further shapes metabolic programming, and ultimately regulates T cell differentiation. In which process, augmenting mitochondrial fission drives the generation of effector T cells through inducing cristae expansion, decreasing electron transport chain efficiency and facilitating glycolysis, while enhancing mitochondrial fusion favours memory T cells formation by promoting OXPHOS and FAO.[58] Mitochondrial fission produces discrete and fragmented mitochondria, which contributes to ROS overproduction.[59] The excess of mitochondrial ROS could activate persistent nuclear factor of activated T cells (NFAT) signalling,[60] which drives terminal exhaustion.[35] Moreover, mitochondrial fission also promotes mitochondrial polarization.[61] Thus, inhibition of fission may prevent ROS production and enforce memory T cell formation.[59, 62] In addition, mitophagy is vital for memory formation, mainly through clearing off dysfunctional mitochondria and maintaining mitochondrial homeostasis.[63, 64]

In summary, persistent antigen stimulation induces T cell exhaustion. The progenitor exhausted T cells rely on mitochondrial FAO and OXPHOS for energy, while persistent antigen stimulation leads to the change of mitochondrial structure and impaired mitochondrial OXPHOS at last. PD-1 pathway blockade affects the progenitor exhausted T cells, but does not reinvigorate terminally exhausted cells.

ANTIGEN STIMULATING SIGNAL AND MICROENVIRONMENTAL CYTOKINES AFFECTING T CELLS METABOLISM

Antigen stimulation signal strength

T cell receptor (TCR) signalling triggers multiple signalling cascades involved in effector functions and metabolic reprogramming (Figure 2). TCR together with costimulatory signal activates phosphatidylinositol 3-kinase (PI3K), subsequently activates AKT and mammalian target of rapamycin (mTOR) signalling, then activates transcription factors including hypoxia-inducible factor-1 α (HIF-1α) and cellular-myelocytomatosis oncogene (c-Myc) to promote glycolysis.[65, 66] C-Myc consequently facilitates a variety of cellular processes involved in cell proliferation and metabolism .[66, 67] The PI3K-AKT–mTOR signalling pathway and adenosine monophosphate-activated protein kinase (AMPK) pathway triggered by TCR signalling are the main pathways that regulate T cell metabolism.[65, 68, 69]

PI3K and AKT inhibitors could increase FAO metabolism and drive the formation of memory T cells.[70, 71] mTOR inhibitor (rapamycin) treatment enforces the formation of memory precursors during the expansion phase of the T-cell response, and accelerates the memory T-cell differentiation program during the contraction phase.[72] Rapamycin has been shown to enhance memory T cell formation by driving glucose-dependent metabolism into FAO.[72] In addition, AMPK inhibits mTOR activation[73] and determines memory T cell formation by reprogramming metabolism from glycolysis to OXPHOS.[74] Metformin is an activator of AMPK and favours memory T cell formation by enhancing FAO.[31]

During chronic infection, persistent antigen exposure gradually impairs memory T cells formation and cytokine secreting potential, and leads to exhaustion.[1, 2] Moreover, exhausted T cells display low and strongly inhibited TCR signalling although antigen is abundant,[75] which enhances the dysfunction of exhausted T cells. The PD-1/PD-L1 interaction promotes the dephosphorylation of key proteins downstream of TCR, resulting in the inhibition of TCR signal transduction.[76]

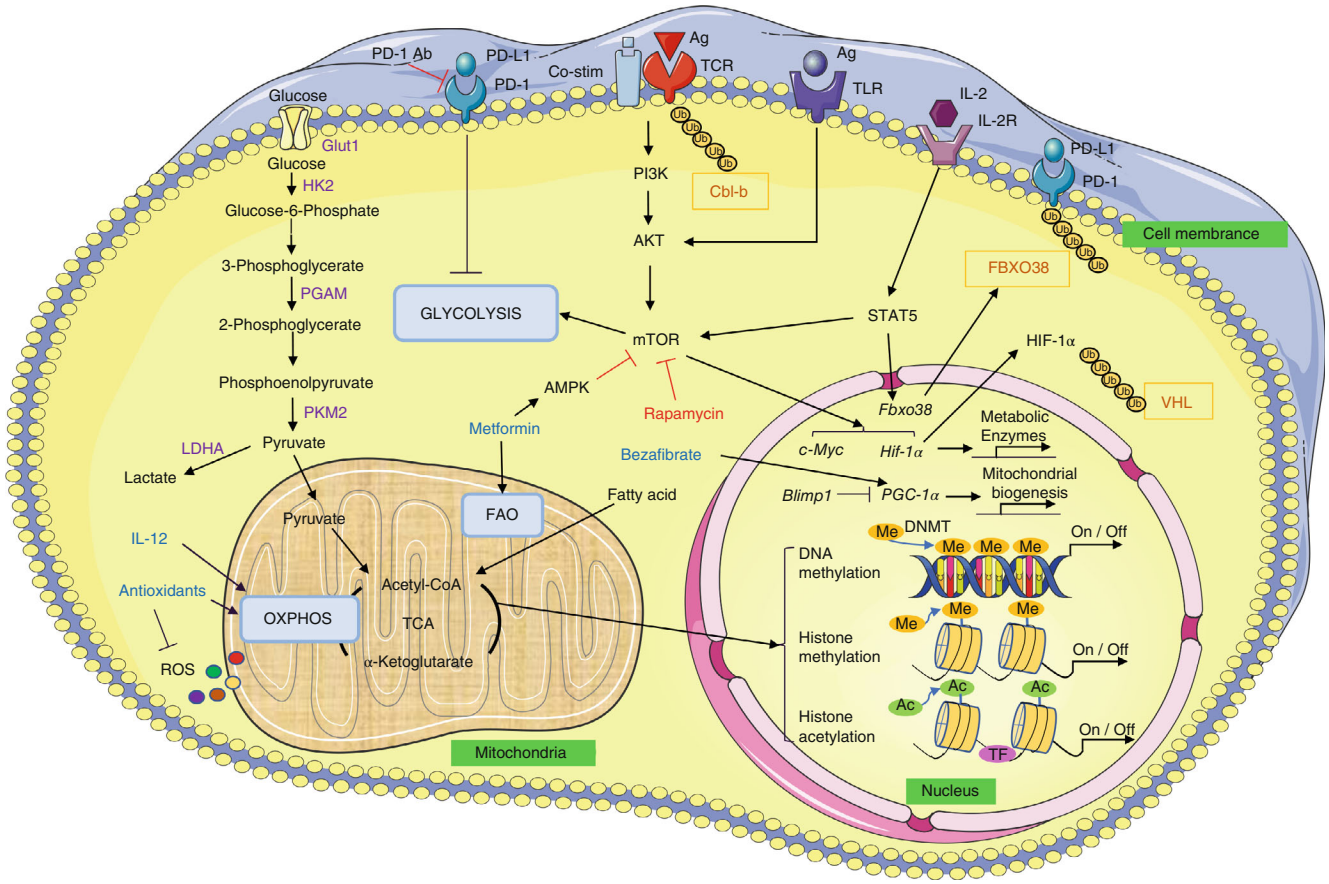

FIGURE 2 Factors involved in metabolism regulation along T cell exhaustion. Antigen stimulation signals strength, cytokine regulation and epigenetic modification can affect the development of T cell exhaustion. During chronic infections, antigens activate TCR together with costimulatory signalling, consequently activate PI3K-AKT–mTOR signalling, and upregulate HIF-1α and c-Myc, leading to metabolic reprogramming, which in turn regulates the gene expression through epigenetics. Ac, acetyl group; Ag, antigen; DNMT, DNA methyltransferase; Glut1, glucose transporter 1; HK2, hexokinase 2; LDHA, lactate dehydrogenase A; Me, methyl group; PGAM, phosphoglycerate mutase; PKM2, pyruvate kinase muscle isoenzyme 2; TCR, T cell receptor; TF, transcription factor; TLR, Toll-like receptor; VHL, von Hippel–Lindau protein. [Correction added on 18, November 2022, after first online publication: In Figure 2, the repeated figure has been deleted.]

Cytokine regulation

T cell metabolism and differentiation are regulated by various cytokines including IL-2, IL-7, IL-10, IL-12 and IL-21 (Figure 2). Among them, IL-2, IL-7 and IL-21 are the members of γ-chain cytokine family and play an important role in antagonizing exhaustion.[77] Both IL-2 and IL-7 are critical for T cell differentiation and homeostasis by regulating metabolism. IL-2 treatment could promote glycolysis by activating the mTOR pathway, thereby altering cell differentiation and effector functions.[33, 78] IL-7 promotes glucose transporter Glut1 expression and glucose uptake via STAT5-mediated Akt activation to maintain T-cell survival.[79] IL-7 also improves T cell survival by enhancing proliferation, decreasing apoptosis and exhaustion via promoting higher Glut1 transport.[80] In addition, IL-7 inhibits the expression of suppressor of cytokine signalling 3 (SOCS3), which is dramatically upregulated in exhausted T cells,[81] and reinvigorates T cell response during chronic LCMV infection.[82] In addition, IL-10 improves the effector function of exhausted T cells by promoting OXPHOS.[83] IL-12 increases the mitochondrial potential and restores the anti-viral effector function of exhausted HBV-specific T cells.[50] IL-21 shifts T-cell metabolism from glycolysis toward FAO, contributing to the induction of T_{CM}-like cells and reduced PD-1 expression.[84] Furthermore, IL-21 together with rapamycin could induce the formation of antigen-specific T_{SCM}-like cells.[85]

In short, T cell metabolism is regulated by antigen stimulation signal strength and cytokines, et al. Identifying the molecular links between metabolism and T-cell-mediated immune responses may help understand the pathogenesis of T cell exhaustion.

METABOLISM REGULATES T CELL DIFFERENTIATION VIA EPIGENETICS

Epigenetic regulations including DNA methylation and histone modifications (such as acetylation, methylation and phosphorylation) affect the chromatin accessibility, and play critical roles in regulating the expression of genes related to T cell differentiation[86] (Figure 2). Naïve T cells show low chromatin accessibility and most genes are kept closed. Effector T cells display increased chromosome accessibility and most genes are turned on, while some genes of memory T cells are in a poised state.[86] This 'poised state' in memory $CD8^+$ T cells helps to keep some genes in an unexpressed state, but induces a rapid response upon re-stimulation.[87] During chronic LCMV, HIV and HCV infection, exhausted T cells display significantly greater chromatin accessibility than that in memory T cells, especially the chromatin regions associated with inhibitory receptors such as PD-1.[88] Moreover, progenitors and terminally exhausted T cells show different chromatin accessibility landscapes.[89] Progenitor exhausted T cells exhibit more chromatin accessibility in genes encoding memory molecules, while terminally exhausted T cells show more chromatin accessibility in effector-related genes.[41]

Cell metabolism may serve as the driving force that influences epigenetic modifications by regulating the production of essential substrates for epigenetic modification, and the activity of DNA- and histone-modifying enzymes.[90, 91] Several metabolites such as s-adenosylmethionine (SAM), α-ketoglutarate and acetyl-CoA could be used as substrates and cofactors for modifying the chromatin and nucleic acids.[92]

DNA methylation and histone methylation

SAM, produced by the one-carbon metabolism of methionine, is the universal source of methyl groups for histone and DNA methyltransferases.[93] SAM regulates the methylation of histone H3 lysine 4 (H3K4me3), thereby affecting chromatin accessibility.[94] α-ketoglutarate (α-KG) is the substrate of demethylases Jumonji domain-containing protein D3 (JMJD3) and ten-eleven translocation (TET), which could, respectively, erase DNA and histone methylation. TCA intermediates fumarate and succinate downregulate the HIF target gene expression via inhibiting the catalytic activity of TET enzymes.[95] 2-hydroxyglutarate (2-HG) is an antagonist of α-KG and a competitive inhibitor of α-KG-dependent TET enzymes.[96, 97] 2-HG is produced by $CD8^+$ T cells in response to TCR triggering and enhances CD62L transcription by inhibiting TET2, which indicates that 2-HG may drive memory T cells formation and inhibit effector differentiation.[98] In addition, DNA methylation at specific genomic loci such as *Pdcd1* (encoding the protein PD-1), *Ifng* and *Tcf7* (encoding the protein TCF-1) involves in the formation of T cell exhaustion. The PD-1 promoter remains completely demethylated in HIV-specific $CD8^+$ T cells during chronic HIV infection, resulting in the high expression of PD-1.[99] The demethylase TET deficiency leads to the enhanced memory T cells formation in LCMV infected mice.[86, 100] As epigenetic regulation can modulate T cell differentiation, it suggests that targeting epigenetic pathways might be a way to promote the formation of memory T cells.

Histone acetylation

Because acetyl-CoA is the only source of acetyl groups for acetylation, limited glucose availability or glycolysis might affect acetyl-CoA production, alter chromatin accessibility and impair T-cell immunity.[93] Glycolysis enhances the expression of IFN-γ by regulating histone acetylation. LDHA deficiency inhibits glycolysis and leads to diminished acetyl-CoA levels, which consequently decreases the acetylation of histone H3 lysine 9 (H3K9Ac) at the *Ifng* locus and represses IFN-γ expression in T cells.[101] During chronic glucose restriction, acetate could be converted to acetyl-CoA, which further restores IFN-γ gene transcription and IFN-γ secretion by enhancing histone acetylation and chromatin accessibility.[102] Moreover, treating $CD8^+$ T cells with histone deacetylase inhibitor (HDACi) drives functional memory T cells formation.[86, 103] Overall, histone acetylation is known to generate more accessible chromatin regions and thereby increase gene transcription, while histone methylation or deacetylation correlates with more tightly bound chromatin regions and a decrease in gene transcription.[104]

Ubiquitination modification

Besides acetylation and methylation, ubiquitination modification plays a critical role in epigenetic regulation. Ubiquitin modifications contribute to regulating the levels of factors related to T cell differentiation (Figure 2). The E3 ubiquitin ligases could recognize and target specific proteins, which further undergo proteasome-mediated degradation. Tumour necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase, negatively regulates activation of TCR[105] and drives CD8 T_{CM} development upon bacterial infection by enhancing fatty acid

metabolism.[31] TRAF6-deficient T cells exhibit defects in FAO, impairing the formation of T_{CM}.[105] Another E3 ubiquitin ligase Casitas B-lineage lymphoma b (Cbl-b) is considered as a TCR signalling inhibitor molecule and upregulated in exhausted T cells.[106] *Cbl-b* deficiency restores the effector function of exhausted T cells during chronic viral infection and tumour.[107] In addition, the E3 ubiquitin ligase FBXO38 could downregulate the expression of PD-1 in tumour-infiltrating T cells by lysine 48-linked poly-ubiquitination and proteasome degradation.[108] Von Hippel–Lindau (VHL), another E3 ubiquitin ligase, is a negative regulator of HIF-1α. *Vhl* deletion in $CD8^+$ T cells shows elevated HIF-1α activity, which further induces high glycolysis and low OXPHOS metabolic profile, and enhances the control of persistent viral infection .[27, 109, 110]

In summary, metabolites impact T cell differentiation by controlling gene expression via epigenetics. Understanding the epigenetic regulation mechanism is instructive for preventing T cell exhaustion and enhancing immunity. Exploring the metabolism and epigenetic regulation mechanisms has the potential to offer novel metabolic-based therapeutic approaches for T cell exhaustion.

