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Original Article

# Fas/FasL and Complement Activation are Associated with Chronic Active Epstein-Barr Virus Hepatitis

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## Abstract

***Background and Aims:*** Chronic active Epstein-Barr virus hepatitis (CAEBVH) is a rare and highly lethal disease characterized by hepatitis and hepatomegaly. This study aimed to investigate the clinicopathological features and pathogenic mechanisms of CAEBVH. ***Methods:*** Ten patients with confirmed Epstein-Barr virus hepatitis infection were enrolled. The clinicopathological characteristics of these patients were summarized and analyzed. Flow cytometry was utilized to detect peripheral blood immune cell phenotypes and whole exome sequencing was used to explore pathogenic genetic mechanisms. Lastly, immunohistochemical staining was employed to verify pathogenic mechanisms. ***Results:*** Clinical features observed in all Epstein-Barr virus hepatitis patients included fever (7/10), splenomegaly (10/10), hepatomegaly (9/10), abnormal liver function (8/10), and $CD8^+$ T cell lymphopenia (6/7). Hematoxylin and eosin staining revealed lymphocytic infiltration in the liver. Positive Epstein-Barr virus-encoded small RNA *in-situ* hybridization (EBER-ISH) of lymphocytes of liver tissues was noted. Whole exome sequencing indicated that cytotoxic T lymphocytes and the complement system were involved. The expression of CD8, Fas, FasL, and Caspase-8 expression as well as apoptotic markers was enhanced in the Epstein-Barr virus hepatitis group relative to the controls ($p<0.05$). Lastly, Complement 1q and complement 3d expression, were higher in CAEBVH patients relative to controls ($p<0.05$). ***Conclusions:*** CAEBVH patients developed fever, hepatosplenomegaly, and lymphadenopathy. Histopathological changes were a diffuse lymphocytic sinusoidal infiltrate with EBER-ISH positivity. Fas/FasL and complement activation were involved in CAEBVH patients.

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**Keywords:** Chronic active EB virus hepatitis; Fas/FasL; Complement.

**Abbreviations:** CAEBV, chronic active Epstein-Barr virus; CAEBVH, chronic active Epstein-Barr virus hepatitis; WES, whole exome sequencing; EBER-ISH, Epstein-Barr virus-encoded small RNA in-situ hybridization; C1q, complement 1q; C3d, complement 3d; C4d, complement 4d; HLH, hemophagocytic lymphohistiocytosis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function; CC, cellular component; BP, biological process; IHC, immunohistochemistry; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; AI, apoptotis index; LSECs, liver sinusoidal endothelial cells; GCs, Glisson's capsules.

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## Introduction

In 1964, Epstein and Barr discovered a virus in the lymphocytes of children with Burkitt's lymphoma in Africa, which later became known as the Epstein-Barr virus (EBV). EBV is a double-stranded DNA, type 4 herpes virus that is characterized by viral particles consisting of a nucleus, a membrane shell, shell particles, and an envelope. EBV is a common virus in humans, affecting more than 90% of people.[1] Most infected individuals are asymptomatic, but adolescents often present with infectious mononucleosis, which is a self-limiting disease that presents with symptoms such as fever, pharyngitis, lymphadenopathy, hepatomegaly, and splenomegaly. In rare cases of EBV infection, symptoms may persist and recur. Such cases are referred to as chronic active EBV (CAEBV) infection.

According to the annual report of the research group Measures against Intractable Diseases of the Ministry of Health, Labor and Welfare of Japan, the rate of onset of CAEBV in Japan was 23.8 cases per year.[2] CAEBV infection is a rare and highly lethal disease that is characterized by multisystem inflammation, abnormally elevated EBV-associated antibodies in the blood, and Epstein-Barr virus-encoded small RNA (EBER) in tissues.[3] CAEBV infection has attracted the attention of scientists worldwide and as such, related research reports have been increasing annually. Most studies primarily investigate CAEBV infection from the aspect of systemic multisystem damage.[4] In contrast, few studies have reported the mechanisms of injury associated with chronic active EBV hepatitis (CAEBVH). CAEBVH has been primarily reported in East Asia and Latin America, suggesting a genetic predisposition in its pathogenesis.[5] Previous studies have correlated whole exome sequencing (WES)-mapped susceptibility phenotypes with CAEBVH.[6] We thus aimed to determine the susceptibility to infection using WES. Secondly, we aimed to explore the pathogenic mechanisms of CAEBVH.

## Methods

### *Ethics*

Informed consent was obtained from each patient included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (sixth revision, 2008) as reflected in *a priori* approval by the institution's human research committee. The institutional review board approved this study of 900TH Hospital of the Joint Logistic Support Force (No. 2020023).

### *Participant selection and data collection*

Patients with CAEBV infection were defined as those patients that had an estimated age at disease who met the criteria for the diagnosis of systemic EBV-T-LPD according to the 2016 World Health Organization (WHO) classifications of lymphoid neoplasms.[7] All patients with CAEBV satisfied the following diagnostic criteria: (1) sustained or recurrent infectious mononucleosis-like symptoms lasting more than 3 months, including fever (≥38.3°C or ≥101°F), liver dysfunction (elevated liver enzymes), lymphadenopathy, hepatosplenomegaly, cytopenia, interstitial pneumonia, hydroa vacciniforme, and hypersensitivity to mosquito bites; (2) increased quantities of EBV in affected tissues by detection of EBV DNA in tissues or peripheral blood by Southern blot hybridization or EBER-positive cells detected in affected tissues by microscopy (≥10 cells/high-power field)] or in peripheral blood, EBV DNA detected in plasma ($\geq 2\times10^2$ copies/mL in plasma); and (3) no evidence of any previous immunological abnormalities or any other infections that could otherwise explain the condition. Patients with CAEBVH were defined as those who met the CAEBV diagnostic criteria and following criteria: (1) elevated liver enzymes; (2) characteristic histopathological changes; (3) positive EBV–PCR or EBER–ISH in the liver tissue.[8] Hemophagocytic lymphohistiocytosis (HLH) was diagnosed by the HLH 2004 guidelines.[9] A diagnosis of HLH was established if criterion 1 or 2 was fulfilled. That is, a molecular diagnosis consistent with HLH or the presence of five of the eight following conditions: Fever; Splenomegaly; Cytopenia affecting two of the following three lineages in peripheral blood, hemoglobin <90 g/L in adults or <100 g/L in infants <4 weeks of age, and/or platelets $<100\times10^9$/L and/or neutrophils $<1.0\times10^9$/L; Hypertriglyceridemia and/or hypofibrinogenemia, or fasting triglycerides ≥3.0 mmol/L (≥265 mg/dL) and/or fibrinogen ≤ 1.5 g/L; Hemophagocytosis in bone marrow or spleen or lymph nodes with no evidence of malignancy; Low or no natural killer (NK) cell activity following the local laboratory reference; Ferritin ≥500 μg/L; and sCD25 soluble interleukin (IL)-2 receptor ≥2,400 U/mL. Ten patients who meet the inclusion criteria were diagnosed with CAEBVH and were selected for subsequent analysis; 2 mL of whole blood was collected intravenously from each patient and stored at −80°C. Additionally, four healthy patients with partial hepatectomy for liver trauma were included as the control group. Patients in the control group who presented with liver damage caused by viruses, drugs, autoimmune diseases, etc., were excluded. Clinical and pathologic data such as age, sex, laboratory examination, radiologic findings, therapeutic regimens, follow-up data, and liver samples were collected between January 1, 2011 and December 30, 2019. Follow-up started on the day of diagnosis of CAEBVH, and the end of follow-up was December 30, 2019, the date of death, or the date of loss to follow-up.

