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CD8 T-cell heterogeneity during T-cell exhaustion and PD-1-targeted immunotherapy

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REVIEW

Abstract

Persistent antigenic stimulation results in loss of effector function or physical deletion of antigen-specific CD8 T cells. This T-cell state is called T-cell exhaustion and occurs during chronic infection and cancer. Antigen-specific CD8 T cells during T-cell exhaustion express the inhibitory receptor PD-1, the expression of which plays a major role in T-cell dysfunction. PD-1 blockade re-invigorates CD8 T-cell immunity and has been proven effective against many different types of human cancer. To further improve the efficacy of PD-1-targeted immunotherapy in cancer patients, a better understanding of T-cell exhaustion is required. Recent studies have revealed that antigen-specific CD8 T cells during T-cell exhaustion are heterogeneous and have also uncovered the detailed mechanisms for PD-1-targeted immunotherapy. Here, we review the CD8 T-cell subsets that arise during T-cell exhaustion, the lineage relationship among these individual subsets and the role of each subset in PD-1 blockade. Also, we discuss potential strategies to enhance the efficacy of PD-1-targeted immunotherapy.

Keywords: cancer, chronic infection, immune checkpoint inhibitors, T-cell exhaustion, T-cell heterogeneity

Introduction

CD8 T cells are known as cytotoxic T lymphocytes (CTLs) and play an important role in not only controlling infection with intracellular pathogens but also anti-cancer immunity (1–5). After antigen stimulation, naive CD8 T cells undergo clonal expansion, acquire cytotoxic function and differentiate into effector T cells that kill target cells (1, 4). During acute infection, effector CD8 T cells eliminate pathogens and further differentiate into long-lived memory T cells that can quickly respond to re-exposure to the same pathogens (1, 4). This process in which effector T cells differentiate into memory CD8 T cells occurs in the absence of antigen, and the generated memory CD8 T cells persist in an antigen-independent manner. On the other hand, during chronic infection, CD8 T cells fail to clear pathogens, and the fate of these CD8 T cells stimulated with persistent antigens is totally different from that during acute infection (2, 5).

By the late 1980s, it was known that immunocompetent adult mice chronically infected with lymphocytic choriomeningitis virus (LCMV) had no detectable levels of CTLs measured by the chromium-release assay (6–9). However, it was unclear whether the loss of CTLs during chronic LCMV infection was due to deletion of virus-specific CD8 T cells or due to impaired function of these cells because the chromium-release assay can only detect functional CD8 T cells that possess cytotoxic activity.

In 1993, Moskophidis *et al*. published a paper to address this important issue about the relationship between virus persistence and virus-specific CD8 T cells (10). To count the number of virus-specific CD8 T cells without using the chromium-release assay, TCR-transgenic CD8 T cells that recognize an LCMV CD8 T-cell epitope were adoptively transferred into recipient mice. The authors found that the population of the transferred TCR-transgenic CD8 T cells expanded after chronic LCMV infection, but then rapidly declined and were physically deleted within 1 month after infection (10). On the basis of this observation, the paper concluded that deletion of LCMV-specific CD8 T cells was responsible for the loss of CTLs during chronic LCMV infection and proposed physical deletion of antigen-specific CD8 T cells as the mechanism of T-cell exhaustion (10). Although this concept was supported by other studies (11–13), subsequent research revealed that the T-cell deletion was not the solo mechanism of T-cell exhaustion.

In 1998, Zajac *et al*. investigated non-transgenic endogenous virus-specific CD8 T-cell responses after chronic LCMV infection using MHC tetramers (14), the method of

which was published by Altman *et al.* in 1996 (15). This study showed that two distinct mechanisms worked to silence antiviral CD8 T-cell immunity during chronic LCMV infection. Similar to what was observed in the above study conducted by Moskophidis *et al.* (10), endogenous CD8 T cells specific for one of the dominant LCMV epitopes were deleted within approximately 1 month after infection (14). However, antigen-specific CD8 T cells recognizing another dominant epitope were maintained without reduction (Fig. 1). Importantly, these persisting LCMV-specific MHC-tetramer$^+$ CD8 T cells had impaired cytotoxic activity as well as an impaired ability to produce cytokines upon stimulation (14), indicating that functional impairment of antigen-specific CD8 T cells is an additional mechanism of T-cell exhaustion. This dysfunctional phenotype was more pronounced without CD4 T-cell help and the severity of compromised function was associated with the prolonged presence of high levels of virus (14). This was the first reported evidence of the physical existence of dysfunctional antigen-specific CD8 T cells during chronic infection and these cells are now widely recognized as exhausted T cells (Fig. 1). Furthermore, this study opened novel possibilities to control chronic infection by using these dysfunctional T cells as therapeutic targets.