METABOLIC AND EPIGENETIC INTERVENTIONS TO REVERSE T CELL EXHAUSTION

Besides targeting immune checkpoints, targeting T cell metabolism and epigenetics has emerged as an approach to reverse T cell exhaustion. The methods that have been applied up to date are as follows (Table 1).

Enhancing the ability of mitochondrial OXPHOS

IL-12,[50] IL-10,[83] IL-21,[84] antioxidant, PGC-1α, Bezafibrate, Metformin, et al., have been reported to enhance the mitochondrial function of exhausted T cells. Among them, antioxidants, acting as ROS scavengers, scavenge excessive ROS and attenuates oxidative damage effects.[111] N-acetylcysteine (NAC), a cell permeable antioxidant, is also reported to rescue T cell proliferation and effector function of exhausted T cells by increasing glutathione synthesis, which can neutralize intracellular ROS.[55] Mitochondria-targeted antioxidants mitoquinone (MitoQ) and the piperidine-nitroxide MitoTempo significantly improve the mitochondrial function of exhausted HBV-specific CD8 T cells by limiting ROS increase.[112]

Additionally, mitochondrial OXPHOS capacity can be compensated by enhancing mitochondrial biogenesis and glycolysis, contributing to rescuing the function of exhausted T cells. PGC-1α, a master regulator in mitochondrial metabolism, could be negatively regulated by PD-1. PD-1 pathway blockade increases the glucose uptake and the expression of PGC-1α[53]. Overexpression of PGC-1α could boost OXPHOS by enhancing glycolysis and mitochondrial respiration.[51, 52] Bezafibrate, an agonist of PGC-1α, promotes FAO, OXPHOS and mitochondrial SRC, and thereby improves the antitumor efficacy of PD-1 blockade.[25, 113] Blimp-1 (encoded by *Prdm1*, a transcriptional repressor) represses mitochondrial biogenesis by downregulating PGC-1α transcription. Removing Blimp-1 in exhausted T cells in tumour restores the mitochondrial mass and cytokines (such as IL-2 and TNF-α) secretion.[35, 51] Moreover, since PGC-1α could downregulate ROS, overexpression of PGC-1α reduces ROS and ultimately alleviates T cell exhaustion.[35]

Metformin, an activator of AMPK, could block mTOR signalling to inhibit glycolysis and restore mitochondrial FAO to improve mitochondrial function, and thereby reinvigorate $CD8^+$ T cell exhaustion.[114, 115] Metformin functions as an adjunctive agent for anti-tuberculosis therapy.[116] Metformin treatment could also reduce intratumoral hypoxia by inhibiting oxygen consumption of tumour cells and enhancing the anti-tumour effect of PD-1 blockade.[117] Metformin may help therapeutic vaccine to improve the multifunctionality of tumour-infiltrating CD8 T cells and prevent T cell exhaustion.[118] These data indicate that metabolic reprogramming could shape the efficacy of CD8 T cells against chronic infections.

Enhancing glycolysis of exhausted T cells

Enhancing impaired glycolysis is another strategy for reinvigorating the function of exhausted T cells. The potential measures include upregulating the expression of glucose transporter 1 (Glut1), regulating the activity of glycolytic enzymes, increasing TCR signalling and activating mTOR signalling pathway. Enhancing glycolysis might be achieved by increasing the expression of Glut1, which partially restores the function of exhausted T cells.[119] Enhancing TCR signalling or mTOR signalling pathway could stimulate glycolysis-enhancing pathways and reinvigorate the function of exhausted T cells.[120] Toll-like receptors (TLRs)-induced T cell activation enhances T cell effector function by upregulating cellular glycolysis.[121] For example, TLR2 agonist treatment

TABLE 1 Metabolic and epigenetic interventions to reverse T cell exhaustion

Strategy	Major changes	Intervention methods	Intervention mechanism
Antibody blockade	PD-1 ↑	PD-1 antibody	Decreasing PD-1 expression
	LAG-3, TIM-3 ↑	LAG-3 antibody, TIM-3 antibody	Decreasing inhibitory receptors expression
Enhancing glycolysis	TCR, TLR, PI3K, AKT, HIF-1α, c-Myc ↓	Cbl-b inhibitor, TLR agonist, PI3K enhancer, AKT enhancer, HIF-1α enhancer, c-Myc enhancer	Enhancing TCR signalling and promoting the transcription of glycolytic genes
	Glut1, HK2, PGAM, PKM2, LDHA ↓	Glut1 enhancer, HK2 enhancer, PGAM enhancer, PKM2 enhancer, LDHA enhancer	Upregulating the expression of Glut1 and the activity of glycolytic enzymes
Enhancing mitochondrial OXPHOS	OXPHOS ↓	IL-12	Enhancing mitochondrial OXPHOS
	ROS ↑	Antioxidant	Decreasing the level of ROS
	FAO ↓	Metformin, IL-21	Enhancing mitochondrial FAO
	PGC-1α ↓	Bezafibrate (PGC-1α agonist)	Promoting the transcription of mitochondrial OXPHOS genes
Epigenetic regulation	Histone deacetylation ↑	Histone deacetylase inhibitor	Decreasing histone deacetylation
	DNA methylation ↑	DNA methyltransferase inhibitor	Decreasing DNA methylation
	FBXO38 ubiquitination ↓	IL-2	Rescuing *Fbxo38* transcription and decreasing PD-1 expression
	VHL ubiquitination ↑	VHL inhibitor	Decreasing VHL ubiquitination to promote HIF-1α activity

significantly prevents chronically stimulated T cells from exhaustion in chronic *M. tuberculosis*-infected mice.[122]

Glycolysis can also be regulated by metabolism-related transcription factors and cytokines. Transcription factors c-Myc and HIF-1α are critical factors in promoting the gene expression of multiple glycolytic enzymes including hexokinase 2 (HK2), phosphoglycerate mutase (PGAM), pyruvate kinase muscle isoenzyme 2 (PKM2) and LDHA, as well as Glut1.[66, 123] Upregulating AKT activity, blockade of AMPK signalling, or altering the expression of transcription factors HIF-1α and c-Myc are the approaches to enhancing mTOR pathways.[120] Elevated levels of HIF-1α enhance the effector capacity of $CD8^+$ T cells by increasing glycolytic activity,[109] while downregulation of c-Myc promotes T cells exhaustion by inducing a mitochondrial defect.[124]

Epigenetic regulation of T cell exhaustion

As epigenetic regulation can modulate T cell differentiation, targeting epigenetic pathways may alleviate T cell exhaustion. For instance, treatment with histone deacetylase inhibitor restores histone acetylation at the *Ifng* locus, promotes the production of IFN-γ, and ultimately rejuvenates T cells exhaustion.[103, 125] The DNA methyltransferase 3 α (DNMT3α)-mediated DNA methylation drives T cell exhaustion. Knocking out of DNMT3α represses the development of exhaustion. Combining DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine treatment with PD-1 blockade enhances the reinvigoration potential of exhausted T cells.[126] In addition, E3 ubiquitin ligase FBXO38 mediates PD-1 ubiquitination by lysine 48-linked poly-ubiquitination and proteasome degradation. FBXO38 transcription is reduced in tumour-infiltrating T cells. IL-2 treatment rescues *Fbxo38* transcription, subsequently downregulates PD-1 levels, and plays a critical role in anti-tumour immunity.[108]

Altogether, these data suggest that targeting T cell metabolism and epigenetic modifications might potentially reverse T cell exhaustion, which still needs further investigation.

CONCLUSION AND PERSPECTIVES

Understanding the metabolic regulation mechanisms and identifying the molecular links between metabolism and T-cell-mediated immunity may help us better understand the pathogenesis of T-cell exhaustion. Investigating how metabolic fitness underlies exhausted T cells has the potential to offer novel metabolic-based therapeutic approaches for the treatment of chronic diseases. More investigation on the regulation of metabolism variation during T cell exhaustion would provide novel ways to restore T cell exhaustion.

AUTHOR CONTRIBUTIONS

Fei Li wrote the manuscript. Huiling Liu, Dan Zhang, Yanlin Ma and Bingdong Zhu did some revisions. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. McLane LM, Abdel-Hakeem MS, Wherry EJ. CD8 T cell exhaustion during chronic viral infection and cancer. Annu Rev Immunol. 2019;37:457–95.
2. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol. 2015;15:486–99.
3. Wherry EJ. T cell exhaustion. Nat Immunol. 2011;12:492–9.
4. Abdel-Hakeem MS, Manne S, Beltra JC, Stelekati E, Chen Z, Nzingha K, et al. Epigenetic scarring of exhausted T cells hinders memory differentiation upon eliminating chronic antigenic stimulation. Nat Immunol. 2021;22:1008–19.
5. Tabana Y, Moon TC, Siraki A, Elahi S, Barakat K. Reversing T-cell exhaustion in immunotherapy: a review on current approaches and limitations. Expert Opin Ther Targets. 2021;25:347–63.
6. Pace L. Temporal and epigenetic control of plasticity and fate decision during CD8+ T-cell memory differentiation. Cold Spring Harb Perspect Biol. 2021;13:a037754.
7. Tanel A, Fonseca SG, Yassine-Diab B, Bordi R, Zeidan J, Shi Y, et al. Cellular and molecular mechanisms of memory T-cell survival. Expert Rev Vaccines. 2009;8:299–312.
8. Restifo NP, Gattinoni L. Lineage relationship of effector and memory T cells. Curr Opin Immunol. 2013;25:556–63.
9. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol. 2003;4:1191–8.
10. Lugli E, Galletti G, Boi SK, Youngblood BA. Stem, effector, and hybrid states of memory CD8+ T cells. Trends Immunol. 2020;41:17–28.
11. Alampi G, Vignali D, Centorame I, Canu A, Cosorich I, Filoni J, et al. Asymmetric T cell division of GAD65 specific naive T cells contribute to an early divergence in the differentiation fate into memory T cell subsets. Immunology. 2022.
12. Jameson SC, Masopust D. Understanding subset diversity in T cell memory. Immunity. 2018;48:214–26.
13. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol. 2004;22:745–63.
14. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. Eur J Immunol. 2013;43:2797–809.
15. Boyman O, Létourneau S, Krieg C, Sprent J. Homeostatic proliferation and survival of naïve and memory T cells. Eur J Immunol. 2009;39:2088–94.
16. Schenkel JM, Masopust D. Tissue-resident memory T cells. Immunity. 2014;41:886–97.
17. Park SL, Gebhardt T, Mackay LK. Tissue-resident memory Tcells in cancer immunosurveillance. Trends Immunol. 2019;40:735–47.
18. Li Y, Wu D, Yang X, Zhou S. Immunotherapeutic potential of T memory stem cells. Front Oncol. 2021;11:723888.
19. Gao S, Liang X, Wang H, Bao B, Zhang K, Zhu Y, et al. Stem cell-like memory T cells: a perspective from the dark side. Cell Immunol. 2021;361:104273.
20. MacIver NJ, Michalek RD, Rathmell JC. Metabolic regulation of T lymphocytes. Annu Rev Immunol. 2013;31:259–83.
21. van der Windt GJ, Pearce EL. Metabolic switching and fuel choice during T-cell differentiation and memory development. Immunol Rev. 2012;249:27–42.
22. Reina-Campos M, Scharping NE, Goldrath AW. CD8+ T cell metabolism in infection and cancer. Nat Rev Immunol. 2021;21:718–38.
23. Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. Nat Rev Immunol. 2005;5:844–52.
24. Chang CH, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. Cell. 2013;153:1239–51.
25. Kumar A, Chamoto K. Immune metabolism in PD-1 blockade-based cancer immunotherapy. Int Immunol. 2021;33:17–26.
26. O'Sullivan D, van der Windt GJ, Huang SC, Curtis JD, Chang CH, Buck MD, et al. Memory CD8+ T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. Immunity. 2014;41:75–88.
27. Phan AT, Doedens AL, Palazon A, Tyrakis PA, Cheung KP, Johnson RS, et al. Constitutive glycolytic metabolism supports CD8+ T cell effector memory differentiation during viral infection. Immunity. 2016;45:1024–37.
28. Ecker C, Guo L, Voicu S, Gil-de-Gómez L, Medvec A, Cortina L, et al. Differential reliance on lipid metabolism as a salvage pathway underlies functional differences of T cell subsets in poor nutrient environments. Cell Rep. 2018;23:741–55.
29. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting glycolytic metabolism enhances CD8+ T cell

