Original Article

An enhanced level of LAMP-2A participates in CD4$^+$T cell hyperactivity in patients with primary biliary cholangitis

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Background: Primary biliary cholangitis (PBC) is an immune-mediated chronic cholestasis, in which T cell homeostasis plays an important role. Lysosomal-associated membrane protein 2 isoform A (LAMP-2A) has been implicated in the regulation of CD4$^+$T cell responses.

Methods: We comprehensively evaluated the immunobiology of CD4$^+$T cells in patients with PBC (PBC, n=42), chronic hepatitis B (CHB, n=20), and healthy control subjects (HC, n=20) by flow cytometry including activation status and LAMP-2A expression. Additionally, we investigated the activation responses of PBC-naïve CD4$^+$T cells by stimulation *in vitro* and tested the changes caused by deleting the gene encoding LAMP-2A.

Results: Firstly, we found an increased activation status of circulating CD4$^+$T cells from PBC patients compared to the HC subjects, and PBC-naïve CD4$^+$T cells showed enhanced responses after stimulation *in vitro*. Secondly, PBC-naïve CD4$^+$T cells expressed a significantly higher level of LAMP-2A compared to the HC and CHB groups [PBC *vs.* HC, 1,954.74 (1,254.28–3,057.14) *vs.* 1,542.12 (961.18–2,277.98), P=0.03; *vs.* CHB, 1,153.59 (726.87–1,275.48), P=0.02], and the overreactions of PBC-naïve CD4$^+$T cells could be reversed by interfering with LAMP-2A expression *in vitro*. Thirdly, the LAMP-2A expression level of PBC-naïve CD4$^+$T cells was related to disease severity and drug response.

Conclusions: An abnormally increased LAMP-2A expression of PBC-naïve CD4$^+$T cells might be related to excessive activation responses. LAMP-2A could be a novel therapeutic target for the treatment of PBC by reversing excessive responses and consequently reducing biliary injury.

Keywords: Autoimmune diseases; primary biliary cholangitis (PBC); LAMP-2A; T cell

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Introduction

Primary biliary cholangitis (PBC), formerly known as primary biliary cirrhosis (1), is a chronic and slow-progressing liver disease (2,3), which leads to autoimmune-mediated nonsuppurative cholangitis that mainly affects the small-to-medium-sized intrahepatic bile ducts (4,5). The etiology of PBC is complex. In terms of immunopathology, PBC is characterized by the presence of disease-specific

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antimitochondrial antibodies (AMAs) and a high frequency of autoreactive $CD4^+$ (6) and $CD8^+$T cells (7) around the damaged bile ducts. However, data on the mechanisms of biliary tract injury remain insufficient. Therefore, understanding the immunological mechanisms of PBC is critical for seeking more targeted therapeutic options and providing effective care for patients.

The lysosomal-associated membrane protein (LAMP) family is a set of conserved transmembrane glycoproteins mainly located in pre-lysosomes and lysosomes. Human LAMP-2A is an isoform of LAMP-2 derived from alternative splicing (8). LAMP-2 functions in delivering proteins from the cytoplasm to lysosomes (9,10) and is an essential component for regulating the function of immunocytes. It was reported that LAMP-2A contributes to immunological recognition and intracellular antigen presentation through the formation of the lysosomal LAMP-2A-HSC70 complex (11). LAMP-2C was reported to skew the class II major histocompatibility complex molecules (MHC II) presentation of cytoplasmic antigens (12). LAMP-2A has also been shown to participate in $CD4^+$T cell activation, and its expression was found to be upregulated when $CD4^+$T cells from a mouse were stimulated and differentiated into Th1 and Th2 in vitro. Deletion of the gene encoding LAMP-2A in $CD4^+$T cells was demonstrated to reduce activation-induced response, and T cell-specific LAMP-2A-deficient mice showed deficient responses to immunization and Listeria infection (13).

