The immune system also gets tired: Overcoming T-cell exhaustion to potentiate anti-cancer immune responses

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Abstract

Despite the anti-tumor effects of CD8⁺ T-cells, chronic exposure to tumor antigens causes T-cell exhaustion, dampening tumor growth control. A subset of exhausted T-cells with stem-like properties ($_{SL}$ T-cells) has been identified in tumor samples, characterized by the expression of inhibitory molecules (PD-1, CTLA-4), the transcription factor TCF1, and reduced cytotoxic effects. After immune checkpoint inhibition (ICI), $_{SL}$ T-cells rapidly proliferate and differentiate into terminally differentiated/exhausted T-cells ($_{TD}$ T-cells) with increased effector mechanisms, associating with favorable cancer outcomes patients. Moreover, various epigenetic mechanisms have been implicated in the development of exhausted T-cells, in which the transcription factor TOX has a crucial role. Getting a better understanding of T-cell exhaustion is essential to develop novel prognostic and therapeutic strategies that improve clinical responses to cancer immunotherapies. This review focuses on T-cell exhaustion: features, development, and therapeutic strategies to overcome this dysfunctional T-cell condition in cancer.

Key words: T-cell exhaustion. Cancer immunology. Tumor-infiltrating lymphocytes. Stem-like CD8⁺ T-cells. Immune checkpoint inhibitors.

Introduction

The immune system plays an active role in recognizing and attacking cancer cells; however, complex interactions between the tumor microenvironment (TME) and immune components exist. Novel therapies to manipulate the immune response against tumors are being implemented with favorable outcomes; nevertheless, several challenges persist¹.

Particularly, CD8⁺ T-cells can recognize and kill tumor cells due to their cytotoxic activity. Tumor antigen recognition in the TME is determined by the activation or priming of naïve CD8⁺ T-cells in the secondary lymphoid organs (SLOs) by cognate antigens bound to

MHC molecules, which form peptide-MHC (pMHC) complexes. The priming process of naïve CD8⁺ T-cells is mediated by antigen-presenting cells (APCs), mainly dendritic cells (DCs)². DCs are phagocytic cells that capture tumor antigens in the TME, become activated and then migrate to the SLO (e.g., draining lymph nodes), carrying tumor antigens through pMHC complexes. In the SLO, naïve CD8⁺ T-cells that express T-cell receptors (TCRs) recognizing their cognate antigens become activated by costimulatory molecules and cytokines released by DCs². Then, primed CD8⁺ T-cells migrate to the blood and later infiltrate the TME. These cytotoxic T lymphocytes (CTLs) express TCRs that can recognize tumor antigens presented on the surface of

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malignant cells through MHC molecules. After antigen recognition, primed CD8⁺ T-cells release cytotoxic molecules such as perforins and granzymes that act directly on neoplastic cells³.

The anti-tumor effect of CTLs is determined by intrinsic and extrinsic factors in the TME that shape their response against cancer. For example, tumor cells can downregulate MHC molecules' expression, dampening the recognition of tumor antigens by T-cells. In addition, anti-tumor CD8⁺ T-cells may become dysfunctional and tolerant, impairing tumor killing. Different facets of CD8⁺ T-cell dysfunction exist after antigen-specific TCR activation, including anergy, exhaustion, and senescence^{4,5}. Some methods to reduce T-cell dysfunction against cancer, including immune checkpoint inhibitors (ICIs), induce good clinical outcomes; however, some patients remain resistant to these therapies⁶. Thus, acquiring a better understanding of the immune mechanisms leading to dysfunctional T-cells inside tumors can provide better strategies to kill cancer cells and identify elements that may predict clinical responses. This review focuses on T-cell exhaustion: features, development, and therapeutic strategies to overcome this dysfunctional T-cell condition in cancer.

Tumor-infiltrating lymphocytes (TILs)

The presence and localization of tumor-infiltrating lymphocytes (TILs) affect the response to immunotherapies and correlate with clinical outcomes in different tumor types. Evidence suggests that cancer patients with high levels of CD8⁺ TILs survive longer than patients with low levels of CD8⁺ TILs^{7,8}. This might be due to the recruitment of primed CD8⁺ T-cells with cytotoxic activity that effectively recognizes and kills target cells. Moreover, patients with immune-inflamed tumors usually respond better to ICI than patients with immune-excluded tumors^{9,10}. These observations might be explained by the reinvigoration of T-cell effector mechanisms against malignancies.