After the discovery of exhausted CD8 T cells (14), various approaches have been tested to re-activate these cells to control chronic infection (16–19). In 2006, a highly effective strategy was found to restore the function of exhausted CD8 T cells utilizing PD-1, an inhibitory receptor, in a mouse model of chronic LCMV infection (Fig. 1) (20). This work showed that PD-1 was up-regulated in exhausted CD8 T cells and the blockade of the PD-1 pathway enhanced the quantity and effector function of these cells, leading to better control of LCMV infection (20).

Importantly, this finding was quickly extended to not only other human chronic infections such as human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and hepatitis C virus (HCV), but also human cancer (21–25). Since the first U.S. Food and Drug Administration approval

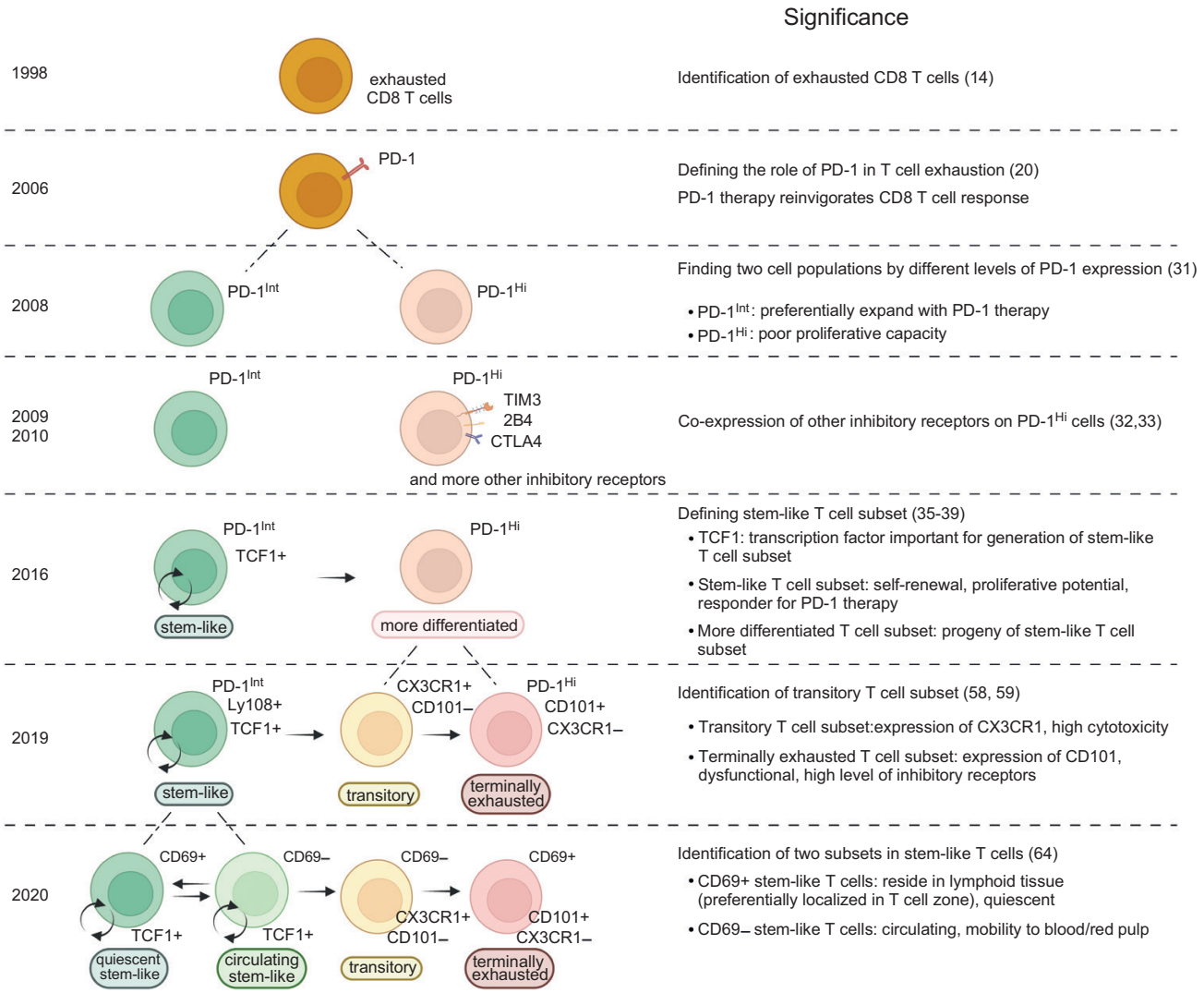

Fig. 1. CD8 T-cell heterogeneity during T-cell exhaustion. Key findings about CD8 T-cell heterogeneity during T-cell exhaustion are shown in chronological order. Created with BioRender.com.

of an anti-PD-1 antibody (which blocks PD-1) for melanoma in 2014 (26), PD-1-targeted immunotherapy has been used and proven effective against many different types of human cancer (27, 28). However, not all cancer patients treated with PD-1 blockade show clinical benefit, and thus there is a critical need to improve the efficacy of this therapy. For this purpose, a better understanding of T-cell exhaustion and the mechanism of action of PD-1-targeted immunotherapy is necessary.

Recent studies revealed that multiple subsets among antigen-specific CD8 T cells exist during T-cell exhaustion and play different roles in PD-1-targeted immunotherapy (29, 30). In this review, we will summarize such cell subsets and discuss their features. Since studies on the heterogeneity of antigen-specific CD8 T cells during T-cell exhaustion are most advanced in the setting of chronic infection, we will focus here on T-cell subsets generated after chronic infection.