### *Flow cytometry*

Peripheral blood mononuclear cells were stained with fluorochrome-conjugated mAbs for flow cytometric analysis. CD3/CD8/CD45/CD4 (Cat# 340499, RRID: AB_400472; BD Biosciences, San Jose, CA, USA) and anti-CD3/CD16$^+$CD56/CD45/CD19 (BD Biosciences Cat# 340500, RRID:AB_400473) were used. Flow cytometry was performed using a FACSAria II (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). CD4$^+$ T lymphocytes were defined as CD45$^+$CD3$^+$CD4$^+$CD8$^-$ cells. CD8$^+$ T lymphocytes were defined as CD45$^+$CD3$^+$CD4$^-$CD8$^+$ cells. NK cells and B lymphocytes were defined as CD45$^+$CD3$^-$CD16$^+$56$^+$CD19$^-$ cells and CD45$^+$CD3$^-$CD16$^-$56$^-$CD19$^+$ cells.

### *WES*

The genomes of the 10 CAEBVH cases, with eligible formalin-fixed live biopsy and peripheral blood samples, were sequenced using the Illumina HiSeq 2500 instrument. Raw data were aligned and analyzed for the detection of insertions/deletions and single-nucleotide variants. Gene Ontology (GO (http://wiki.geneontology.org/index.php/Immunologically_Important_Genes) and used to select immune-associated gene mutations. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment were used to analyze the screened genes. DAVID (https://david.ncifcrf.gov/) was used to conduct GO functional analysis and KEGG pathway enrichment analysis and $p<0.05$ was the cutoff criterion. R (version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria), and Python (version 3.10; Python Software Foundation, Wilmington, DE, USA), were used for running various tools or for local parallelization.

### *Histology*

Biopsy specimens were fixed in 10% formalin and embedded in paraffin. Serial 3–4 μm sections were stained with hematoxylin and eosin for microscopic examination.

### *Epstein-barr virus-encoded small RNA in-situ hybridization*

*In-situ* hybridization (ISH) was conducted on formalin-fixed paraffin sections with a digoxin-labeled oligonucleotide probe complementary to 2 EBER, EBER-1, and EBER-2 (Lot # 20170020; TIB, Xiamen, China). A sense probe (negative control) was then labeled with digoxigenin (DIG) using previously described methods.[10]

### *Immunohistochemistry (IHC) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)*

Immunohistochemical staining of paraffin sections included CD3 (Cat# ZM-0417, RRID:AB_2890105; ZSGB-Bio, Beijing, China), CD4 (ZSGB-Bio Cat# ZM0418,RRID:AB_2890106), CD8 (ZSGB-Bio Cat# ZA-0508, RRID:AB_2890107), CD20 (Cat# MAB-0669, RRID:AB_2890108; Maixin, Fuzhou, China), CD56 (ZSGB-Bio Cat# ZM-0057, RRID:AB_2890109), perforin (Maixin Cat# MAB-0315, RRID:AB_2890110), GrB (Maixin Cat# MAB-0352, RRID:AB_2890111), Fas (Cat# ab82419, RRID:AB_1658628; Abcam, Cambridge, UK), FasL (Abcam Cat# ab10041, RRID:AB_296781), caspase-8 (Abcam Cat#ab25901,RRID:AB_448890), Complement 1q (C1q) (Abcam Cat# ab11861, RRID:AB_298643), Complement 3d (C3d) (Abcam Cat# ab17453, RRID:AB_443879) and Complement 4d (C4d) (Abcam Cat# ab36075, RRID:AB_726920). IHC staining was performed as previously described.[11] ImageJ software was used to scoring the IHC images as follows:

$$H_{score} = \Sigma(i \times P_i)$$

where, i is the staining intensity graded as 0, 1, 2, or 3, and

$P_i$ is the percentage of stained cells.

To assay liver tissue apoptosis, TUNEL staining was performed with a One-Step assay kit (Lot # MA0223; MeilunBio, Dalian, China) performed as previously described.[12] Apoptotic cells were detected by fluorescence, 1,000 were counted, and the apoptotis index (AI) was reported as the percentage of positive cells (Model 80i; Nikon, Tokyo, Japan).

### *Statistical analysis*

Data analysis was performed with GraphPad Prism (version 8.4.3; GraphPad Software, San Diego, CA, USA). Measurement data with a normal distribution are expressed as means±SDs. Measurement data with a non-normal distribution were reported as medians and interquartile range (IQR). Statistical significance was determined with the Student's *t*-tests, and *p*-values <0.05 was considered statistically significant.

