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Original Article

Lymphocytes in Patients with Chronic Active Epstein-Barr Virus Disease Exhibited Elevated PD-1/PD-L1 Expression and a Prevailing Th2 Immune Response

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Competing interests: The authors declare no conflict of Interest.

Abstract. ***Background And Objectives*** **Chronic active Epstein-Barr virus disease (CAEBV) is a proliferative disease of EBV^+ T or natural killer (NK) cells with an unclear pathogenesis. This study aimed to examine the frequency and exhaustion levels of lymphocyte subsets in patients with CAEBV to further investigate the pathogenesis.**
Methods. **Using flow cytometry, we detected the frequency, expression levels of programmed cell death 1 (PD-1) and programmed death ligand 1 (PD-L1), and EBV infection status of peripheral T subsets and NK cells in patients with CAEBV and healthy individuals.**
Results. **24 patients and 15 healthy individuals were enrolled in this study. Patients showed notably higher expression levels of PD-1 and PD-L1 in peripheral T subsets and NK cells compared to healthy individuals ($P < 0.05$). EBV^+ lymphocytes exhibited significantly higher PD-L1 expression levels than EBV^- lymphocytes. Additionally, the frequency of effector memory T (Tem) cells was significantly increased in patients, and the PD-L1 expression level was positively correlated with the EBV load. Besides, helper T cell 2 (Th2) immune bias, also favoring EBV amplification, was found in patients, including increased Th2 cell frequency, enhanced response capacity, and elevated serum levels of associated cytokines. The distribution and PD-1 expression levels of peripheral T subsets returned to normal in patients who responded to PD-1 blockade therapy.**
Conclusions. **The up-regulation of the PD-1/PD-L1 pathway of peripheral T and NK cells and Th2 immune predominance jointly promoted EBV replication and the development of CAEBV. PD-1 blockade therapy reduced the PD-1 expression level of lymphocytes and helped normalize the distribution of the T subsets.**

Keywords: Chronic active Epstein-Barr virus disease; Lymphocyte subsets; Programmed cell death protein 1; Programmed death-ligand 1; Helper T cells 2.

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Introduction. Epstein-Barr virus (EBV), a gamma human herpes virus, infects over 95% of the population. The majority of these infections occur during childhood, typically asymptomatically, and infections in adolescents often present as transient infectious mononucleosis (IM).[1-3] Chronic active EBV disease (CAEBV) is a progressive disease of ≥3 months duration, characterized by significantly elevated blood levels of

EBV-DNA and the infiltration of organs by EBV-infected lymphocytes in the absence of identified immunodeficiency.[4] Owing to the fact that cases affecting B cells are typically associated with primary immunodeficiency, the spectrum of CAEBV disease has been reconfigured to encompass only T and NK cell disorders.[5] The clinical manifestations of CAEBV are heterogeneous, including fever, lymphadenopathy, and hepatosplenomegaly, sometimes associated to thrombocytopenia.[6] Compared to patients with EBV-infected NK cells, those with T-cell infection exhibit shorter survival, more severe systemic symptoms, and elevated blood EBV titers. Conversely, patients with NK cell infections frequently present with severe mosquito bite allergy and elevated serum IgE levels.[4] Furthermore, CAEBV may present concurrently with hemophagocytic lymphohistiocytosis (HLH) or lymphoproliferative disease (LPD)/lymphoma during the disease. CAEBV carries a dismal prognosis, with an overall survival rate of merely 58% at three years post-diagnosis.[7] To date, allogeneic hematopoietic stem cell transplantation (allo-HSCT) remains the exclusive curative approach for CAEBV despite the high treatment-related mortality rates.[7] Consequently, comprehensive and in-depth explorations of the pathogenesis and further discovery of new effective treatments for CAEBV are paramount.

So far, the pathogenesis of CAEBV remains obscure. It is well known that lymphocytes play a crucial role in viral infection defense, however, the investigation of lymphocyte subsets in patients with CAEBV is limited. Lin et al. reported that the frequency of peripheral naïve T (Tn) cells and $CD28^+$ T cells was diminished, while the frequency of effector memory T (Tem) cells, regulatory T (Treg) cells, and $CD38^+$ T cells was elevated in patients with CAEBV (n=64) compared to healthy individuals (n=64) in a retrospective study, suggesting that the distribution of T cell subsets might be implicated in the pathogenesis of CAEBV.[9] Nonetheless, the potential modifications of lymphocyte subsets other than those aforementioned in patients with CAEBV remain unclear.

In recent years, an increasing focus has been placed on the role of immune checkpoints (ICs) in EBV immune escape. The programmed cell death protein 1/programmed cell death-ligand 1 (PD-1/PD-L1) axis plays a pivotal role in maintaining immune tolerance by down-regulating inflammatory responses and inhibiting T-cell activation.[10] However, aberrant upregulation of PD-1/PD-L1 expression has been observed in various EBV-associated diseases, such as NK/T-cell lymphoma, gastric cancer (GC), and nasopharyngeal carcinoma (NPC), and is correlated with poor prognosis.[11-13] Previously, we effectively reduced the EBV-DNA copies in peripheral blood mononuclear cells (PBMCs) of patients with CAEBV through the PD-1 blockade and lenalidomide combination therapy with an overall response rate (ORR) of 54.2%, demonstrating the considerable potential of PD-1 blockade therapy in CAEBV.[14] Interestingly, in that study, we found that the frequency of $CD8^+$Tem cells in the response (R) group was significantly higher than that in the non-response (NR) group, indicating that blocking the PD-1/PD-L1 pathway may affect the distribution of T-cell subsets, which needs to be verified.

Therefore, this study was designed to investigate the distribution of peripheral T-cell subsets and the expression levels of PD-1/PD-L1 in T and NK cells in CAEBV patients before and after PD-1 blockade therapy.

Patients and Methods.

Study Population. This prospective observational study enrolled a total of 24 patients diagnosed with CAEBV and 15 healthy individuals who were admitted to Beijing Friendship Hospital, Capital Medical University, between November 2022 and August 2023. All patients met the diagnostic criteria outlined in the revised 2017 World Health Organization classification: (1) IM-like symptoms persisting for more than three months; (2) elevated levels of EBV-DNA in peripheral blood (PB) or histological evidence of organ involvement as well as detection of virus RNA or proteins in affected tissues; (3) Exclusion of IM, congenital immunodeficiency including X-linked lymphoproliferative diseases (XLP), familial hemophagocytic lymphohistiocytosis (FHL), human immunodeficiency virus (HIV) infection, autoimmune disease, or other underlying conditions requiring immunosuppressive therapy, coinfected with other viruses such as hepatitis B virus (HBV), hepatitis C virus (HCV), and cytomegalovirus (CMV).[15] Before enrollment, of 24 patients, 10 were untreated, 8 had taken dexamethasone, 4 had received final L-DEP (PEG-asparaginase together with liposomal doxorubicin, etoposide, and methylprednisolone) regimen one month earlier, 2 had received final DEP (liposomal doxorubicin, etoposide, and methylprednisolone) regimen one month earlier, and no one had received HSCT. The 15 healthy individuals were HSCT donors who tested negative for EBV-DNA in the week prior to enrollment and exhibited no fever or any signs of infectious disease or history of medications within the past two weeks. To detect the frequency and PD-1/PD-L1 expression levels of peripheral lymphocyte subsets in patients with CAEBV after PD-1 block therapy, 5 patients who responded to PD-1 blockade combined with lenalidomide therapy were included in the study. Of these, 3 had received 6 courses of treatment and 2 had received 3 courses of treatment. The detailed regimen was shown in our previous published study.[14]

This study has been registered with ClinicalTrials.gov under the identifier NCT05841342. The protocol was approved by the Ethics Committee of Beijing Friendship Hospital (ID: 2022-P2-333-02), and informed consent was obtained from all patients prior to

participation in the study. This study was funded by the National Natural Science Foundation of China (No. 82370185).

