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Cutting Edge: Programmed Death-1 Up-Regulation Is Involved in the Attrition of Cytomegalovirus-Specific CD8⁺ T Cells in Acute Self-Limited Hepatitis B Virus Infection¹

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Attrition of heterologous virus-specific CD8⁺ T cells has been demonstrated in murine viral infection; however, little is known regarding this phenomenon in human viral infections. In this study, we observed that CMV-specific CD8⁺ T cells displayed numerical decline and functional impairment in the early phase of acute infection, whereas programmed death-1 (PD-1) expression was significantly up-regulated by these CMV-specific CD8⁺ T cells. This early PD-1 up-regulation was found to be closely associated with the increased apoptotic sensitivity of CMV-specific CD8⁺ T cells. The *in vitro* addition of anti-PD-1 further enhanced the spontaneous apoptosis of CMV-specific CD8⁺ T cells; however, blockade of the PD-1-mediated pathway with anti-PD-L1 significantly restored the CMV-specific CD8⁺ T cell proliferation and IFN- γ production. Thus, PD-1 plays a crucial role in the attrition of CMV-specific CD8⁺ T cells in acute hepatitis B virus infection, which in turn, influences the preexisting homeostatic virus-specific CD8⁺ T cell pool. *The Journal of Immunology*, 2008, 181: 3741–3744.

Memory T cells are capable of stable survival after the resolution of a pathogenic infection, even in environments lacking MHC Ags (1). However, the stable survival of memory CD8⁺ T cells in mice can be disrupted by subsequent heterologous viral or bacterial infections in which the number of the majority of non-cross-reactive memory CD8⁺ T cells decreases via cytokine-dependent mechanisms (2–6). In mice, this phenomenon has been identified as the attrition of memory CD8⁺ T cells (7, 8). The following two models have been proposed to explain this attrition: 1) the passive attrition model, which predicts that old memory T cells are

defeated by newly formed memory T cells while competing for survival niches within the immune system; and 2) the active attrition model, which predicts that the preexisting memory cells undergo direct apoptotic attrition (7–9). However, in humans there is no report on the attrition of memory CD8⁺ T cells, and the mechanisms underlying such attrition remain to be elucidated.

The interaction between programmed death-1 (PD-1)⁴ and its ligand PD-L1 has been reported to play a crucial role in inducing T cell exhaustion (10, 11), anergy (12), and apoptosis (13) in mice and human viral infections (14–22). These studies indicated that PD-1 up-regulation may exhaust virus-specific CD8⁺ T cells, facilitating viral persistence in chronic viral infection. The *in vitro* blockade of PD-1/PD-L1 interaction reversed the exhaustion of the virus-specific T cells (10–12, 14–19). In acute viral infections, PD-1 was transiently up-regulated on virus-specific CD8⁺ T cells in the early phase of the infection, and successful clearance of these viruses often correlated with decreased PD-1 expression and the development of functional CD8⁺ memory T cells (21, 22). Moreover, PD-1-positive virus-specific CD8⁺ T cells showed higher sensitivity to apoptosis in the case of HIV-1 infection (15), and higher *in vitro* PD-1/PD-L1 interaction enhanced the apoptosis of hepatitis B virus (HBV)-specific CD8⁺ T cells during acute HBV (AHB) infection (21). These findings indicate that PD-1-mediated coinhibitory signaling not only inhibits the function but also regulates the survival of virus-specific CD8⁺ T cells during acute and chronic viral infections. Thus far, little information is available regarding the roles of PD-1 in the attrition of heterologous virus-specific CD8⁺ T cells. Notably, a recent study demonstrated that PD-1 is responsible for the depletion of autoreactive CD8⁺ T cells in mice (13).

In this study, we investigated the association between PD-1 expression and the fate of CMV-specific CD8⁺ T cells in acute

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⁴ Abbreviations used in this paper: PD-1, programmed death-1; AHB, acute hepatitis B; HBV, hepatitis B virus; HC, healthy control; HBsAg, hepatitis B surface antigen; PD-L1, PD-1 ligand.

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self-limited HBV infection. HBV infection often results in acute self-limited infection in adults with prominent clinical presentation (20). The majority of the patients in this study had previously been infected with CMV during early childhood and established long-term immune protection against this infection. Our data indicate that PD-1 is involved in the attrition of CMV-specific CD8⁺ T cells in AHB, which, in turn, influences the preexisting homeostatic virus-specific CD8⁺ T cell pool.