## Results

### *Clinical features*

Of the 10 CAEBVH patients enrolled, 7/10 were men and 3/10 were women. The median age was 32 (range: 14–62) years. The most prominent symptom observed among subjects was fever (7/10). Radiologic findings showed splenomegaly (10/10) and hepatomegaly (9/10; Fig. 1A). Laboratory examination showed that most patients had elevated liver function indexes at admission to hospital, indicated by mild to moderate abnormal alanine aminotransferase (ALT) or Aspartate transaminase (AST) levels (8/10). The EBV load was elevated in the blood of all patients and varied from $1.75\times10^4$ to $1.74\times10^7$ IU/mL. An immune cell phenotype was detected in seven patients. The percentage of $CD3^+$ T lymphocytes decreased in four of seven cases and the percentage of $CD4^+$ T lymphocytes decreased in six of seven cases. Six of seven cases had reduced $CD8^+$ T lymphocyte counts. The $CD4^+/CD8^+$ T lymphocyte ratio increased in three of seven cases. The percentage of NK cells increased in three of five cases, and in two of three cases with a abnormal B lymphocyte ratio (Fig. 2B). Inflammatory indicators such as blood ferritin were elevated in five of seven patients and four of seven patients progressed to HLH. Despite these results, just above half of the participants (5/9) had normal C-reactive protein (CRP). Most patients (9/10) received antiviral (ganciclovir) and immunosuppressive therapy. Immunosuppressive regimens included glucocorticoids, immunoglobulin combinations, chemotherapy drugs (cyclophosphamide, etoposide, or vincristine), and calcineurin inhibitor immunosuppressants (cyclosporine A or tacrolimus). Half of the CAEBVH patients (5/10) died, with a median survival of 3 (1.5, 4.0) months. The main causes of death were the onset of secondary severe infections, HLH, disseminated intravascular coagulation, and other complications (Tables 1 and 2).

### *EBER-ISH and liver tissue histology*

The liver is one of the most common target organs of CAEBV infection. Hematoxyin and eosin staining showed varying degrees of steatosis and edema (+ to +++) characterized by mixed macro-vesicular/vesicular steatosis in four of ten cases. and the four cases had different degrees of inflammatory cell infiltration in the portal area and hepatic lobules (+ to +++) accompanied by a small amount of hepatocyte necrosis in the liver tissues in those with CAEBVH. EBER-ISH of liver tissue included positive lymphocytes in patients with CAEBVH, which clarified that lymphocytes were the target cells of EBV infection (Fig. 1C).

### *WES analysis*

GO describes genes from three aspects, molecular function (MF), cellular component (CC), and biological process (BP). In the BP group, genes were primarily enriched in T cell activation, regulation of immune effector processes, and leukocyte proliferation functional groups. In the CC group, genes were primarily enriched in the external side of the plasma membrane, secretory granule membrane, and endocytic vesicles. In the MF group, genes were primarily enriched in immune receptor activity, cytokine receptor binding, and amide binding functional groups (Fig. 2A). KEGG pathway enrichment analysis demonstrated that the genes were primarily associated with human papillomavirus infection, complement and coagulation cascades, and cytokine-cytokine receptor interaction (Fig. 2B).

We additionally detected recurrent mutations activating the complement pathway. Most frequently, we found complement activation gene mutation involved in all cases (10/10; Fig. 2C). Virtually all *C3* mutations clustered in the transcript domain that is essential for dimerization and activation of C3 (Fig. 2D). In most samples, mutations affecting genes encoding for components of the cytotoxic T lymphocyte signaling pathway were detected, including mutations in *CASP8* (9/10), and *GZMB* (10/10), indicating a role of cytotoxic T lymphocyte signaling in the molecular pathogenesis of CAEBVH (Fig. 2C, D).

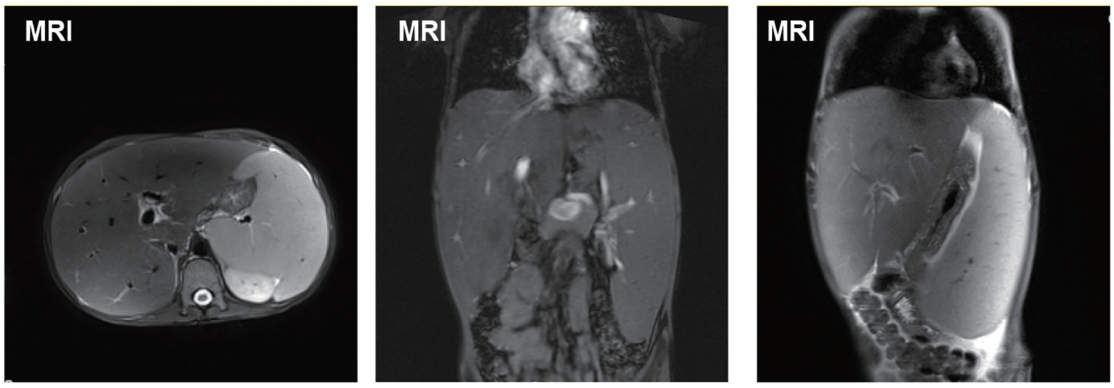
### *Mechanisms of CAEBVH*

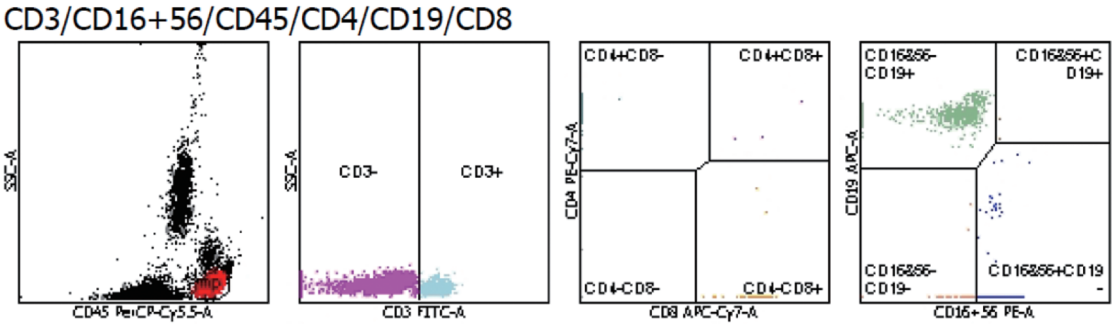
Based on the GO enrichment results, IHC was carried out to determine whether cytotoxic T lymphocyte activation was involved in CAEBVH. CD8 expression in the CAEBVH group was increased relative to that of the control group ($p<0.05$) but there were no significant differences in CD3, CD4, CD56, and CD20 in the two groups ($p>0.05$; Fig. 3). To further investigate the mechanism of immune damage by $CD8^+$ T lymphocytes, we examined the expression of perforin, granzyme B, Fas, and caspase-8 using IHC. The expression of perforin and granzyme B in the two groups did not differ significantly ($p>0.05$), but the expression of Fas, FasL, and caspase-8 was higher in the CAEBVH group relative to the control group ($p<0.05$; Fig. 4A). Additionally, there was a higher proportion of apoptotic cells in the CAEBVH group relative to the control groups as measured by the TUNEL apoptosis index (AI, $p<0.05$; Fig. 4B).