T-cell exhaustion

Although CD8⁺ T-cells can infiltrate tumor tissues, neoplastic cells and their microenvironment may induce T-cell exhaustion and impair tumor rejection¹¹. As explained below, exhausted CD8⁺ TILs share common features with those found in exhausted T-cells associated with chronic viral infections¹².

Characteristics and development of exhausted T-cells

Exhausted T-cells are hyporesponsive, terminally differentiated immune cells with limited proliferative potential. They overexpress inhibitory receptors (PD-1, CTLA-4, TIM-3, and LAG-3) and downregulate the expression of costimulatory molecules. Inhibitory receptors on T-cells induce negative effects on their activation and effector function by either: (1) activating intracellular pathways that interfere with signals from the TCR and costimulatory proteins (e.g., CD28), or (2) by competing with ligands that activate costimulatory pathways (e.g., CTLA4 binding on T-cells to CD80/CD86 on DCs)^{13,14}.

Exhausted T-cells were first observed in transgenic CD8⁺ T-cells bearing lymphocyte choriomeningitis virus (LCMV)-specific TCRs in mice¹⁵. Further evidence suggested that during chronic LCMV infection, CD8⁺ T-cells did not have appropriate antiviral effector mechanisms¹⁶. After acute antigen exposure, naïve CD8⁺ T-cells become activated, differentiating into early effector and memory T-cells. During acute viral infections, early effector T-cells contribute to attacking and controlling infected host cells. In contrast, memory T-cells persist in the circulation and control viral replication if reinfection occurs¹⁷. However, after prolonged antigen exposure, activated T-cells become hyporesponsive and with limited proliferative potential. Hence, T-cell exhaustion may protect tissues from potentially harmful effects of effector CD8⁺ T-cells¹⁸.

In the context of cancer, T-cell exhaustion can be explained by: (1) persistent TCR signaling due to chronic antigen exposure that leads to the sustained expression of inhibitory molecules on T-cells, (2) non-effective interactions with tumor-infiltrating DCs caused by negative costimulatory signals, and (3) due to immunosuppressive effects of cells (e.g., regulatory T-cells) and cytokines (e.g., IL10 and TGFβ) in the TME¹³. Thus, strategies to avoid T-cell exhaustion or reactivate exhausted T-cells might induce tumor control.

Effects of ICI on exhausted T-cells

Blockade of checkpoint molecules has been shown to stimulate anti-tumor T-cell responses and provide clinical benefits in certain types of cancer. Nevertheless, resistance to treatment and the appearance of immune-related adverse events are some of their limitations⁶. Progress is being made to elucidate the mechanisms of ICI against tumors better. This might

facilitate the identification of markers of response/resistance to ICI and the development of therapeutic strategies to achieve durable clinical responses.

Pauken *et al.* showed that ICI induces epigenetic mechanisms that reverse T-cell exhaustion, reacquiring some features of effector T-cells⁹. In contrast, a study in patients with basal cell carcinoma demonstrated that anti-PD1 therapy increased the number of CD8⁺ TILs due to the expansion of anti-tumor clones that were not present in tumors before ICI. Analysis of TCR sequencing in peripheral blood indicated that, instead of reinvigorating exhausted T-cells, anti-PD1 favored the recruitment of new anti-tumor T-cell clones with different antigen specificity¹⁹.

Several groups have recently found that ICI expands a relatively undifferentiated cluster of exhausted CD8⁺ TILs with stem-like properties^{20,21}. These studies provided strong evidence that ICI effects to control tumor growth rely on the expansion of stem-like TILs rather than a reversal of T-cell exhaustion.

Stem-like T-cells and cancer

Single-cell technologies have enabled scientists to characterize TILs better, clustering them according to their phenotypic and transcriptional profiles. The identification of key elements associated with exhausted T-cells might provide novel biomarkers and therapeutic targets in cancer.

A subset of PD1⁺CD8⁺ TILs expressing the transcription factor TCF1 (encoded by the *Tcf7* gene) has been identified in melanoma patients, associating with good therapeutic responses after ICI and with prolonged survival²². This population of cells, known as early/progenitor exhausted or stem-like T-cells (_{SL}T-cells), can self-renew and differentiate into terminally exhausted/terminally differentiated T-cells (_{TD}T-cells)²¹. _{SL}T-cells express intermediate levels of PD1, high levels of co-stimulatory molecules (CD28, ICOS, and SLAMF6), genes associated with survival/proliferation (e.g., IL7R and IL2RA), chemokines (XCL1), and chemokine receptors (CCR7 and CXCR5) that are relevant for T-cell recruitment. However, these _{SL}T-cells lack additional coinhibitory molecules such as TIM-3 and express low levels of effector molecules (e.g., perforins or granzymes). In contrast, _{TD}T-cells do not express TCF1, have low proliferation potential, and express high levels of PD1, TIM3, LAG3, CD39, and granzymes (Fig. 1).