Materials and Methods

Subjects

A total of 16 HLA-A2-positive AHB patients (12 male and four female; mean age, 35.6 ± 8.8 years) were diagnosed as described in our recent report (21). In brief, the serum alanine aminotransferase levels at the clinical onset were at least 10-fold the upper limit of the normal level at the time of the first detection of serum hepatitis B surface Ag (HBsAg) and Ig M anti-hepatitis B core Ag accompanying multispecific anti-HBV CD8⁺ T cell responses (21). All of the AHB patients finally recovered clinically and displayed alanine aminotransferase normalization and HBsAg seroconversion within 6 mo of the initial onset of symptoms. Sixteen healthy subjects were recruited as controls (HCs). All participants were anti-hepatitis A virus, anti-hepatitis C virus, anti-hepatitis D virus, and anti-HIV-1 and anti-HIV-2 Ab-negative. Informed consent was obtained from each participant. The study was approved and has been reviewed by the local medical ethics committee.

Abs and reagents

All Abs were purchased from BD Biosciences, except for the anti-PD-L1-blocking mAb (MIH1) and the anti-human PD-1 Ab (AF 1086), which were purchased from eBioscience and R&D Systems, respectively. A PE-labeled CMV pentamer loaded with the pp65 epitope (495–503, NLVPMVATV) was synthesized by ProImmune. Lymphocyte counts of these patients were determined using an automated differential blood count system. Plasma IFN- α concentration was measured using a standard ELISA kit from BioSource International.

Apoptosis assay

Freshly isolated PBMCs were stained with the PE-labeled CMV pentamer, followed by anti-CD8-PerCP and anti-PD-1-allophycocyanin and finally with annexin V according to our previously reported protocols (21). In some experiments, 1–1.5 × 10⁶ PBMCs were cultured in 24-well plates with or without plate-bound anti-human PD-1 Ab (20 μ g/ml) for 12 h at 37°C. The cells were subsequently stained with annexin V to detect apoptosis.

Intracellular IFN- γ staining assay

PBMCs were stimulated with pp65 495–504 peptide (10 μ g/ml) plus anti-PD-L1 (10 μ g/ml) or isotype control Ab for 6 h. GolgiStop (BD Pharmingen) was added to the cells after stimulation for 2 h. Intracellular IFN- γ staining assay was performed according to our previously reported protocols (16, 21).

Ag-specific cell proliferation

CFSE-based proliferation assays were performed according to our previously described protocols (16, 21). Cells were stimulated with pp65 495–504 peptide (2 μ g/ml), anti-human CD28 (0.5 μ g/ml), and human rIL-2 (20 U/ml) in the presence of anti-PD-L1 (10 μ g/ml) or isotype control Ab in a 24-well plate. On day 6, the cells were supplemented with 0.2 ml of fresh medium containing the above-mentioned cytokines. On day 10, the cells were stained with pentamers.

Statistical analysis

All data were analyzed using SPSS software. Comparison between various groups was performed using the Mann-Whitney *U* test. For the blockade assay, statistical comparisons were performed using the Wilcoxon matched pairs *t* test. The correlation between variables was evaluated using the Spearman's rank correlation test. For all two-tailed tests, *p* < 0.05 was considered significant.

Results and Discussion

The attrition of preexisting virus-specific CD8⁺ T cells following heterologous viral infections has been well demonstrated in mice models (2–9). However, whether this attrition occurs in human viral infections is debatable; in particular, it is unknown