Discovery of a CD8 T-cell subset that responds to PD-1-targeted therapy during T-cell exhaustion

Although PD-1 blockade had been shown to restore CD8 T-cell responses during chronic infection (20), it was unclear whether PD-1-expressing antigen-specific CD8 T cells uniformly responded to the therapy or whether the T cells that proliferated after PD-1 blockade arose from a subpopulation of antigen-specific CD8 T cells.

One of the earlier studies addressed this question and showed that antigen-specific CD8 T cells contained a cell population that preferentially responds to PD-1-targeted immunotherapy (31). This work found that, during chronic LCMV infection, antigen-specific CD8 T cells that expressed intermediate levels of PD-1 (PD-1^{Int}) were better rescued by PD-1 blockade compared to counterparts with high levels of PD-1 expression (PD-1^{Hi}) (Fig. 1) (31). Subsequent studies further characterized these PD-1^{Int} and PD-1^{Hi} cell populations by examining other inhibitory receptors (32, 33). PD-1^{Hi} CD8 T cells expressed higher levels of multiple inhibitory receptors such as 2B4, LAG-3, CD160 and TIM3 than did PD-1^{Int} CD8 T cells in mice chronically infected with LCMV (Fig. 1) (32, 33). Co-expression of multiple inhibitory receptors on PD-1^{Hi} CD8 T cells was associated with more severe dysfunction regarding proliferation and production of cytokines (32, 33). In addition to inhibitory receptors, Paley *et al.* demonstrated qualitative differences in antigen-specific CD8 T cells marked by expression of transcription factors T-bet and Eomes (34). T-bet^{Hi} Eomes^{Low} CD8 T cells were relatively functional and expressed intermediate levels of PD-1 whereas T-bet^{Low} Eomes^{Hi} CD8 T cells showed a dysfunctional phenotype with high PD-1 expression and were the progeny of T-bet^{Hi} Eomes^{Low} CD8 T cells (34). Thus, these earlier studies clearly established that the antigen-specific CD8 T cells generated during chronic infection were functionally and phenotypically heterogeneous.

