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PD-1 and CTLA-4 Mediated Inhibitory Signaling for T cell Exhaustion during Chronic Viral Infections

Chunyang Li#, Xiaoyan Xu#, Hongyan Wang* and Bin Wei*

Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China
#These authors contributed equally to this review

Abstract

Emerging studies show that T cell exhaustion correlates well with increased expression levels of inhibitory receptors including Programmed cell death receptor 1 (PD-1) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) during chronic infections. Both inhibitory molecules play similar but non-redundant role in T cell exhaustion. Engagement of PD-1 and CTLA-4 by their ligands inhibits T cell proliferation, cytokine secretion, and attenuates immune responses. Blockade of PD-1 and CTLA-4 restores effector function of exhausted T cells. PD-1 and CTLA-4 could both recruit Src homology 2-containing tyrosine phosphatase 2 (SHP2) and inhibit activation of Akt. Nevertheless, PD-1 and CTLA-4 also target distinct signaling molecules to inhibit T cell function. In this review, we will discuss current understanding of PD-1 and CTLA-4 initiated signaling pathways, their regulatory roles in a variety of chronic viral infections, and their promising potential as targets to enhance T cell function for antiviral therapy.

Keywords: PD-1; CTLA-4; T cells; Chronic viral infection; Inhibitory signaling

Introduction

Chronic viral infections, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), are great threats to human health, and developing effective therapies against those infections is a big challenge. During chronic viral infections, persistent viral load triggers continual stimulation signals via TCR to virus specific T cells, resulting in gradually loss of effector functions or even deletion of these T cells [1,2]. Recent studies have revealed that inhibitory receptors, programmed cell death receptor 1 (PD-1) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), are involved in the process of T cell exhaustion during chronic viral infections. PD-1, a 50-55 kDa membrane protein that belongs to the immunoglobulin superfamily, was first discovered in 1992 from a T cell hybridoma undergoing apoptosis [3]. PD-1 is expressed on a subset of thymocytes, activated T cells, B cells and myeloid cells [4-6], while CTLA-4, a type-one transmembrane glycoprotein, is only expressed on $CD4^+$ and $CD8^+$ T cells [7]. CTLA-4 is 30% homologous to CD28 and shares the same ligands CD80/86 with CD28 in antigen presenting cells (APCs) [8,9]. In the following sections, we will discuss PD-1 and CTLA-4 mediated signaling pathways, their regulatory roles in a variety of chronic viral infections, and their possible applications for antiviral therapy.

Function and Expression of PD-1

PD-1 deficiency results in development of progressive arthritis and lupus-like glomerulonephritis in aged C57BL/6 mice and autoimmune dilated cardiomyopathy in BALB/c mice [10,11]. Further *in vitro* studies show that signaling through PD-1 inhibits cell proliferation and cytokine secretion such as IFN-γ, TNF-α and IL-2, in both $CD4^+$ and $CD8^+$ T cells [12]. These reports suggest that PD-1 regulates peripheral T cell tolerance. Importantly, PD-1 deficient $CD8^+$ TCR transgenic T cells show potent tumor rejection *in vivo* [13]. Furthermore, PD-1 plays a key role in cytotoxic T lymphocyte (CTL) exhaustion during chronic viral infections, indicating that PD-1 negatively regulates $CD8^+$ T cell effector function.

PD-1 is not expressed on resting T cells, while it could be quickly induced upon T cell activation [4]. Previous reports have suggested that transcription factors including NFAT, T-bet and c-Fos, regulate PD-1 expression. NFATc1 or the activator protein 1 (AP-1) subunit c-Fos directly binds to regulatory element at the *pdcd1* promoter to upregulate PD-1 expression upon stimulation [14,15]. By contrast, T-bet directly represses transcription of PD-1 [16]. Recently, two reports show that epigenetic modulation via DNA methylation might affect PD-1 expression. DNA demethylation at the *pdcd1* promoter by 5-Zac contributes to PD-1 overexpression on lymphoid cell line-Molt-4 cells [17]. Consistently, lack of DNA remethylation in the *Pdcd1* regulatory region of exhausted $CD8^+$ T cells from acute and chronic viral infections in human and mice may leave the *Pdcd1* locus poised for rapid expression [18] (Figure 1).