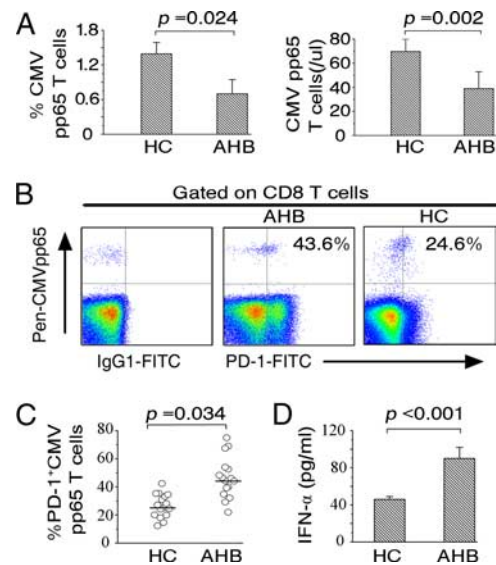


FIGURE 1. Attrition of CMV-specific CD8⁺ T cells at the clinical onset of acute HBV infection. *A*, The percentages and numbers of CMV-pp65 CD8⁺ T cells in AHB patients and HCs. The numbers of circulating CMV-pp65 CD8⁺ T cells were calculated as the lymphocyte count (cells/microliter) × percentage of CD8 × percentage of CMV-pp65 pentamer. *B*, Representative dot plots of PD-1 expression on CMV-pp65 CD8⁺ T cells. Values in the upper right quadrants indicate the percentages of CMV-pp65 CD8⁺ T cells that express PD-1. *C*, Pool data of PD-1 expression on CMV-pp65 CD8⁺ T cells. *D*, Pool data of the plasma IFN- α concentration.

whether acute HBV infection affects the preexisting CMV-specific CD8⁺ T cell pools in AHB patients. To address the issue, we first found that both the percentages and absolute number of CMV-specific CD8⁺ T cells were significantly reduced at the clinical onset of AHB in patients compared with those of HCs (Fig. 1*A*). In contrast, multispecific anti-HBV CD8⁺ T cell responses at such an early phase were observed in the patients (21). Thus, these data primarily indicate that acute HBV infection may induce the attrition of CMV-specific CD8⁺ T cells during the early stage of HBV infection in humans. This early attrition of CMV-specific CD8⁺ T cells might make room for newly formed HBV-specific T cells within the immune system and facilitate the mounting of a strong anti-HBV T cell response.

PD-1 has been demonstrated to regulate the survival of virus-specific CD8⁺ T cells during acute and chronic viral infections (15, 21). To investigate the association between PD-1 expression and the attrition of CMV-specific CD8 T cells in AHB infections, we serially analyzed the PD-1 expression on these CMV-specific CD8 T cells. Our data indicated that PD-1 expression on CMV-specific CD8⁺ T cells was significantly up-regulated in AHB patients compared with that in HCs (Fig. 1*B*). The pool data further confirmed this observation (Fig. 1*C*). The underlying mechanism of PD-1 up-regulation on these preexisting virus-specific CD8⁺ T cells is not yet clear. A study has suggested that PD-1 expression occurs before the appearance of early T cell activation markers, including CD69 and CD25, and that PD-1 expression may be controlled by early responding cytokines in addition to TCR signaling (12). In this regard, CMV was not observed to be reactivated, and no cross-reactivity was observed between CMV and HBV in these AHB patients. Interestingly, we found that PD-1 was also up-regulated by influenza-specific CD8⁺ T cells in AHB patients