In 2016, multiple groups reported more detailed characteristics of antigen-specific CD8 T-cell heterogeneity during chronic infection (35–39). They found two distinct antigen-specific CD8 T-cell subsets that can be identified by TIM3 and TCF1 expression: TCF1^{+} Tim3^{-} and TCF1^{-} Tim3^{+} cell subsets (Fig. 1). The TCF1^{+} Tim3^{-} cell subset had the ability to self-renew as well as differentiate into the other subset (TCF1^{-} Tim3^{+}), and was essential for maintaining the size of the antigen-specific CD8 T-cell population during chronic infection (Fig. 1) (35–39). Thus, preventing generation of this subset by knocking out TCF1 showed a striking loss of antigen-specific CD8 T cells over time after infection (35, 37, 38).

PD-1 was expressed on both subsets but its expression on the TCF1^{+} TIM3^{-} subset was lower than that on the TCF1^{-} Tim3^{+} subset. PD-1 blockade induced substantial proliferation of the TCF1^{+} Tim3^{-} cell subset and increased the differentiation of this subset into the TCF1^{-} Tim3^{+} subset. On the other hand, PD-1 blockade had minimal effect on the TCF1^{-} Tim3^{+} subset because this subset exhibited a more severe dysfunctional phenotype characterized by high expression of multiple inhibitory receptors, impaired proliferative potential and poor cytokine production (Fig. 1).

Therefore, these studies demonstrated that the TCF1^{+} Tim3^{-} cell subset plays an essential role in antigen-specific CD8 T-cell responses as well as PD-1-targeted immunotherapy during chronic infection (35–39). After identification of the TCF1^{+} Tim3^{-} cell subset in a mouse model of chronic infection, a similar subset was found in virus-specific CD8 T cells during chronic human viral infection (38, 40–42). Although tumor-infiltrating CD8 T cells contain not only tumor-specific T cells but also other antigen-specific T cells recognizing non-tumor epitopes, for example, virus-specific T cells, earlier studies observed the TCF1^{+} Tim3^{-} cell subset in human cancer (43–48). Furthermore, more recent studies demonstrated the presence of the TCF1^{+} Tim3^{-} cell subset in tumor-specific CD8 T cells obtained from tumor-infiltrating lymphocytes (49, 50).

The TCF1^{+} TIM3^{-} antigen-specific CD8 T cell subset during chronic infection is referred to by various names in published papers. Although the name 'memory-like T cells' is sometimes used in literature to describe TCF1^{+} TIM3^{-} antigen-specific CD8 T cells (37, 51), the overall transcriptional and epigenetic signature of this subset is very different from that of memory T cells generated during acute infection (52–55). The name 'progenitor of exhausted T cells' or 'precursor of exhausted T cells' is also often used because this subset can give rise to TIM3^{+} T cells with an exhausted T-cell phenotype (5, 56). In addition to this differentiation capability into TIM3^{+} cells, the TCF1^{+} TIM3^{-} antigen-specific CD8 T cells have one more important ability: self-renewal (35). Because the properties of self-renewal and generation of differentiated daughter cells are typical characteristics of stem cells, the TCF1^{+} TIM3^{-} cell subset is also referred to as 'stem-like T cells' (2, 35). The stemness of the TCF1^{+} TIM3^{-} T cell subset is a critical feature for maintaining the antigen-specific CD8 T-cell population during chronic infection. Therefore, in this review, this cell subset will henceforth be referred to as 'stem-like'.

Another unique phenotype of stem-like CD8 T cells is expression of CXCR5 and their localization in secondary lymphoid tissues (35, 36). Because of a similar phenotype to follicular helper CD4 T cells, some papers refer to these cells as follicular CD8 T cells (36, 38, 40, 41, 57). However, quantitative analysis by microscopy showed that stem-like CD8 T cells were predominantly (over 50%) localized in the T-cell zone, whereas less than 30% of stem-like CD8 T cells were

detected in the B-cell zone during chronic LCMV infection (35). Therefore, the name 'follicular CD8 T cell' does not appropriately represent the entire scope of stem-like CD8 T cells in LCMV-infected mice.