Analysis of Lymphocyte Subsets by Flow Cytometry. Peripheral blood samples were collected and anticoagulated with ethylenediaminetetraacetic acid (EDTA). Within 2 h, PBMCs were isolated using a lymphocyte isolation solution (P8610, Solarbio, Beijing, China) and subsequently incubated with Fc Block reagent (564219, BD Biosciences, Franklin Lakes, NJ USA) to prevent nonspecific staining. To concurrently assess the EBV infection status along with the detection of frequencies and ICs levels of lymphocyte subsets, we employed the primerFlow RNA assay kit (88-18005-210, Invitrogen, Waltham, MA, USA) containing AF647 fluorescent probes for EBERs 1-2 (337601-000, Invitrogen). The following operations were conducted following the instructions provided with the kit. PBMCs were equally divided into five tubes, four of which were stained with different surface antibodies. Tube 1 (Blank): No antibodies were added. Tube 2 (T, NK, NKT and Treg cells): CD3-BV510 (317332, Biolegend, San Diego, CA, USA), CD4-BB700 (566392, BD), CD25-PE-Cy7 (302612, Biolegend), CD56-APC-Cy7 (318332, Biolegend), PD-1-BV421 (562516, BD), PD-L1-BB515 (564554, BD); Tube 3 (naïve-memory T cells): CD3-BV510 (317332, Biolegend), CD4-BB700 (566392, BD), CD8-APC-Cy7 (557834, BD), CD45RO-PE (304206, Biolegend), CCR7-PE-Cy7 (557648, BD), PD-1-BV421 (562516, BD), PD-L1-BB515 (564554, BD); Tube 4 (Th1, Th2, Th17 cells and PD-1): CD3-BV510 (317332, Biolegend), CD4-PE-Cy7 (557852), CD8-APC- Cy7 (557834, BD), CXCR3-BB700 (566532, BD), CCR4-PE (359412, BD), CCR6-BB515 (564479, BD), PD-1-BV421 (562516, BD); Tube 5 (Th1, Th2, Th17 cells and PD-L1): Antibodies to CD3, CD4, CD8, CXCR3, CCR4, and CCR6 were all consistent with those in the tube 4, PD-L1-BV421 (563738, BD). After incubation at 4°C for 30 minutes under light protection, all tubes were sequentially washed, fixed, and permeabilized, followed by the application of Foxp3-PE (560046, BD) intracellular staining only to tube 2. After the second fixation, all tubes, excluding the blank tube, were incubated with the EBERs 1-2 probe for incubation at 40°C for 2 h under light protection. Following the washing process, the samples were subsequently stored in a light-free environment at 4°C for an overnight duration. On the second day, the signal amplification steps were executed. The samples were sequentially incubated first with pre-amp mix solution for 1.5 h, then with amp mix solution for 1.5 h, and finally with the label probes for 2 h in the dark at 40°C. At last, cells were resuspended and detected using a FACSCantoIIflow cytometer (BD). The obtained data was subjected to analysis using the Flowjo software.

The cell subpopulation labeling schemes are as follows: NKT cells ($CD3^+CD56^+$), Treg cells ($CD3^+CD4^+CD25^+Foxp3^+$), $CD4^+$ Tn cells ($CD3^+CD4^+CD45RO^-CCR7^+$), $CD4^+$ central-memory T (Tcm) cells ($CD3^+CD4^+CD45RO^+CCR7^+$), $CD4^+$ Tem cells ($CD3^+CD4^+CD45RO^+CCR7^-$), $CD8^+$ Tn cells ($CD3^+CD8^+CD45RO^-CCR7^+$), $CD8^+$ Tcm cells ($CD3^+CD8^+CD45RO^+CCR7^+$), $CD8^+$ Tem cells ($CD3^+CD8^+CD45RO^+CCR7^-$), helper T cell 1 (Th1) ($CD3^+CD4^+CXCR3^+CCR4^-CCR6^-$), Th2 cells ($CD3^+CD4^+CXCR3^-CCR4^+CCR6^-$), Th17 cells ($CD3^+CD4^+CXCR3^-$ $CCR4^+CCR6^+$).

Detection of Cytokines. In this study, we investigated the serum levels of 9 cytokines in both healthy individuals and patients, including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), C-X-C chemokine ligand 9 (CXCL9), interleukin 18 (IL-18), cluster of differentiation 163 (CD163), suppression of tumorigenicity 2 (ST2), interleukin-1 receptor antagonist (IL-1RA), interleukin-10 (IL-10), interleukin-17A (IL-17A). The employed methods comprised the cytokine microsphere assay from Becton Dickinson and the Luminex Assay Platform System.

Immune Response Capacity of Th Cell Subsets to PHA Stimulation. PBMCs from patients of CAEBV or healthy individuals were seeded in 24-well plates at a density of 10^6 cells/mL and then stimulated with PHA (00-4977-93, eBioscience, MA, USA) for 72 h. GolgiPlug™ Protein Transport Inhibitor (555029, BD) was added 9h before the end of stimulation. After stimulation, PBMCs were collected and centrifuged at 1200 rpm for 5 min. After being washed twice using PBS buffer, PBMCs were stained with surface antibodies, including CD3-BV510 and CD4-BB700. Intracellular staining was performed after the cells were fixed and permeabilized using the Fixation/Permeabilization Kit (554714, BD) according to the instructions. Antibodies used for intracellular staining included IFN-γ-PE-Cy7 (557643, BD), IL-4-APC (560671, BD), IL-17A-BV421 (562933, BD), IL-10-PE (506804, BD). Subsequently, cells were washed and resuspended according to the instructions. Finally, the percentage of cytokine-positive cells among $CD4^+$ T cells in the samples was detected using the FACSCantoIIflow cytometer.

Statistical Analysis. Statistical analysis was executed using GraphPad Prism 8 software. The measurement data conforming to the normal distribution and homogeneity of variance were represented by the mean ± standard deviation (SD) and analyzed with a two-sided Student's t-test. The measurement data that did not conform to the normal distribution were represented by M (P25, P75), and a comparison between the two groups was conducted using the Mann-Whitney U test. The χ^2

Table 1. Clinical characteristics of the study population.

Characteristics	CAEBV[1] group (N=24)	Control group (N=15)	p-Value
Age (years), median (range)	28 (15-67)	37 (14-55)	0.66
Gender-no. (%)			
Female	13 (54.17)	9 (60)	0.72
Male	11 (45.83)	6 (40)	
Previous complicated with HLH[2]-no. (%)	10 (41.67)	-	-
Fever-no. (%)	21 (87.5)	-	-
Lymphadenopathy-no. (%)	19 (79.17)	-	-
Splenomegaly-no. (%)	14 (58.33)	-	-
Hepatomegaly-no. (%)	2 (8.33)	-	-
White blood cell count ($\times10^9$/L)	4.22 ± 2.48	6.37 ± 1.32	0.004
Lymphocyte count ($\times10^9$/L)	0.74 (0.36-1.37)	1.85 (1.15-2.37)	0.0009
Neutrophil count ($\times10^9$/L)	2.62 ± 1.89	3.97 ± 1.1	0.02
Hemoglobin (g/L)	116 (90.25, 124)	124 (120, 130)	0.03
Platelet ($\times10^9$/L)	181.5 ± 96.43	254.7 ± 54.14	0.01
EBV-DNA (PBMCs[3], copies/mL), median (range)	1.9×10^5 (4.3×10^4-1.38×10^6)	-	-
Organ infiltration (EBER$^+$)[4] -no. (%)	18 (75)	-	-

[1]CAEBV, chronic active Epstein-Barr virus disease; [2]HLH, hemophagocytic lymphohistiocytosis; [3]PBMCs, peripheral blood mononuclear cells; [4]EBER, EBV-encoded RNA.