$CD4^+$T cells from patients with PBC show differential T cell receptor (TCR) repertoires and T cell activation (14), but the mechanism through which specific T cell subsets participate in the natural progression of the disease remains obscure (15,16). To address this issue, we designed the present study to evaluate the activation status of $CD4^+$T cells in PBC and to investigate the role of LAMP-2A in this process. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-2427).

Methods

Patient enrollment and sampling

In this study, 42 newly diagnosed patients with PBC, 20 healthy control subjects (HC), and 20 patients with chronic hepatitis B (CHB) were enrolled from January to October 2019 at Xijing Hospital of Digestive Diseases. The Institutional Research Ethics Committee of Xijing Hospital of Digestive Diseases approved this study (KY20173316-1). The study conformed to the provisions of the Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from each participant. The diagnostic criteria were based on the 2018 guidelines of the American Association for the Study of Liver Disease (AASLD) (3) and the 2017 guidelines of the European Association for the Study of the Liver (EASL) (17). No patients were diagnosed with a PBC-autoimmune hepatitis (PBC-AIH) overlap (18), other autoimmune disease, or other liver-related disease. No patients had taken glucocorticoids or immunosuppressive drugs within 6 months prior to diagnosis.

Blood was collected from 42 patients with PBC, 20 healthy controls (HC), and 20 patients with CHB. Paraffin-embedded liver tissues of 35 PBC patients were made available by ultrasound-guided needle liver biopsies. These 35 patients were divided into stages I–II and stages III–IV based on Ludwig's classification (19), or categorized as non-cirrhotic or cirrhotic patients. The demographic and clinical features of the PBC patients at baseline are listed in *Table 1*.

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized whole blood by gradient centrifugation with Lymphoprep™ (Stemcell Technologies, Vancouver, BC, Canada) within 6 hours, stored with autologous serum and 10% dimethyl sulfoxide (DMSO) at −80 ℃ for 24 hours, and then transferred to liquid nitrogen for long-term preservation. These standard procedures for PBMC preparation and storage were designed to ensure the uniformity of sample preparation between controls and disease cases.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD8, APC-Cy7-conjugated anti-HLA-DR, phycoerythrin (PE)-conjugated anti-ICOS, peridinin chlorophyll protein (PerCP)-conjugated anti-CD45RA, PerCP-Cy5.5-conjugated anti-IFN-γ, Brilliant Violet 421-conjugated donkey anti-rabbit IgG, and APC-Cy7/PE-Cy7-conjugated anti-CD3 were purchased from BioLegend (San Diego, CA, USA); Brilliant Violet 510-conjugated anti-IL-17 was purchased from BD Biosciences (San Diego, CA, USA); PE-Cy7-conjugated anti-IL-4 was purchased from eBioscience (San Diego, CA, USA); and rabbit anti-human LAMP-2A was purchased from Abcam (Cambridge, UK).

Table 1 Clinical features of patients with PBC included in the study

	PBC (n=42)	HC (n=20)	CHB (n=20)	Normal reference ranges
Age	58.14±10.29	57.60±12.87	55.15±11.71	–
Gender (F/M)	35/7	16/4	15/5	–
ALT (IU/L)	76.00 (34.00–122.00)	20.00 (16.00–37.00)	47.50 (31.50–83.75)	9.00–50.00
AST (IU/L)	97.00 (58.00–137.00)	19.00 (17.00–22.00)	53.50 (33.25–76.50)	15.00–40.00
ALP (IU/L)	165.00 (101.00–323.00)	67.00 (59.00–76.00)	83.00 (74.75–131.00)	45.00–125.00
GGT (IU/L)	186.00 (63.00–393.00)	21.00 (14.00–34.00)	39.00 (18.25–84.75)	10.00–60.00
TBil (μmol/L)	20.00 (15.00–54.70)	11.70 (9.40–16.70)	18.75 (16.45–25.35)	3.40–20.50
Alb (g/L)	38.60 (32.20–40.40)	47.40 (46.20–49.30)	42.80 (35.50–45.60)	40.00–55.00
AMA (+)/(-)	34/8	–	–	–
AMA-M2 (+)/(-)	28/14	–	–	–
IgM (g/L)	2.54 (1.72–4.22)	–	–	–
Peripheral blood usage	42	20	20	–
Liver biopsy usage	35	–	–	–
Early stage (I-II)	20	–	–	–
Advanced stage (III-IV)	15	–	–	–
Clinical stage				–
Non-cirrhotic	24	–	–	–
Cirrhotic	11	–	–	–