_{SL}T-cells have been identified in different tumors, based on the expression of CXCR5 (lung cancer)²³, or PD1 and TCF1 coexpression (melanoma and kidney

cancer)^{21,24}, representing a minority of CD8⁺ TILs. Similarly, an analogous population of virus-specific TCF1⁺ T-cells with high CXCR5 expression levels has been found in lymphoid tissues during chronic viral infections²⁵.

Functional relevance of _{SL}TILs

Analysis in human tumors has shown that _{SL}CD8⁺ TILs have more proliferation and differentiation potential than _{TD}CD8⁺ TILs after antigen stimulation^{24,26}. Moreover, recent murine studies have demonstrated that _{SL}T-cells are important for tumor control, and ICIs burst the proliferation of intratumoral _{SL}T-cells expressing TCF1 and expand the pool of _{TD}T-cells, limiting tumor growth (Fig. 2).

Siddiqui *et al.* observed that tumor growth was increased in tumor-bearing mice receiving adoptively transferred tumor-specific T-cells lacking TCF1 compared to mice treated with TCF1-expressing T-cells. They found that _{SL}TILs (TCF1⁺PD1⁺) proliferate and differentiate in response to ICI, increasing the pool of _{SL}TILs and _{TD}TILs (TCF1⁺PD1⁺). Although TCF1 was not essential to generate _{SL}TILs, it was required for _{SL}TIL proliferation and differentiation²¹.

Similar results were found by Kurtulus *et al.* They observed that TIM3 and PD1 checkpoint blockade in a colon carcinoma model expanded _{SL}TILs (PD1⁺TIM3⁻). Adoptive transfer of _{SL}TILs into tumor-bearing mice without lymphocytes (RAG^{-/-}) showed that _{SL}TILs can self-renew and act as precursors of _{TD}TILs (PD1⁺TIM3⁺)²⁷.

In contrast to _{SL}T-cells, exhausted _{TD}T-cells have been associated with anti-PD1 resistance²⁵. However, the blockade of additional pathways in _{TD}T-cells (e.g., anti-TIM3 and anti-CD39) has been shown to decrease tumor growth and prolong survival in melanoma models²². Thus, exhausted _{SL}TILs primarily respond to anti-PD-1 therapy compared to _{TD}TILs.

Furthermore, it has been shown that _{SL}TILs associate with improved clinical outcomes. Lung cancer patients responding to ICI have higher _{SL}TILs in pre-treatment biopsies compared to non-responders²³. Additional studies showed that an increased ratio of CD8⁺TCF1⁺ to CD8⁺TCF1⁻ TILs before ICI predicts better therapeutic responses to anti-PD1 and overall survival in melanoma patients²². Similarly, melanoma patients with increased amounts of _{SL}TILs experience a longer duration of response to ICI²⁰. These observations indicate that ICI is more likely to provide clinical responses in patients who are not able to generate _{TD}TILs from _{SL}TILs before treatment. Thus, strategies to expand and

Figure 1. Exhausted progenitor T_{SL} -cells originate after chronic antigen exposure.

It has been recently shown that some tumors have a population of stem-like (SL) $CD8^+$ -T-cells ($TCF1^+PD1^{int}TIM3^+CD28^+$), able to replicate and differentiate into terminally differentiated (TD) cytotoxic T-cells ($TCF1^+PD1^{hi}TIM3^+CD28^+$). T_{SL} -cells express high levels of survival genes (e.g., IL7R and IL2RA), costimulatory molecules (CD28, ICOS, and SLAMF6), chemokines (XCL1), and chemokine receptors (CCR7 and CXCR5). In contrast, T_{TD} -cells express higher levels of effector molecules (perforins and granzymes) and checkpoint molecules (PD1 and TIM3). Similar populations of T_{SL} -cells and T_{TD} -cells have also been described during chronic viral infections, suggesting their formation after chronic antigen exposure. Remarkably, exhausted progenitor T_{SL} -cells share similar features with memory progenitor T-cells ($PD1^+TCF1^+$), including the increased expression of Lef1, Bcl6, and IL7R. Memory progenitor T-cells develop after acute antigen exposure and facilitate the differentiation and persistence of memory $CD8^+$ T-cells. | Figure created in ©BioRender - biorender.com.

preserve T_{SL} TILs might represent an important approach to improve anti-tumor immune responses.