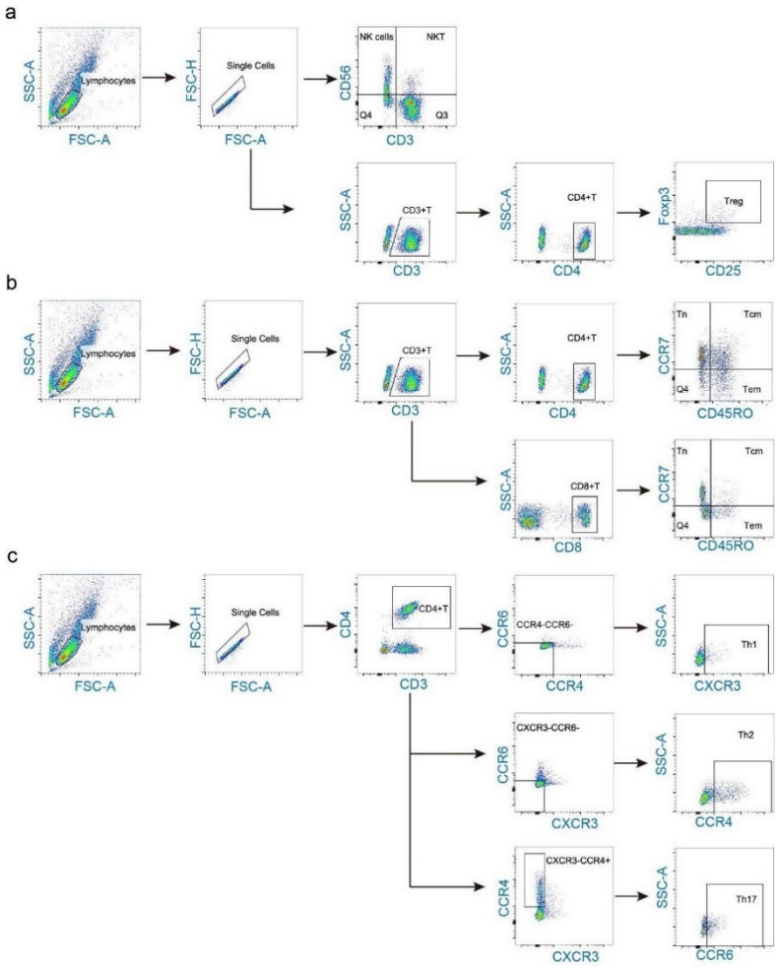

Figure 1. The gating strategy to set T-cell subsets and NK cells by flow cytometry. (**a**) Representative dot plot showing the gating strategy for $CD3^-CD56^+$ NK, $CD3^+CD56^+$ NKT, $CD3^+$ total T, and $CD3^+CD4^+CD25^+Foxp3^+$ Treg cells. (**b**) Gate strategy to set $CD3^+CD4^+$ Th, $CD3^+CD8^+$ Tc, $CD45RO^-CCR7^+$ Tn, $CD45RO^+CCR7^+$ Tcm, and $CD45RO^+CCR7^-$ Tem cells. (**c**) Gate strategy to set $CXCR3^+CCR4^-$ $CCR6^-$ Th1, $CXCR3^-CCR4^+CCR6^-$ Th2, and $CXCR3^-CCR4^+CCR6^+$ Th17 cells.

test was employed for the processing of categorical data. The statistical significance was determined when the P value was less than 0.05.

Results.

General Characteristics of Participants. The CAEBV group consisted of 13 females and 11 males, with a median age of 28 years. The healthy control group included 9 females and 6 males, and the median age is 37 years. Age and sex did not differ significantly between the two groups. In the CAEBV group, 10/24 (41.67%) had a previous history of HLH, 21/24 (87.5%) had fever, 19/24 (79.17%) presented with lymphadenopathy, 14/24 (58.33%) exhibited splenomegaly, and hepatomegaly was observed in 2 patients (8.33%). Leukopenia, anemia, and thrombocytopenia were common findings in patients with CAEBV. The clinical characteristics of all participants are shown in **Table 1**.

Patients with CAEBV Exhibited Increased PD-1/PD-L1 Expression Levels on Peripheral T Cell Subsets and NK Cells. The gating strategy to set $CD3^+$ total T, $CD3^+CD4^+$ Th, $CD3^+CD8^+$ cytotoxic T (Tc), $CD3^+CD56^+$ NKT, and $CD3^-CD56^+$ NK cells by flow cytometry was illustrated in **Figure 1a, b**. There were no significant differences in the frequencies of T cells and NK cells in peripheral lymphocytes between healthy individuals and patients with CAEBV, and there were no significant differences in the frequencies of Th, Tc, and NKT cells in T cells between the two groups (**Figure 2a, b)**. Patients exhibited significantly elevated expression levels of PD-1 in T, Th, Tc, and NK cells compared to those in the control group (44.2% [30.93-55.2%] vs. 20.8% [12.5-35.4%], 50% [35.58-58.78%] vs. 24.2% [16.4, 26.6%], 31.95% [22.65-49.5%] vs. 21.6% [14.4-26.7%], 4.64% [2.31-10.86%] vs. 1.57% [0.69-3.68%], **Figure 2c, d**). In addition, PD-L1 expression levels showed a similar trend in T, Th, Tc, NKT, and NK cells across both groups (2.2% [1.28-4.04%] vs. 0.64% [0.1-0.97%], 1.49% [0.91- 5.28%] vs. 0.22% [0.19-2.57%], 2.37% [1.26-3.7%] vs. 0.28% [0.18-1.01%], 4.91% [3.6-7.72%] vs. 0.96% [0.47-2.8%], 11.85% [5.45-24.45%] vs. 1.11% [0.29-2.43%], **Figure 2c, e**).

To investigate the potential association between the increased expression level of PD-1 or PD-L1 on lymphocytes and EBV infection, we categorized patients' lymphocytes into $EBER^+$ and $EBER^-$ and compared the expression levels of PD-1/PD-L1 between two groups. Results showed that there was no significant difference in PD-1 expression levels between $EBER^+$ T cells and $EBER^-$ T cells (38.75% [8.32-57.13%] vs. 47.55% [35.29- 55.49%], **Figure 3a, c**), or $EBER^+$ NK cells and $EBER^-$ NK cells (1.32% [0.19-8.97%] vs. 4.47% [2.58-8.4%], **Figure 3a, c**). Notably, PD-L1 expression levels of $EBER^+$ T and $EBER^+$ NK cells were significantly

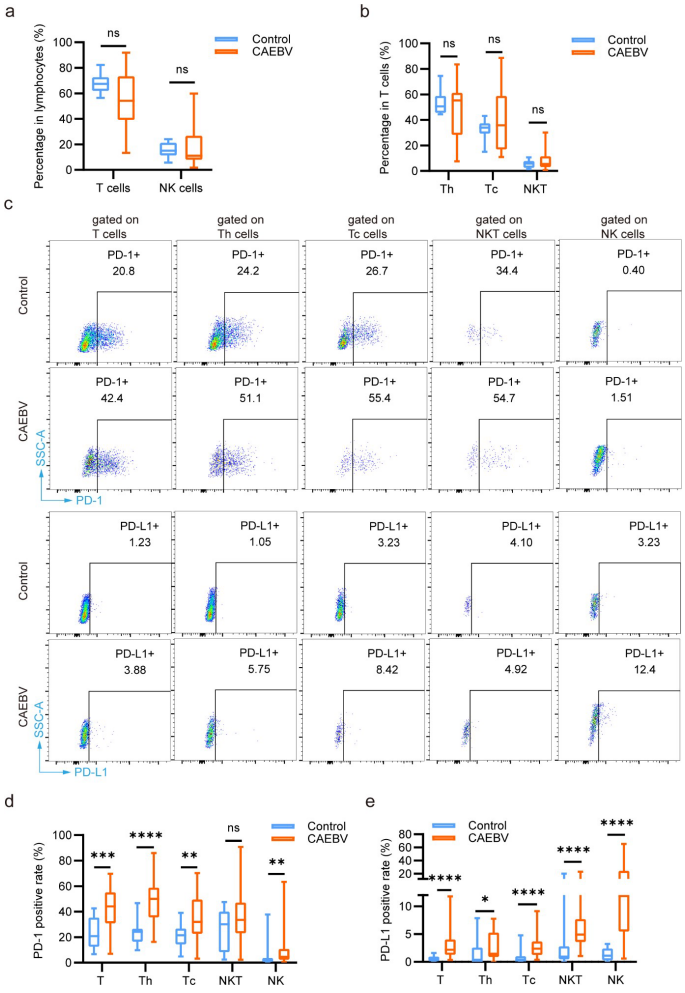

Figure 2. Patients with CAEBV showed significantly higher expression levels of PD-1 and PD-L1 in T cell subsets and NK cells. Percentages of T (**a**) and NK (**b**) cells in lymphocytes in the CAEBV group and the control group. n=15 for the control group, and n=24 for the CAEBV group. Representative flow profiles (**c**) and summarized positive percentages of PD-1 (**d**) and PD-L1 (**e**) in T, Th, Tc, NKT, and NK cells in the CAEBV group and the control group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, not significant.

higher than those of the corresponding $EBER^-$ cells (28.6% [13.39-53.02%] vs. 2.54% [0.9-4.46%], 21% [7.39-44.27%] vs. 10.33% [4.48-11.88%], **Figure 3b, d**), indicating that EBV induces PD-L1 expression in host lymphocytes in CAEBV.

Next, we distinguished $EBER^+$ T cells based on the surface marker CD45RO. We found that 58.75% of the infected T cells exhibited a $CD45RO^+$ phenotype, suggesting that EBV may ensure its long-term existence by infecting memory T (Tm) cells (**Figure 3e, f**).