Notably, virus-induced PD-1 upregulation has been reported. Nef is an HIV-1 accessory protein and plays an important role in the pathogenesis of both HIV-1 infected humans and primates. It has been reported that Nef induces PD-1 transcription, while specific inhibitor against p38/MAPK inhibits Nef activity and effectively blocks PD-1 upregulation. This suggests that Nef-mediated PD-1 expression is dependent on p38/MAPK activation [19]. In HBV infected patients, PD-1 expression is increased on $CD4^+$ T cells from peripheral blood compared to those in healthy volunteers. In addition, there is a positive correlation between serum HBV DNA levels and the PD-1 expression levels on $CD4^+$ T cells in the immune clearance phase. *In vitro* assay further shows that Hepatitis B Core Antigen (HBcAg) induces PD-1

***Corresponding authors:** Dr. Bin Wei, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China, E-mail: binweiwhy@gmail.com

Dr. Hongyan Wang, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China, Tel: +86 (0) 21-54921086; E-mail: hongyanwang@sibcb.ac.cn

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expression on T cells, and inhibitors against JNK, ERK and PI3K/AKT significantly decrease HBcAg-induced PD-1 expression on $CD4^+$ T cells [20]. Interestingly, several reports demonstrate that anti-viral cytokine IFN-α promotes the induction and maintenance of PD-1 expression through an association of IFN-responsive factor 9 (IRF9) to the IFN stimulation response element on the *pdcd1* promoter in T cells [21]. IFN-α also mediates upregulation of PD-1 in macrophages, which is dependent on interferon-sensitive responsive element (ISRE) and STAT1 and STAT2 [22]. Taken together, these observations suggest that both viral proteins and signaling proteins from host cells could regulate PD-1 expression (Figure 1).

Function and Cellular Trafficking of CTLA-4

Multiple studies have shown that signals induced by CTLA-4 dampen T cell responses. Engagement of CTLA-4 together with TCR and CD28 decreases T cell proliferation, delays T cell cycle transition from G0 to G1 phase and reduces IL-2 production as well as IL-2 receptor expression [23-25]. T cells from CTLA-4 deficient mice display activated phenotype ($CD44^{hi}$, $CD69^+$) and undergo robust proliferation [26,27]. Autoimmune diseases developed in CTLA-4-deficient mice provide compelling evidence to demonstrate the crucial inhibitory role of CTLA-4, which results from accumulation of T cell blasts in spleen and LN, and depleting T cells in CTLA-4 deficient mice prevents these diseases. Consistently, CTLA-4 overexpression on cell surface impairs T-cell responses *in vivo* and *in vitro* as shown in CTLA-4 transgenic mice [28].

In addition, recent findings also show that the biological function of CTLA-4 is tightly related to the elegant regulation of CTLA-4 trafficking and localization. Newly synthesized CTLA-4 is transported from Golgi to cell membrane that is dependent on ARF-ribosylation factor 1 and phospholipase D [29,30]. In resting T cells, the unphosphorylated YVKM motif in the cytoplasmic tail of CTLA-4 interacts with the clathrin adapter protein AP, leading to rapid internalization of CTLA-4 in a clathrin-dependent way [31,32]. Upon TCR stimulation, both Y-165 and Y-182 in the cytoplasmic tail of CTLA-4 are phosphorylated by activated Src family kinases to abolish the association of CTLA-4 with AP2. This results in a large fraction of CTLA-4 retained on cell surface [33,34].