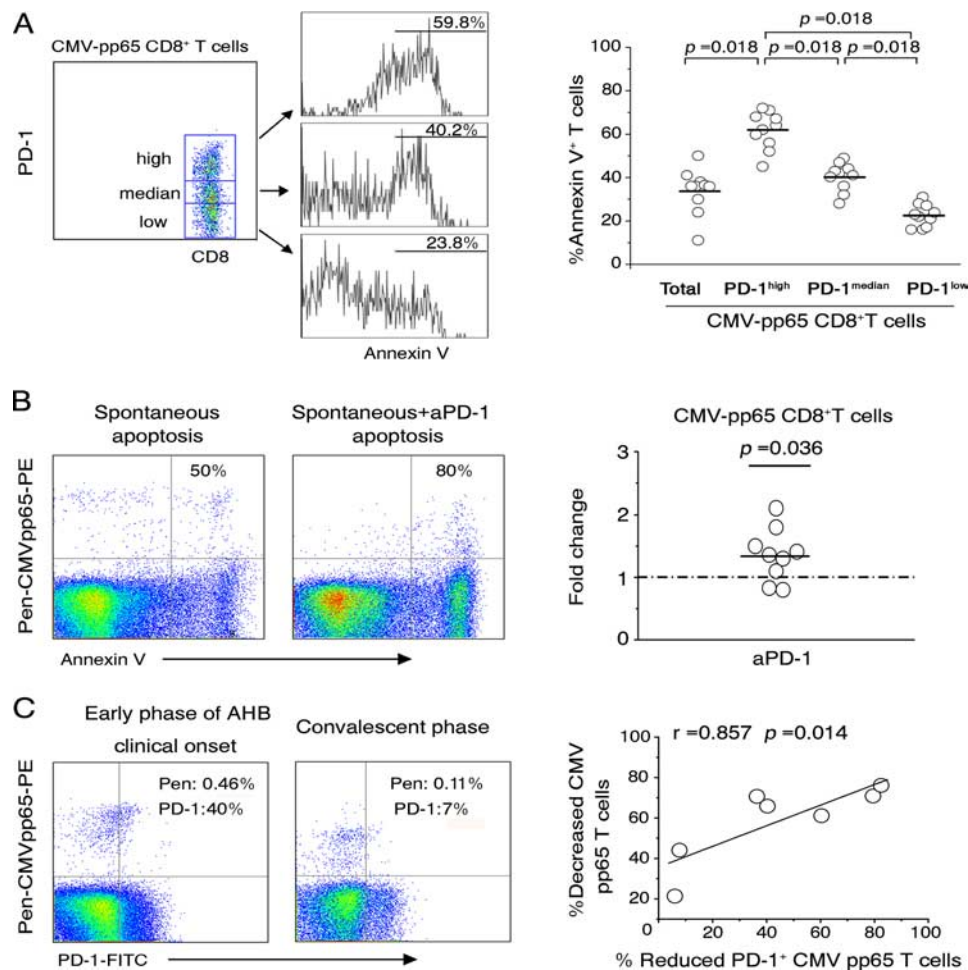


FIGURE 2. PD-1 up-regulation is associated with the apoptosis of CMV-specific CD8⁺ T cells in AHB patients. *A*, Representative histograms depict the annexin V positivity in PD-1^{high}, PD-1^{median}, and PD-1^{low} CMV-pp65 CD8⁺ T cells at the clinical onset in an AHB patient. Pool data indicate the percentages of annexin V-positive cells in different subsets of CMV-pp65 CD8⁺ T cell populations of 10 AHB patients. Horizontal bars present the median percentages of annexin V-positive cells. *B*, Representative dot plots of annexin V staining in CMV-pp65 CD8⁺ T cells in the presence and absence of plate-bound anti-PD-1 Ab (aPD-1). Values in the upper right quadrants indicate the percentages of annexin V-positive CMV-pp65 CD8⁺ T cells. The fold change in the percentage of CMV-pp65 CD8⁺ T cells is shown. *C*, Representative dot plots represent the kinetics of PD-1 expression on CMV-pp65 CD8⁺ T cells in acute HBV infections. The correlation between the decrease in CMV-pp65 CD8⁺ T cells and the reduction in PD-1-positive CMV-pp65 CD8⁺ T cells was analyzed. Pen, Pentamer.

compared with those of HCs (data not shown). Thus, PD-1 up-regulation on CMV-specific CD8 T cells seems to be less likely due to CMV-specific TCR signaling, provided that influenza infection does not result in a viral persistence. Therefore, further studies on the factors inducing PD-1 up-regulation on CMV-specific CD8 T cells are warranted.

Notably, there is a difference in the time at which the responses were observed in the AHB patients of this study and in the study performed on mice models. The IFN-induced attrition observed in mice models occurred at the first few days of infection before Ag-specific T cells in response to the acute infection; this attrition can be easily detected (2–9). By contrast, AHB patients are usually identified between 10 and 15 wk after HBV infection, when they become clinically symptomatic; HBV-specific T cells are detectable at this stage (21). Thus, although we also found that the serum IFN- α level was significantly higher at clinical onset of infection than that in healthy subjects (Fig. 1D), this time difference suggested that the attrition of CMV-specific CD8 T cells in AHB patients may be a different phenomenon than the IFN-induced attrition observed in mice. Future studies should elucidate the mechanisms responsible for the attrition in AHB patients.