Patients with CAEBV Showed Elevated Frequency and PD-L1 Expression Levels of Tem Cells. We investigated the frequency and the PD-1/PD-L1 expression level of naive-memory subpopulations of T cells in the PB of patients with CAEBV and healthy donors. The results demonstrated a significant reduction in the frequencies of both $CD4^+$ Tn and $CD8^+$ Tn cells (10.44% [4.32-

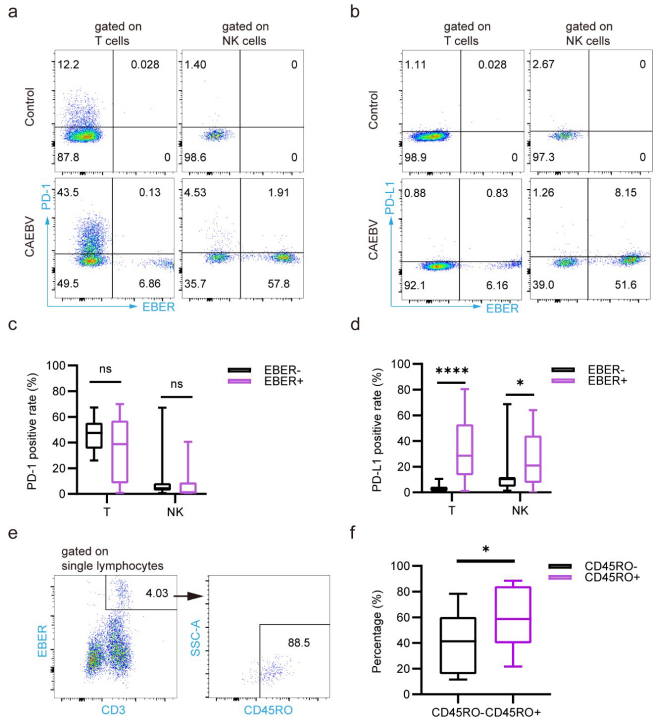

Figure 3. Elevated PD-L1 expression levels of patients with CAEBV were associated with direct EBV infection. Representative flow profiles (**a**) and summarized positive percentages (**c**) of PD-1 in EBER- and EBER+ T and NK cells in patients with CAEBV. Representative flow profiles (**b**) and summarized positive percentages (**d**) of PD-L1 in EBER- and EBER+ T and NK cells in patients with CAEBV. n=16 for the EBER- and EBER+ T group, n=21 for the EBER- and EBER+ NK group. (**e**) Representative dot plot showing the gating strategy for the frequencies of CD45RO+ memory and CD45RO- non-memory cells in EBER+CD3+ T cells. (**f**) Summarized frequencies of CD45RO+ memory and CD45RO- non-memory cells in EBER+CD3+ T cells. n=16 for the CD45RO- and CD45RO+ group. *P < 0.05, ****P < 0.0001. ns, not significant.

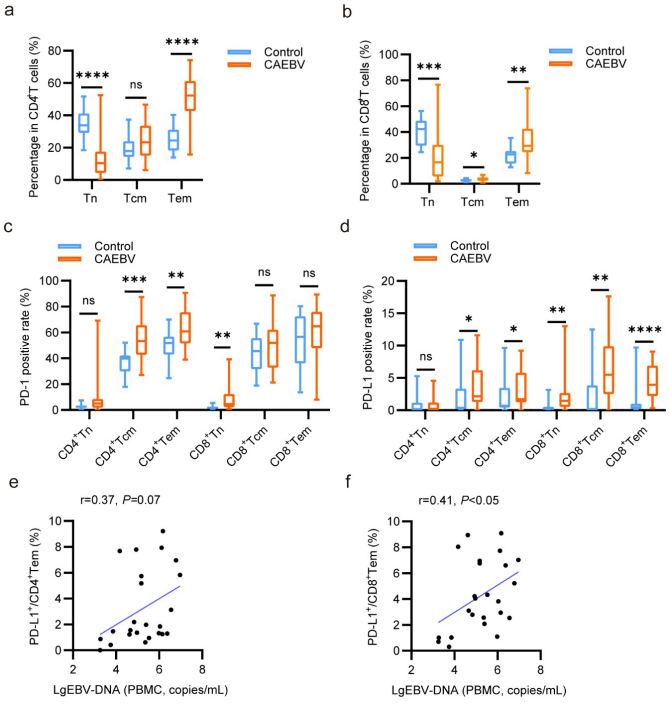

Figure 4. Patients with CAEBV showed notably decreased frequency of Tn cells and increased frequency of Tem cells, the PD-L1 expression level of Tem cells was positively correlated with the EBV-DNA copies in PBMCs. Frequencies of Tn, Tcm, and Tem cells in CD4+ Th (**a**) and CD8+ Tc (**b**) cells in the CAEBV and control groups. Positive rates of PD-1 (**c**) and PD-L1 (**d**) in CD4+ and CD8+ Tn, Tcm, and Tem cells in the CAEBV and the control group. The correlation between PD-L1 positive rates of CD4+ Tem (**e**) or CD8+ Tem (**f**) cells and EBV DNA copies in PBMCs. n=15 for the control group, and n=24 for the CAEBV group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, not significant.

17.55] vs. 33.8% [29.2-41.2], 16.65% [5.51-30.2] vs. 42.3% [29.4-48.8]), concurrent with a significant increase in the frequencies of both $CD4^+$ Tem and $CD8^+$ Tem cells (52.3% [42.7-61.35] vs. 24.5% [18.3-31.1], 29.25% [24.33-42.58] vs. 22.6% [15.6-25.3]) in patients compared to healthy individuals (**Figure 4a, b**). Besides, patients showed significantly elevated frequencies of $CD8^+$ Tcm cells (3.75% [2.37-4.92] vs. 2.71% [1.87-3.37] (**Figure 4b**).

Moreover, the PD-1 expression levels of $CD4^+$ Tcm, $CD4^+$ Tem, and $CD8^+$ Tn cells were markedly elevated in patients with CAEBV than in healthy individuals (53.35% [42.73-65.68] vs. 39.7% [29.9-42.1], 60.75% [51.48-75.63] vs. 51.8% [42.8-56.7], 4.65% [2.17-12.39] vs. 1.82% [0.83-2.5], **Figure 4c**). Similarly, the PD-L1 expression levels of $CD4^+$ Tcm, $CD4^+$ Tem, $CD8^+$ Tn, $CD8^+$ Tcm, and $CD8^+$ Tem cells were significantly higher in patients with CAEBV than in healthy individuals (2.15% [1.3-6.21] vs. 0.38% [0.21-3.34], 1.7% [1.23-5.81] vs. 0.68% [0.4-3.46], 1.47% [0.54-2.67] vs. 0.27% [0.16-0.54], 5.5% [2.49-9.9] vs. 0% [0-3.85], 3.93% [2.2-6.91] vs. 0.43% [0.17-0.99], **Figure 4d**). Of note, the PD-L1 expression levels of $CD4^+$ and $CD8^+$ Tem cells were both positively correlated with EBV-DNA copies in PBMCs (r=0.37, p=0.07 and r=0.41, p<0.05, respectively, **Figure 4e, f**).

Reduced Th1/Th2 Ratio Was Positively Correlated with the EBV-DNA Copies in PBMCs in CAEBV. Additionally, we measured the frequency and PD-1/PD-L1 expression levels of Th1, Th2, Th17, and Treg cells in PB between patients with CAEBV and healthy individuals. As presented in **Figure 5a**, patients exhibited a notable decrease in the frequency of Th1 cells (2.92% [1.51-4.33] vs. 6.02% [1.89-11.33]) and a significant increase in the frequency of Th2 cells (25.43% [18.35-29.35] vs. 16.39% [10.93-21.68]) compared to healthy individuals. Consequently, the Th1/Th2 ratio in patients with CAEBV was significantly lower than that in healthy individuals (0.13 [0.06-0.19] vs. 0.49 [0.12-0.77], **Figure 5b**).

Furthermore, the EBV-DNA copies in PBMCs were found to be negatively correlated with the Th1 frequency (r=-0.4, p<0.05, **Figure 5c**) and the Th1/Th2 cell ratio (r=-0.41, p<0.05, **Figure 5e**), while positively correlated with the Th2 frequency (r=0.23, p=0.27, **Figure 5d**) in the CAEBV group.