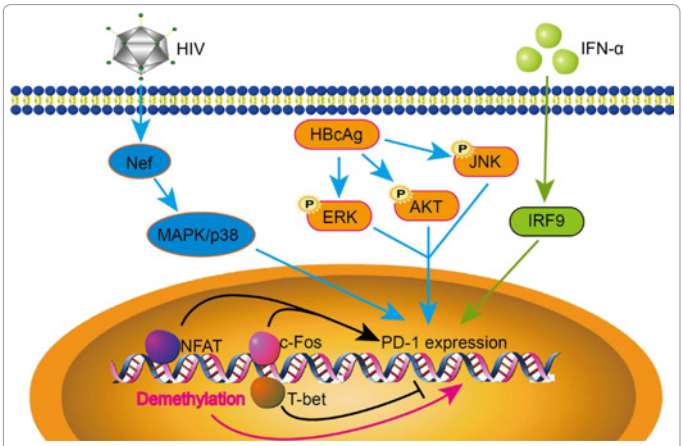

Figure 1: Different mechanisms regulate PD-1 expression in T cells. (1) NFATc1 or c-Fos induces PD-1 expression upon stimulation, while T-bet represses transcription of PD-1 (black lines). (2) HIV Nef induced PD-1 transcription is dependent on p38/MAPK activation. Hepatitis B Core Antigen (HBcAg) induces PD-1 expression on T cells, which depends on the phosphorylation of JNK, ERK and AKT (blue lines). (3) IFN-α promotes the induction and maintenance of PD-1 expression through an association of IFN-responsive factor 9 (IRF9) to the IFN stimulation response element on the pdcd1 promoter (green lines). (4) DNA demethylation at the pdcd1 promoter increases PD-1 expression (pink line).

Ligands of PD-1 and CTLA-4

PD-1 and CTLA-4 have been reported to commit their biological function through ligation with individual ligands. PDL-1 (B7-H1, also termed CD274) and PDL-2 (B7-DC, also termed CD273), two ligands of PD-1, show different expression patterns on different cell types [35,36]. PDL-1 is ubiquitously expressed in multiple cells, including T, B cells, DCs, macrophages, bone marrow-derived mast cells, and many nonhematopoietic cells. By contrast, PDL-2 is mainly expressed on DCs, macrophages and bone marrow-derived mast cells [5]. Both PDL-1 and PDL-2 could engage PD-1 to deliver inhibitory signaling to PD-1 expressing cells, and also deliver reverse signaling to PDL-1 and PDL-2 expressing cells [37,38].

CTLA-4 and the costimulator CD28 share the same ligands B7-1 (CD80) and B7-2 (CD86) [39]. Contrast to inhibitory signals delivered by CTLA-4, engagement of CD28 favors T cell activation, proliferation and effective cytokines secretion. Without CD28 ligation, TCR signals alone lead to T cell anergy. The topology of CTLA-4 homodimer allows for its bivalent binding to B7, while only monovalent binding occurs in CD28-B7 interaction [40]. Therefore, CTLA-4 binds these ligands with greater affinity and avidity compared to CD28, suggesting that CTLA-4 might block CD28 signaling by competitive binding to B7s. It is reported that CTLA-4 engagement of B7-1 is functionally equivalent to engagement of B7-2 [41]. However, CTLA-4 dominantly binds B7-1 since B7-1 expression is rapidly enhanced to reach peak levels after activation [42], while B7-2 expression level is still very low at that period. Interestingly, B7-1 could also interact with PDL-1 through their IgV-like domain and deliver bidirectional inhibitory signals [43]. Therefore, there might be a crosstalk between PD-1 and CTLA-4 signaling in certain conditions.

PD-1 Inhibitory Signaling Pathway

While both PD-1 and CTLA-4 use same key downstream signaling molecules to restrain T cell activation, biased signal cascades are mediated by PD-1 or CTLA-4 engagement. PD-1 consists of a single IgV-like extracellular domain, a transmembrane domain, and a cytoplasmic domain. Its cytoplasmic domain contains an immunotyrosine-based inhibitory motif (ITIM) and an immunotyrosine-based switch motif (ITSM). ITSM plays an essential role for PD-1 inhibitory function and mutation of ITSM results in dysfunction of PD-1 [44,45]. Upon TCR stimulation and engagement of PD-1, tyrosines in the cytoplasmic domain of PD-1 might be possibly phosphorylated by Lck in T cells [46] or by Lyn in B cells [47], which in turn recruits SH2-domain containing tyrosine phosphatase 1 (SHP1) and SHP2 [44,48]. Due to stronger interaction of SHP2 with PD-1 than SHP1, PD-1 functions mainly by recruitment of SHP2 [46,49] (Figure 2). In the case of T cells interacting with antigen presenting cells (APCs), immunological synapse (IS) is formed at the contact site, which is also named supermolecular activation cluster (SMAC) [50,51]. Interestingly, PD-1 accumulates at the synapse extensively when T cells interact with dendritic cells (DCs) expressing high levels of PDL-2 [52]. Further, PD-1 forms microclusters with TCR at the center of SMAC in a ligand binding-dependent manner [53]. SHP2 is immediately but transiently recruited to the PD-1 microclusters, to decrease the phosphorylation of TCR proximal signaling molecules, such as CD3ξ, ZAP70, PKCθ and PI3K, to attenuate TCR signaling [45,46,53]. PD-1 also inhibits Erk activation, which could be rescued by cytokines IL-2, IL-7 and IL-15 via the activation of STAT5.