Based on the sequence data, we investigated whether the complement pathway was involved in the pathogenesis of CAEBVH. Expression of C1q, C3d, and C4d, essential component of the complement pathway, was detected in liver sinusoidal endothelial cells (LSECs) and Glisson's capsules (GCs) in liver tissue. The expression of C1q in LSEC and GC and C3d in LSECs was higher in CAEBVH patients relative to the healthy controls ($p<0.05$). The expression of C4d in LSECs and GC and C3d in GC was similar between the two groups ($p>0.05$; Fig. 5).

## Discussion

Our study summarized the clinicopathologic features and pathogenic mechanisms of CAEBVH. In addition, we represented a comprehensive genetic analysis of CAEBVH patients. Several identified genetic alterations may be useful to guide novel therapeutic strategies for CAEBVH patients. While direct targeting of Complement and Fas/FasL pathway proteins remains challenging, functional studies should address the potential therapeutic inhibition in the feature. EBV is predominantly present in B, T cells, and NK cells, and has




| Parameter | Percent | Value/AbsCnt |
|---|---|---|
| Lymph Events | | 3679 |
| CD3+ | 25.17 | 0 |
| CD3+CD8+ | 7.04 | 0 |
| CD3+CD4+ | 16.93 | 0 |
| CD3+CD4+CD8+ | 0.08 | 0 |
| CD16+CD56+ | 53.44 | 0 |
| CD19+ | 19.92 | 0 |
| CD45+ | | 0 |
| 4/8 Ratio | | 2.41 |

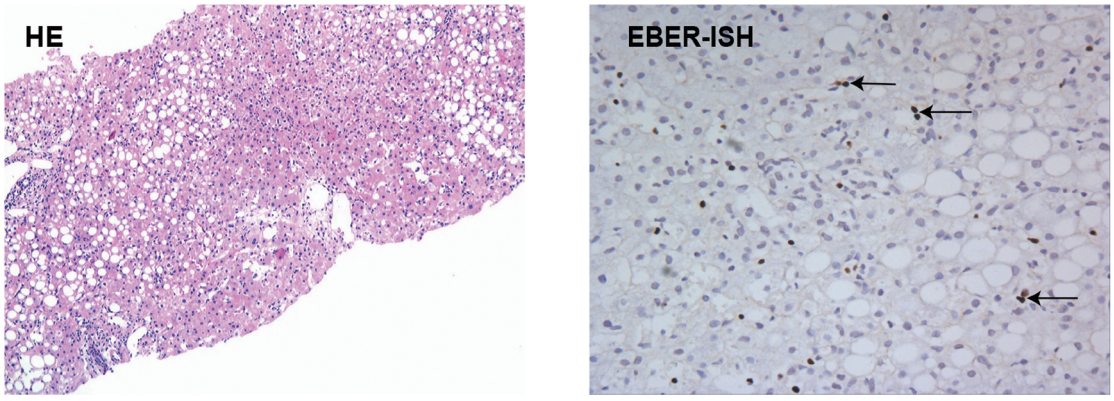



**Fig. 1. Clinical and histologic features.** (A) Magnetic resonance imaging of the upper abdomen in cross-sectional, coronal, and sagittal views. It showed splenomegaly and hepatomegaly in a representative example of chronic active Epstein-Barr virus hepatitis (CAEBVH) patients. (B) Flow cytometric assay of immunocytes in peripheral blood in a representative example of CAEBVH patients. The percentage of $CD4^+$ T lymphocytes was 16.9% ($CD45^+CD3^+CD4^+CD8^-$ cells), The percentage of $CD8^+$ T lymphocytes was 7.0% ($CD45^+CD3^+CD4^-CD8^+$ cells). The percentage of natural killer cells ($CD45^+CD3^-CD16^+56^+CD19^-$ cells) and B lymphocytes ($CD45^+CD3^-CD16^-56^-CD19^+$ cells) were 53.4% and 19.9%. (C) Hematoxylin and eosin staining and Epstein-Barr virus-encoded small RNA in-situ hybridization (EBER-ISH) in a representative example of CAEBVH patients (×200). Hematoxylin and eosin staining revealed lymphocytic infiltration in the liver. Positive EBER-ISH of lymphocytes of liver tissues was noted (black arrows).