Moreover, the PD-1 expression levels of Th1, Th2,

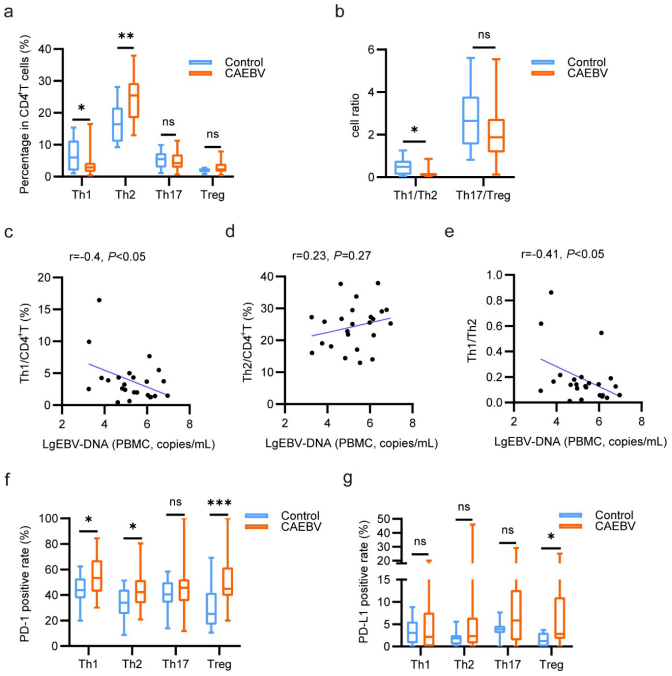

Figure 5. The reduced Th1/Th2 ratio was positively correlated with EBV-DNA copies in PBMCs in CAEBV. (**a**) Frequencies of Th1, Th2, Th17, and Treg cells in CD4+ Th cells in the CAEBV and the control group. (**b**) The Th1/Th2 ratio and the Th17/Treg ratio in the CAEBV and control groups. The correlation between Th1 cell frequency (**c**), Th2 cell frequency (**d**), Th1/Th2 ratio (**e**), and EBV DNA copies in PBMCs. Positive rates of PD-1 (**f**) and PD-L1 (**g**) in Th1, Th2, Th17, and Treg cells in the CAEBV and control groups. n=15 for the control group, and n=24 for the CAEBV group. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

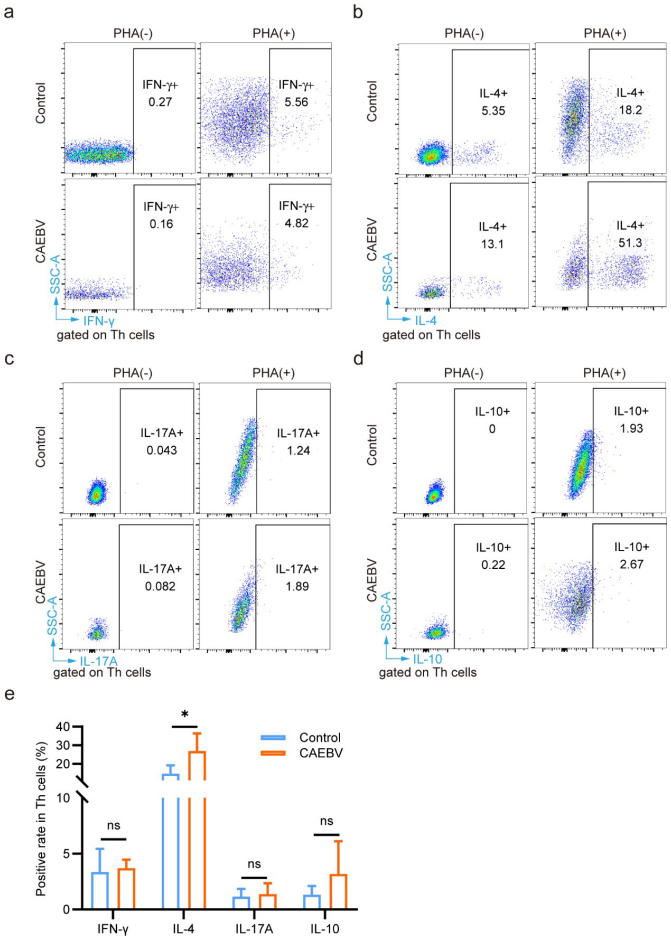

Figure 6. Patients with CAEBV showed significantly increased percentages of IL-4+ T cells after PHA stimulation. Classic dot plot showing the IFN-γ (**a**), IL-4 (**b**), IL-17A (**c**), and IL-10 (**d**) positive rate in CD4+ Th cells in patients with CAEBV and healthy individuals before and after PHA stimulation for 72 h. (**e**) Summarized positive percentages of IFN-γ, IL-4, IL-17A, and IL-10 cells in CD4+ Th cells in two groups. *P < 0.05. ns, not significant.

and Treg cells in the CAEBV group were significantly elevated compared to the control group (53.25% [42.63-67.35] vs. 43.8% [37.3-53.1], 42.25% [33.6-51.78] vs. 34% [25.1-44.3], 44.9% [39.4-61.68] vs. 25.2% [16.7-41.7], **Figure 5f**). As for the PD-L1 expression levels, no significant differences were detected in Th1 or Th2 cells between the two groups. However, the PD-L1 expression level of Treg cells was significantly increased in the patients with CAEBV compared to healthy individuals (2.81% [1.7-11.05] vs. 1.25% [0-3.08], **Figure 5g**). There was no significant difference in frequency, PD-1, or PD-L1 expression levels of Th17 cells between the two groups.

Patients with CAEBV Showed Enhanced Response Capacity of Th2 Cells after PHA Stimulation. To assess the response capacity of Th1, Th2, Th17, and Treg cells, we measured the frequency of IFN-γ, IL-4, IL-17A, and IL-10 positive cells in $CD4^+$ T cells of healthy individuals and patients with CAEBV after 72 h of stimulation with PHA. The results revealed that the percentage of $IL\text{-}4^+$ cells in $CD4^+$ T cells was significantly higher in patients than in healthy people after PHA stimulation (26.95±9.41% vs. 14.71±4.5%, **Figure 6b, e**). However, the proportions of IFN-γ, IL-17A, and IL-10. Positive cells in patients' $CD4^+$ T cells were not significantly different from those of healthy individuals (**Figure 6a, c, d, e**). These results suggest that the response capacity of Th2 cells in patients with CAEBV is significantly higher than that of healthy individuals. The Th2 immune bias in patients with CAEBV included both elevated frequency and enhanced response capacity of Th2 cells.

Serum CD163 Levels Were Positively Correlated with Th2 Cell Frequency and EBV-DNA Copies in PBMCs in CAEBV. To better characterize the immune profile of patients with CAEBV, we measured the serum levels of 9 cytokines, including TNF-α, IFN-γ, CXCL9, IL-18, CD163, ST2, IL-1RA, IL-10, and IL-17A, in 21 patients using a Luminex 200 instrument (**Table 2**). Based on the biological functions, these cytokines were categorized as Th1-associated (TNF-α, IFN-γ, CXCL9), M1 macrophage-associated (IL-18), Th2-associated (ST2, IL-10), M2 macrophage-associated (CD163, IL-1RA), and Th17- associated (IL-17A). The percentages of patients exhibiting abnormally elevated Th1/M1-related cytokines were as follows: IL-18 (85.71%), IFN-γ

Table 2. Serum cytokine levels in patients with CAEBV.

	TNF-α[1]	IFN-γ[2]	CXCL9[3]	IL-18[4]	CD163[5]	IL-1RA[6]	ST2[7]	IL-10[8]	IL-17A[9]
R[10] (pg/mL)	0-25	0-62	0-5213	0-629	0-2874775	0-1868	0-21437	0-12	0-49
P[11]	4.5	20.4	1207	2676	2838398	851.3	109424	17.8	1.4
P2	6.3	21.8	1501	763.3	2971448	1309	32004	1.5	2
P3	2.3	8.6	581.6	339.8	7080291.2	670.2	40231	1.4	4.4
P4	3.3	7.2	1043	854.9	1549792	628.2	3623	2.2	2.2
P5	41.4	26.2	2816.7	18072.3	7593810	6976	80679.2	16.1	4.4
P6	7	24.2	666.5	959.1	1375153.7	1732.4	6618.1	2.3	1.2
P7	15	46.4	3357.1	3990.8	2782847.1	4832.9	75759.9	20.9	2
P8	5.5	8.4	398.4	300.3	1319365.3	984.1	7269.6	1.4	1.1
P9	10.5	15.9	1028.4	951.4	3778786.2	1065.8	32310.9	3.3	1.6
P10	2.8	7.9	516.7	228	493141.5	331.7	4743.5	1.4	1.2
P11	23.9	458.6	6611.1	7845.7	6343361	7822.1	139253.3	78.1	2.9
P12	9.9	54.3	801.2	4519.5	4717122.9	2057.8	75829.1	5.6	2.2
P13	9.7	33.2	52015.5	3012.1	2524943.4	3211.3	93578.5	27.3	4.4
P14	5.4	17.5	1251.2	4552.5	4911452.8	1022.9	119820	4.7	4.7
P15	12.8	153.8	2415.4	5324.7	4245931.9	4548.4	105357.2	5.2	2.6
P16	19.4	25.6	3364.6	4834.4	3767712.1	2912.7	12017.2	4.7	2.6
P17	4.1	13.3	715.1	1660.8	3852027.8	848.8	48602.8	1.4	1
P18	15.9	18.8	904.9	1455.3	596107.1	1955	14789.8	6.9	4.4
P19	2.5	14.9	198.7	116.6	974420	566.6	9186.7	1.4	4.4
P20	9.7	21.2	1625.7	755	2645752.8	4252.1	32540.6	8.8	4.4
P21	5.6	313.8	1799.9	1442.2	2705793.4	3594.3	23804.7	5.5	0.6

[1]TNF-α, Tumour necrosis factor alpha; [2]IFN-γ, interferon (IFN)-gamma; [3]CXCL9, C-X-C chemokine ligand (CXCL) 9; [4]IL-18, interleukin 18; [5]CD163, cluster of differentiation 163; [6]IL-1RA, interleukin-1 receptor antagonist; [7]ST2, suppression of tumorigenicity 2; [8]IL-10, interleukin-10; [9]IL-17A, interleukin-17A; [10]R, reference range; [11]P, patient.