memory and antitumor function. J Clin Invest. 2013;123:4479–88.
30. He J, Shangguan X, Zhou W, Cao Y, Zheng Q, Tu J, et al. Glucose limitation activates AMPK coupled SENP1-Sirt3 signalling in mitochondria for T cell memory development. Nat Commun. 2021;12:4371.
31. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, et al. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature. 2009;460:103–7.
32. Sipula IJ, Brown NF, Perdomo G. Rapamycin-mediated inhibition of mammalian target of rapamycin in skeletal muscle cells reduces glucose utilization and increases fatty acid oxidation. Metabolism. 2006;55:1637–44.
33. Hermans D, Gautam S, García-Cañaveras JC, Gromer D, Mitra S, Spolski R, et al. Lactate dehydrogenase inhibition synergizes with IL-21 to promote CD8+ T cell stemness and antitumor immunity. Proc Natl Acad Sci U S A. 2020;117:6047–55.
34. Lombardi A, Villa S, Castelli V, Bandera A, Gori A. T-cell exhaustion in mycobacterium tuberculosis and nontuberculous mycobacteria infection: pathophysiology and therapeutic perspectives. Microorganisms. 2021;9:2460.
35. Scharping NE, Rivadeneira DB, Menk AV, Vignali PDA, Ford BR, Rittenhouse NL, et al. Mitochondrial stress induced by continuous stimulation under hypoxia rapidly drives T cell exhaustion. Nat Immunol. 2021;22:205–15.
36. Crawford A, Angelosanto JM, Kao C, Doering TA, Odorizzi PM, Barnett BE, et al. Molecular and transcriptional basis of $CD4^+$ T cell dysfunction during chronic infection. Immunity. 2014;40:289–302.
37. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol. 2003;77:4911–27.
38. Shin H, Blackburn SD, Intlekofer AM, Kao C, Angelosanto JM, Reiner SL, et al. A role for the transcriptional repressor Blimp-1 in CD8+ T cell exhaustion during chronic viral infection. Immunity. 2009;31:309–20.
39. Quigley M, Pereyra F, Nilsson B, Porichis F, Fonseca C, Eichbaum Q, et al. Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. Nat Med. 2010;16:1147–51.
40. Kao C, Oestreich KJ, Paley MA, Crawford A, Angelosanto JM, Ali MA, et al. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. Nat Immunol. 2011;12:663–71.
41. Kagoya Y. Dissecting the heterogeneity of exhausted T cells at the molecular level. Int Immunol. 2022. Online ahead of print.
42. Paley MA, Kroy DC, Odorizzi PM, Johnnidis JB, Dolfi DV, Barnett BE, et al. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. Science. 2012;338:1220–5.
43. Utzschneider DT, Charmoy M, Chennupati V, Pousse L, Ferreira DP, Calderon-Copete S, et al. T cell factor 1-expressing memory-like $CD8^+$ T cells sustain the immune response to chronic viral infections. Immunity. 2016;45:415–27.
44. Siddiqui I, Schaeuble K, Chennupati V, Fuertes Marraco SA, Calderon-Copete S, Pais Ferreira D, et al. Intratumoral Tcf1+PD-1+CD8+ T cells with stem-like properties promote tumor control in response to vaccination and checkpoint blockade immunotherapy. Immunity. 2019;50:195–211.e10.
45. Miller BC, Sen DR, Al Abosy R, Bi K, Virkud YV, LaFleur MW, et al. Subsets of exhausted CD8+ T cells differentially mediate tumor control and respond to checkpoint blockade. Nat Immunol. 2019;20:326–36.
46. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. Nature. 2016;537:417–21.
47. Hudson WH, Gensheimer J, Hashimoto M, Wieland A, Valanparambil RM, Li P, et al. Proliferating transitory T cells with an effector-like transcriptional signature emerge from PD-1+ stem-like CD8+ T cells during chronic infection. Immunity. 2019;51:1043–58.e4.
48. Chen Z, Ji Z, Ngiow SF, Manne S, Cai Z, Huang AC, et al. TCF-1-centered transcriptional network drives an effector versus exhausted CD8 T cell-fate decision. Immunity. 2019;51:840–55.e5.
49. Adams WC, Chen YH, Kratchmarov R, Yen B, Nish SA, Lin WW, et al. Anabolism-associated mitochondrial stasis driving lymphocyte differentiation over self-renewal. Cell Rep. 2016;17:3142–52.
50. Schurich A, Pallett LJ, Jajbhay D, Wijngaarden J, Otano I, Gill US, et al. Distinct metabolic requirements of exhausted and functional virus-specific CD8 T cells in the same host. Cell Rep. 2016;16:1243–52.
51. Scharping NE, Menk AV, Moreci RS, Whetstone RD, Dadey RE, Watkins SC, et al. The tumor microenvironment represses T cell mitochondrial biogenesis to drive intratumoral T cell metabolic insufficiency and dysfunction. Immunity. 2016;45:374–88.
52. Bengsch B, Johnson AL, Kurachi M, Odorizzi PM, Pauken KE, Attanasio J, et al. Bioenergetic insufficiencies due to metabolic alterations regulated by the inhibitory receptor PD-1 are an early driver of CD8+ T cell exhaustion. Immunity. 2016;45:358–73.
53. Siska PJ, Beckermann KE, Mason FM, Andrejeva G, Greenplate AR, Sendor AB, et al. Mitochondrial dysregulation and glycolytic insufficiency functionally impair CD8 T cells infiltrating human renal cell carcinoma. JCI. Insight. 2017;2:e9341.
54. Pallett LJ, Schmidt N, Schurich A. T cell metabolism in chronic viral infection. Clin Exp Immunol. 2019;197:143–52.
55. Vardhana SA, Hwee MA, Berisa M, Wells DK, Yost KE, King B, et al. Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T cells exposed to persistent antigen. Nat Immunol. 2020;21:1022–33.
56. van der Windt GJ, Everts B, Chang CH, Curtis JD, Freitas TC, Amiel E, et al. Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. Immunity. 2012;36:68–78.
57. van der Windt GJ, O'Sullivan D, Everts B, Huang SC, Buck MD, Curtis JD, et al. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. Proc Natl Acad Sci U S A. 2013;110:14336–41.
58. Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, et al. Mitochondrial dynamics controls

T cell fate through metabolic programming. Cell. 2016;166: 63–76.
59. Yu T, Robotham JL, Yoon Y. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci U S A. 2006;103:2653–8.
60. Sena LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, et al. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. Immunity. 2013;38:225–36.
61. Campello S, Lacalle RA, Bettella M, Mañes S, Scorrano L, Viola A. Orchestration of lymphocyte chemotaxis by mitochondrial dynamics. J Exp Med. 2006;203:2879–86.
62. Lanna A, Dustin ML. Mitochondrial fusion fuels T cell memory. Cell Res. 2016;26:969–70.
63. Murera D, Arbogast F, Arnold J, Bouis D, Muller S, Gros F. CD4 T cell autophagy is integral to memory maintenance. Sci Rep. 2018;8:5951.
64. Xu X, Araki K, Li S, Han JH, Ye L, Tan WG, et al. Autophagy is essential for effector CD8+ T cell survival and memory formation. Nat Immunol. 2014;15:1152–61.
65. Frauwirth KA, Riley JL, Harris MH, Parry RV, Rathmell JC, Plas DR, et al. The CD28 signaling pathway regulates glucose metabolism. Immunity. 2002;16:769–77.
66. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, et al. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity. 2011;35: 871–82.
67. Marchingo JM, Sinclair LV, Howden AJ, Cantrell DA. Quantitative analysis of how Myc controls T cell proteomes and metabolic pathways during T cell activation. Elife. 2020;9:e53725.
68. Bishop EL, Gudgeon N, Dimeloe S. Control of T cell metabolism by cytokines and hormones. Front Immunol. 2021;12: 653605.
69. Beckermann KE, Dudzinski SO, Rathmell JC. Dysfunctional T cell metabolism in the tumor microenvironment. Cytokine Growth Factor Rev. 2017;35:7–14.
70. Bowers JS, Majchrzak K, Nelson MH, Aksoy BA, Wyatt MM, Smith AS, et al. PI3Kδ inhibition enhances the antitumor fitness of adoptively transferred CD8+ T cells. Front Immunol. 2017;8:1221.
71. Crompton JG, Sukumar M, Roychoudhuri R, Clever D, Gros A, Eil RL, et al. Akt inhibition enhances expansion of potent tumor-specific lymphocytes with memory cell characteristics. Cancer Res. 2015;75:296–305.
72. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, et al. mTOR regulates memory CD8 T-cell differentiation. Nature. 2009;460:108–12.
73. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell. 2008;30:214–26.
74. Blagih J, Coulombe F, Vincent EE, Dupuy F, Galicia-Vázquez G, Yurchenko E, et al. The energy sensor AMPK regulates T cell metabolic adaptation and effector responses in vivo. Immunity. 2015;42:41–54.
75. Sandu I, Cerletti D, Claassen M, Oxenius A. Exhausted CD8+ T cells exhibit low and strongly inhibited TCR signaling during chronic LCMV infection. Nat Commun. 2020;11: 4454.
76. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008; 26:677–704.
77. Peña-Asensio J, Calvo H, Torralba M, Miquel J, Sanz-de-Villalobos E, Larrubia JR. Gamma-chain receptor cytokines & PD-1 manipulation to restore HCV-specific CD8+ T cell response during chronic hepatitis C. Cell. 2021;10:538.
78. Ross SH, Cantrell DA. Signaling and function of interleukin-2 in T lymphocytes. Annu Rev Immunol. 2018;36:411–33.
79. Wofford JA, Wieman HL, Jacobs SR, Zhao Y, Rathmell JC. IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T-cell survival. Blood. 2008;111:2101–11.
80. He C, Zhou Y, Li Z, Farooq MA, Ajmal I, Zhang H, et al. Co-expression of IL-7 improves NKG2D-based CAR T cell therapy on prostate cancer by enhancing the expansion and inhibiting the apoptosis and exhaustion. Cancers (Basel). 2020;12:1969.
81. Kleinsteuber K, Heesch K, Schattling S, Sander-Juelch C, Mock U, Riecken K, et al. SOCS3 promotes interleukin-17 expression of human T cells. Blood. 2012;120:4374–82.
82. Pellegrini M, Calzascia T, Toe JG, Preston SP, Lin AE, Elford AR, et al. IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology. Cell. 2011;144:601–13.
83. Guo Y, Xie YQ, Gao M, Zhao Y, Franco F, Wenes M, et al. Metabolic reprogramming of terminally exhausted CD8+ T cells by IL-10 enhances anti-tumor immunity. Nat Immunol. 2021;22:746–56.
84. Loschinski R, Böttcher M, Stoll A, Bruns H, Mackensen A, Mougiakakos D. IL-21 modulates memory and exhaustion phenotype of T-cells in a fatty acid oxidation-dependent manner. Oncotarget. 2018;9:13125–38.
85. Jeza VT, Li X, Chen J, Liang Z, Aggrey AO, Wu X. IL-21 augments rapamycin in expansion of alpha fetoprotein antigen specific stem-cell-like memory T cells in vitro. Pan Afr Med J. 2017;27:163.
86. Tough DF, Rioja I, Modis LK, Prinjha RK. Epigenetic regulation of T cell memory: recalling therapeutic implications. Trends Immunol. 2020;41:29–45.
87. Dogra P, Ghoneim HE, Abdelsamed HA, Youngblood B. Generating long-lived CD8+ T-cell memory: insights from epigenetic programs. Eur J Immunol. 2016;46:1548–62.
88. Sen DR, Kaminski J, Barnitz RA, Kurachi M, Gerdemann U, Yates KB, et al. The epigenetic landscape of T cell exhaustion. Science. 2016;354:1165–9.
89. Jadhav RR, Im SJ, Hu B, Hashimoto M, Li P, Lin JX, et al. Epigenetic signature of PD-1+ TCF1+ CD8 T cells that act as resource cells during chronic viral infection and respond to PD-1 blockade. Proc Natl Acad Sci U S A. 2019;116:14113–8.
90. Dimeloe S, Burgener AV, Grählert J, Hess C. T-cell metabolism governing activation, proliferation and differentiation; a modular view. Immunology. 2017;150:35–44.
91. Gray SM, Kaech SM, Staron MM. The interface between transcriptional and epigenetic control of effector and memory $CD8^+$ T-cell differentiation. Immunol Rev. 2014;261:157–68.
92. Reid MA, Dai Z, Locasale JW. The impact of cellular metabolism on chromatin dynamics and epigenetics. Nat Cell Biol. 2017;19:1298–306.

93. Franco F, Jaccard A, Romero P, Yu YR, Ho PC. Metabolic and epigenetic regulation of T-cell exhaustion. Nat Metab. 2020;2:1001–12.
94. Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, Ratanasirintrawoot S, et al. Influence of threonine metabolism on S-adenosylmethionine and histone methylation. Science. 2013;339:222–6.
95. Laukka T, Mariani CJ, Ihantola T, Cao JZ, Hokkanen J, Kaelin WG Jr, et al. Fumarate and succinate regulate expression of hypoxia-inducible genes via TET enzymes. J Biol Chem. 2016;291:4256–65.
96. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. Cancer Cell. 2011;19:17–30.
97. Chowdhury R, Yeoh KK, Tian YM, Hillringhaus L, Bagg EA, Rose NR, et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. EMBO Rep. 2011;12:463–9.
98. Tyrakis PA, Palazon A, Macias D, Lee KL, Phan AT, Veliça P, et al. S-2-hydroxyglutarate regulates $CD8^+$ T-lymphocyte fate. Nature. 2016;540:236–41.
99. Youngblood B, Oestreich KJ, Ha SJ, Duraiswamy J, Akondy RS, West EE, et al. Chronic virus infection enforces demethylation of the locus that encodes PD-1 in antigen-specific CD8+ T cells. Immunity. 2011;35:400–12.
100. Carty SA, Gohil M, Banks LB, Cotton RM, Johnson ME, Stelekati E, et al. The loss of TET2 promotes CD8+ T cell memory differentiation. J Immunol. 2018;200:82–91.
101. Peng M, Yin N, Chhangawala S, Xu K, Leslie CS, Li MO. Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism. Science. 2016;354:481–4.
102. Qiu J, Villa M, Sanin DE, Buck MD, O'Sullivan D, Ching R, et al. Acetate promotes T cell effector function during glucose restriction. Cell Rep. 2019;27:2063–74.e5.
103. Zhang F, Zhou X, DiSpirito JR, Wang C, Wang Y, Shen H. Epigenetic manipulation restores functions of defective $CD8^+$ T cells from chronic viral infection. Mol Ther. 2014;22:1698–706.
104. Veliz K, Beier UH. Transcriptional regulation of T cell metabolism and metabolic control of T cell gene expression. Curr Opin Genet Dev. 2021;70:83–8.
105. King CG, Kobayashi T, Cejas PJ, Kim T, Yoon K, Kim GK, et al. TRAF6 is a T cell-intrinsic negative regulator required for the maintenance of immune homeostasis. Nat Med. 2006;12:1088–92.
106. Ou R, Zhang M, Huang L, Moskophidis D. Control of virus-specific CD8+ T-cell exhaustion and immune-mediated pathology by E3 ubiquitin ligase Cbl-b during chronic viral infection. J Virol. 2008;82:3353–68.
107. Kumar J, Kumar R, Kumar Singh A, Tsakem EL, Kathania M, Riese MJ, et al. Deletion of Cbl-b inhibits CD8+ T-cell exhaustion and promotes CAR T-cell function. J Immunother Cancer. 2021;9:e001688.
108. Meng X, Liu X, Guo X, Jiang S, Chen T, Hu Z, et al. FBXO38 mediates PD-1 ubiquitination and regulates anti-tumour immunity of T cells. Nature. 2018;564:130–5.
109. Doedens AL, Phan AT, Stradner MH, Fujimoto JK, Nguyen JV, Yang E, et al. Hypoxia-inducible factors enhance the effector responses of CD8+ T cells to persistent antigen. Nat Immunol. 2013;14:1173–82.
110. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 2006;3:177–85.
111. Poljsak B, Šuput D, Milisav I. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxid Med Cell Longev. 2013;2013:956792.
112. Fisicaro P, Barili V, Montanini B, Acerbi G, Ferracin M, Guerrieri F, et al. Targeting mitochondrial dysfunction can restore antiviral activity of exhausted HBV-specific CD8 T cells in chronic hepatitis B. Nat Med. 2017;23:327–36.
113. Chowdhury PS, Chamoto K, Kumar A, Honjo T. PPAR-induced fatty acid oxidation in T cells increases the number of tumor-reactive CD8+ T cells and facilitates anti-PD-1 therapy. Cancer Immunol Res. 2018;6:1375–87.
114. Russell SL, Lamprecht DA, Mandizvo T, Jones TT, Naidoo V, Addicott KW, et al. Compromised metabolic reprogramming is an early indicator of CD8+ T cell dysfunction during chronic *Mycobacterium tuberculosis* infection. Cell Rep. 2019;29:3564–79.e5.
115. Verdura S, Cuyàs E, Martin-Castillo B, Menendez JA. Metformin as an archetype immuno-metabolic adjuvant for cancer immunotherapy. Onco Targets Ther. 2019;8:e1633235.
116. Vashisht R, Brahmachari SK. Metformin as a potential combination therapy with existing front-line antibiotics for tuberculosis. J Transl Med. 2015;13:83.
117. Scharping NE, Menk AV, Whetstone RD, Zeng X, Delgoffe GM. Efficacy of PD-1 blockade is potentiated by metformin-induced reduction of tumor hypoxia. Cancer Immunol Res. 2017;5:9–16.
118. Eikawa S, Nishida M, Mizukami S, Yamazaki C, Nakayama E, Udono H. Immune-mediated antitumor effect by type 2 diabetes drug, metformin. Proc Natl Acad Sci U S A. 2015;112:1809–14.
119. Siska PJ, van der Windt GJ, Kishton RJ, Cohen S, Eisner W, MacIver NJ, et al. Suppression of glut1 and glucose metabolism by decreased Akt/mTORC1 signaling drives T cell impairment in B cell leukemia. J Immunol. 2016;197:2532–40.
120. Sears JD, Waldron KJ, Wei J, Chang CH. Targeting metabolism to reverse T-cell exhaustion in chronic viral infections. Immunology. 2021;162:135–44.
121. Li Q, Yan Y, Liu J, Huang X, Zhang X, Kirschning C, et al. Toll-like receptor 7 activation enhances CD8+ T cell effector functions by promoting cellular glycolysis. Front Immunol. 2019;10:2191.
122. Chodisetti SB, Gowthaman U, Rai PK, Vidyarthi A, Khan N, Agrewala JN. Triggering through toll-like receptor 2 limits chronically stimulated T-helper type 1 cells from undergoing exhaustion. J Infect Dis. 2015;211:486–96.
123. Phan AT, Goldrath AW. Hypoxia-inducible factors regulate T cell metabolism and function. Mol Immunol. 2015;68:527–35.
124. Liu YN, Yang JF, Huang DJ, Ni HH, Zhang CX, Zhang L, et al. Hypoxia induces mitochondrial defect that promotes T cell exhaustion in tumor microenvironment through MYC-regulated pathways. Front Immunol. 2020;11:1906.
125. McCaw TR, Li M, Starenki D, Liu M, Cooper SJ, Arend RC, et al. Histone deacetylase inhibition promotes intratumoral

CD8+ T-cell responses, sensitizing murine breast tumors to anti-PD1. Cancer Immunol Immunother. 2019;68:2081–94.