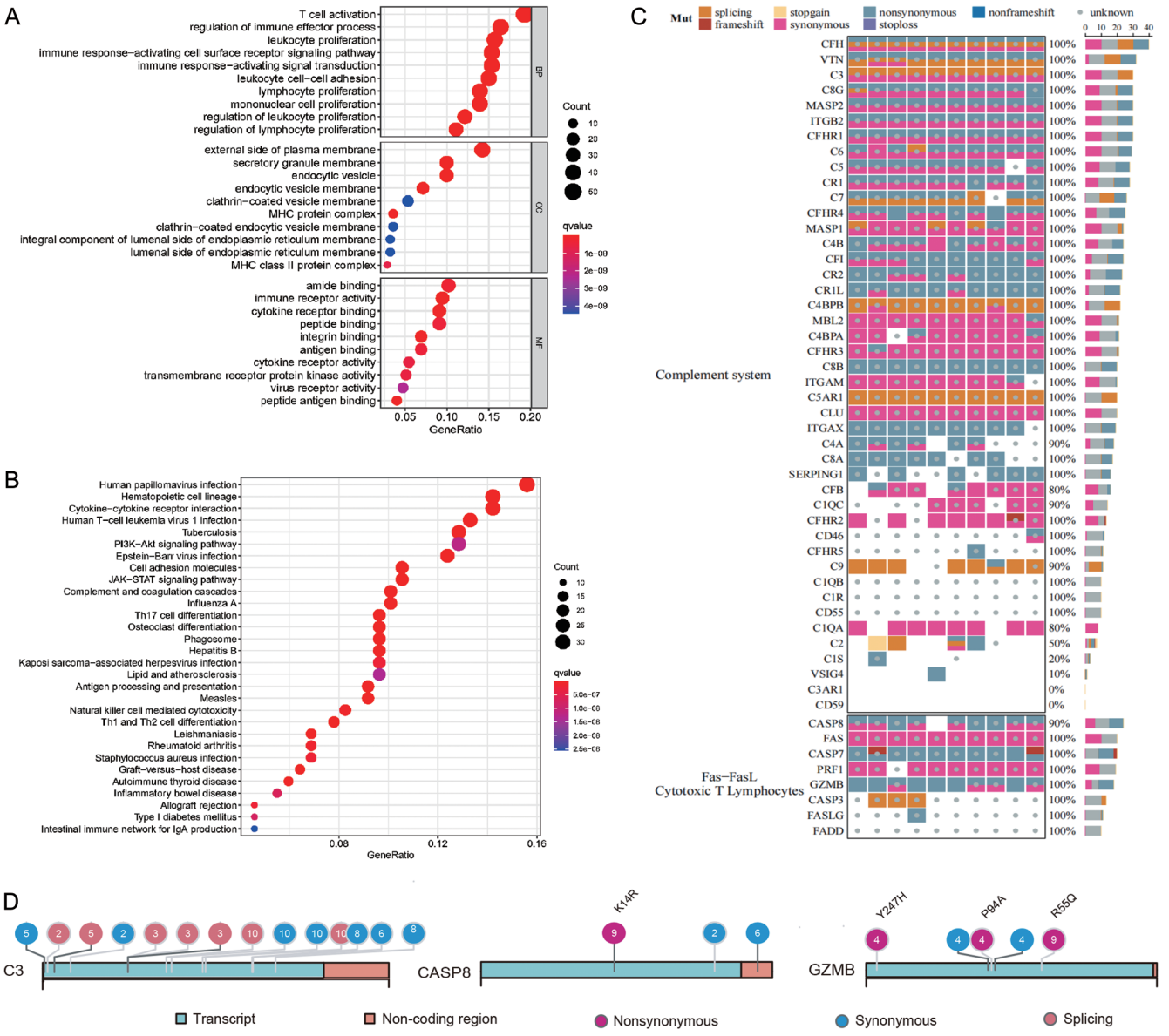



**Fig. 2. Analysis of whole exome sequencing.** (A) Gene Ontology (GO) enrichment analysis shown in a bubble chart. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis in the bubble chart q-value, enrichment significance. Whole exome sequencing indicated an enrichment of genes corresponding to T cell activation and complement and coagulation cascades. Number of enriched genes, gene ratio, ratio of the number of genes enriched to the target pathway to the total number of genes. (C) Co-occurrence of mutations of complement and cytotoxic T lymphocyte pathways. For each pathway, samples are presented in their corresponding waterfall sort order by binary gene mutation status. The bar graph on the left shows the ratio of splicing (brown), stop gain (yellow), non-synonymous (gray), non-frameshift (blue), frameshift (red), synonymous (pink), and stop loss (purple) mutations per gene. (D) Mutation location distribution map. The distribution of detected mutations on protein level for the selected *C3* (NM_000064), *CASP8* (NM_001080125) and *GZMB* (NM_004131). Numbers in circles indicate the number of samples with mutations.

a geographical distribution that favors certain cell types over another. In Asian countries, nearly 60% of cases of CAEBV are of the T cell type.[13] This study followed the T cell types of CAEBV infection, including fever, hepatomegaly, splenomegaly, and elevated EBV DNA viral load. In our study, several patients had progressed to HLH and half the CAEBVH patients died. However, because of the small number of cases, a correlation between the outcome of HLH and/or death and flow cytometry, liver histology/IHC, TUNEL, or gene analysis data could not be identified.

Clinically, CAEBVH patients present with more apparent symptoms, faster progression, and worse prognosis than patients without liver damage.[14] Therefore, this study aimed to investigate the mechanism of CAEBVH. Three main features were noted from the histopathology of patient liver samples. Firstly, diffuse hepatocyte damage. Secondly, the inflammatory cells, mainly lymphocytes, were observed to have infiltrated the confluent area and hepatic sinusoids. Thirdly, the same lymphocytes were EBER-ISH positive. The histopathological changes are consistent with the characteristics reported in previous studies, diffuse lymphocytic sinusoidal infiltrate; hepatocytes with mild swelling, vacuolation, and

**Table 1. Clinical characteristics of patients with CAEBVH**

| Case | Sex/age, years | | Imaging findings | Treatment | Pro-gress | Follow-up in months |
|---|---|---|---|---|---|---|
| 1 | M/25 | Fever | Hepatomegaly and splenomegaly | Ganciclovir, GC, Ig | – | D (4.0) |
| 2 | M/14 | – | Hepatomegaly and splenomegaly | Ganciclovir, Ig | – | A (58.0) |
| 3 | M/16 | Fever | Hepatomegaly and splenomegaly | Ganciclovir, GC | – | D (0.5) |
| 4 | M/33 | Fever | Hepatomegaly and splenomegaly | Ganciclovir | – | A (34.0) |
| 5 | M/31 | Fever | Hepatomegaly and splenomegaly | Ganciclovir, chemotherapy, GC, Ig | HLH | D (1.5) |
| 6 | F/52 | Fever | Hepatomegaly and splenomegaly | Ganciclovir, chemotherapy, GC | HLH | D (3.0) |
| 7 | F/27 | Fever | Hepatomegaly and splenomegaly | – | – | A (14.0) |
| 8 | M/38 | Fever | Hepatomegaly and splenomegaly | Ganciclovir, GC, Ig | HLH | D (12.0) |
| 9 | M/35 | – | Hepatomegaly and splenomegaly | Ganciclovir, GC | HLH | A/R (15.0) |
| 10 | F/62 | – | Splenomegaly | Ganciclovir | – | A (6.5) |

M, male; F, female; GC, glucocorticoid; Ig, immunoglobulin; INF, interferon A; A, alive; D, dead; R, recurrence; HLH, hemophagocytic lymphohistiocytosis; –, None.

steatosis with focal apoptosis, and EBER positivity in lymphocytes.[14]

Immune cell phenotyping by flow cytometry revealed decreased $CD8^+$ T lymphocytes in CAEBVH patients, and WES found that cytotoxic T lymphocyte genes were enriched in CAEBVH patients relative to healthy controls. Previous studies have reported a reduced number and impaired functionality of EBV-specific $CD8^+$ T lymphocytes in CAEBVH patients.[15,16] In contrast to these data, IHC found an increased frequency of $CD8^+$ T lymphocytes was observed in our CAEBVH patients relative to the control group. However, no differences in the frequency of $CD3^+$ T, $CD4^+$ T, B lymphocytes, and NK cells in CAEBVH patients relative to healthy controls was observed.