(14.29%), TNF-α (4.76%), and CXCL9 (9.52%), while the proportions of patients with elevated Th2/M2-related cytokines were as follows: ST2 (66.67%), CD163 (57.14%), IL-1RA (52.38%), and IL-10 (23.81%). None of these patients showed abnormally elevated serum IL-17A. Notably, 4 cytokines were found abnormally elevated in more than half of these patients: IL-18, CD163, ST2, and IL-1RA, and the latter 3 were associated with Th2/M2. In particular, serum CD163 levels were negatively correlated with Th1 cell frequency ($r=-0.45$, $p<0.05$, **Figure 7a**), and positively correlated with both Th2 cell frequency ($r=0.48$, $p<0.05$, **Figure 7b**) and EBV-DNA copies in PBMCs ($r=0.57$, $p<0.05$, **Figure 7c**).

Additionally, as presented in **Figure 7d** and **Figure 7e**, both the serum levels of CD163 and IL-1RA were positively associated with the serum levels of ST2 ($r=0.75$, $p = 0.0001$; $r = 0.53$, $p < 0.05$, respectively).

CAEBV Patients Showed Normalized PD-1 Expression Levels and Distribution of Lymphocyte Subsets after PD-1 Blockade. Our team has previously investigated the efficacy of PD-1 blockade in combination with lenalidomide in patients with CAEBV and found that this regimen achieved an ORR of 54.2% and significantly reduced the EBV-DNA copies number in PB. In order to investigate whether the frequency and the PD-1/PD-L1 expression levels of lymphocyte subsets in patients return to the normal range after PD-1 blockade therapy, the above indicators were detected in 5 patients with CAEBV who received this regimen and then compared with the results of healthy individuals. All 5 patients achieved partial response (PR), of which 3 had received 6 courses of treatment, and 2 received 3 courses of treatment.

First, the PD-1 expression levels of lymphocyte subpopulations were all decreased in patients with CAEBV after PD-1 blockade therapy. The PD-1 expression levels of several T cell subsets were significantly lower than those of healthy individuals, including T, Th, Tc (2.39% [1.34-15.59] vs. 20.8% [12.5-35.4], 4.21% [2.58-17.17] vs. 24.2% [16.4-26.6], 3.51% [2.07-19.29] vs. 21.6% [14.4-26.7], **Figure 8a, b**), $CD4^+$ Tcm, $CD4^+$ Tem, $CD8^+$ Tem (5.89% [3.18-26.1] vs. 39.7% [29.9-42.1], 5.4% [3-31.92] vs. 51.8% [42.8-56.7], 2.07% [1.34-36.15] vs. 56.5% [36.1-72.5], **Figure 9a**), Th1, Th2, and Th17 cells (4.61% [2.99-30.55] vs. 43.8% [37.3-53.1], 0.41% [0.18-16.37] vs. 34% [25.1-44.3], 1.19% [0.44-15.64] vs. 40.5% [33.9-50], **Figure 9c**). The PD-1 expression levels of other lymphocyte

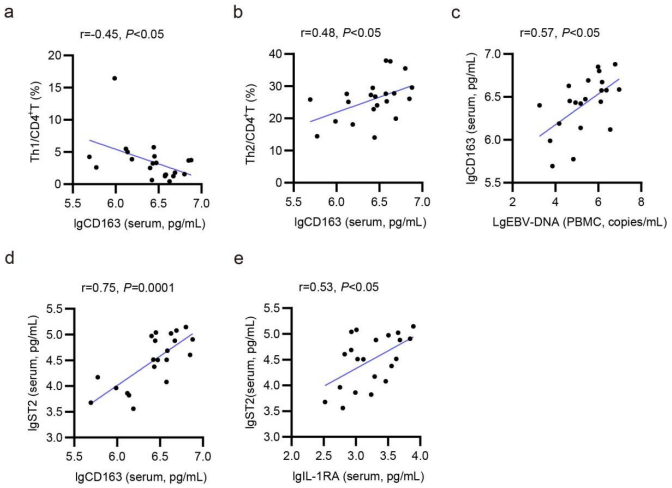

Figure 7. Serum CD163 levels in CAEBV patients was positively correlated with Th2 cell frequency and EBV-DNA copies in PBMCs. The correlation between the Th1 cell frequency (**a**), the Th2 cell frequency (**b**), EBV-DNA copies in PBMCs (**c**), and serum CD163 levels in patients with CAEBV. The correlation between serum CD163 levels (**d**), serum IL-1RA levels (**e**), and serum ST2 levels. n=21 for the CAEBV group.

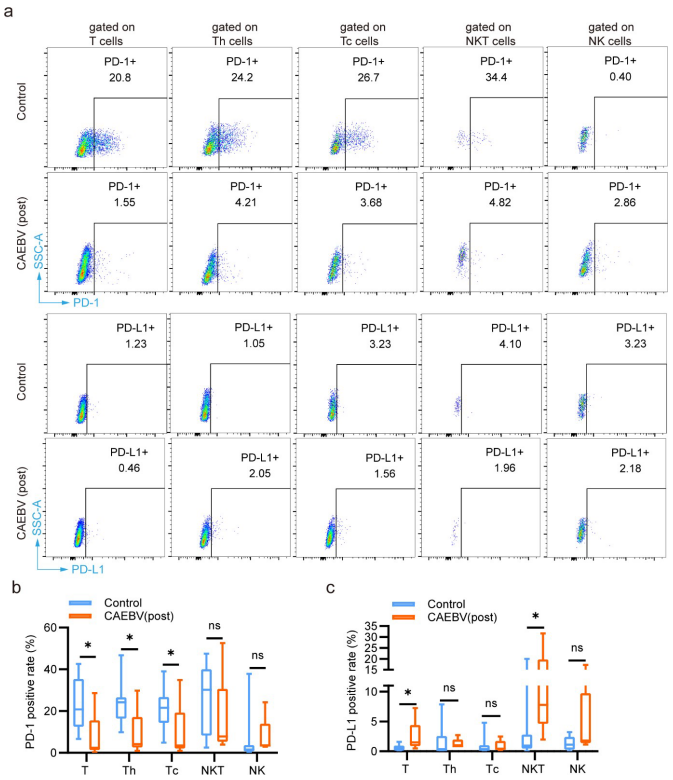

Figure 8. The PD-1 expression level of T cells was significantly decreased in patients with CAEBV responded to the PD-1 blockade therapy. (**a**) Classic dot plot showing the PD-1 and PD-L1 positive rates in T, Th, Tc, NKT, and NK cells of healthy individuals and patients with CAEBV who were responded to the PD-1 blockade therapy. Summarized positive percentages of PD-1 (**b**) and PD-L1 (**c**) in T, Th, Tc, NKT, and NK cells of healthy individuals and patients with CAEBV who were responded to the PD-1 blockade therapy. n=15 for the control group. n=5 for the CAEBV (post) group. *P < 0.05. ns, not significant.

subsets were not significantly different from those of healthy individuals. These results suggest that the anti-PD-1 monoclonal antibody (mAb) can effectively bind to PD-1 expressed on the surface of lymphocytes and thus block the PD-1/PD-L1 pathway. However, the expression levels of PD-L1 in several T cell subsets of patients were still significantly higher than those of healthy individuals, including T, NKT (1.49% [0.87-4.41] vs. 0.64% [0.1-0.97], 1.79% [1.38-9.74] vs. 1.11% [0.29-2.43], **Figure 8a, c**), Th1 and Treg cells (6.75% [5.12-12] vs. 3.04% [0.74-5.61], 13% [6.35-19.25] vs. 1.25% [0-3.08], **Figure 9d**), which is justified by the fact that the anti-PD-1 mAb could not bind PD-L1 expressed on the surface of lymphocytes.