Continuous data are presented as means ± standard deviation (SD) or as medians with interquartile range; F/M, female and male; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; TBil, total bilirubin; Alb, albumin; AMAs, anti-mitochondrial antibodies.

Flow cytometry

The Fc receptors on the human cells were blocked with Human TruStain FcX™ (BioLegend, San Diego, CA, USA) and incubated with fluorochrome-conjugated monoclonal antibodies for surface markers (CD3, CD4, CD8, HLA-DR, ICOS, and CD45RA) for 30 minutes (5 μL antibody in 100 μL) then fixed and permeabilized with the Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA, USA). Subsequently, the cells were blocked and incubated with an excess amount (1 μL antibody in 200 μL) of primary polyclonal antibody against LAMP-2A overnight at 4 ℃ (20). After further incubation with secondary fluorochrome-conjugated antibody for 30 minutes (2 μL antibody in 100 μL), the cells were acquired with the BD FACSCanto™ II flow cytometer. For intracellular cytokine staining, PBMCs were stimulated by the Cell Stimulation Cocktail (eBioscience, San Diego, CA, USA) including PMA, ionomycin, and GolgiStop for 4 hours at 37 ℃ in 5% CO_2 before staining. The data were analyzed by FlowJo V10 (Tristar Inc., San Carlos, CA, USA), and positive thresholds were set by fluorescence minus one control. The gating strategy is shown in Figures S1,S2.

Cell purification and culture

Naïve $CD4^+$T cells were negatively selected from PBMCs with the human Naïve $CD4^+$T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the isolated cell population was more than 90%. The isolated cells were cultured in X-VIVO15 (Lonza, Basel, Switzerland) in a 5% CO_2 incubator at 37 ℃. Where appropriate, the cells were stimulated to activate with ImmunoCult™ Human CD3/CD28 T Cell Activator (Stemcell Technologies, Vancouver, BC, Canada) for 4 days.