$CD8^+$ T lymphocytes are essential immune cells that have a critical role in fighting viral infections.[17] Previous studies have shown that perforin, granzyme B, and the Fas/FasL apoptosis pathways have a key role in $CD8^+$ T lymphocyte-mediate host protection.[18–21] Furthermore, Katano *et al*.[22] reported, using WES, the presence of multiple mutations in the perforin gene of CAEBVH patients absent in healthy controls, indicating a protective role of perforin within this model. In this study, multiple mutations in the granzyme B genes were present. In contrast to these studies, no differences in granzyme B and perforin expression between CAEBVH patients and healthy controls was observed. However, the observation may result from the low number of subjects assessed in the current study. Future studies with larger sample sizes may be sufficiently powered to robustly discriminate perforin and granzyme mutations in diseased individuals and correlate these with clinical outcomes.

We found mutations in *CASP*, *CASP7*, and *Fas* genes. Nomura *et al*.[23] identified three Japanese CAEBVH patients who presented with Fas pathway-associated mutations that were lacking in healthy controls. Thus, the Fas/FasL pathway may lead to CAEBVH. Next, we investigated the role of the caspase-dependent apoptotic pathway in CAEBVH. Fas, FasL, and caspase-8 were highly expressed in the CAEBVH group relative to the healthy controls (Fig. 5A). Nomura *et al*.[24] reported that caspase-3, a critical apoptosis pathway protein, was highly expressed in CAEBVH patients. Additionally,

**Table 2. Laboratory examinations in the blood of 10 CAEBVH patients**

| Case | Blood lymphocyte analysis | | | | | | Liver function | | | EBV | Inflammation | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD3 (%) | CD4 (%) | CD8 (%) | CD4/ CD8 | B (%) | NK (%) | ALT (U/L) | AST (U/L) | TBIL (μmol/L) | DNA (IU/mL) | Ferritin (ng/mL) | CRP (ug/mL) |
| 1 | 95 | 28.7 | 16.9 | 1.7 | – | – | 292 | 313 | 98.6 | $1.23\times10^6$ | 118 | <3.3 |
| 2 | 25.1 | 16.9 | 7.0 | 2.4 | 19.9 | 53.4 | 74 | 238 | 83.8 | $5.28\times10^4$ | 52.5 | <3.3 |
| 3 | 26.9 | 20.3 | 5.8 | 3.5 | – | – | 80.8 | 125.2 | 151.2 | $1.74\times10^7$ | – | 2.08 |
| 4 | 66.6 | 38.7 | 15.3 | 2.5 | 11.5 | 18.9 | 83.1 | 57 | 20.6 | $6.83\times10^6$ | – | – |
| 5 | – | – | – | – | – | – | 136.1 | 234.3 | 50.2 | $2.43\times10^5$ | >1,888 | 100 |
| 6 | – | – | – | – | – | 6.6 | 25.4 | 42.6 | 5.3 | $1.15\times10^5$ | 1,102 | 17.88 |
| 7 | 47.4 | 29.3 | 16.9 | 1.7 | – | 49 | 5.8 | 26.5 | 8.5 | $1.99\times10^6$ | – | <7.2 |
| 8 | 16.9 | 7.5 | 8.6 | 0.9 | 2.1 | 80.5 | 73.9 | 43 | 9.7 | $1.02\times10^7$ | 1,129 | 15.6 |
| 9 | 95 | 30.7 | 62.5 | 0.5 | – | – | 16.1 | 21.6 | 14.2 | $1.75\times10^4$ | 1,502 | <7.2 |
| 10 | – | – | – | – | – | – | 93.3 | 151.3 | 57.5 | $2.30\times10^6$ | 322.5 | 11.8 |

Liver function index was determined at admission to hospital. $CD3^+$ T cells, reference range (59–80.4%); $CD4^+$ T cells, reference range (35.5–52.9%); $CD8^+$ T cells, reference range (20.2–36.4%); CD4/CD8, reference range (1.2–1.8%); NK cells, reference range (8.1–25, 6%); B cells, reference range (7.5–18.2%); ALT, reference range (7–50 U/L); AST, reference range (13–40 U/L); TBIL, reference range (0–21 μmol/L); EBV DNA, reference range (<500 IU/mL); Ferritin, reference range (men, 20–400 U/L, women, 13–150 U/L); CRP, reference range (0–8 mg/L). NK, natural killer; ALT, alanine aminotransferase; AST, aspartate transaminase; TBIL, total bilirubin; CRP, C-reactive protein.