Second, the frequencies of peripheral lymphocyte subsets in patients with CAEBV returned to normal after PD-1 blockade treatment (**Figure 10**). It is worth noting that in the naïve-memory T cell subsets, patients exhibited elevated frequencies of Tn cells and decreased frequencies of Tem cells after PD-1 blockade treatment; all results were not significantly different from healthy individuals (**Figure 10c, d**). In addition, it is noteworthy that the peripheral Th1/Th2 cell ratio returned to normal in patients with CAEBV after PD-1 blockade therapy (**Figure 10e, f**). The exact mechanism by which PD-1 blockade therapy affects the distribution of peripheral lymphocyte subsets in patients with CAEBV is unknown and requires in-depth study.

Discussion. We observed a significant alteration in the distribution of peripheral T cell subsets and a notable increase in the expression levels of PD-1 and PD-L1 in T cell subsets and NK cells in patients with CAEBV compared to healthy individuals, implying the abnormal distribution of T cell subsets and the exacerbated depletion of T and NK cells might jointly contribute to the pathogenesis of CAEBV.

Lin et al. reported a decreased frequency of Tn cells ($CD45RA^+CD62L^+$) and an increased frequency of Tem ($CD45RO^+CD62L^-$) and Treg cells ($CD4^+CD25^+$) in patients with CAEBV compared to healthy individuals in a retrospective study.[9] Although different staining methods were employed, the present study also observed a reduction in the frequency of Tn cells ($CD45RO^-CCR7^+$) and an increase in the frequency of Tem cells ($CD45RO^+CCR7^-$) in PB of patients with CAEBV. Nonetheless, the Treg frequency of patients did not display any significant disparity compared to that of healthy individuals within this study, which may be partly due to variations in case numbers and staining protocols. Thus, further investigation is necessary to reveal the exact rationale behind the difference. Especially, a decrease in the frequency of Th1 cells and an increase in the frequency of Th2 cells in patients with CAEBV were found for the first time in the present study. The reversed Th1/Th2 ratio was positively correlated with the EBV-DNA copies number in PBMCs, suggesting that the Th2 immune bias contributes to the development of CAEBV.

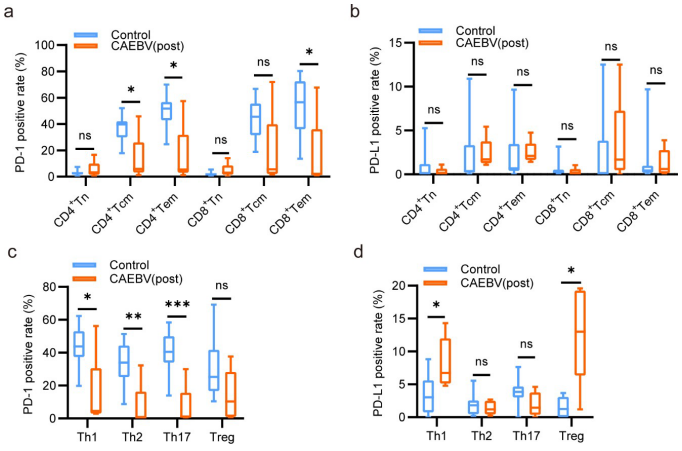

Figure 9. The PD-1 expression levels of T cell subsets was significantly decreased in patients with CAEBV responded to the PD-1 blockade therapy. Summarized positive percentages of PD-1 (**a**) and PD-L1 (**b**) positive rates in $CD4^+$ and $CD8^+$ Tn, Tcm, and Tem cells of healthy individuals and patients with CAEBV who were responded to the PD-1 blockade therapy. Summarized positive percentages of PD-1 (**c**) and PD-L1 (**d**) in Th1, Th2, Th17, and Treg cells of healthy individuals and patients with CAEBV who were responded to the PD-1 blockade therapy. n=15 for the control group. n=5 for the CAEBV (post) group. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

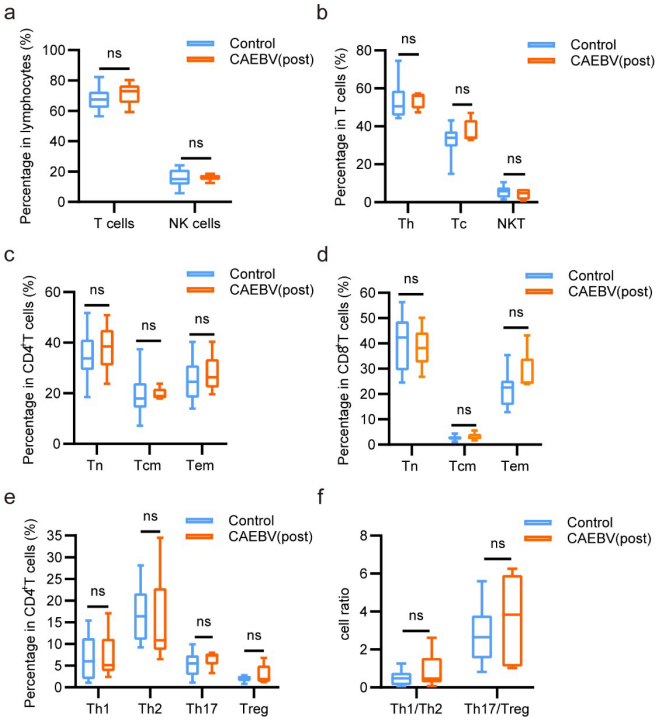

Figure 10. The distribution of T cell subsets returned to normal in patients with CAEBV responded to the PD-1 blockade therapy. Summarized percentages of T and NK cells in lymphocytes (**a**), Th, Tc and NKT cells in T cells (**b**), Tn, Tcm, and Tem cells in CD4+ T cells (**c**), Tn, Tcm, and Tem cells in CD8+ T cells (d), Th1, Th2, Th17, and Treg cells in CD4+ T cells (**e**) in healthy individuals and patients with CAEBV who were responded to the PD-1 blockade therapy. (**f**) Summarized Th1/Th2 and Th17/Treg cell ratios in healthy individuals and patients with CAEBV who were responded to the PD-1 blockade therapy. n=15 for the control group. n=5 for the CAEBV (post) group. ns, not significant.

In addition, we observed significantly elevated expression levels of PD-1 and PD-L1 in peripheral T cell subsets and NK cells of patients with CAEBV, and the increased expression of PD-L1 was partly due to the direct EBV infection. By leveraging the innovative approach introduced by Fournier et al., we classified patients' lymphocytes into $EBER^+$ and $EBER^-$ subgroups and comparatively assessed the expression levels of PD-1 and PD-L1 in two groups.[16] The findings unraveled a significant upregulation of PD-L1 expression in $EBER^+$ T cells and NK cells compared to their $EBER^-$ counterparts, suggesting that EBV promotes the expression of PD-L1 in host cells after infection. A previous study reported that EBV-miR-BART11 and EBV-miR-BART17-3p upregulated the expression of PD-L1 through respectively inhibiting FOXP1 and PBRM1, inhibitors of the expression of PD-L1 in NPC and GC cell lines in vitro.[11] EBV-encoded circBART2.2 promoted transcription of PD-L1 by binding the helicase domain of retinoic acid-inducible gene I (RIG-I) and activating transcription factors IRF3 and NF-κB in NPC cell line in vitro.[13] Whether EBV promotes PD-L1 expression of host T cells and NK cells through the above mechanisms in patients with CAEBV requires further study and validation.

Although Fournier et al. initially reported that the majority of $EBER^+$ T cells display an effector memory phenotype ($CD27^-$ $CD45RA^-$) in two patients with CAEBV, a more substantial cohort of participants is required for further validation and confirmation.[16] Upon dividing $EBER^+$ T cells into $CD45RO^+$ memory and $CD45RO^-$ non-memory types from 16 patients with CAEBV, we observed a significantly higher proportion of $CD45RO^+$ Tm cells in the infected cells, which verified the correctness of the results reported by Fournier et al. We provided a laboratory basis for the pathophysiological study of CAEBV. EBV predominantly infects Tm cells, presumably to ensure its long-term persistence in patients with CAEBV. Consequently, EBV escapes the immune response by upregulating PD-1 and PD-L1 expression in T and NK cells and potentially achieves long-term presence by infecting Tm cells in patients with CAEBV.