Recently, an inhibitory loop by PD-1 signaling to suppress T cell proliferation has been demonstrated in human $CD4^+$ T cells (Figure 2, green colour) [54]. PD-1 engagement inhibits TCR-induced activation of the PI3K-Akt and Ras-MEK/ERK pathways, and suppresses SKP2 transcription, which results in accumulation of p27kip1, *an inhibitor of cyclin-dependent kinases,* and inhibition of CDK2 activity. Impaired CDK2 activity then acts on downstream effectors, which eventually suppresses SKP2 expression to inhibit cell cycle in a feed-back loop [54]. Alternatively, other findings have indicated that PD-1 could also inhibit T cell proliferation and effector function by upregulating the expression level of basic leucine transcription factor ATF-like (BATF) in exhausted $CD8^+$ T cells from human and mice. BATF is a transcription factor in the AP-1 family. Overexpression of BATF markedly impairs T cell proliferation and cytokine secretion, while depletion of BATF reduces PD-1 mediated inhibition function. More importantly, silencing BATF rescues the function of exhausted HIV-specific T cells [55].

Except for key signaling molecules, miRNA has also been recently reported to play important roles in PD-1 signaling pathway. Silencing or deficiency of PD-1 upregulates miR-21 expression and enhances STAT5 binding to the promoter of miR-21 in T-cells. In addition, miR-21 regulates the expression of programmed cell death 4 (PDCD4) [56]. Collectively, PD-1 deficiency activates a signaling cascade mediated by STAT5, miR-21, and PDCD4, which results in hyperproliferation of T cells and enhanced secretion of IFN-γ and IL-17.

CTLA-4 Inhibitory Signaling Pathway

Similar to PD-1, CTLA-4 is translocated to the cSMAC to stabilize its binding to the ligands CD80/CD86 upon TCR stimulation [57,58]. Ligation of CTLA-4 reduces the contact time between DC and T cells and prevents immunological synapse formation. This leads to a major reduction in Ca^{2+} influx/mobilization and an abrogation of ZAP70 microcluster formation [59]. It was also suggested that CTLA-4 regulates T cell adhesion and promotes T cell mobility, possibly in a RAP1-dependent way [60]. Different from typical ITIM and ITSM motifs in PD-1, the YVKM motif in the cytoplasmic tail of CTLA-4 is phosphorylated by kinases Fyn, Lyn and Lck [61,62], and recruits the SH2 domain of PI3K to enhance PI3K activity upon CTLA-4 ligation [63,64]. Activated PI3K phosphorylates the pro-apoptotic factor BAD and enhances BcL-XL activity [65]. This decreases FasL expression to prevent T cell apoptosis and also sustains T cell in an anergy state through inhibiting phosphorylation of Forkhead transcription factor FKHRL1 [66]. Notably, the tail of CTLA-4 binds to the regulatory subunit of serine/threonine phosphatase PP2A (also called PP2AA) to inhibit Akt phosphorylation and activation (Figure 2, purple colour) [45,67]. Differently, PD-1 could also inhibit AKT activity via its ITSM motif in the cytoplasmic tail [45].

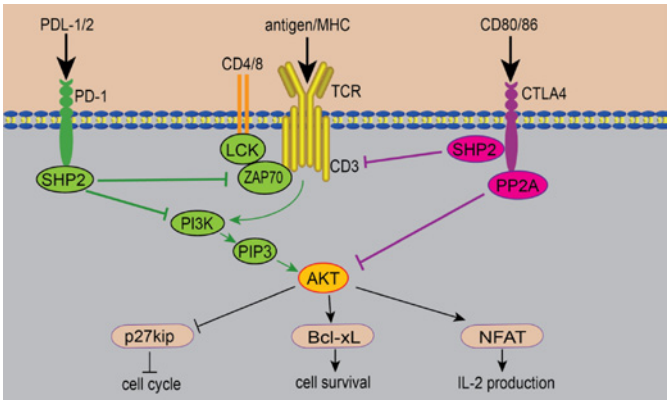

Figure 2: PD-1 and CTLA-4 target different molecules to inhibit T cell activation. Upon T cell conjugates with APC, PD-1 is located in the immune synapse at the T-APC interface and recruits SHP2 to inhibit TCR-induced activation of the PI3K-Akt and Ras-MEK/ERK pathways. PD-1 also suppresses transcription of SKP2 to result in accumulation of p27kip1, which is an inhibitor of cyclin-dependent kinases to block cell cycle and proliferation. Ligation of CTLA-4 dephosphorylates TCRζ chain and other signaling molecules including CD3ε, ZAP70 and Fyn. CTLA-4 inhibits Akt phosphorylation and activation by recruiting PP2A to its cytoplasmic tail. Ligation of CTLA-4 phosphorylates the pro-apoptotic factor BAD and enhances BcL-XL activity to prevent T cell apoptosis.