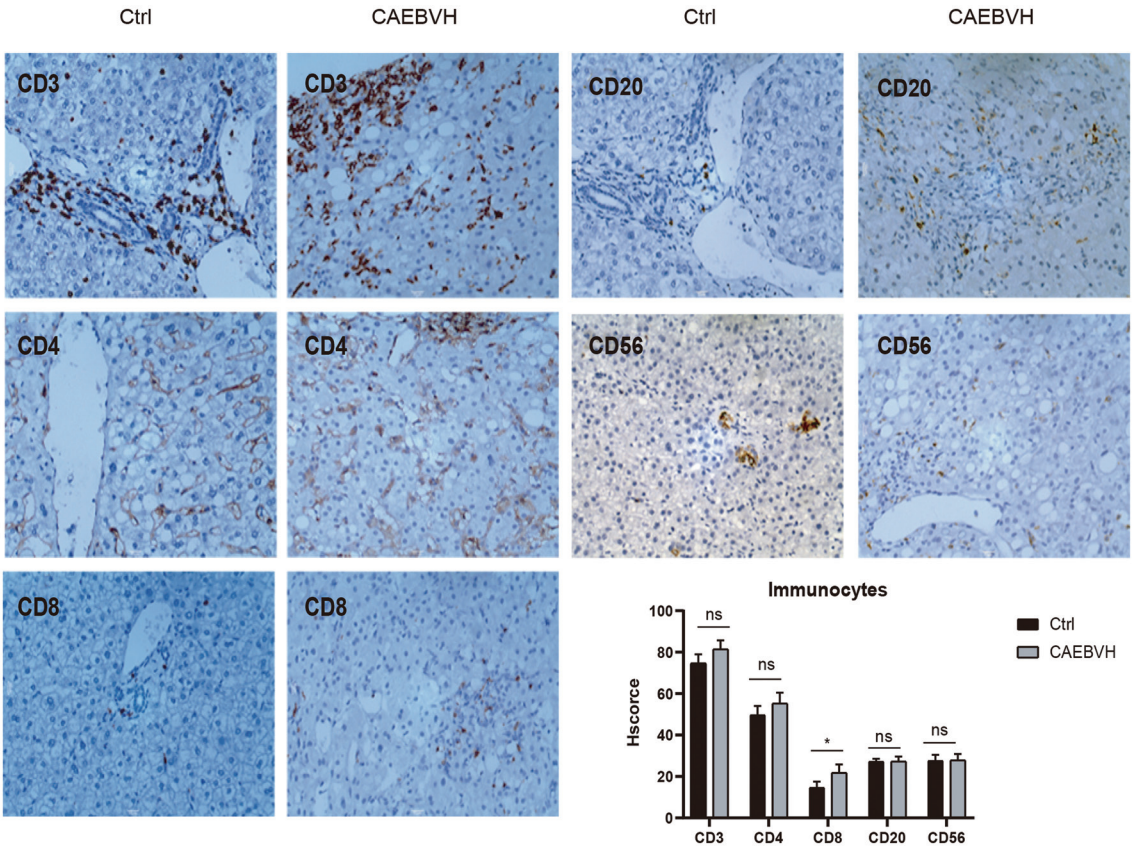



**Fig. 3. CAEBVH immunophenotype.** Immunohistochemistry (IHC) staining of CD3, CD4, CD8, CD20, and CD56 in CAEBVH and control groups (×200). The expression of CD8 in the liver tissue of the CAEBVH group was higher relative to the controls. $n$=4, *$p$<0.05 **$p$<0.01.

previous studies showed that when Fas in lymphocytes was combined with FasL in tissue cells, caspase-8 was activated in tissue cells becomes and initiated caspase-dependent apoptosis.[25,26] In our study, the high expression of caspase-8 in lymphocytes suggests that lymphocytes were undergoing excessive apoptosis due to abnormal proliferation.[27] It is also understood that activated caspase-8 promotes the secretion of inflammatory cytokines that can exacerbate tissue damage.[28]

WES analysis suggested that complement activation was involved in CAEBVH pathogenesis. Here, we reported a higher expression of C1q in LSECs and that GC was in the CAEBVH group relative to the controls. It is understood that C1q is activated when bound to the immune complex and subsequently initiates the classical complement pathway.[29] Both the classical pathway and the lectin pathway, activate C4d. Additionally, C3d participates in the classical, lectin, and alternative pathways.[30] Further, C3d expression was higher in the LSECs of the CAEBVH group relative to the healthy controls. C4d expression was not observed in either group. Together, the data indicate that the alternative pathway, and neither the lectin nor the classical pathway, were essential to establish CAEBVH. The imbalance of C3-derived fragments is key to autoimmune and neoplastic diseases.[31,32] Therefore, we hypothesize that the complement pathway could be involved in CAEBVH and highlight this pathway as a novel target for CAEBVH drug development.

The study has some limitations. Firstly, liver puncture biopsy is an invasive test that resulted in a relatively small number of subjects for this study. Future studies should focus on increasing the sample size of active CAEBVH patients to better identify key differences that may explain CAEBVH pathogenesis.

We conclude that our CAEBVH patients were mainly shown to have T cell types consistent with developing fever, hepatosplenomegaly, and splenomegaly. Histopathological changes included diffuse lymphocytic sinusoidal infiltrates; mild swelling, vacuolation, and steatosis of hepatocytes; and EBER positivity in lymphocytes. Fas/FasL and complement activation were involved in CAEBVH patients, suggesting that function studies should address the potential therapeutic inhibition to relieve the progression of CAEBVH.

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## Conflict of interest

The authors have no conflict of interests related to this publication.