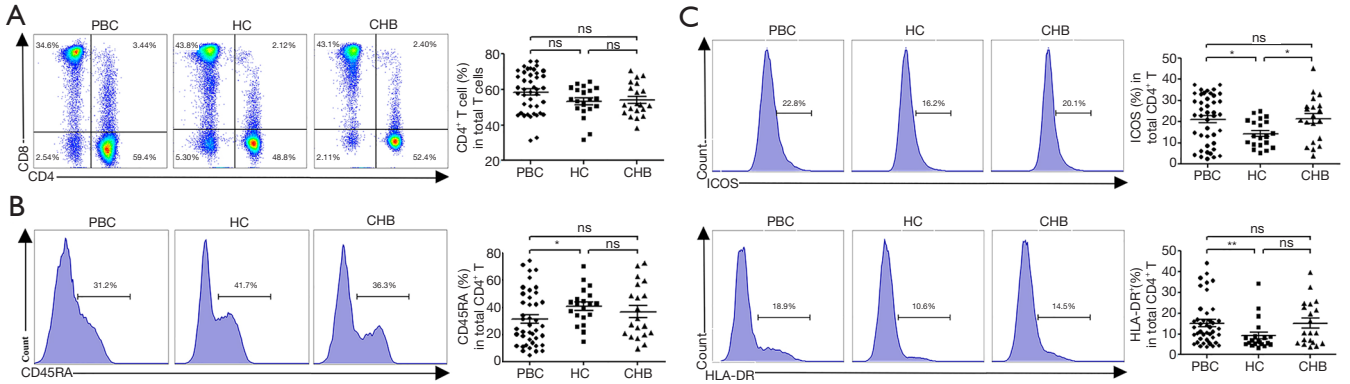

Figure 1 The increased phenotypic activation of CD4$^+$T cells from PBC patients. (A) The comparison of the proportion of CD4$^+$T cells in total T cells in PBC (n=42), chronic hepatitis B (CHB) (n=20) and healthy controls (HC) (n=20), with representative plots of flow cytometry for each group in the left and scatter plot in the right. (B) The comparison of the proportion of naïve CD4$^+$T cells in total CD4$^+$T cells in the PBC and HC groups with representative plots of flow cytometry and scatter plot. (C) In the PBC group, the ratios of activated (HLA-DR$^+$/ICOS$^+$) CD4$^+$T cells were higher than those in the HC group. *, P<0.05; **, P<0.01; ns, P>0.05.

Transfection of T cells

HEK293T cells were seeded in a cell culture dish in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) 1 day before transfection. Cells were co-transfected with the pHelper 1.0, pHelper 2.0 plasmid, and either the GV493 vector or an empty vector using Lipofectamine (Genechem, Shanghai, China). The supernatant of HEK293T cells was collected 48 hours after transfection. Human naïve CD4$^+$T cells were transfected with the supernatant using the T Cell-Specific Lentivirus Transfection Kit (Genechem, Shanghai, China) according to the manufacturer's instructions.

Proliferation and apoptosis assay

The CFSE Cell Division Tracker Kit (BioLegend, San Diego, CA, USA) and the Annexin V-PE Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) were used according to the manufacturer's instructions.

Enzyme-linked immunosorbent assays.

Cell supernatants were collected and measured with the Human IFN-γ and IL-2 ELISA Kits (4A Biotech, Beijing, China) according to the manufacturer's instructions

Statistical analysis

Continuous variables with normal distribution are expressed as the mean ± standard deviation, and comparisons were made between groups using Student's *t*-tests. Non-normal data distributions are presented as the median and 25th–75th interquartile range, and were analyzed with the Mann-Whitney U-test. Intra-individual comparisons were performed with Wilcoxon's matched-pairs test. Statistical analyses were performed with SPSS 23 (IBM, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). A P value <0.05 was considered statistically significant.

Results

CD4$^+$T cells from patients with PBC showed an increased activation status

As shown in *Figure 1A*, there was no significant difference in the percentage of CD4$^+$T cells in the total T cells of PBMCs from PBC patients compared to that of the HC and CHB groups [PBC *vs.* HC, 67.91% (59.98–71.64%) *vs.* 54.52% (49.20–60.66%), P=0.09; *vs.* CHB, 51.44% (47.24–62.84%), P=0.11, respectively]. As shown in *Figure 1B*, the proportion of naïve CD4$^+$T cell subsets from the PBC patients showed a dramatic decrease compared with that of the HC subjects [33.14% (20.67–50.63%) *vs.* 42.54% (32.93–47.96%), P=0.03] but was not statistically different to that of the CHB patients [33.14% (20.67–50.63%) *vs.* 29.74% (20.39–55.15%), P=0.26]. Mid- and late-activation