On the other hand, during HIV and SIV infection, virus-specific CXCR5$^+$ CD8 T cells were detected more in the B-cell follicular zone than in the extrafollicular region (38, 40, 41, 57). This discrepancy between LCMV infection and HIV/SIV infection may be explained by differences in the cells that the virus targets for infection. LCMV rarely infects CD4 T cells, but CD4 T cells are the primary target of HIV and SIV. CXCR5$^+$ CD8 T cells may migrate to the B-cell follicular zone to control HIV-infected or SIV-infected follicular helper CD4 T cells. Thus, stem-like CD8 T cells may change their localization in a manner dependent on antigen distribution, and it is important to investigate the localization of stem-like CD8 T cells in individual settings, for example, cancer and other chronic infections.

Identification of a transitory CD8 T-cell subset that has the ability to control infection and is generated from stem-like T cells during chronic infection

It was reported that TCF1$^-$ TIM3$^+$ CD8 T cells generated from stem-like T cells can be divided into two subpopulations by the expression of the glycoprotein CD101 and the chemokine receptor CX3CR1: transitory T cells (CX3CR1$^+$ CD101$^-$) and terminally exhausted T cells (CX3CR1$^-$ CD101$^+$) (Fig. 1) (58, 59). Transitory CD8 T cells were shown to be a proliferating cell subset generated from stem-like CD8 T cells. These transitory cells expressed high levels of effector molecules and had anti-viral functions to prevent excessive viral growth (58, 59). In contrast, CX3CR1$^-$ CD101$^+$ terminally exhausted CD8 T cells showed impaired effector functions and rarely proliferated, and were progeny of transitory CD8 T cells (59).

There are at least two identified factors either one of which can result in the differentiation of stem-like CD8 T cells into CX3CR1$^+$ transitory CD8 T cells. Zander *et al.* demonstrated that the generation of CX3CR1$^+$ transitory CD8 T cells was dependent on IL-21 produced from antigen-specific CD4 T cells (58). These results are consistent with previous findings that IL-21 secreted by antigen-specific CD4 T cells improves effector function of antigen-specific CD8 T cells in controlling viral infection in chronically infected mice (60–63). In addition to CD4 T-cell help, another way to promote the generation of transitory CD8 T cells is anti-PD-1 therapy. PD-1 blockade substantially increased the number of CX3CR1$^+$ transitory CD8 T cells even without CD4 T-cell involvement (58, 59). This indicates that the quantity of transitory CD8 T cells generated by PD-1 blockade is one of the key parameters for the success of this immunotherapy since these cells have the highest effector functions among antigen-specific CD8 T cells during T-cell exhaustion.

One more subset and lineage relationship during T-cell exhaustion

Beltra *et al.* further investigated antigen-specific CD8 T-cell responses during chronic viral infection (64). Since Ly108 is a surrogate marker of TCF1 (58, 65), stem-like T cells were distinguished from transitory and terminally exhausted T cells by Ly108 expression. The authors found that Ly108$^+$ stem-like CD8 T cells could be divided into two subsets: CD69$^+$ and CD69$^-$ stem-like T cells (Fig. 1) (64).

A series of experiments including phenotypic and transcriptional analyses as well as adoptive transfer of each cell subset revealed that CD69$^+$ stem-like CD8 T cells had features of quiescent progenitors and gave rise to CD69$^-$ stem-like T cells that further differentiated into transitory CD8 T cells (Fig. 1) (64). Importantly, CD69$^-$ stem-like CD8 T cells were able to de-differentiate into the progenitors (CD69$^+$ stem-like T cells) (64). Since CD69 promotes lymphocyte retention in lymphoid tissue (66), CD69$^+$ stem-like T cells were not detected in the blood and were predominantly localized in the white pulp of spleen (64). Such residency of CD69$^+$ stem-like T cells was independently confirmed by Im *et al.* in parabiosis experiments (67). In contrast, a portion of CD69$^-$ stem-like T cells was localized in the blood-accessible splenic red pulp, and these cells were able to circulate in the blood (64).

Beltra *et al.* also showed that the formation of CD69$^-$ stem-like CD8 T cells as well as transitory CD8 T cells was promoted by CD4 T-cell help or PD-1 blockade (64). It is currently unclear which stem-like CD8 T cells will be the primary target of anti-PD-1 therapy to induce proliferation of antigen-specific CD8 T cells during chronic infection. However, it appears that at least CD69$^+$ stem-like T cells can respond to PD-1 blockade, since PD-1 blockade increases antigen-specific CD8 T cells even in CD4 T-cell-depleted chronically infected mice in which CD69$^+$ stem-like CD8 T cells are predominant (35, 59, 64).