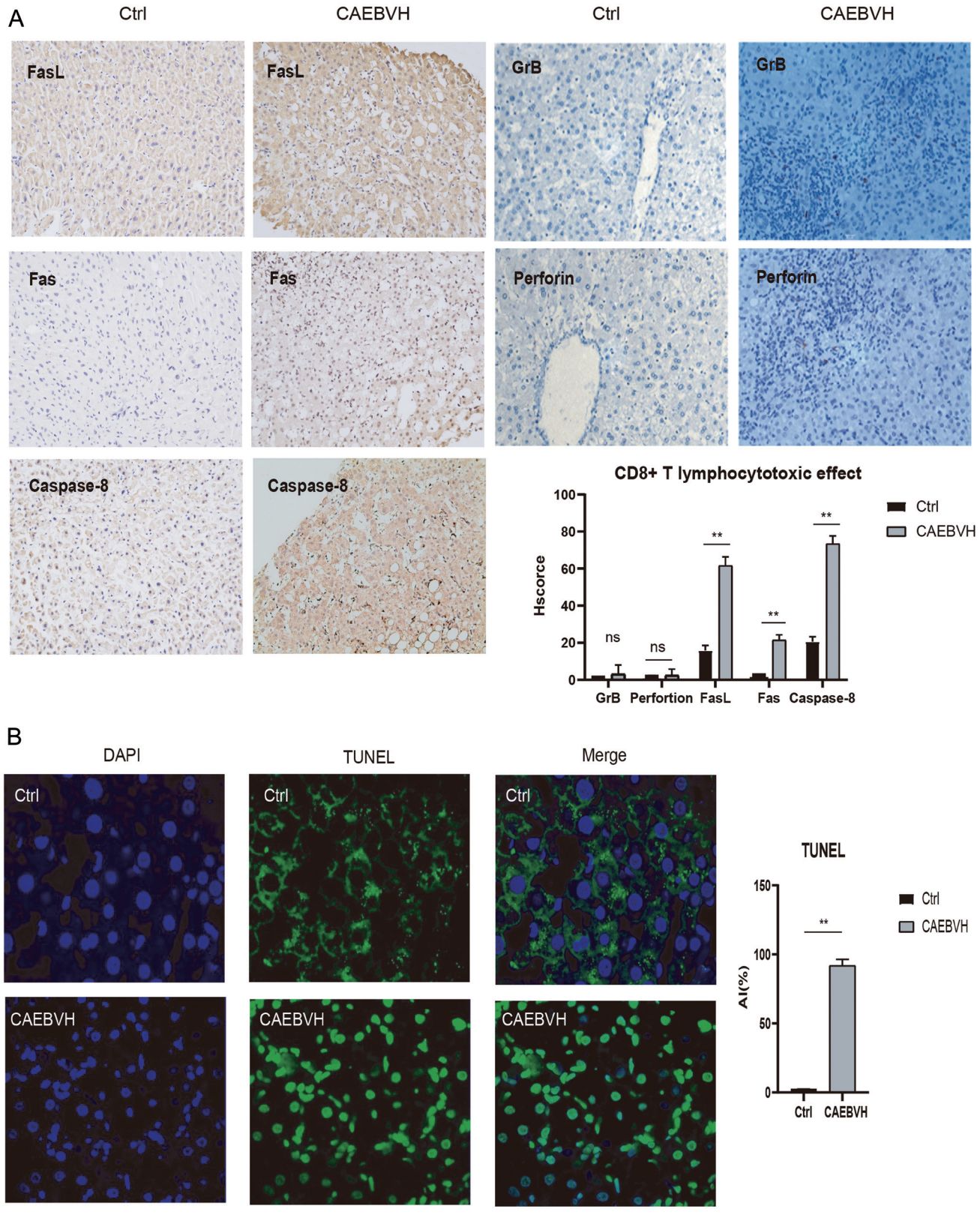


**Fig. 4. T cell immune mechanism of CAEBVH.** (A) IHC staining of GrB, perforin B, Fas, FasL, and caspase-8 (×200). Fas, FasL, and Caspase-8 expression were enhanced in the CAEBVH group relative to the controls. (B) Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay of apoptosis CAEBVH and the control group. The TUNEL index in the CAEBVH group was higher relative to the controls. $n$=4, *$p$<0.05, **$p$<0.01.

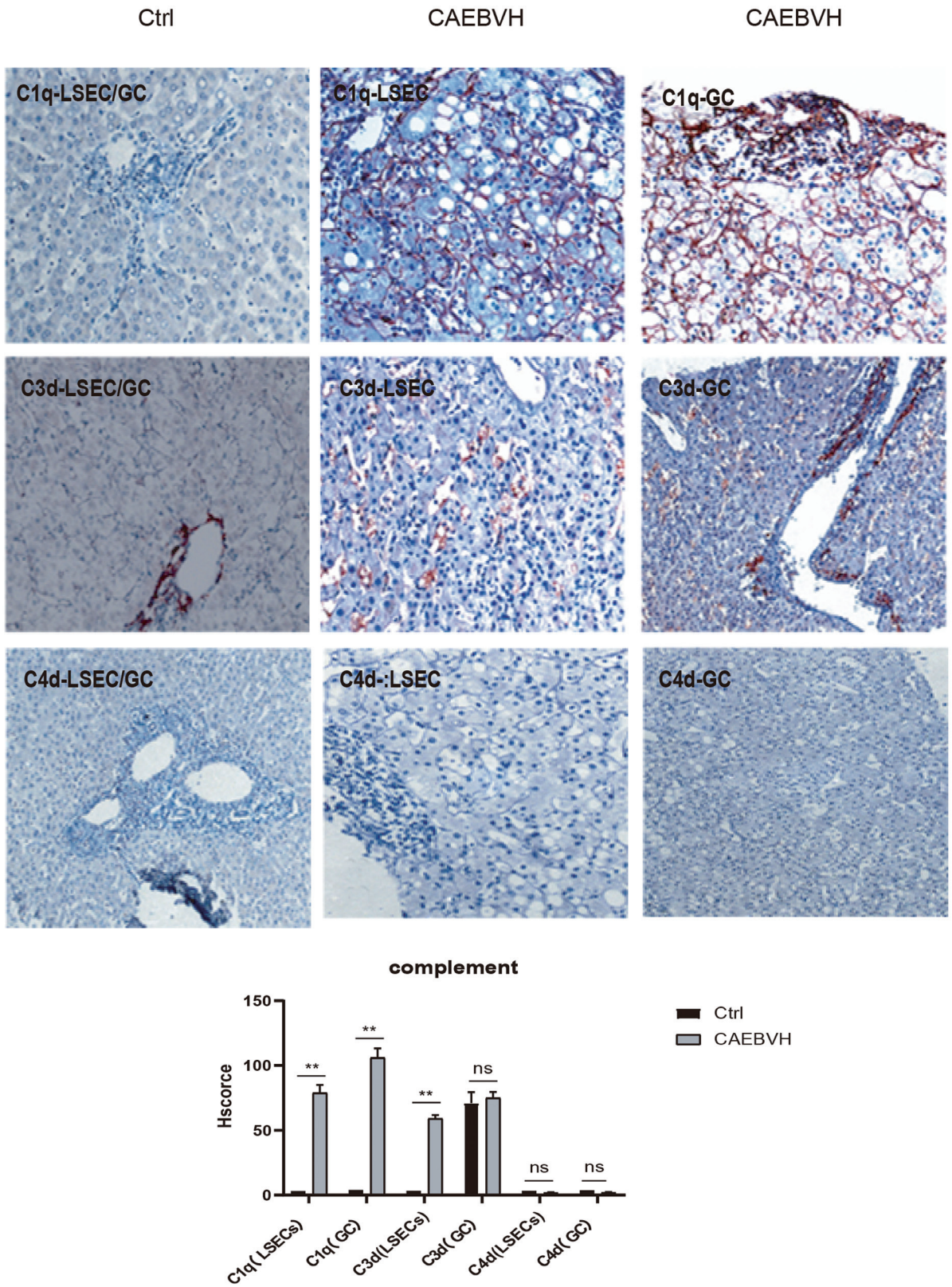


**Fig. 5. Complement activation of CAEBVH.** Expression of Complement 1q (C1q), complement 3d (C3d), and complement 4d (C4d) in liver sinusoidal endothelial cells (LSECs) and the Glisson's capsule (GC) of CAEBVH and control groups. C1q and C3d expression were higher in CAEBVH patients relative to controls. $n$=4, *$p$<0.05. **$p$<0.01.

## Author contributions

Participated in manuscript drafting (JL), completed the experimentation (JL, MFS), collected and analyzed clinical data (JLZ), checked the article quotation smoothness, corrected misspelled words, and organized the literature (LG, HCW XW, HYL and ZXW), and data interpretation and manuscript revision (DLL). All authors read and approved the final manuscript. All authors approved the final version of the article, including the authorship list.

## Ethical statement

This study was conducted in accordance with the ethical principle of that Declaration of Helsinki and was approved by the Institutional Review Board of 900TH Hospital of the Joint Logistic Support Force (Review number:2020023).

## Data sharing statement

The data used for this study are not freely available because of human participants. Interested researchers can contact the corresponding authors through e-mail for more detailed information.

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