Ligation of CTLA-4 specifically dephosphorylates TCRζ chain [68]. Furthermore, other signaling molecules including CD3ε, ZAP70 and Fyn are hyperphosphorylated in CTLA-4 deficient mice [69,70]. These observations indicate that CTLA-4 may recruit phosphatases such as SHP2 to dephosphorylate these signaling proteins, resulting in blockade of TCR signals [71]. Although the typical binding motif I/VxYxxI/V/L for SHP2 is absent in CTLA-4, CTLA-4 could co-immunoprecipitate with SHP2 and CD3ε *in vitro* [72]. It indicates that SHP2 might be associated with CTLA-4 through unknown adaptor proteins. However, there is a debate about the inhibition role of the CTLA-4-SHP2 complex. In naive $CD8^+$ T cells where CTLA-4 has no inhibitory function, CTLA-4 is still associated with SHP2 [73,74].

Recently, new evidences have been provided to explain the inhibitory ability of CTLA-4. CTLA-4 is found to constitively expressed on regulatory T cells (Tregs) and contributes to the suppressive function of Tregs. Tregs are crucial for maintaining peripheral tolerance, by suppressing proliferation and immune responses of effector T cells. Deficiency and functional alteration in Tregs cause autoimmune diseases. There are currently at least four mechanisms to explain the suppressive function of Tregs: 1) production of inhibitory cytokines such as IL10 and TGFβ to constrain effector T cells; 2) secretion of granzymes to cytolysis effector T cells; 3) down-modulation of DC function or maturation to inactivate effector T cells; 4) consumption of metabolite such as IL-2 and cAMP to deprive metabolite essential for effector T cell survival. CTLA-4 in Tregs could modulate CD80/CD86 expression on DCs in foxo3-dependent way [75]. Downregulation of CD80/CD86 expression decreases the potency of DCs to activate T cells. In addition, the production of indoleamine 2, 3-diooxygenase (IDO), an enzyme secreted by APC, could be upregulated by CTLA-4. Soluble CTLA-4Ig protein induces IDO expression by binding to ligands CD80/CD86 [76,77]. Consistently, less IDO is secreted in CTLA-4 deficient Tregs than those from WT mice [78]. IDO has been shown to suppress T cells and simultaneously promote DC death due to tryptophan depletion [79]. Therefore, it is proposed that CTLA-4 might also use IDO to commit its inhibitory function.

To summary the above findings, distinct signaling transduction profiles between PD-1 and CTLA-4 are mainly caused by their different expression profile, different ligands, and distinct downstream signaling molecules. In addition, a possible crosstalk between PD-1 and CTLA-4 pathways support their synergizing inhibitory effect in some settings. Further studies are needed to demonstrate the precise signaling pathways transduced by the crosstalk of PD-1 and CTLA-4.

Different Role of PD-1 and CTLA-4 for $CD8^+$ T cells Exhaustion during Chronic Viral Infections

Increased levels of PD-1 and CTLA-4 were observed in various

chronic viral infections, and their regulatory functions have been extensively studied on different T cell subsets during chronic viral infections. The behavior of $CD8^+$ effector T cells is different in acute and chronic viral infections. During acute virus infection, $CD8^+$ effector T cells are activated immediately and clear virus efficiently. Most of these virus-specific effector $CD8^+$ T cells then undergo apoptosis, leaving behind a small number of long-lived memory cells against secondary infection. However, during chronic or persistent viral infection, long-term antigenic stimulation results in gradually lose of effector T cells, and generates exhausted T cells that unable to clear virus effectively. $CD8^+$ T cell exhaustion was observed in persistent LCMV, HIV, HBV and HCV infections in human patients and mouse models [1,2].