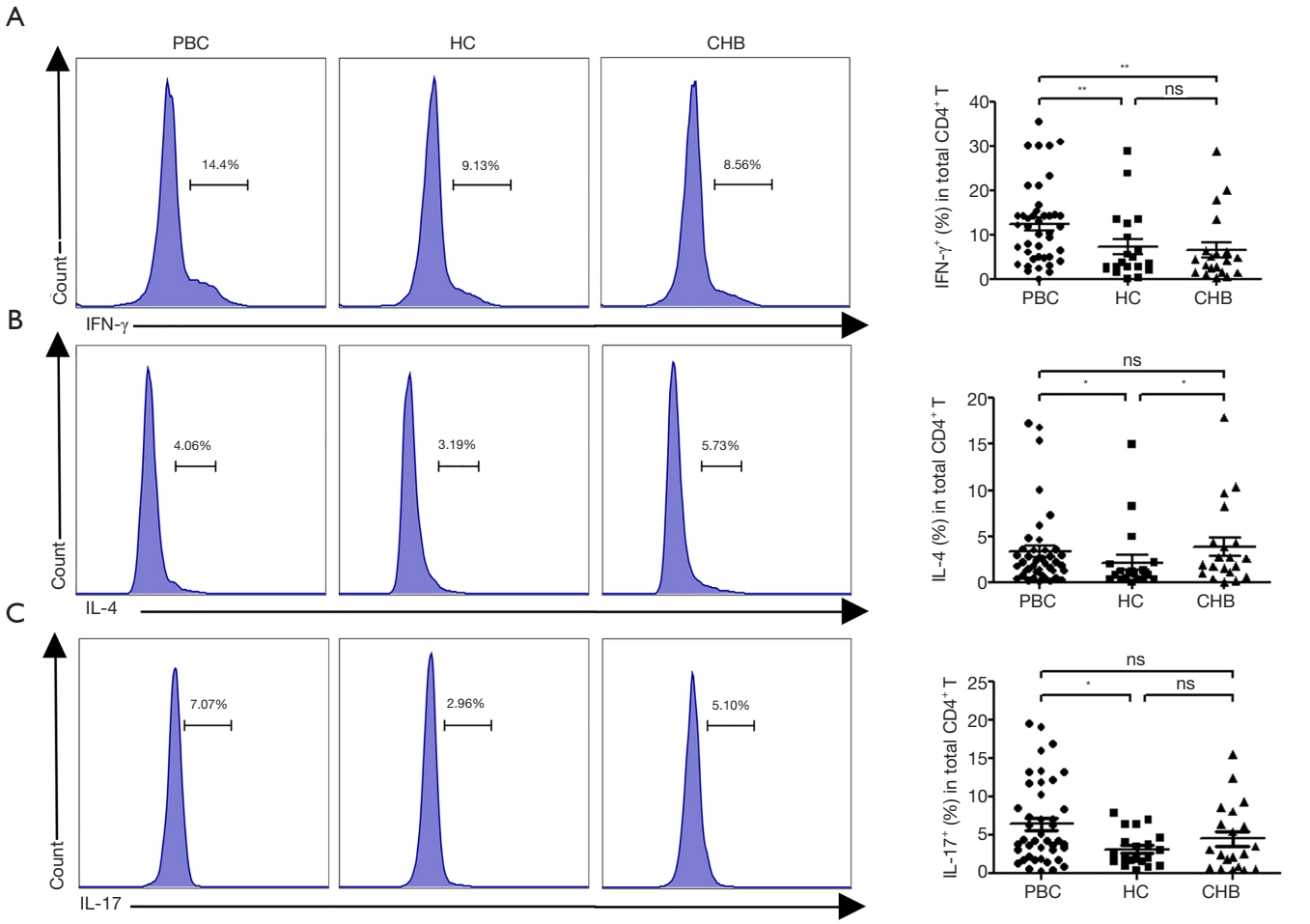

Figure 2 The increased multiple-cytokine activation of CD4⁺T Cells from patients with PBC. The comparison of the proportion of Th1 (IFN-γ⁺CD4⁺T cells) (A), Th2 (IL-4⁺ CD4⁺T cells) (B), and Th17 (IL-17⁺CD4⁺T cells) (C) in total CD4⁺T cells in the PBC, HC, and CHB groups with representative plots of flow cytometry and scatter plot. *, P<0.05; **, P<0.01; ns, P>0.05.