T_{SL} -cells and APC-enriched niches

Recent studies have identified a subpopulation of T_{SL} $CD8^+$ TILs that preferentially locate within MHC-II dense regions inside the stromal barrier of human tumors (APC niches)²⁴, which also contain blood and lymphatic vessels (Fig. 3). The presence of APC niches correlates with the number of $CD8^+$ TILs, associating with good outcomes in cancer patients²⁴ and improved responses to ICI^{20,22}. This is consistent with the requirement of CD28 costimulation to promote $PD1^+CD8^+$ T-cell expansion after ICI²⁸.

It has been suggested that these APC niches might protect T_{SL} TILs from exposure to high levels of tumor antigens and provide costimulatory signaling to enhance T_{SL} TIL proliferation and differentiation in response to ICI²⁹. In contrast, T_{TD} T-cells preferentially interact with tumor cells, and ICI may enhance their cytotoxic effects.

Regulation of T_{SL} and T_{TD} T-cell programming as a therapeutic strategy against cancer

Epigenetic modifications during the differentiation process of exhausted T-cells can determine the potential of T_{SL} TILs to exert sustained anti-tumor responses³⁰.

Figure 2. Immune checkpoint inhibitors and modification of epigenetic mechanisms expand the pool of stem-like and terminally differentiated T-cells in tumors.

Available evidence suggests that after priming, early effector T-cells infiltrate non-lymphoid tissues, where they become exhausted after chronic antigen exposure. However, recent studies in humans and mice indicate that undifferentiated tumor-specific $CD8^+$ T-cells can infiltrate the TME, acquiring exhaustion features and persist within tumor tissue. Here, tumor-specific $CD8^+$ T-cells expand and differentiate into TDT-cells in response to ICI, increasing the amount of tumor-specific T-cells. Subsequently, $CD8^+$ T-cells migrate and interact with tumor cells, where anti-PD1 will enhance their effector functions, contributing to tumor regression. Moreover, inhibition of DNA methylation (e.g., disruption of DNA methyltransferase 3 alpha; DNMT3A) in combination with ICI enhances the proliferation of tumor-specific $CD8^+$ T-cells and polyclonal $PD1^{hi}$ TILs, causing significant control of tumor growth. | Figure created in ©BioRender - biorender.com.

Exhaustion induced by TOX

TOX is a transcription factor that activates epigenetic programs associated with $CD8^+$ T-cell exhaustion. TOX is induced through calcineurin/NFAT after persistent TCR stimulation, causing chromatin remodeling, over-expression of inhibitory receptors, and reduction of cytokine production³¹.

It has been shown that TILs from cancer patients express high levels of TOX, correlating with the expression of exhaustion markers (PD1 and TIM3)³². Moreover, the loss of TOX in tumor-specific $CD8^+$ T-cells failed to persist in tumors and remained dysfunctional even when the exhaustion program was abrogated (i.e., cells showed high expression of TCF1 and inhibitory receptors were not upregulated)³². Another study confirmed the lack of TD -T-cell responses when TOX was completely depleted; however, the transfer of partially TOX-deficient ($TOX^{+/-}$) T-cells into tumor-bearing mice reduced tumor growth³¹. Moreover, a variable correlation between TOX and TCF1 has been observed^{31,32}. Thus, partially reducing TOX activity during adoptive cell transfer might reduce exhaustion without decreasing the number of functional T-cells within tumors;

however, further studies are required to determine this effect.

Exhaustion-associated DNA methylation programs

Furthermore, the acquisition of *de novo* DNA methylation has been identified in exhausted $CD8^+$ TILs. Ghoneim *et al.* observed new methylated regions (suppression) at *Tcf7*, *Ccr7*, and *IFN γ* loci in $PD1^{hi}$ TD TILs, while $PD1^{int}$ SL TILs showed demethylation (expression) of *Ccr7* and *Tcf7* loci, suggesting that *de novo* DNA methylation at specific loci induces terminal differentiation of exhausted T-cells³³. Moreover, these exhaustion-associated epigenetic programs were preserved in the progeny of TD TILs and could not be modified after anti-PD-1 monotherapy in cancer patients³³. However, inhibition of DNA methylation using decitabine combined with ICI induced the proliferation of polyclonal $PD1^{hi}$ TILs and tumor-specific $CD8^+$ T-cells within the TME, causing significant control of tumor growth³³. Thus, differences in SL and TD T-cells associate with distinct DNA methylation programs, which could be manipulated to improve ICIs effect on cancer patients.