PD-1 has been demonstrated to play a non-redundant role to induce $CD8^+$ T cells exhaustion during viral infections. PD-1 was first reported to be selectively upregulated by exhausted T cells during chronic LCMV infection [80]. Further investigations have reported that PD-1 expression is upregulated in HIV, HBV, and HCV specific $CD8^+$ T cells in patients, which is correlated with viral load, and blockade of PD-1 increases virus specific T cell functions [81-87]. *In vivo* blockade of PD-1 and PD-L1 interaction with antibody could enhance T cell responses with a great reduction of viral burden. Furthermore, even in persistently infected mice lacking $CD4^+$ T cell help, blockade of the PD-1 pathway could restore the ability of 'helpless' $CD8^+$ T cells to undergo proliferation, secrete cytokines, kill infected cells and decrease viral load. These studies identify importance of PD-1 to $CD8^+$ T cell exhaustion and viral control, and purpose a potential effective strategy for the treatment of chronic viral infections.

By contrast, the role of CTLA-4 in $CD8^+$ T cell responses against viral infection is controversial. Although CTLA-4 mRNA is increased on exhausted $CD8^+$ T cell in chronic LCMV infection, blockade of the CTLA-4 inhibitory pathway shows no effect on either $CD8^+$ T-cell function or viral control [80]. Consistently, CTLA-4Ig transgenic mice (blocking B7-CD28 interaction) infected with LCMV show no alternation of the function of virus specific cytotoxicity T lymphocytes (CTLs) [88]. In HIV infection, CTLA-4 expression is not altered on $CD8^+$ T cells isolated from patients [89]. However, other reports suggest that CTLA-4 regulates CTL function during HBV or VSV infection. Upregulated CTLA-4 has been found on $CD8^+$ T cells from HBV infected patients. HBV-specific $CD8^+$ T cells with excessive CTLA-4 are prone to apoptosis due to enhanced expression of Bim, a proapoptotic protein. Abrogation of CTLA-4-mediated inhibition reduces Bim expression and simultaneously increases expansion of IFN-γ-producing HBV-specific $CD8^+$ T cells [90]. Other supporting studies show that non-replicating or poorly replicating VSV infection dramatically impairs the proliferation of primed CTLs during acute phases in CTLA-4Ig transgenic mice [88]. Blockade of CTLA-4 in both the primary and secondary infection could increase memory $CD8^+$ T cell expansion with enhanced effective function to clear foreign invaders without changing TCR repertories [91].

Different Role of PD-1 and CTLA-4 for $CD4^+$ T cell Exhaustion in Chronic Viral Infection

Although $CD4^+$ T cells could produce IL-2 and IFN-γ and assist activation of $CD8^+$ CTLs against viral infection, $CD4^+$ T cells exhaustion also exists during chronic viral infections. CTLA-4 has a profound, non-redundant role in regulating virus specific-$CD4^+$ T cells function. During HIV infection, the expression level of CTLA-4 is positively correlated with virus load. Excessive CTLA-4 may contribute to impairment of $CD4^+$ T cell proliferation and reduction of IL-2 expression [92]. CTLA-4 blockade restores HIV-specific $CD4^+$ T cell proliferation and effective cytokine secretion, including IFN-γ and IL-2. Importantly, blockade of CTLA-4 decreases the production of TGF-β and IL-10, but increases IFN-γ production by HIV-specific $CD8^+$ T cells [93]. Depletion of $CD4^+$ T cells abrogates the impact of CTLA-4 on HIV-specific $CD8^+$ T cells. This indicates that interestingly, CTLA-4 on HIV-specific $CD4^+$ T cells confers negative roles to HIV-specific $CD8^+$ T cells.

By contrast, the role of PD-1 for $CD4^+$ T cell exhaustion is not consistent during different viral infections. In HIV infected patients, PD-1 expression is unregulated in $CD4^+$ T cells, which is correlated with viral load and $CD4^+$ T cell numbers [94]. However, some studies show controversial data that PD-1 may not play an important role for $CD4^+$ T cell exhaustion. In an inducible transgenic mouse system in which antigen presentation is controlled, $CD4^+$ T cell exhaustion does not require PD-1 expression. Further, successful tolerance is induced in PD-1 deficient $CD4^+$ TCR transgenic cells, demonstrating that PD-1 signaling is not required for either the induction, or the maintenance of peptide-induced tolerance [95]. Therefore, further investigation is needed to answer if PD-1 involved tolerance-inducing mechanisms in $CD4^+$ T cell exhaustion is probably dependent on different types of APCs or antigen doses [96].