PD-1 was originally cloned from apoptotic cell lines (22) and could increase the apoptotic sensitivity of HBV-specific CD8 T cells in acute HBV infections (21). Subsequently, we examined whether PD-1 up-regulation affects the numbers of CMV-specific CD8⁺ T cells in AHB patients. Both PD-1^{high} and PD-1^{median} CMV-specific CD8⁺ T cell subsets were observed to possess higher levels of annexin V staining than the PD-1^{low} subsets (Fig. 2A), suggesting that the higher levels of PD-1 expression were associated with the greater apoptosis sensitivity of CMV-specific CD8⁺ T cells. More importantly, we observed that plate-bound stimulatory anti-PD-1 directly enhanced the apoptosis of CMV-specific CD8⁺ T cells. Pooled data confirmed that the results were consistent for seven of nine patients (Fig. 2B). We also longitudinally analyzed the association between PD-1 expression and the depletion of CMV-specific CD8⁺ T cells in seven AHB patients and found that the decreases in CMV-specific CD8⁺ T cells was positively correlated with the reduction in PD-1-expressing CMV-specific CD8⁺ T cells (Fig. 2C). These results indicate that PD-1 up-regulation might mediate the apoptosis of CMV-specific CD8⁺ T cells in acute HBV infections.

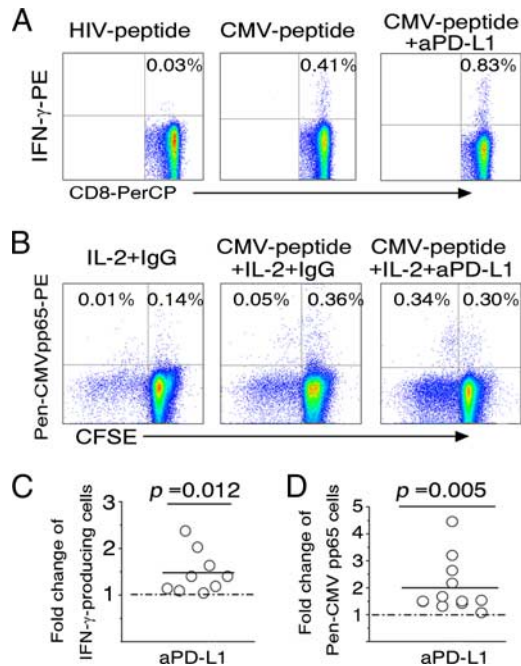


FIGURE 3. PD-1 up-regulation impairs the function of CMV-specific CD8⁺ T cells in the early phase of acute HBV infection. *A*, Representative dot plots represent the intracellular IFN- γ staining of CMV-pp65 CD8⁺ T cells in an AHB patient at the clinical onset. Values in the upper right quadrants indicate the percentages of IFN- γ -producing CD8⁺ T cells. *B*, Representative dot plots represent CFSE staining in an AHB patient. Values in the upper left quadrant indicate the percentage of CFSE^{low} pentamers. *C*, Summary data for the fold change in the percentage of IFN- γ -producing CMV-pp65 CD8⁺ T cells are shown. *D*, Summary data for the fold change in the percentage of CFSE^{low} pentamers are shown. Horizontal bars in *C* and *D* indicate the median fold changes. aPD-11, anti-PD-11; Pen, pentamer.

Persistent PD-1 up-regulation can exhaust virus-specific T cells during established chronic HIV, hepatitis C virus, and HBV infections (10–19). In this section, we further investigated whether the interference of PD-1 with PD-L1 influenced the function of CMV-specific CD8⁺ T cells following stimulation with a cognate peptide. We found that blockade of PD-1/PD-L1 interaction by using an anti-PD-L1 blocking Ab significantly increased IFN- γ production (Fig. 3, *A* and *C*) and the accumulation of CFSE^{low} pentamers (Fig. 3, *B* and *D*) among these CMV-specific CD8⁺ T cells. However, due to the high apoptotic sensitivity of the PD-1^{high} CMV-specific CD8⁺ T cells, this expansion of CMV-specific CD8⁺ T cells in the presence of anti-PD-L1 may be a direct result of enhanced CD8⁺ T cell proliferation, decreased apoptosis, or a combination of the two effects. These data indicated that PD-1 may also participate in the functional attrition of CMV-specific CD8⁺ T cells in acute HBV infections.

In summary, this study provides the first indication that PD-1 up-regulation, at least in part, contributes to the attrition of CMV-specific CD8⁺ T cells in acute HBV infections. These findings, therefore, extended the attrition of memory T cells from mouse viral infections to human viral infections and presented a mechanism that influences the homeostasis in the pre-existing virus-specific CD8⁺ T cell pool.

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Disclosures

The authors have no financial conflict of interest.

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