126. Ghoneim HE, Fan Y, Moustaki A, Abdelsamed HA, Dash P, Dogra P, et al. De novo epigenetic programs inhibit PD-1 blockade-mediated T cell rejuvenation. Cell. 2017;170: 142–57.e19.

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RESEARCH ARTICLE

WILEY

T-cell exhaustion: A potential target biomarker of the tumour microenvironment affecting oesophageal adenocarcinoma

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Abstract

Background: Oesophageal adenocarcinoma (EAC) is one of the most common malignant tumours, and the number of patients is increasing year by year. T-cell exhaustion (TEX) is an important risk factor for tumour immunosuppression and invasion, but its underlying mechanism in the pathogenesis of EAC is not clear.

Methods: Unsupervised clustering was performed to screen relevant genes based on Gene Set Variation Analysis scores of the three pathways of the HALLMARK gene set IL2/IFNG/TNFA. Multiple enrichment analyses and data combinations were used to depict the relationship between TEX-related risk models and CIBERSORTx immune infiltrating cells. In addition, to explore the impact of TEX on EAC therapeutic resistance, we assessed the impact of TEX risk models on the therapeutic sensitivity of various novel drugs using single-cell sequencing and searched for their potential therapeutic targets and cellular communication.

Results: Four risk clusters of EAC patients were identified by unsupervised clustering and searched for potential TEX-related genes. Based on this, LASSO regression and decision trees were used to construct risk prognostic models containing a total of three TEX-associated genes in EAC. The results showed that TEX risk scores were significantly associated with the survival prognosis of EAC patients in both the Cancer Genome Atlas dataset and the independent validation set of Gene Expression Omnibus. Immune infiltration and cell communication analyses identified mast cell resting as a protective factor in TEX, and pathway enrichment analyses showed that the TEX risk model was highly associated with multiple chemokines as well as inflammation-associated pathways. In addition, higher TEX risk scores were associated with a weak responsiveness to immunotherapy.

Conclusion: We describe the immune infiltration, prognostic significance and potential possible mechanisms of TEX in the EAC patient population. This is a novel attempt to promote the development of novel therapeutic modalities and immunological target construction for oesophageal adenocarcinoma. It is expected to make a potential contribution to advancing the exploration of immunological mechanisms and the opening of target drugs in EAC.

KEYWORDS

oesophageal adenocarcinoma, individualised medicine, predictive model, T-cell exhaustion, tumour environment

1 | INTRODUCTION

According to the Global Cancer Statistics Report 2021, there were 604,100 new cases of oesophageal cancer worldwide in 2021, accounting for 3.1% of new malignancies, and more than 544,076 oesophageal cancer-related deaths, accounting for 5.5% of all deaths.[1,2] Patients with oesophageal cancer have a poor prognosis, especially those with advanced stages, with a 5 year survival rate of ~20%.[3–5] Oesophageal squamous cell carcinoma (ESCC) and EAC are the most predominant pathological types of oesophageal cancer, with ESCC accounting for more than 90% of the incidence.[6,7] Although the incidence of EAC is relatively low, there is a large incidence of underlying esophageal disease. Traditional treatments for EAC include surgical resection, chemotherapy and radiotherapy. However, in view of the low survival rate with traditional treatment modalities, some new treatment modalities such as immunotherapy and molecular targeted therapy are emerging as the future hope for EAC treatment.[8,9]

During tumourigenesis and invasion, antigen-specific T cells, although initially acquiring appropriate effector functions, gradually diminish to the point of loss with prolonged antigen stimulation, failing to convert to non-antigen-dependent memory T cells, a dysfunctional state representing a unique state of T-cell differentiation called TEX.[10,11] It has been shown that TEX plays an important role in tumour development, invasion, metastasis and chemoresistance.[12,13] This suggests that perhaps TEX is an important immunological element of the tumour microenvironment that mediates poorer treatment outcomes and poor prognosis in oesophageal cancer. Immune checkpoint inhibitor therapy has been widely used in clinical trials as a new breakthrough point in the treatment of oesophageal cancer. However, its therapeutic response in EAC remains highly controversial.[14] Considering that PD-1 therapy does not utilise endogenous T cells, perhaps this poor therapeutic responsiveness is closely related to the important role played by TEX in the evolution of the tumour microenvironment in EAC.[15,16]

As one of the main effector cells of anticancer immunity, CD8+ T cells are usually deregulated in tumour microenvironment infiltration.[17,18] Exhausted CD8+ T cells showed impaired viability and proliferative capacity, increased apoptotic rate and decreased effector cytokine production.[19] CD8+ T cells account for a large proportion of anticancer immune cells in the cancer microenvironment and have been the focus of research in cancer.[20] A stratified loss of expression of the effector cytokines interleukin-2 (IL-2), tumour necrosis factor-α and interferon-γ was observed in exhausted CD8+ T cells in cancer.[20,21] A decrease in cell viability, cell lysis and cell proliferation was also observed. An increasing number of studies have shown that, based on prognostic criteria associated with CD8+ T-cell depletion, it plays an important role in the prognosis of cancers such as liver, colorectal and breast cancers.[22,23] However, the prognostic features associated with CD8+ T-cell depletion have not been established in oesophageal adenocarcinoma.

The main immunological effects of TEX are manifested by a sustained increase in the expression of inhibitory receptors PD-1, CTLA-4, TIM-3 and LAG-3 or the simultaneous coexpression of multiple inhibitory receptors,[24] a progressive decrease in effector functions, including cytokine release (interferon-γ, IL-2) and perforin and granzyme production, and proliferation.[25] These potential mechanisms may lead to a reduction in the EAC's ability to proliferate and self-renew (IL-7 and IL-15 dependent) and metabolic changes, with reduced mitochondrial activity in depleted T cells, restricted glycolysis and reduced metabolic reserve capacity.[26] These potential mechanisms may contribute to the development of EAC. In addition, local infiltrative invasion of oesophageal cancer in the oesophageal region, for example, can induce T cells to enter a depleted state in order to evade host immune mechanisms and contribute to tumour invasion and metastasis and immune rejection.

In addition, with the development of science and technology, it is not sufficient to explore the tumour microenvironment and related mechanisms solely on the basis of bulk mRNA sequencing data. Although the identification of oncogenic drivers using this approach has led to some important therapeutic breakthroughs in a number of tumour types, it is still largely limited by the analytical techniques available.[27] Single-cell RNA sequencing (scRNA-seq) reveals the gene expression patterns of each individual cell and deciphers the intercellular signalling network of each cell.[28,29] Exploring the more specific features provides clear insights into the entire tumour ecosystem, such as the mechanisms of intra- and inter-tumour heterogeneity and cell-to-cell interactions via ligand–receptor signalling.[30–32] For this reason, an increasing number of studies have examined the tumor microenvironment of cancer cells on a single-cell basis.[33–37] A prospective single-cell sequencing experiment showed that ESCC and EAC differ in their cellular transcriptome profiles, leading to extensive intratumoural cellular heterogeneity and different treatment strategies for the two oesophageal cancers.[38] Therefore, to shed new light on EAC patients, an increasing number of studies are beginning to explore novel therapeutic strategies and mechanisms for EAC based on the single-cell transcriptome level.[39–41]

In the present study, we explored the microscopic mechanisms and immune regulatory features of TEX in the development of EAC. Through the analysis of transcriptomic data from large samples and single-cell sequencing data, we have drawn a blueprint for the regulation and impact of TEX on the overall immune environment of EAC. The relationship between TEX-related risk models and CIBERSORTx immune infiltrating cells was mapped through multiple enrichment analyses and data combinations. Furthermore, to explore the impact of TEX on therapeutic resistance in EAC, we used single-cell sequencing to assess the impact of TEX risk models on the therapeutic sensitivity of multiple novel drugs and to search for their potential therapeutic targets and cellular communication. This study provides important insights to promote the construction of immune biomarkers for EAC, and to explore the significance of TEX in the tumour microenvironment-related immune regulation of EAC.

2 | MATERIALS AND METHODS

2.1 | Data preparation

The primary data for this study came from the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases. We included oesophageal adenocarcinoma gene expression data and complete clinical data from TCGA, including age, gender, tumour stage, tumor, node, metastasis (TNM) stage, and survival status. GSE19417 was included as an external validation set for a total of 70 oesophageal adenocarcinoma patients. The inclusion and exclusion criteria for this study were as follows: inclusion criteria—(1) pathologically confirmed primary oesophageal adenocarcinoma, (2) reliable clinical follow-up time of no less than 30 days, (3) inclusion of mRNA and miRNA gene expression level-related data and (4) complete clinicopathological information and follow-up information; exclusion criteria—(1) secondary oesophageal tumours or other types of oesophageal tumours, (2) combined primary tumours elsewhere in the body and (3) incomplete follow-up information. Data used in this study were publicly available.

2.2 | Data preprocessing

The R "limma" package was used to normalise all datasets against the expression matrix, and the R "sva" package was used to remove batch effects from the datasets. Data from bulk transcriptomes were normalised to account for the lengths of genes and the number of aligned reads. In order to analyse further, log2 (FPKM + 1) values were transformed into fragments per kilobase per million fragments mapped (FPKM) expression values.

2.3 | Consensus clustering analysis of TEX-associated gene sets and exploration of differential genes

Consensus clustering analysis of the TCGA-EAC cohort was performed to identify TEX-associated gene clusters. The R package "ConsensusClusterPlus" (version 1.56.0) was used together with key parameters including maxK = 6 and duplicates = 500 for stable identification. By identifying different patterns of RNA modification regulation through consensus clustering, we further performed differential gene expression analysis. Unsupervised clustering was performed based on the Gene Set Variation Analysis (GSVA) scores of the three pathways, IL2/IFNG/TNFA, from the HALLMARK gene set. The R package "LIMMA" (version 3.48.3) was applied for pairwise comparison of gene expression of different patterns. LMFIT and EBayes functions were used to ensure accuracy. TEX genes were obtained from Gene Ontology (GO), the MSigDB database and previous studies in total, and differential TEX genes were the intersection of TEX genes with differentially expressed genes (DEGs). Subsequently, we screened for DEGs and used an adjusted p-value <0.05 and an absolute value of logFC >1 as the selection criteria.

We wanted to analyse in depth the differences between different subgroups of clusters and plotted Kaplan–Meier (K-M) survival curves for different clusters with a survival marker of 50%. The K-M survival curves for paired subgroups were used to analyse the differences in survival between the different clusters obtained by unsupervised clustering. In addition, we assessed the abundance of 22 immune cell species in the different cluster samples, using the CIBERSORTx algorithm. After an in-depth analysis of cell infiltration and inter-sample associations, we performed Cox regression analysis to explore the prognostic value of the different cells in each TEX cluster. The EAC was divided into high and low differential expression groups based on the median immune cell content, and differences in survival between the most different groups were assessed. In addition, we also mapped the differential gene analysis volcanoes for the four TEX clusters.

2.4 | Weighted coexpression network analysis of TEX-related genes and risk model construction

Weighted correlation network analysis (WGCNA) is a systems biology approach for characterising patterns of gene association between samples that can be used to identify highly synergistic gene sets and to identify candidate biomarker genes or therapeutic targets based on the endogenous nature of gene sets and the correlation between gene sets and phenotypes.[42] Based on these two conditions, gene networks can be divided into different modules according to expression similarity to identify hub genes: (1) genes with similar expression patterns may be co-regulated, functionally related, or in the same pathway; and (2) gene networks conform to scale-free distributions. The coexpression network of DEGs was constructed using the "WGCNA" package to transform differentially expressed genes into an adjacency matrix with a soft threshold of four and then into a topological overlap matrix to identify gene modules. The minimum number of genes in a gene module was 30, and the cut height threshold for merging similar gene modules was set to 0.25. The analysis consisted of searching an initial set of six modules and further investigation using the grey module that showed the strongest association.

Based on this, we began to identify the genes of interest with the highest correlation to TEX and used them for risk model construction for EAC. Univariate Cox regression analysis was performed in the TCGA training set, extracting prognosis-related genes from our in-gene set with a p-value <0.01. To obtain a quantitative description of each patient's survival risk, we further used least absolute shrinkage and selection operator (LASSO) regression analysis to calculate the patient's TEX index via the R package glmnet 4.1.3, with the dependent variable for LASSO regression being the number of days the patient survived. The results were validated using a survival random forest of 1000 trees and the importance of genes obtained from the LASSO regression with the R package random survival forest version 3.6.4 was ranked. With overall survival as the outcome event, a

p-value less than 0.05 was considered statistically significant. Efficacy assessment and functional exploration of TEX-associated gene risk models were performed. The TEX-related gene score was calculated using the following formula:

$$TEXriskscore = Coef_1 \times Geneexpression_1 + Coef_2 \times Geneexpression_2 + \cdots Coef_n \times Geneexpression_n$$

where Coef represents the prognostic value of each gene in Cox regression analysis and Gene expression values represent the expression values of the corresponding modelled genes.