Different Role of PD-1 and CTLA-4 in Regulatory T cells (Tregs)

Upon virus infection, Tregs are activated and proliferate at the sites of infection, which are double-edged sword in virus eradication. From the beneficial side, Tregs restrain excessive immune responses to minimize tissue damages. On the other hand, Tregs suppress the function of effector T cells, which leads to enhanced virus survival. CTLA-4 is found constitutively expressed on Tregs and its specific transcription factor Foxp3 activates CTLA-4 expression [97]. CTLA-4 has been confirmed to regulate the suppressive function of Tregs. CTLA-4 deficiency impairs the suppressive activity of Tregs characterized by its incapability to downregulate the ligands CD80/86 on APC, leading to massive conventional T cells proliferation and excessive IL-2 and IFN-γ production [75].

Similar to the controversial role of PD-1 in $CD4^+$ T-cell exhaustion, the role of PD-1 in Tregs is not consistent during different viral infections. Some studies report that PD-1-PDL-1 interaction has a pivotal role in the development, maintenance, and function of induced Tregs (iTregs). PDL-1 deficient APCs minimally convert naive $CD4^+$ T cells into iTregs. In a naïve $CD4^+$ T cell adoptive transfer model, PDL-1 and PDL-2 double knock-out mice markedly reduce the conversion of iTregs and rapidly develop a fatal inflammatory phenotype. It has been suggested that PDL-1 downregulates phospho-Akt, mTOR, S6, ERK2 and upregulates PTEN to induce iTregs [98]. Consistently, PDL-1 enhances foxp3 expression and increases the suppressive function of iTregs. However, other studies show that PD-1 dampers Treg function in chronic infection. In the livers of patients chronically infected with HCV, PD-1 expression is enhanced in Tregs and this is coincided with decreased expansion of Tregs than effector T cells. Blockade of PD-1 signaling enhances the expansion and function of Tregs from HCV patients via upregulation of STAT-5 phosphorylation *ex vivo* [99]. This indicates that PD-1 negatively regulates Tregs by limiting STAT-5 phosphorylation in patient infected with HCV.

Targeting PD-1 and CTLA-4 for Antiviral Therapy

It has been about 20 years since PD-1 and CTLA-4 were discovered. Extensive studies have been made to understand their transcriptional regulation mechanisms, detailed signaling pathways, and their regulatory roles in chronic viral infections. Taken together, it seems that PD-1 plays a more convincing role for $CD8^+$ T cell exhaustion than CTLA-4. By contrast, CTLA-4 plays non-redundant role in regulating the suppressive function of Tregs. PD-1 may serve as both a marker of disease progression and a therapeutic target to reverse function of exhausted $CD8^+$ CTLs. Many studies have shown that PD-1 blockade is a promising way to enhance effector T cell function in chronic infections [100]. PD-1 blockade using antibody in an SIV-macaque model was reported to enhance HIV-specific immune responses [101]. On the other hand, it is also important to consider $CD4^+$ T-cell exhaustion and Treg function during antiviral therapy. Suppression of Treg function in short time may enhance anti-viral responses. Ipilimumab, a human monoclonal IgG1 antibody against CTLA-4, has been clinically used in the treatment of cancer and supports a possible usage of anti–CTLA-4 antibody in the context of infectious diseases. However, it is still unclear how CTLA-4 blockade influences Treg or $CD4^+$ T-cell function *in vivo*. Further studies will elucidate the effect of anti- CTLA-4 therapy *in vivo* during chronic viral infections. It is also important and possible to consider the combination of PD-1 and CTLA-4 blockade for immunotherapy to enhance antiviral immune responses [102,103].

However, it is still a long way to go for the success clinical trial on PD-1 and CTLA-4 involved antiviral therapies. Since both PD-1 and CTLA-4 control the balance between an adequate protective immune response and suppression of immunopathology, a key issue is that blockade of PD-1/CTLA-4 may cause side effects or autoimmune diseases. Manipulation of PD-1 downstream signaling may be an alternative option to overcome the problem. Further investigations on PD-1 and CTLA-4 signaling pathways and the molecular mechanisms of T-cell exhaustion may provide new therapeutic opportunities to improve T cell mediated immune responses against chronic viral infections.

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