Toward improving PD-1-targeted immunotherapy

Stem-like CD8 T cells have been found among tumor-infiltrating lymphocytes and findings about the heterogeneity of antigen-specific CD8 T cells from chronic-infection studies can be applied to human cancer (43–48, 50). There are multiple possible ways to improve PD-1-targeted immunotherapy but we would like to focus here on the CD8 T cells. The key factors that should be manipulated to enhance the effectiveness of PD-1 blockade are the quantity, durability and functionality of transitory cells (Fig. 2) that are induced by the treatment since these are the main cells for targeting and destroying infected cells and tumor cells as discussed above.

How can we augment the number of transitory CD8 T cells after PD-1 therapy? One important way is to enhance the quantity of available stem-like CD8 T cells (Fig. 2A). Since the cells that proliferate and differentiate into transitory CD8 T cells after PD-1 blockade are primarily stem-like T cells (35, 59, 64), an increase in stem-like T cells should result in robust induction of transitory T cells after anti-PD-1 therapy.

In addition to the quantity, improving the quality of stem-like CD8 T cells, specifically their proliferative capacity, may result in a greater number of transitory T cells (Fig. 2B). The proliferative capacity of stem-like CD8 T cells is superior to that of transitory and terminally exhausted T cells but inferior to that of memory CD8 T cells generated during acute infection (64). If the proliferative capacity of stem-like CD8 T cells can be increased to the same extent as that of memory T cells, PD-1 blockade should yield more transitory CD8 T cells (Fig. 2B). To develop