We divided patients into high-risk and low-risk groups based on the formula generated by the risk model. Meanwhile, K-M survival curves were plotted for the high- and low-risk groups. Receiver operating characteristics (ROCs) were plotted to further analyse the predictive power of the TEX-related prognostic model for survival in EAC. Multifactor Cox regression analysis was used to determine the independent prognostic significance of the risk scores. To ensure the value of external generalisation of the prognostic model, we used another EAC dataset, GSE19417, for validation. Gene Ontology functional enrichment analysis was performed on the modules obtained from the weighted gene coexpression network analysis and risk model building blocks associated with the vital status of EAC patients to clarify the biological processes, molecular functions and cellular localisation of the genes involved in the modules, together with the Kyoto Encyclopedia of Genes and Genomes (KEGG). The GO annotation analysis was performed using the R package "clusterProfiler" (version 4.0.5) with a false discovery rate of <0.05 to identify significant enrichment pathways. Gene Set Enrichment Analysis (GSEA) software version 4.1.0 (Broad Institute, Cambridge, MA, USA) was used to identify genomic cohorts that were significantly altered between predefined Clusters in consensus clusters. A *p*-value of <0.05 and a false discovery rate of <0.25 were considered statistically significant. Based on the GSEA results obtained above, several gene sets were selected for GSVA analysis, where Microarray and RNA-seq data gene sets were enriched under parameter-free and unsupervised conditions. The gene–sample data matrix (microarray data, FPKM, RPKM, etc.) is converted into a gene-set–sample matrix. Based on this matrix, the enrichment of gene sets (e.g. KEGG pathway) in individual samples can be further analysed to explore the relevance of the TEXScore to multiple metabolic and tumour pathways.

The CIBERSORTx algorithm was used to analyse 22 different types of immune cell infiltration in the high- and low-risk groups. The R package "maftools" was used to display the detailed mutation status of the high- and low-risk groups in order to compare the two groups. Based on the expression matrix, CIBERSORT used a deconvolution algorithm to assess the composition and abundance of immune cells in mixed cells. Based on the expression matrix and the immune cell Marker gene set, ssGSEA calculated enrichment scores for single samples and gene set pairs to determine the degree of immune infiltration. mCP-counter quantified the abundance of eight immune cells and two stromal cells using transcriptomic data. To analyse the relationship between our constructed TEX prognostic risk model and EAC immune infiltration at the genetic level, we analysed the relationship between TEXScores and EAC-associated gene mutations. The type of RNA mutation and the probability of mutation in tumour cells of patients with oesophageal cancer in the TCGA-EAC database were determined (using the R package "maftools") to more closely relate clinical treatment choices to TEX. With the IMvigor210 software package, we analysed gene expression and immunotherapy efficacy in the IMvigor210 cohort in both high- and low-risk groups of the TEX risk model.

2.5 | Evaluation of TEX-associated gene models at the single-cell sequencing level and functional exhibition

Single-cell sequencing data from EAC obtained in public databases were subsequently processed using the Seurat R package (version 3.0.2) and the standard downstream processing package for this analysis. In addition, genes detected in fewer than three cells and genes detected in less than 200 cells were excluded, and the percentage of mitochondria detected was limited to no more than 20% of the total number of genes. The *t* data were then normalised using LogNormalize. *t*-Distributed random neighbourhood embedding was used for unsupervised clustering and unbiased viewing of cell populations on a two-dimensional map following principal component analysis. A cell population score of at least 0.25 was used in both populations to identify marker genes in each cluster using the "FindAllMarkers" function. The filtering criterion was an absolute log2-fold change (FC) ≥1 filtering value. In addition, the expression pattern of each marker gene within a cluster was visualised in Seurat using the "DotPlot" function to visualise its expression pattern. The SingleR package (version 1.0.0) was then used to annotate cell types based on marker-based information. The spatial distribution of several immune cells in EAC cells was localised and data on regional expression levels of the genes of interest were obtained. The expression of TEX risk genes in EAC patients was compared in tumour and normal tissues via the GEPIA2 online website (http://gepia2.cancer-pku.cn/#index) and the overall survival rates of the high- and low-expression groups were analysed. In addition, the functional enrichment process of major gene expression in the development of EAC was analysed by GSEA.

2.6 | Statistical analysis

Independent *t*-tests and Mann–Whitney *U* tests were used to determine statistical significance when comparing two groups of variables with normal and nonnormal distributions. One-way analysis of variance (ANOVA) and Kruskal–Wallis tests were used to compare differences between more than two groups. Cox regression analysis was used to determine prognostic factors. Overall survival and riskScore were determined using the R package survivor and cut-off values were determined before all survival curves were generated using the R package survivor. All heatmaps were plotted using the R package ComplexHeatmap 2.4.3. Data comparisons were visualised using the

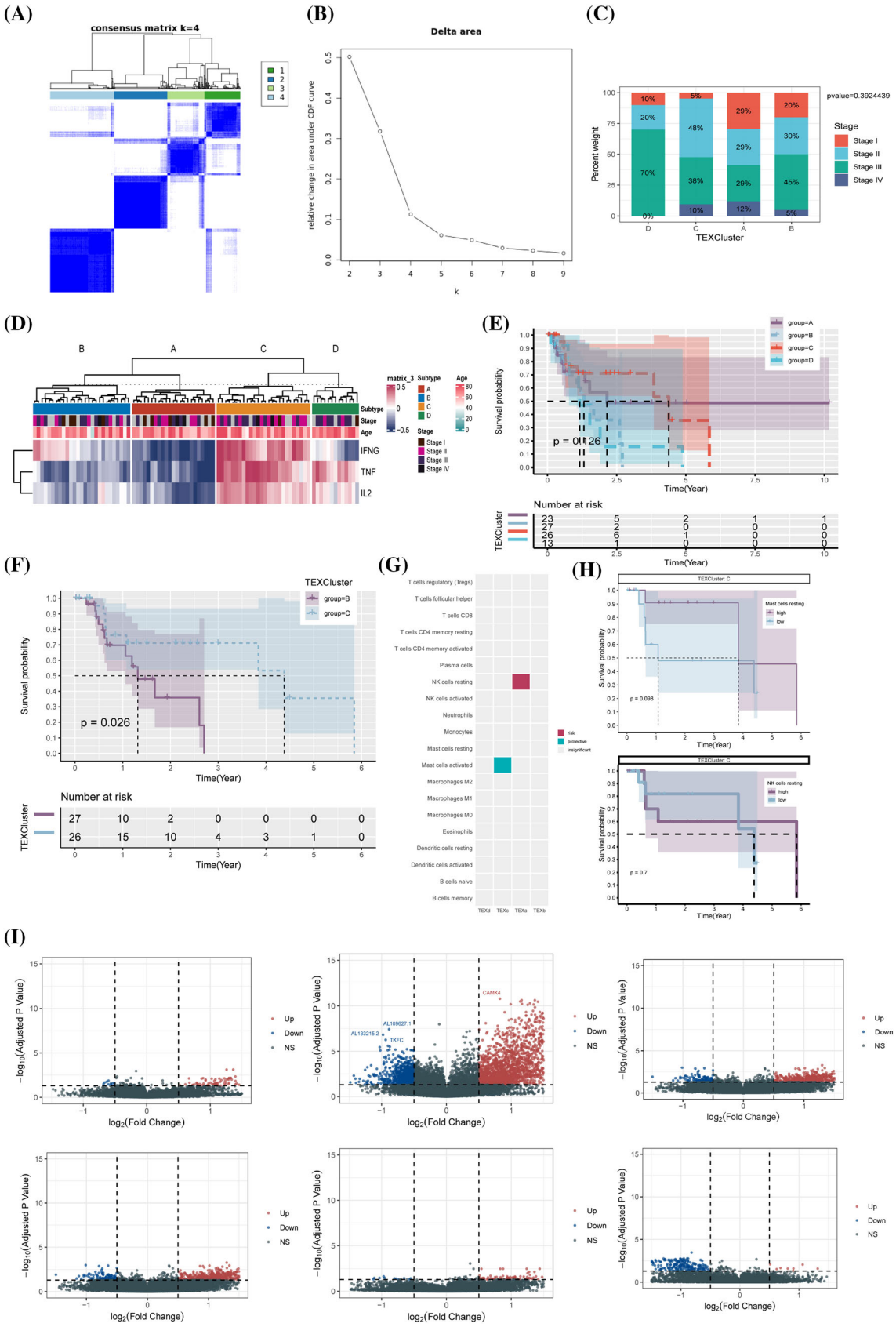

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FIGURE 1 Consensus clustering analysis of TEX-related gene sets and differential gene acquisition. (A) Unsupervised clustering analysis of TEX-related gene bases with consensus matrix K = 4 as the best clustering criterion. (B) Scatter plot of area change under the CDF curve for unsupervised clustering curves with different K-values sets. (C) Hierarchical histogram analysis of the tumour stage distribution of oesophageal adenocarcinoma (EAC) patients with four TEX clusters. (D) Correlation heat map comparing the four TEX-related clusters in terms of stage, age distribution and expression of the three gene pathways. (E) K-M curve survival analysis of four cluster comparisons. (F) K-M curve survival analysis between Cluster B and Cluster C. (G) Expression levels of different immune cell types obtained by CIBERSORTX on four prognostic values of TEX subsets. (H) K-M survival curve analysis of groups with different expression levels of mast cell resting and NK cell resting in Cluster C. (I) Volcanic plots comparing differential gene expression in different clusters.

R package ggplot2. All statistical analyses were performed using two-sided statistical analysis with R software. Statistical significance was defined as a *p*-value of <0.05.

3 | RESULTS

3.1 | Consensus clustering analysis of TEX-associated gene sets and differential gene acquisition

We identified TEX-related pathways and gene expression patterns by consensus clustering analysis, with the *k*-values of the consensus matrix set to 1–6, respectively. Finally, patients were divided into four clusters based on the GSVA scores of the three pathways of the HALLMARK gene set IL2/IFNG/TNFA, with optimal conditions (Figure 1A–B). Cluster C had the highest score for the IL2/IFNG/TNFA pathway and cluster D had the lowest score. In addition, we also analysed the tumour stage distribution of EAC patients in the four TEX clusters using a hierarchical histogram (Figure 1C). Cluster D was dominated by patients with Stage III, accounting for 70% of the entire Cluster population, and there were no patients with Stage IV in this Cluster; ClusterA had a significantly lower distribution of Stage IV for Clusters B and C; the distribution of Stage IV patients was more similar. In Figure 1D, we compare the Stage and Age distributions of the four TEX-related clusters and the expression of the three gene pathways by correlation heat map. For TNF and IL2, Clusters C and D had significantly higher expression than Clusters A and B. In addition, we plotted K-M survival curves for the overall survival of the four clusters and there were no statistical differences in survival between the four clusters ($p = 0.126$). In addition, we also analysed the K-M curves for the four clusters in two-by-two comparisons (Figure 1E). Interestingly, we found that the survival analysis of the four TEX clusters revealed differences in survival, with differences concentrated between Clusters B and C (Figure 1F). The two-by-two survival curves for several other clusters are shown in the Supplementary Material (Figure S1). For several other clusters, there were no statistically significant survival differences between the comparisons. This result suggests that there is a factor between subgroup B and subgroup C that makes them significantly different in survival, and this is the goal of our study.

After obtaining the cellular infiltration of each sample using CIBERSORTx, the prognostic value of the various cells in each TEX cluster was explored by Cox regression analysis (Figure 1G). It was found that mast cell resting was protective in TEXc, while natural killer (NK) cell resting was a risk factor for TEXb. This suggests to us that perhaps the regulation of immune infiltration of TEX genes in EAC is associated with NK cells and mast cells. We also delineated the different expression levels of the two cell groups in Cluster C and found that the expression levels of mast cell and NK cells were not associated with the survival status of Cluster C by K-M curves (Figure 1H). To further analyse the transcriptome differences between the different clusters, we compared the different clusters using differential gene analysis with analysis criteria greater than or equal to $|\log FC| = 0.5$, corrected for *p*-values less than 0.05 and mapped volcano maps used to show the results of the analysis (Figure 1I).

3.2 | Weighted coexpression network analysis of TEX-associated genes and risk model construction

Subsequently, we further analysed the four TEX-associated clusters using WGCNA analysis. The results revealed (Figure 2A–C) that the yellow and green-yellow groups were most associated with survival-related information, and two groups were selected for subsequent analysis. There were 225 genes in the yellow and green-yellow modules that were selected as pivotal genes because they had absolute values of module affiliation [MM] greater than 0.5 and absolute values of gene significance [GS] greater than 0.5 within the modules. Variables were selected based on minimum depth values below threshold (0.001) and above threshold (0.2). By the random forest algorithm, three genes were obtained (*ALS2CL*, *LINC02864* and *PLAAT3*) as initial candidate EAC prognostic markers (Figure 2D, E). This suggests that TEX-related genes are closely associated with the survival of EAC patients.

To facilitate clinical risk assessment of TEX-related genes as well as practical applications, we constructed a riskScore system containing three TEX-related genes by LASSO analysis. The risk score formula was: TEXriskScore = (−0.114763752**ALS2CL* + 0.095103745**LINC02864* − 0.003715602**PLAAT3*). To further enrich the clinical generalisability of the model, a multifactorial Cox analysis was used (Figure 3A). We assessed the clinical and pathological information of EAC patients as well as the prognostic predictive ability of riskScore, which showed that the predictive ability of riskScore for the survival of EAC patients was significantly higher than other clinical indicators ($p < 0.05$, risk ratio, 1.720 [1.119–2.642]). TCGA-EAC patients were divided into a high-risk group and a low-risk group according to each patient's risk score. K-M survival curves between the high-risk and low-risk groups showed that the overall survival rate was significantly lower in the high-risk group than in the