markers (ICOS and HLA-DR, respectively) were used to determine the activated CD4⁺T cell phenotype. As shown in *Figure 1C*, results indicated that the proportion of ICOS⁺ and HLA-DR⁺ CD4⁺T cells in the PBC patients was significantly higher than that in the HC subjects [ICOS, 23.22% (13.12–32.51%) *vs.* 12.93% (8.94–20.31%), P=0.02; HLA-DR, 9.88% (6.19–13.97%) *vs.* 6.21% (5.11–8.82%), P<0.01], but was not statistically different to that in the CHB patients [ICOS, 23.22% (13.12–32.51%) *vs.* 21.80% (10.77–27.51%), P=0.86; HLA-DR, 9.88% (6.19–13.97%) *vs.* 10.56% (5.69–24.03%), P=0.98].

Additionally, the frequencies of CD4⁺T cells producing interferon-γ (IFN-γ), interleukin-4 (IL-4), and IL-17 (defined as Th1/Th2/Th17 subsets, respectively) were measured. As shown in *Figure 2*, CD4⁺T cells from PBC patients showed multiple-cytokine activation when compared to the HC group [Th1, 11.92% (7.46–14.59%) *vs.* 3.76% (2.61–11.96%), P<0.01; Th2, 1.98% (0.93–2.73%) *vs.* 1.05% (0.45–1.88%), P=0.02; and Th17, 6.07% (3.35–12.05%) *vs.* 2.32% (1.53–4.56%), P=0.02]. As shown in *Figure 2A*, the percentage of Th1 from PBC patients was higher than that from CHB patients [11.92% (7.46–14.59%) *vs.* 4.47% (1.70–6.49%), P<0.01], but there were no significant differences between Th2 and Th17 [Th2, 1.98% (0.93–2.73%) *vs.* 2.32% (1.08–4.34%), P=0.72; Th17, 6.07% (3.35–12.05%) *vs.* 2.82% (0.67–7.68%), P=0.15].

The LAMP-2A expression level of non-activated CD4⁺T cells was increased in patients with PBC

The mean fluorescence intensity (MFI) in certain T cell subsets is proportional to the amount of total cellular LAMP-2A (20). As shown in *Figure 3A*, the MFI in ICOS⁺ CD4⁺T cells was significantly greater than that in the ICOS⁻CD4⁺T cells, in PBC patients, CHB patients, and HCs [PBC, 1,298.05 (880.03–2,324.88) *vs.* 1,167.88 (828.72–1,893.41), P<0.0001; HC, 1,082.62 (880.69–1,858.91) *vs.* 937.79 (719.90–1,381.72), P<0.0001; CHB, 1,087.15 (807.50–1,547.17) *vs.* 969.07 (731.40–1,258.47), P<0.01]. For HLA-DR–marked cells, the results showed a consistent trend [PBC, 1,914.05 (1,201.15–3,307.09) *vs.* 1,255.26 (855.50–2,078.62), P<0.0001; HC, 1,333.00 (855.21–2,301.29) *vs.* 930.58 (709.79–1,547.00), P<0.0001; CHB, 1,223.76 (875.02–1,957.52) *vs.* 922.32 (687.21–1,321.10), P<0.0001], as shown in *Figure 3B*. This indicated that LAMP-2A may participate in the initiation and maintenance of the CD4⁺T cell activation responses.

Interestingly, we found that the LAMP-2A expression in HLA-DR⁻CD4⁺T cells from the PBC patients was significantly higher than that in the HC subjects and the CHB patients [PBC *vs.* HC, 1,255.26 (855.50–2,078.62) *vs.* 930.58 (709.79–1,547.00), P=0.02; *vs.* CHB, 922.32 (687.21–1,321.10), P=0.02], and ICOS⁻CD4⁺T cells showed the same trend, as shown in *Figure 3C*. Furthermore, the level of LAMP-2A in naïve CD4⁺T cells from the PBC patients was significantly higher than that in the HCs and the CHB patients [PBC *vs.* HC, 1,954.74 (1,254.28–3,057.14) *vs.* 1542.12 (961.18–2,277.98), P=0.03; *vs.* CHB, 1,153.59 (726.87–1,275.48), P=0.02], as shown in *Figure 3D*.