Figure 3. Antigen-presenting cell (APC) niches inside tumors appear to maintain stem-like T-cell proliferation and differentiation into more terminally differentiated effector T-cells. *Created using data from Jansen CS, et al.²⁴* Evidence indicates that $_{SL}$ T-cells are present in immune-inflamed tumors, which have increased numbers of $_{TD}$ T-cells. In contrast, tumors with poor CD8⁺ T-cell infiltration have fewer $_{TD}$ T-cells, while $_{SL}$ T-cells were still present, suggesting that $_{SL}$ T-cells cannot differentiate into $_{TD}$ T-cells in immune-desert tumors. Further analysis has shown that $_{SL}$ T-cells preferentially locate within MHC-II dense regions inside the stromal barrier of tumors (APC niches), which also contain blood and lymphatic vessels. In contrast, tertiary lymphoid structures (TLSs) locate outside the stromal barrier and do not correlate with the number of CD8⁺ TILs. Immunofluorescence analyses have shown that APC niches closely resemble extrafollicular regions of lymphoid tissue, while TLS looks more like follicular regions. Furthermore, the presence of APC niches correlates with the number of CD8⁺ TILs and inversely correlates with tumor progression in cancer patients. Evidence also suggests that CD28 costimulation is needed to induce the proliferation and differentiation of $_{SL}$ T-cells after anti-PD1 therapy. Thus, APCs expressing CD80/CD86 might facilitate $_{SL}$ T-cell differentiation and protect them from chronic exposure to tumor antigens. In response to anti-PD1, APCs might provide costimulatory signaling to enhance $_{SL}$ T-cell proliferation and differentiation to $_{TD}$ T-cells. In contrast, $_{TD}$ T-cells preferentially interact with tumor cells, and ICI enhances their cytotoxic effects. | Figure created in ©BioRender - biorender.com.

Decreasing CAR T-cell exhaustion in solid tumors

Modified T-cells expressing chimeric antigen receptors (CAR T-cells) have been effective against advanced hematological malignancies because they target cancer antigens that do not require peptide binding MHC molecules. However, these therapies' success in solid tumors has been limited due to several factors, including exhaustion³⁴. Increasing the effector and memory functions of CAR T-cells can promote their persistence and improve their

anti-tumor response. This has been shown in pre-clinical models using CAR T-cells with a stem-like memory phenotype^{35,36}.

Stem-like cells can be induced from naïve T-cells by triggering Wnt signaling using GSK3 β inhibitors³⁷, or after their activation with anti-CD3/CD28 antibodies and the addition of IL7 and IL15 *in vitro*³⁸. IL15 can also expand human CXCR5⁺CD8⁺ TILs *ex vivo*, preserving their stem-like properties compared to IL2²⁶.

CAR T-cells stimulated with anti-CD3/CD28 and cultured with IL7 and IL15 can promote a stem-like phenotype with low expression of exhaustion markers

(PD1/LAG3), increased cytotoxicity, proliferation, and survival³⁶. Similarly, the adoptive transfer of CXCR5⁺TIM3⁻_{SL} TILs has been shown to promote tumor control in tumor-bearing mice³⁹. Thus, generating CAR T-cells with stem-like properties are a promising strategy to enhance anti-tumor effects and improve their expansion and survival in solid tumors.

Conclusions and perspectives

T-cell exhaustion represents a major limitation to anti-tumor immune responses. ICI has been shown to enhance cytotoxic effects against malignant cells. One of these mechanisms involves the expansion of the pool of stem-like and terminally differentiated T-cells in the TME. Thus, manipulating epigenetic programs in T-cells to prevent or revert exhaustion might represent a potential therapeutic option in cancer. For example, modified T-cells with stem-like properties could be genetically engineered to express CARs. It can be suggested that CARs developed using reprogrammed T-cells with stem-like properties can provide sustained immune responses against solid tumors due to their proliferative and differentiation capacities into cells with effector functions and the reduced expression of exhaustion markers. The characterization and functional evaluation of _{SL}TILs to develop and test CAR constructs in preclinical models might determine the possibility to translate these approaches into clinical studies.

Future work will be required to identify intracellular pathways associated with coinhibitory molecules that cause exhaustion, such as TIM3, and to elucidate the mechanisms by which single or combined ICI strategies work. Identifying the mechanisms by which APC niches are formed in tumors and how do different tumor types interfere with their formation is also needed. Stimulating the growth of these niches might enhance the preservation of _{SL}T-cells or CAR T-cells within tumor tissue, improve the anti-tumor immune responses of ICI, and increase T-cell infiltration to the TME. Moreover, work to standardize the identification of _{SL}TILs using different approaches will be necessary to optimize their clinical applicability.

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Conflicts of interest

The author declares that they have no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

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