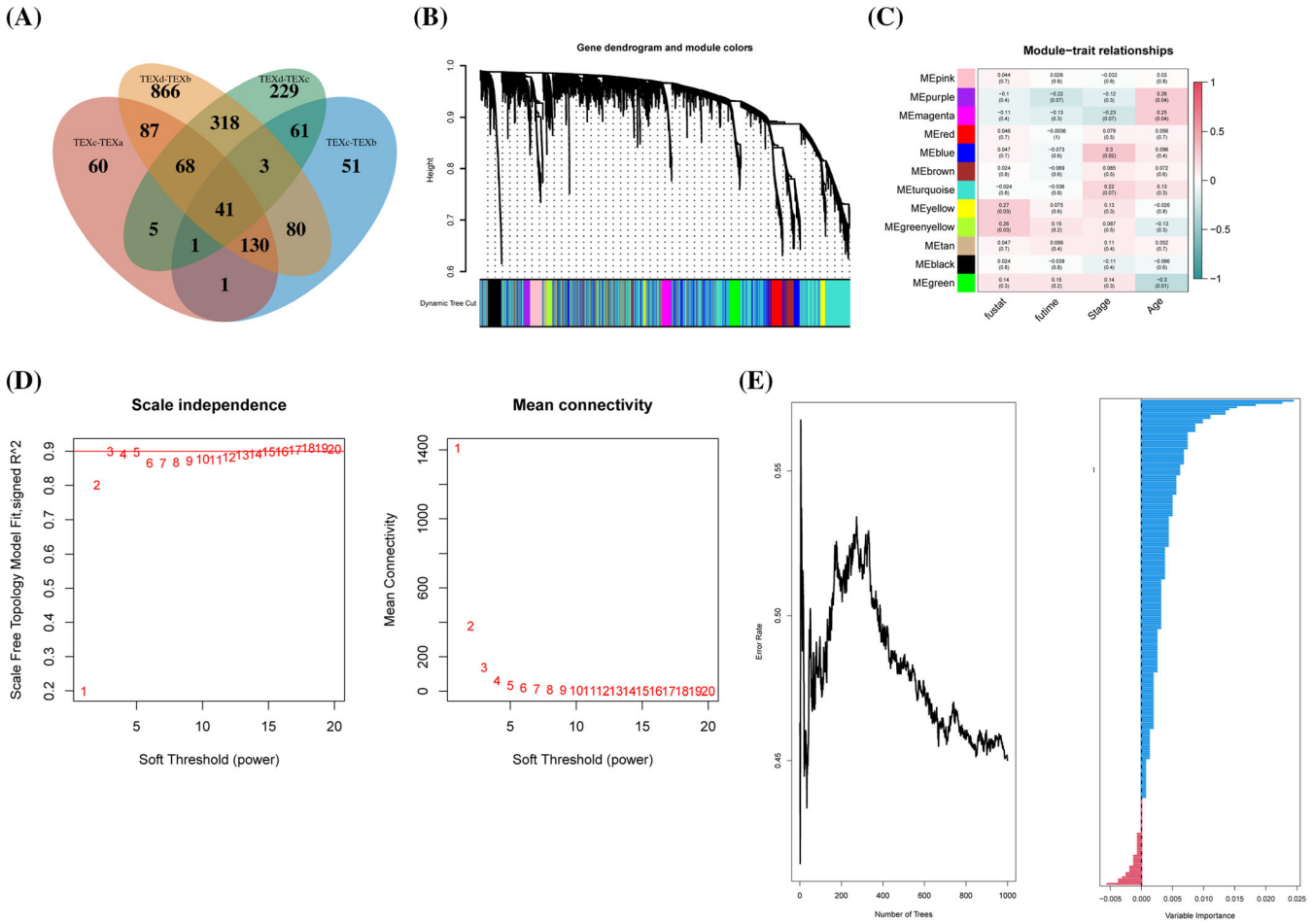

FIGURE 2 Weighted coexpression network analysis and random forest-based gene selection for TEX-related gene sets. (A) Analysis of Wayne butterfly plots for weighted correlation network analysis (WGCNA) analysis after demerging four clustered differential genes; (B) distribution of gene dendrogram and module colours plots in the four clusters of TEX; (C) module–trait relationship colour configuration plots for the four clustered differential gene plots; (D) preconstruction of risk model parameter configuration and soft threshold analysis for the decision tree; and (E) histogram of survival risk gene composition selected by survival random forest analysis in the yellow and green-yellow datasets.

low-risk group ($p < 0.005$) (Figure 3B). In Figure 3C, we evaluate the survival predictive ability of the risk score for EAC patients using ROC curves, with 1, 3 and 5 year survival prediction levels of 0.605, 0.632 and 0.704 for EAC patients, respectively. In addition, we also analysed the prognostic predictive ability of the TEX-related risk score in the independent validation set of the GSE19417 database ability. It was found that in Figure 3D, the model had equally good prognostic stratification ability even in the independent data validation set. In the GEO database, the survival prediction for EAC patients at 1, 3 and 5 years showed better results, with 0.785, 0.776 and 0.744, respectively (Figure 3E).

3.3 | Efficacy assessment and biological function exploration of TEX-associated genetic risk models

To enrich the biological function of high-risk patients, we used GSEA, KEGG and GO datasets to marker high-risk patients. In the GSEA analysis (Figure 3F), the top five enriched signature pathways were hallmark interferon-alpha response, interferon-gamma response, allograft rejection and epithelial mesenchymal. The enriched GO pathways (Figure 3G) are mainly pancreas beta cells, the P53 pathway, apoptosis, complement and TNFA signalling via NFKB. In the high-risk group of patients, the five major enriched KEGG pathways were allograft rejection, epithelial mesenchymal transition, inflammatory response, interferon-gamma response, and myogenesis (Figure 3H). We also assessed KEGG enrichment in the low-risk group of patients, with the top five enriched KEGG pathways including cell adhesion molecules cams, chemokine signalling pathway, haematopoietic cell lineage, leishmania infection and primary immunodeficiency (Figure 3I). Functional analysis of these pathways showed that TEX-associated high-risk patients were highly associated with many tumour proliferation and metabolism-related pathways, suggesting targeting TEX primarily through tumour proliferation and metabolism pathways influencing the survival of EAC patients.

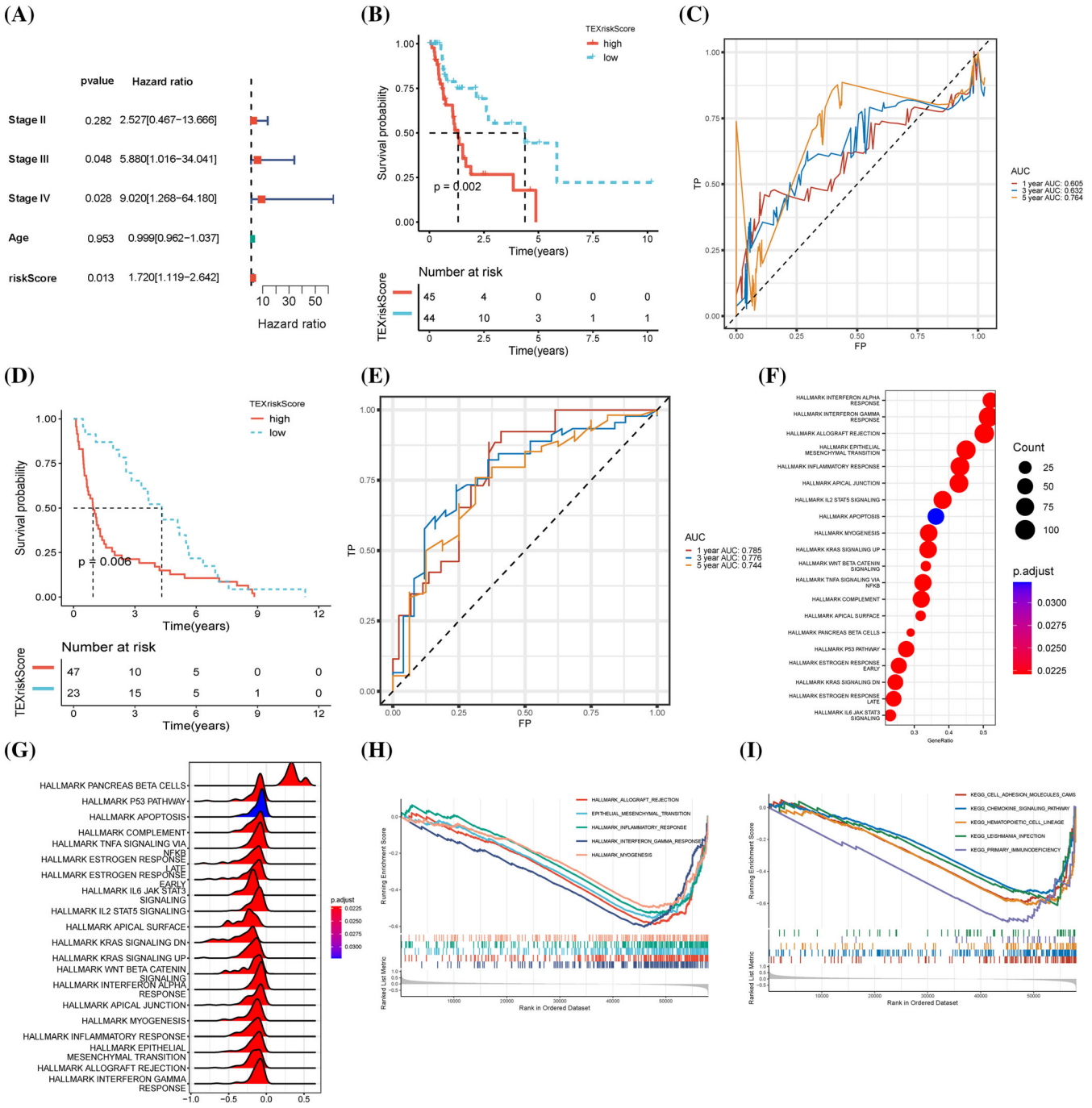

FIGURE 3 Prognostic survival model construction and biological function enrichment analysis of TEX-related genes. (A) Forest plots from multifactorial Cox regression analysis comparing the risk prediction function of riskScore with multiple clinical indicators; (B) K-M survival curves for high-risk vs. low-risk subgroups according to TEX risk scores; (C) ROC curves for 1, 3 and 5 year survival rates applying risk scores in TCGA-EAC; (D) GSE19417 dataset of K-M survival curves for high-risk vs. low-risk subgroups according to TEX risk scores; (E) ROC curves for 1, 3 and 5 year survival with application of risk socres in GSE19417; (F) landmark pathway analysis for high-risk patients with TEX risk scores using Gene Set Enrichment Analysis (GSEA) analysis; (G) landmark pathway analysis for high-risk patients with TEX risk scores using Gene Ontology (GO) analysis signature pathway analysis; and (H and I) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis for patients in the low- and high-risk groups of the TEX risk score.

On this basis, several gene sets were selected for GSVA analysis based on the above GSEA results and TEXScore was found to be associated with multiple metabolic as well as tumour pathways. The specific results are shown in Figure 4A and B, mainly enriched in pancreatic cancer, Fc gamma R-mediated phagocytosis, the B-cell receptor signalling pathway, acute myeloid leukaemia, basal cell carcinoma and the hedgehog signalling pathway pathway. These enriched pathways not only have similar malignant tumour diseases,

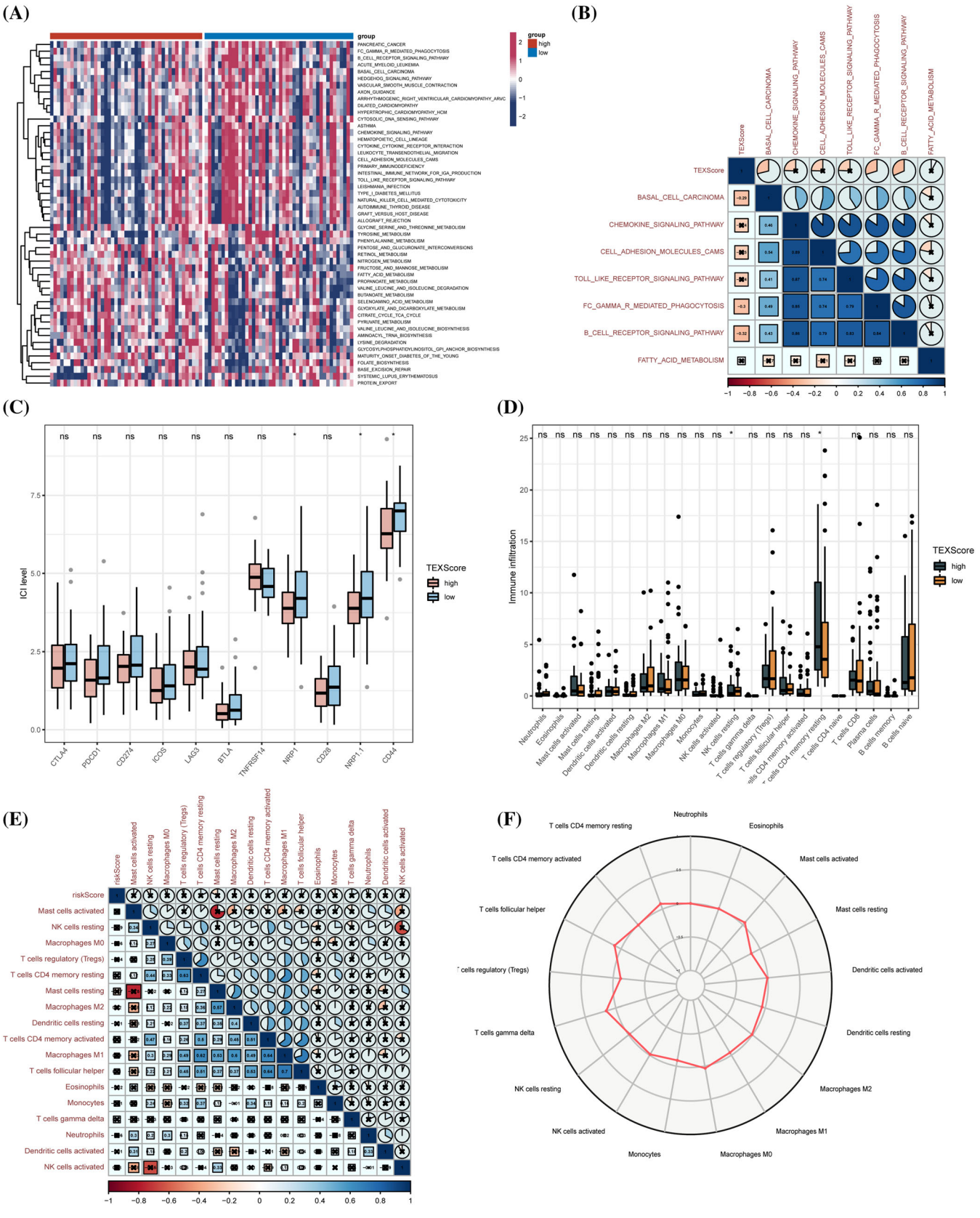

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FIGURE 4 Pathway enrichment and immune infiltration assessment of TEX-associated gene risk models. (A) GSVA analysis of the multigene set to assess the correlation of TEXScore with multiple metabolic as well as tumour pathways; (B) correlation heat map analysis of the correlation of TEXScore with multiple metabolic and tumour pathways; (C) box plot analysis of the expression levels of 11 immune checkpoints in the high-risk and low-risk groups; (D) immune infiltration analysis between 22 immune cells and TEXScore of box plot; (E) correlation heat map analysing the correlation of immune infiltration between two immune cells and TEXScore; (H) radar plot further showing the correlation of TEXScore with the content of major immune cells in the high-risk group.

but are also enriched in immune-related diseases or reactions, indicating that the genes we screened have research significance and value. Potential pathways through which TEX functions in patients with EAC are highlighted. Subsequently, we further explored the value of TEX in immunotherapy, based on an analysis of the potential association between TEX and immune infiltration in previous studies. The expression levels of 11 immune checkpoints in the high-risk and low-risk groups were analysed and boxed histograms were plotted. The results showed higher expression of *CD44* in patients in the high-risk group for the risk score of TEX, suggesting that the study of inhibition of the binding of TEX pathway genes to *CD44* may be a novel potential target for future tumour immunotherapy (Figure 4C). The results for 22 immune cell infiltrations showed more resting CD4 cells and fewer activated NK cells in the high-risk group (Figure 4D). The TEX risk score was further correlated with 22 immune cells as shown in Figure 4E. Radar plots further show the correlation between TEXScores and the content of major immune cells in the high-risk group (Figure 4F), suggesting that TEX may influence the prognosis of EAC by regulating T cells gamma delta.