The underlying mechanisms contributing to the alteration in the frequency of navïe-memory T cells in patients with CAEBV could be attributed to the following two aspects: Firstly, since Tcm and Tem cells are differentiated from Tn cells, the decreased frequency of Tn cells and increased frequency of Tem cells may be due to the continued differentiation of Tn cells into Tem cells caused by prolonged exposure to EBV antigens, which could be regarded as immune compensatory changes. Patients with other viral infections, such as HCV and COVID-19, also exhibit a diminished frequency of Tn cells in PB,[17-20] which might be able to corroborate our speculation. Secondly, the frequency of Tn cells might be curtailed by Th2 cells. The crucial role of thymic epithelial cells and the thymic

microenvironment in the proliferation and differentiation of Tn cells has been extensively demonstrated.[21] Shen et al. demonstrated that Th2 cells play a vital role in inhibiting the development of embryonic thymocytes through the key factor IL-4 and that even the lowest concentration of Th2 cells significantly reduces the total number of thymocytes in mice.[22] Inspired by this, we calculated and discovered a negative correlation between the $CD3^+$ Tn cell frequency and the Th2 cell frequency in patients with CAEBV in this study, suggesting Th2 cells may also have an inhibitory effect on the development of thymocytes and Tn cells in humans, which need to be further corroborated. The decline in the frequency of Tn cells may result in decreased T cell counts among patients with CAEBV, and the exclusive differentiation into EBV-specific T cells may lead to a diminished ability to cope with infections by other pathogens. A positive correlation was observed between the PD-L1 expression level in $CD8^+$ Tem cells and the EBV- DNA copies in PBMCs, indicating that despite the presence of a compensative elevated frequency, the severe depletion status of Tem cells still caused a compromised capacity to combat EBV infection.

It is widely acknowledged that Th1 cells and their corresponding factors, notably TNF-α and IFN-γ, play a crucial role in combating viral infections, while Th2 cells and their factors, including IL-4, IL-5, and IL-13, are primarily engaged in countering helminths and venoms and facilitating tissue repair.[23,24] These two cell types exhibit antagonistic interactions. We observed a Th2 immunity bias concerning cell frequency, response capacity, and serum levels of related cytokines in CAEBV: Firstly, the Th1/Th2 cell ratio was negatively correlated with the EBV-DNA copies in PBMCs in patients with CAEBV, suggesting the reduced Th1/Th2 ratio contribute to the EBV replication. Secondly, after PHA stimulation, the proportion of $IL\text{-}4^+$ cells in $CD4^+$ T cells of patients with CAEBV was significantly higher than that of healthy individuals, indicating an enhanced immune response capacity of Th2 cells in patients. Last, among the 21 CAEBV serum samples, a relatively lower proportion of patients presented abnormal elevations of Th1 cell-related cytokines, whereas a relatively higher proportion of patients exhibited abnormal elevations of Th2 cell-related cytokines. IL-18, an IFN-γ inducer secreted by M1 macrophages, is known to trigger Th1 immunity and is frequently employed as a marker to assess the activity and severity of HLH.[25] In this study, 18/21 (85.71%) patients with CAEBV exhibited abnormally elevated serum IL-18 levels, and those concurrent with HLH exhibited significantly higher serum levels of IL-18 compared to those without (5161±5378 vs. 1517±1520, $P < 0.05$). However, its downstream cytokine, IFN-γ, was abnormally elevated in only 3/21 (14.29%) patients. The proportion of abnormally elevated TNF-α is even lower (1/21, 4.76%). CXCL9, the recruitment factor for $CXCR3^+$ Th1 cells, was also observed to be abnormally elevated in only 2/21 (9.52%) patients. Combined with the increased PD-1 expression level of Th1 cells, these findings suggest a deficiency in Th1 immunity in patients with CAEBV.

In contrast, serum levels of Th2 cell-related factors were generally elevated. ST2, the specific receptor of IL-33, is selectively expressed on the surface of various cells including Th2 cells. ST2 can promote the secretion of IL-5 and IL-13 by Th2 cells, as well as IL-13-induced M2 macrophages polarization upon binding to IL-33.[26-30] Also, the IL-33/ST2 axis could be activated by HBV X protein (HBx) to inhibit the secretion of TNF-α and IFN-γ from human Th1 cells in vitro.[31] 66.67% (14/21) of patients showed abnormally elevated serum levels of ST2, indicating the IL-33/ST2 axis may be activated and contribute to the Th2 immunity bias in CAEBV. CD163, a specific marker for M2 macrophages, was found abnormally elevated in 12/21 (57.14%) serum samples from patients with CAEBV. Meanwhile, CD163 serum levels were negatively correlated with Th1 frequency and positively correlated with Th2 cell frequency, serum ST2 levels, and EBV-DNA copies in PBMCs, more directly indicating that the Th2/M2 immune bias favoring EBV amplification in patients with CAEBV. IL-1RA, secreted by M2 macrophages, is also an important anti-inflammatory factor.[32] IL-1RA was found abnormally elevated in 11/21 (52.38%) patients and was also positively correlated with serum levels of ST2. Previous studies showed that blocking IL-1 enhanced the Th2 immunity in mice, indicating a potential promotion of IL-1RA to the Th2 immunity,[33,34] however, its effect on Th2 immunity in humans requires further investigation. Therefore, although the mechanism of Th2 immunity predominance in CAEBV remains unclear, targeted suppression of Th2 immunity could potentially serve as a novel treatment approach for CAEBV.

There was no significant difference in the frequency, response capacity, and PD-1/PD-L1 expression levels of peripheral Th17 cells between patients and healthy individuals, and serum IL-17A levels of patients were not elevated abnormally. Consequently, the contribution of Th17 cells in the pathogenesis of CAEBV might be minimal. Tregs possess immunosuppressive properties. In the current study, we observed a significant increase in expression of PD-1 and PD-L1 in Treg cells from patients with CAEBV compared to healthy people, indicating that the impaired immunosuppressive function of Tregs might be contributing to the persistence of inflammation in CAEBV.

It was satisfying that the expression levels of PD-1 in peripheral T cell subsets of patients with CAEBV were significantly decreased, even obviously lower than those of healthy individuals after PD-1 blockade therapy, demonstrating that anti-PD-1 mAb can effectively bind PD-1 and block PD-1/P-L1 pathway, which is the

biological basis for the effectiveness of PD-1 blockade therapy. It is worth noting that the distribution of peripheral T cell subsets of patients with CAEBV returned to normal after PD-1 blockade treatment. The normalized frequencies of Tn and Tem cells in patients after PD-1 blockade may be mainly due to the weakened stimulation of EBV antigen in Tn differentiation caused by the elimination of the depletion state of lymphocytes and subsequent enhancement of anti-EBV ability. Besides, the potential role of the PD-1/PD-L1 pathway in the differentiation of Tm cells is unclear; whether blocking the PD-1/PD-L1 pathway affects the frequency of Tm cells deserves further investigation. In addition, the Th1/Th2 cell ratio returned to normal in CAEBV patients after PD-1 blockade therapy, which may be related to the suppression of the EBV-induced Th2 immune predominance caused by the enhanced anti-EBV ability of patients after PD-1 blockade, which needs to be further verified. Further investigation to understand the mechanisms is important for improving the efficacy of PD-1 blockade therapy and the application of combination therapy.

Although the current study characterized the distribution and the PD-1/PD-L1 expression levels of peripheral T cell subsets in patients with CAEBV, there are still several subsets and ICs were not detected due to the limited amount of blood drawn from patients and the maximum 8-color limit of the flow cytometry. Furthermore, the precise mechanism by which EBV triggers PD-L1 expression in host T and NK cells after infection remains obscure, as does how EBV induces PD-1 expression in uninfected T cells. Additionally, due to the constraints of the existing platform, we were unable to assess the serum levels of other Th2 cell-related factors, including IL-4, IL-5, IL-13, and IL-33. Determining the effects of these cytokines on the Th1/Th2 balance holds great significance for further elucidating the pathogenesis of CAEBV.

Conclusions. This study revealed significantly elevated PD-1/PD-L1 expression in peripheral T cell subsets and NK cells, and Th2 immunity predominance jointly facilitates the EBV proliferation and the development of CAEBV. Most EBV-infected T cells are Tm cells, which may contribute to the long-term persistence of EBV. Anti-PD-1 mAb effectively blocked the PD-1/PD-L1 pathway in T cells and contributed to the normalization of T cell subset distribution in some CAEBV patients. These findings provide insights into a novel pathogenesis of CAEBV and pave the way for the development of innovative therapeutic approaches.

Author Contributions. Conceptualization, Zhao Wang, Kang Sun; methodology, Chaofan Wu, Kang Sun; software, Kang Sun; resources, Qi Kong, Junxia Hu, Lin Shi, Yubo Pi, Deli Song, Tingting Cui, Leilei Chen, Xiaodan He, Zhengyang Song, Lin Wu, Jingshi Wu; writing, Kang Sun; supervision, Jingshi Wang, Zhao Wang; funding acquisition, Zhao Wang. All authors have read and agreed to the published version of the manuscript.

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