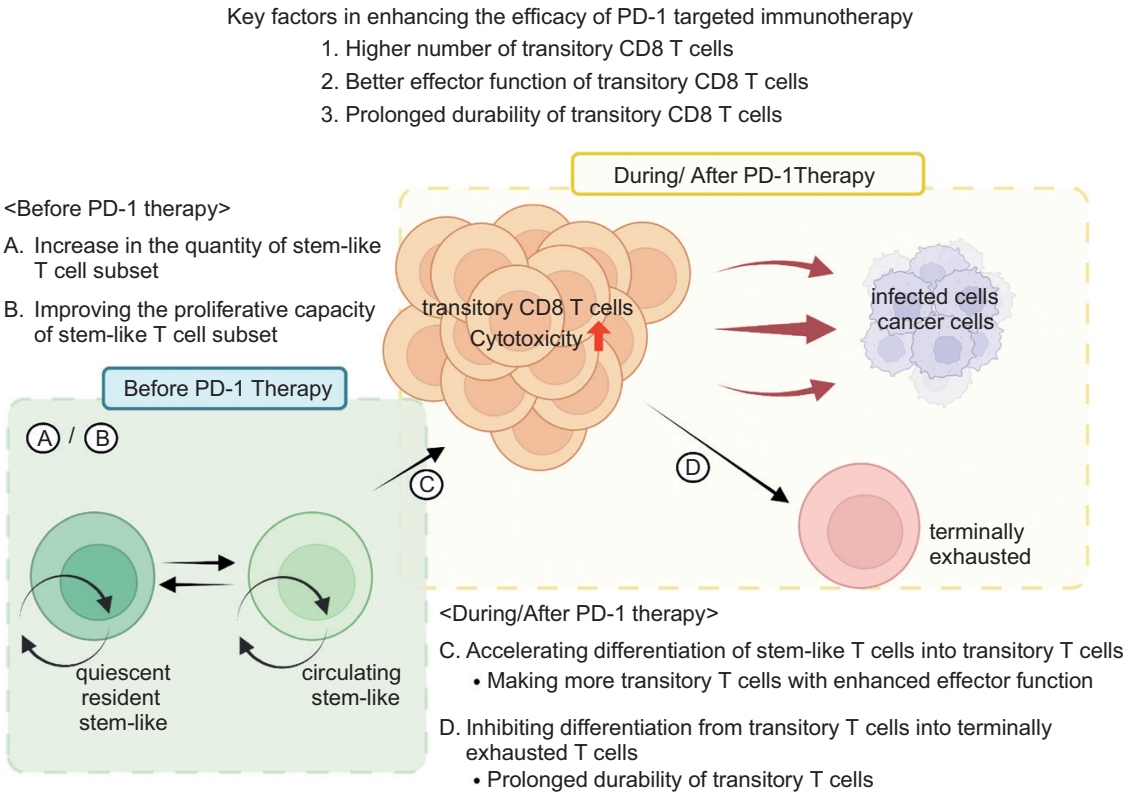

Fig. 2. Potential strategies to improve the efficacy of PD-1-targeted immunotherapy. Three key factors that should be manipulated to enhance the efficacy of PD-1 blockade are (1) number, (2) effector function and (3) durability. To manipulate these factors, four potential strategies are proposed. (A and B) Increase in the quantity (A) and/or improve the proliferative capacity (B) of the stem-like T-cell subset before PD-1 therapy. These should result in the generation of a higher number of transitory CD8 T cells after PD-1 blockade. (C) Accelerating the differentiation of stem-like T cells into transitory T cells during PD-1 blockade. This may be achieved by combination therapy and induces more transitory T cells with enhanced effector function. (D) Inhibiting differentiation from transitory T cells into terminally exhausted T cells during and/or after PD-1 therapy. This approach will prolong the durability of transitory T cells. Created with BioRender.com.

methods to improve the quantity and quality of stem-like CD8 T cells, further studies are required to gain a better understanding of the generation and maintenance of these cells.

In addition to manipulating stem-like CD8 T cells, the process of T-cell differentiation can be targeted to enhance the efficacy of PD-1 blockade. Differentiation of stem-like T cells into transitory T cells occurs in the steady state but PD-1 blockade accelerates this process, leading to the formation of a high number of transitory T cells (35, 59, 64). Further stimulation of this differentiation process during PD-1 blockade would be expected to increase the amount of transitory T cells even more (Fig. 2C). To this end, combination therapy may be a useful approach since a substantial increase in antigen-specific CD8 T cells was achieved after PD-1 combination therapy including IL-2, adoptive transfer of antigen-specific CD4 T cells, Treg cell depletion and blocking other inhibitory receptors (27, 68–72). Furthermore, it will be more ideal if such combination therapy improves the quality of transitory T cells compared with PD-1 blockade monotherapy (Fig. 2C). One of the essential qualitative features of transitory T cells is their effector function such as cytotoxic activity and cytokine production (58, 59, 64). Enhancing the effector function of transitory T cells may be beneficial for controlling tumor growth (Fig. 2C).

Another important quality is the durability of transitory T cells. Transitory T cells gradually lose their effector functions and differentiate into terminally exhausted T cells (59, 64). If combination therapy promotes the generation of more durable transitory T cells, the therapeutic effect would be improved. It is also possible to prolong the durability of transitory T cells by inhibiting their differentiation into terminally exhausted T cells (Fig. 2D). To establish the methods to improve the quality of transitory T cells, it will be important to elucidate the molecular and cellular mechanisms that regulate the differentiation of stem-like T cells into transitory T cells and terminally exhausted T cells.

Conclusions

It is now clear that antigen-specific CD8 T cells during chronic viral infection are heterogeneous and can be distinguished into at least four subsets: $CD69^{+}$ quiescent stem-like T cells, $CD69^{-}$ circulating stem-like T cells, transitory T cells and terminally exhausted T cells. The lineage relationship among these subsets has been clarified and also great progress has been made in understanding how PD-1 blockade modulates each subset. In this review, we propose potential approaches that target antigen-specific CD8 T cells to improve the effect of PD-1 blockade on the basis of knowledge obtained from

chronic infection studies. However, a further understanding of tumor-specific CD8 T cells may be needed to apply such approaches to cancer patients. As we discussed, individual pathogens target different types of cells and tissues, so the localization of pathogen-specific stem-like CD8 T cells can vary with different pathogens. Since the differences in the microenvironment between chronic infection and tumors are greater than those between individual chronic infections, the tumor microenvironment may influence the generation, differentiation, maintenance and localization of tumor-specific CD8 T cells, potentially resulting in unique features of T-cell exhaustion in cancer patients. Such tumor-specific features may represent new therapeutic targets that enhance PD-1 therapy. Thus, it will be crucial to further investigate the similarities and differences in antigen-specific CD8 T cells of chronic infection and tumors.

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