Immediately afterwards, we sought to analyse the potential close association between TEX-related mutations and EAC tumour development at the nucleotide level. In Figure 5A and B, we assessed the gene mutations in patients in the high-risk and low-risk TEX groups, respectively. As seen from the images, 42 and 44 genes were mutated in the two groups of patients, respectively, with the highest frequency of *TP53* and TTN mutations in the HIGH group. We sought to explore the major mutation patterns of TEX-related gene mutations in EAC patients. A missense mutation was found to be the most common gene mutation type in EAC, SNP was the most common gene mutation type, and C > T mutation was the main single nucleotide variants (SNV) mutation classification (Figure 5C). We wanted to further evaluate the direct association between the main clinical treatments and TEX gene expression. By comparing the efficacy of immunotherapy and chemotherapy in high- and low-risk groups, we performed a subgraph analysis of the TCGA-EAC dataset and the IMvigor210 dataset. The response rate to immunotherapy was lower in the high-risk group (Figure 5D) and the TEXScore was higher in the low-responder group than in the responder group, with a statistically significant difference between the two (Figure 5E). Overall survival was also found to be lower in the high-risk group than in the low-risk group when receiving immunotherapy, irrespective of the median risk score, as revealed by the survival curves (Figure 5F). These results suggest that the TEX risk score has an important role in predicting the efficacy of immunotherapy and the prognosis of patients receiving immunotherapy. This may account for the close association between the TEX risk score and immune infiltration.

3.4 | Evaluation of TEX-related gene models at the single-cell level and functional exhibition

To further explore the internal mechanisms underlying the therapeutic role of TEX in the EAC patient population, a single-cell sequencing dataset of T cells was used to search for potential therapeutic target cell interactions. We identified 13 cell subtypes (B, CD4 Tconv, CD8T cells, CD8Tex, DC, NK, Tproif and Treg; Figure 6A). We identified the key gene of the risk model, ALS2CL, which is mainly expressed in endothelial, malignant and macro cells (Figure 6B, C), as a potential therapeutic target. The cell communication results showed (Figure 6D and E) that TEX interacts mainly with CD8T cells. This reveals a new explanatory theory and therapeutic target for TEX deficiency in EAC. The GSEA analysis revealed that TEX cells are predominantly enriched in immunodeficiency pathway processes (Figure 6F).

4 | DISCUSSION

Oesophageal adenocarcinoma is gradually gaining attention as more and more patients with oesophageal cancer are identified during follow-up. Considering the complexity of the treatment of EAC, the burden of survival for patients with oesophageal cancer has been exacerbated.[43,44] The clinical value of early screening for primary oesophageal cancer has now been affirmed and several relevant screening models and evaluation methods have been proposed. A number of studies have reported the risk of developing EAC.[45] However, most of them have not screened for and assessed high-risk influencing factors, and there is a relative gap in the analysis of its main pathogenic mechanisms and immunological infiltration indicators.

Given the increasing focus on EAC, it is necessary to build a transcriptome immune infiltration risk prediction model for EAC on the basis of a large number of cases. We identified potential TEX-associated genes in EAC patients by unsupervised clustering, which all showed good performance in our analysis. To facilitate the clinical risk assessment and practical application of TEX-associated genes, we constructed a risk model containing three TEX-associated genes (ALS2CL, LINC02864 and PLAAT3) by LASSO analysis. These three genes play a greater or lesser role in the development and progression of cancer, but our experiment is the first to combine these

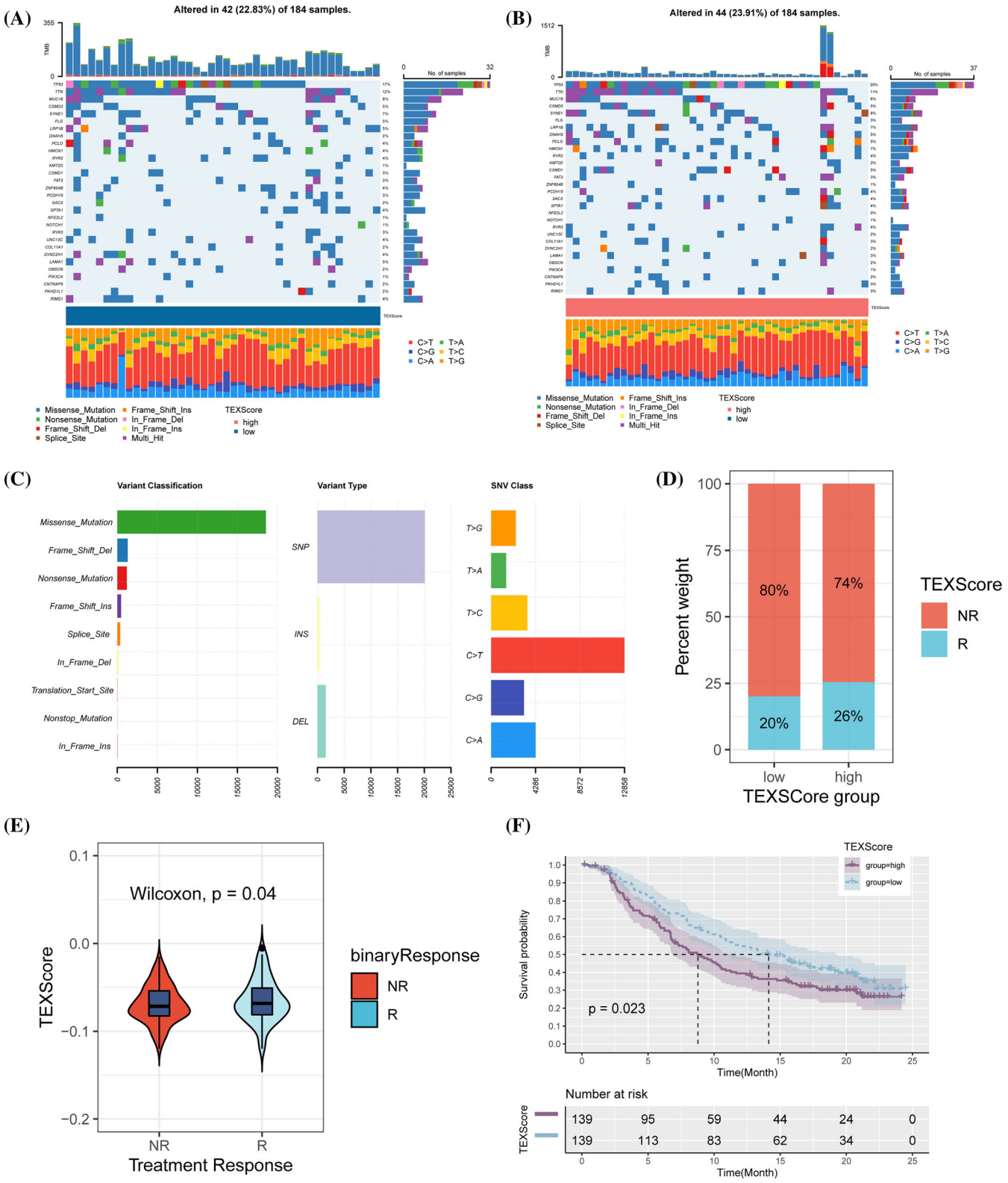

FIGURE 5 Gene mutation analysis and treatment response evaluation of TEX-related genetic risk model. (A) Exhibition plot of mutations in patients in the high-risk group of TEX-associated gene risk assessment score; (B) exhibition plot of mutations in patients in the low-risk group of TEX-associated gene risk assessment score; (C) histogram of mutation classification, species and SNV Class in EAC patients; (D) histogram of TEXScore distribution in different treatment response groups in the IMvigor210 dataset; (E) violin plot reveals the distribution of TEXScore in different treatment response groups; (F) K-M survival curves depicting significant differences in survival across TEX risk subgroups.

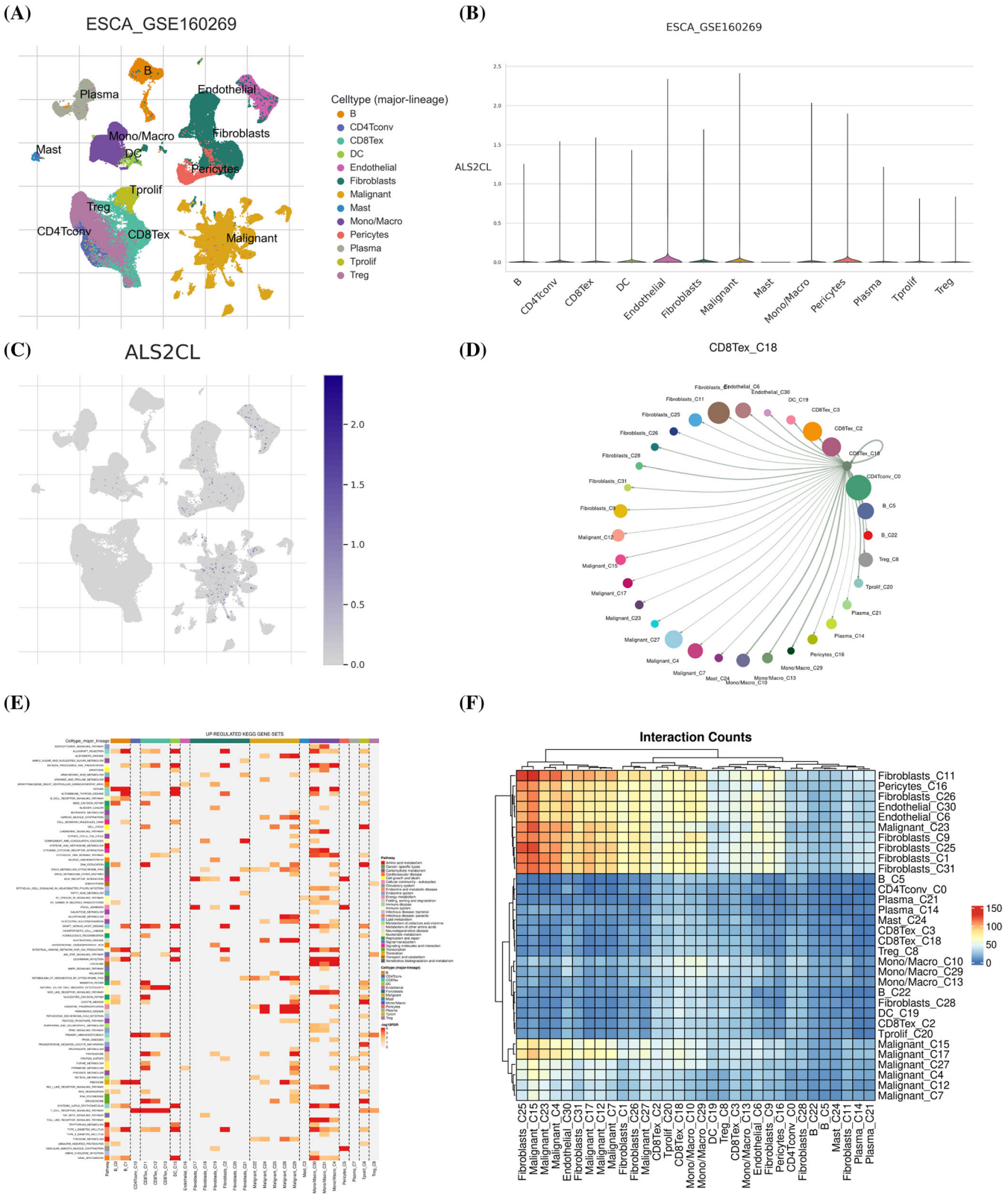

FIGURE 6 Assessment and functional exhibition of TEX-related gene models at the single-cell level. (A) Immune cell distribution map from single-cell sequencing reveals the main immune infiltration distribution of EAC cells; (B) exhibition of the distribution of ALS2CL, the main risk gene in the TEX risk model, in different immune cells; (C) visualisation of the distribution of ALS2CL in different immune cells from single-cell sequencing; (D) cellular communication reveals the main interaction of TEX with CD8 somatic cells; (E and F) GSEA analysis shows that TEX cells are mainly enriched in immunodeficiency pathway processes.

three genes as a global risk model. In addition, there are many risk models with multiple genes used to predict the prognosis of cancer patients in clinical practice. For example, the National Comprehensive Cancer Network clinical practice guidelines for breast cancer strongly recommend 21 gene expression testing in breast cancer patients.[46] However, the risk model we investigated consisted of three genes, representing a more convenient test for clinical use. Subsequently, we deeply explored and analysed the biological function and pathway-related enrichment of the constructed TEX gene model. At the same time, the perinatal relationship between the TEX pathway and tumour immunity was investigated by immune infiltration and cell communication analysis. In addition, single-cell sequencing data also delineated the predominant distribution of risk genes for TEX at the single-cell level. This suggests that TEX may play an important role in the development of future EACs and in exploring new goals.

We chose to perform an unsupervised cluster analysis based on the three closest TEX signalling pathways (IL2/IFNG/TNFA) in 184 LUAD patients and classified patients into four clusters. PD-1 overexpression resulted in inhibition of signalling and induction of TEX, leading to tumour immune escape, suggesting that TEX-related pathways could be used to influence and interfere with PD-1 therapeutic resistance. Analysis of immune cell infiltration in four TEX clusters22 by CIBERSORTx showed that increased mast cells in TEXa and TEXc, and CD4 T-cell resting were associated with a poorer prognosis. We used GSEA, KEGG and GO datasets for marker labelling of high-risk patients. In the GSEA analysis, the main enriched signature pathways were hallmark interferon-alpha response, interferon-gamma response, allograft rejection and epithelial mesenchymal transition. The main enriched GO pathways were pancreas beta cells, the P53 pathway, apoptosis, complement and TNFA signalling via NFKB. In the high-risk group of patients, the main KEGG pathways are allograft rejection, epithelial mesenchymal transition, inflammatory response, interferon gamma response and myogenesis. This suggests that these metabolic pathways can be used for TEX and EAC cross-linking interference.

The present study also has some drawbacks and limitations. First, the data applied in this study were obtained from the public database TCGA, and although its reliability and data richness have been adequately studied for inflammation, future rich experimental exploration and mechanistic studies at the cellular level are needed. Secondly, the study included more limited molecular and clinical information on patients with EAC, and these pathological factors are relevant to clinical treatment decisions and the role of the TEX pathway. We hope that we can include more molecular pathological and clinical information related to EAC in future studies, to deeply analyse the interaction between TEX-related genes and the immunological pathway of EAC, and to comprehensively explore and analyse the developmental mechanisms of the TEX pathway in EAC in depth.

Through in-depth bioinformatics analysis with single-cell sequencing and transcriptome analysis, we confirmed the high relevance of TEX-related genomic models to the immune infiltration and immunological pathways in EAC. This suggests that the interaction between the TEX pathway and immune infiltration and immune cells may be a potential target to facilitate the development of novel tumour therapies for EAC.

However, our experiments still have some limitations. Our risk model for EAC was developed based on TEX-associated genes, but has not been further validated from cell experiments and *in vitro* experiments. These results help to further demonstrate the usability of our study. However, our study also has certain advantages. Unlike traditional risk models, our model is based on bulk transcriptome data and single-cell sequencing data and has an external validation set, and our research results are meaningful.

5 | CONCLUSIONS

The potential role of TEX in tumour development has received increasing attention. We describe the immune infiltration, prognostic significance and potential possible mechanisms of TEX in a patient population with oesophageal adenocarcinoma. This is a novel attempt to promote the development of novel therapeutic modalities and immunological target construction for oesophageal adenocarcinoma, although more molecular biology experiments are needed to further solidify this idea in the future. This is expected to make a potential contribution to advancing the exploration of immunological mechanisms and the opening of targeted drugs in oesophageal adenocarcinoma.

AUTHOR CONTRIBUTIONS

Shiyu Peng was responsible for the construction of the article framework, information extraction and data processing. Xiaojiang Han and Geng Wenbin were responsible for the inclusion of the dataset. Zhao-Lifang reviewed the whole manuscript.

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We sincerely appreciate all members who participated in data collection and analysis.

CONFLICT OF INTEREST STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the online article material.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONSENT FOR PUBLICATION

All authors agreed to publish.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249. doi:10.3322/caac.21660
2. Vineis P, Wild CP. Global cancer patterns: causes and prevention. *Lancet (London, England)*. 2014;383(9916):549-557. doi:10.1016/S0140-6736(13)62224-2
3. Thrift AP. Global burden and epidemiology of Barrett oesophagus and oesophageal cancer. *Nat Rev Gastroenterol Hepatol*. 2021;18(6):432-443. doi:10.1038/s41575-021-00419-3
4. Peters Y, al-Kaabi A, Shaheen NJ, et al. Barrett oesophagus. *Nat Rev Dis Primers*. 2019;5(1):35. doi:10.1038/s41572-019-0086-z
5. Coleman HG, Xie S, Lagergren J. The epidemiology of esophageal adenocarcinoma. *Gastroenterology*. 2018;154(2):390-405. doi:10.1053/j.gastro.2017.07.046
6. Singh S, Sharma AN, Murad MH, et al. Central adiposity is associated with increased risk of esophageal inflammation, metaplasia, and adenocarcinoma: a systematic review and meta-analysis. *Clin Gastroenterol Hepatol*. 2013;11(11):1399-1412.e7. doi:10.1016/j.cgh.2013.05.009
7. Zhang HY, Spechler SJ, Souza RF. Esophageal adenocarcinoma arising in Barrett esophagus. *Cancer Lett*. 2009;275(2):170-177. doi:10.1016/j.canlet.2008.07.006
8. Lagergren J, Smyth E, Cunningham D, Lagergren P. Oesophageal cancer. *Lancet (London, England)*. 2017;390(10110):2383-2396. doi:10.1016/S0140-6736(17)31462-9
9. Joseph A, Raja S, Kamath S, et al. Esophageal adenocarcinoma: a dire need for early detection and treatment. *Cleve Clin J Med*. 2022;89(5):269-279. doi:10.3949/ccjm.89a.21053
10. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol*. 2015;15(8):486-499. doi:10.1038/nri3862
11. Chow A, Perica K, Klebanoff CA, Wolchok JD. Clinical implications of T cell exhaustion for cancer immunotherapy. *Nat Rev Clin Oncol*. 2022;19(12):775-790. doi:10.1038/s41571-022-00689-z
12. Blank CU, Haining WN, Held W, et al. Defining 'T cell exhaustion'. *Nat Rev Immunol*. 2019;19(11):665-674. doi:10.1038/s41577-019-0221-9
13. Good CR, Aznar MA, Kuramitsu S, et al. An NK-like CAR T cell transition in CAR T cell dysfunction. *Cell*. 2021;184(25):6081-6100. doi:10.1016/j.cell.2021.11.016
14. Van Der Kraak L, Goel G, Ramanan K, et al. 5-fluorouracil upregulates cell surface B7-H1 (PD-L1) expression in gastrointestinal cancers. *J Immunother Cancer*. 2016;4:1-8.
15. Huang AC, Postow MA, Orlowski RJ, et al. T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. *Nature*. 2017;60-65.
16. Pauken KE, Sammons MA, Odorizzi PM, et al. Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. *Science*. 2016;354(6316):1160-1165. doi:10.1126/science.aaf2807
17. Dolina JS, Van Braeckel-Budimir N, Thomas GD, Salek-Ardakani S. CD8+ T cell exhaustion in cancer. *Front Immunol*. 2021;12:715234. doi:10.3389/fimmu.2021.715234
18. Ma X, Bi E, Lu Y, et al. Cholesterol induces CD8+ T cell exhaustion in the tumor microenvironment. *Cell Metab*. 2019;30(1):143-156.e5. e145. doi:10.1016/j.cmet.2019.04.002
19. Hashimoto M, Kamphorst AO, Im SJ, et al. CD8 T cell exhaustion in chronic infection and cancer: opportunities for interventions. *Annu Rev Med*. 2018;69(1):301-318. doi:10.1146/annurev-med-012017-043208
20. He Q-F, Xu Y, Li J, Huang Z-M, Li X-H, Wang X. CD8+ T-cell exhaustion in cancer: mechanisms and new area for cancer immunotherapy. *Brief Funct Genomics*. 2019;18(2):99-106. doi:10.1093/bfgp/ely006
21. McLane LM, Abdel-Hakeem MS, Wherry EJ. CD8 T cell exhaustion during chronic viral infection and cancer. *Annu Rev Immunol*. 2019;37(1):457-495. doi:10.1146/annurev-immunol-041015-055318
22. Guan Y, Kraus SG, Quaney MJ, Daniels MA, Mitchem JB, Teixeiro E. FOLFOX chemotherapy ameliorates CD8 T lymphocyte exhaustion and enhances checkpoint blockade efficacy in colorectal cancer. *Front Oncol*. 2020;10:586. doi:10.3389/fonc.2020.00586
23. Wang X, He Q, Shen H, et al. TOX promotes the exhaustion of antitumor CD8+ T cells by preventing PD1 degradation in hepatocellular carcinoma. *J Hepatol*. 2019;71(4):731-741. doi:10.1016/j.jhep.2019.05.015
24. Barsch M, Salié H, Schlaak AE, et al. T-cell exhaustion and residency dynamics inform clinical outcomes in hepatocellular carcinoma. *J Hepatol*. 2022;77(2):397-409. doi:10.1016/j.jhep.2022.02.032
25. Whiteside TL. Exosomes and tumor-mediated immune suppression. *J Clin Invest*. 2016;126(4):1216-1223. doi:10.1172/JCI81136
26. Xu J, Fang Y, Chen K, et al. Single-cell RNA sequencing reveals the tissue architecture in human high-grade serous ovarian cancer. *Clin Cancer Res*. 2022;28(16):3590-3602. doi:10.1158/1078-0432.CCR-22-0296
27. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364(26):2507-2516. doi:10.1056/NEJMoa1103782
28. Xing X, Yang F, Huang Q, et al. Decoding the multicellular ecosystem of lung adenocarcinoma manifested as pulmonary subsolid nodules by single-cell RNA sequencing. *Sci Adv*. 2021;7(5):eabd9738. doi:10.1126/sciadv.abd9738
29. Hwang B, Lee JH, Bang D. Single-cell RNA sequencing technologies and bioinformatics pipelines. *Exp Mol Med*. 2018;50(8):1-14. doi:10.1038/s12276-018-0071-8
30. Finotello F, Eduati F. Multi-omics profiling of the tumor microenvironment: paving the way to precision immuno-oncology. *Front Oncol*. 2018;8:430. doi:10.3389/fonc.2018.00430
31. Saliba A-E, Westermann AJ, Gorski SA, Vogel J. Single-cell RNA-seq: advances and future challenges. *Nucleic Acids Res*. 2014;42(14):8845-8860. doi:10.1093/nar/gku555
32. Kolodziejczyk AA, Kim JK, Svensson V, Marioni JC, Teichmann SA. The technology and biology of single-cell RNA sequencing. *Mol Cell*. 2015;58(4):610-620. doi:10.1016/j.molcel.2015.04.005
33. Xiao Z, Dai Z, Locasale JW. Metabolic landscape of the tumor microenvironment at single cell resolution. *Nat Commun*. 2019;10(1):3763. doi:10.1038/s41467-019-11738-0
34. Qian J, Olbrecht S, Boeckx B, et al. A pan-cancer blueprint of the heterogeneous tumor microenvironment revealed by single-cell profiling. *Cell Res*. 2020;30(9):745-762. doi:10.1038/s41422-020-0355-0
35. Massalha H, Bahar Halpern K, Abu-Gazala S, et al. A single cell atlas of the human liver tumor microenvironment. *Mol Syst Biol*. 2020;16(12):e9682. doi:10.15252/msb.20209682
36. Ren X, Zhang L, Zhang Y, Li Z, Siemers N, Zhang Z. Insights gained from single-cell analysis of immune cells in the tumor microenvironment. *Annu Rev Immunol*. 2021;39(1):583-609. doi:10.1146/annurev-immunol-110519-071134
37. Sun D, Wang J, Han Y, et al. TISCH: a comprehensive web resource enabling interactive single-cell transcriptome visualization of tumor microenvironment. *Nucleic Acids Res*. 2021;49(D1):D1420-D1430. doi:10.1093/nar/gkaa1020
38. Wu H, Yu J, Li Y, et al. Single-cell RNA sequencing reveals diverse intratumoral heterogeneities and gene signatures of two types of esophageal cancers. *Cancer Lett*. 2018;438:133-143. doi:10.1016/j.canlet.2018.09.017
39. Wu H, Chen S, Yu J, et al. Single-cell transcriptome analyses reveal molecular signals to intrinsic and acquired paclitaxel resistance in esophageal squamous cancer cells. *Cancer Lett*. 2018;420:156-167. doi:10.1016/j.canlet.2018.01.059

40. Yang L, Zhang X, Hou Q, et al. Single-cell RNA-seq of esophageal squamous cell carcinoma cell line with fractionated irradiation reveals radioresistant gene expression patterns. *BMC Genomics*. 2019;20(1):611. doi:10.1186/s12864-019-5970-0
41. Chen Z, Zhao M, Liang J, et al. Dissecting the single-cell transcriptome network underlying esophagus non-malignant tissues and esophageal squamous cell carcinoma. *EBioMedicine*. 2021;69:103459. doi:10.1016/j.ebiom.2021.103459
42. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinf*. 2008;9(1):559. doi:10.1186/1471-2105-9-559
43. Maity AK, Stone TC, Ward V, et al. Novel epigenetic network biomarkers for early detection of esophageal cancer. *Clin Epigenetics*. 2022;14(1):23. doi:10.1186/s13148-022-01243-5
44. Snider EJ, Kaz AM, Inadomi JM, Grady WM. Chemoprevention of esophageal adenocarcinoma. *Gastroenterol Rep*. 2020;8(4):253-260. doi:10.1093/gastro/goaa040
45. Maret-Ouda J, Konings P, Lagergren J, Brusselaers N. Antireflux surgery and risk of esophageal adenocarcinoma: a systematic review and meta-analysis. *Ann Surg*. 2016;263(2):251-257. doi:10.1097/SLA.0000000000001438
46. Sparano JA, Gray RJ, Makower DF, et al. Adjuvant chemotherapy guided by a 21-gene expression assay in breast cancer. *N Engl J Med*. 2018;379(2):111-121. doi:10.1056/NEJMoa1804710

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Strategies and Considerations for the Characterization and Deeper Immunophenotyping of T Cells

BD Biosciences

T cells develop distinct surface markers and cytokine signatures during their development. Different types of T cell subsets have been identified based on their phenotypic and functional characteristics. Carefully designed flow cytometry panels with the combination of some basic surface markers can be used for characterizing T cells at a high level. But for deeper characterization of the different subtypes, a basic panel is not sufficient. Development of larger multicolor panels with additional drop-in fluorochromes is needed. Expansion of an existing flow cytometry panel by adding new fluorochromes can be challenging as the drop-ins can introduce spillover. The spillover spread may compromise panel resolution, ultimately requiring a complete redesign of a new panel, influencing cost, time and the ability to define cell biology in depth.

How are standalone panels and pre-optimized backbone panels different?

Several standalone panels are available for T cell characterization. However, these panels are not specifically designed to accommodate drop-ins. Expansion of these panels can cause suboptimal resolution of backbone and/or drop-in channels if panel design rules are not strictly followed. In addition, the standalone panels incorporate some of the most commonly available fluorochromes instead of leaving them open for drop-in flexibility.

A strategically designed pre-optimized backbone panel eliminates these disadvantages. Both standalone and backbone panels use different approaches, but while both rely on the fundamental rules of panel design, such as considerations to fluorochrome brightness and spillover and antigen density and co-expression, they differ in several practical aspects:

Considerations for building backbone panels

When building a backbone panel, the following four important criteria should be considered:

- Backbone panel should clearly resolve all relevant cell subsets
- Panel should not impact resolution of the recommended drop-in fluorochromes
- Drop-in fluorochromes should not impact resolution of the backbone panel-Drop-in fluorochromes should not impact each other

Standalone Panel	Backbone Panel
Designed for use without modification or additions	Prospectively designed to be expanded with different drop-ins
Easy to design—utilize spectrally separated fluorochromes	Challenging to design—utilizes challenging fluorochromes with high spillover
Difficult to expand—challenging selection of remaining fluorochromes	Easy to expand—spectrally separated fluorochromes are left for drop-ins
Panel redesign may be required to add new markers	Minimal panel design is required to add new markers in the backbone

The newly developed BD Horizon™ Human T Cell Backbone Panel from BD Biosciences

BD Biosciences has developed the BD Horizon™ Human T Cell Backbone Panel, which contains five individual vials of fluorochrome-conjugated antibodies against core T cell markers—CD3, CD4, CD8, CD45RA, CD197

(CCR7)—conventionally used to assess T cell maturation and identify naïve, central memory (CM), effector memory (EM) and effector memory RA (EMRA) subsets.[1,2] The kit also contains the BD Horizon™ Brilliant Stain Buffer for optimal performance.

The BD Horizon™ Human T Cell Backbone Panel could be used as a starting point for a deeper dive into, for example, CD4+ T helper cells and further identification of regulatory T cells through the addition of specific drop-ins.

Advantages of using the pre-optimized BD Horizon™ Human T Cell Backbone Panel

The BD Horizon™ Human T Cell Backbone Panel is strategically designed to be complemented with up to five recommended drop-in fluorochromes paired with your antigens of choice, depending on instrument configuration, with minimal panel design effort and loss of resolution.

It is vigorously tested and provides application data that can be used to gauge the performance of the panel before using it. By reducing panel design effort and providing tested and pre-optimized panels, it helps in building efficiency and confidence in your experiments.

- Strategic design of a backbone panel allows for expansion with recommended fluorochrome drop-ins, without impacting resolution
- The panel design process for addition of new markers is simplified, as spillover spread and antigen coexpressionare not of concern
- Proper matching of fluorochrome brightness and antigen density is still required to ensure optimal resolution
- The number of recommended drop-in fluorochromes may change depending on flow cytometer configuration
- Application data demonstrate the flexibility and clear resolution of a properly designed backbone panel

If you are interested in learning more about the T cell backbone panel, reviewing performance data, accessing relevant protocols and getting additional tips and tricks, download our BD Horizon™ Human T Cell Backbone Panel e-book.

REFERENCES

1. Hamann D, Baars PA, Rep MH, et al. Phenotypic and functional separation of memory and effector human CD8+T cells. *J Exp Med.* 1997;186(9):1407-1418. doi: 10.1084/jem.186.9.1407

2. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 1999;401(6754):708–712. doi: 10.1038/44385