An abnormally increased LAMP-2A expression of naïve CD4⁺T cells was responsible for CD4⁺T cell hyperactivation in PBC

We found that naïve CD4⁺T cells from PBC patients showed a greater ability to proliferate compared with HCs after stimulation by the CD3/CD28 T Cell Activator *in vitro* [75.10% (66.80–90.10%) *vs.* 55.00% (44.00–80.00%), P=0.03], as shown in *Figure 4A*, but there were no significant difference in apoptosis [10.40% (7.60–12.90%) *vs.* 10.30% (7.80–13.20%), P=0.71], as shown in *Figure 4B*. Additionally, activation induced cytokine production in the cell supernatant; for example, IL-2 levels were significantly higher in cells from the PBC patients compared with HCs [9.13 (8.36–13.27) *vs.* 7.35 (5.50–8.62) ng/mL, P=0.02], and although IFN-γ levels were not significantly different they showed a consistent trend [12.14 (9.21–16.27) *vs.* 9.00 (6.84–13.12) ng/mL, P=0.07], as shown in *Figure 4C*.

We subsequently transfected naïve CD4⁺T cells from the PBC patients with a retrovirus expressing a control (PBC-Scr) or a LAMP-2A-specific short hairpin RNA (PBC-ShL2A) and puromycin resistance, which was used to select the cells. After activation *in vitro*, silencing of the LAMP-2A expression significantly reduced activation-induced IFN-γ and IL-2 production [IFN-γ, 12.14 (9.21–16.27) *vs.* 1.50 (0.31–3.13) ng/mL, P<0.0001; IL-2, 9.13 (8.36–13.27) *vs.* 0.78 (0.27–0.92) ng/mL, P<0.0001] and proliferation ability [75.10% (66.80–90.10%) *vs.* 26.00% (22.00–32.00%), P<0.0001], as shown in *Figures 4A,C,D*. The impaired T cell responses caused by silencing the LAMP-2A mRNA were not due to increased apoptosis [10.40% (7.60–12.90%) *vs.* 11.20% (10.00–14.60%), P=0.27], as shown in *Figure 4B*.

LAMP-2A expression in naïve CD4⁺ T cells from PBC patients was related to the disease severity and ursodeoxycholic acid (UDCA) response

The relationship between the LAMP-2A expression in naïve CD4⁺T cells and PBC clinical features was investigated in our research. Given that PBC predominantly occurs in middle–aged and elderly women, we investigated the influence of sex and age profiles on the LAMP-2A expression in naïve CD4⁺T cells, but these differences were statistically insignificant [Sex, 2,313.18 (1,038.82–2,745.45) *vs.* 1,425.91 (1,067.29–3,057.14), P=0.69; Age, 1,817.09 (1,095.56–2,786.40) *vs.* 2,284.57 (1,364.68–2,914.94), P=0.31], as shown in *Figure 5A*. Hepatic biochemical indices were closely related to the prognoses of PBC patients. However, we found that the LAMP-2A expression in naïve CD4⁺T cells was independent of hepatic biochemical indices, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), total bilirubin (TBil), and albumin (Alb), as shown in the Figures S1-S3.

Interestingly, the LAMP-2A expression in naïve CD4⁺T cells was higher in stage III–IV patients than in stage I–II patients [2,284.57 (1,425.91–3,057.14) *vs.* 1,360.46 (1,123.09–2,313.18), P=0.01], and higher in the cirrhotic group than in the non-cirrhotic group [2,730.97 (1,907.15–3,127.62) *vs.* 1,946.14 (1,068.02–2,745.45), P<0.001],

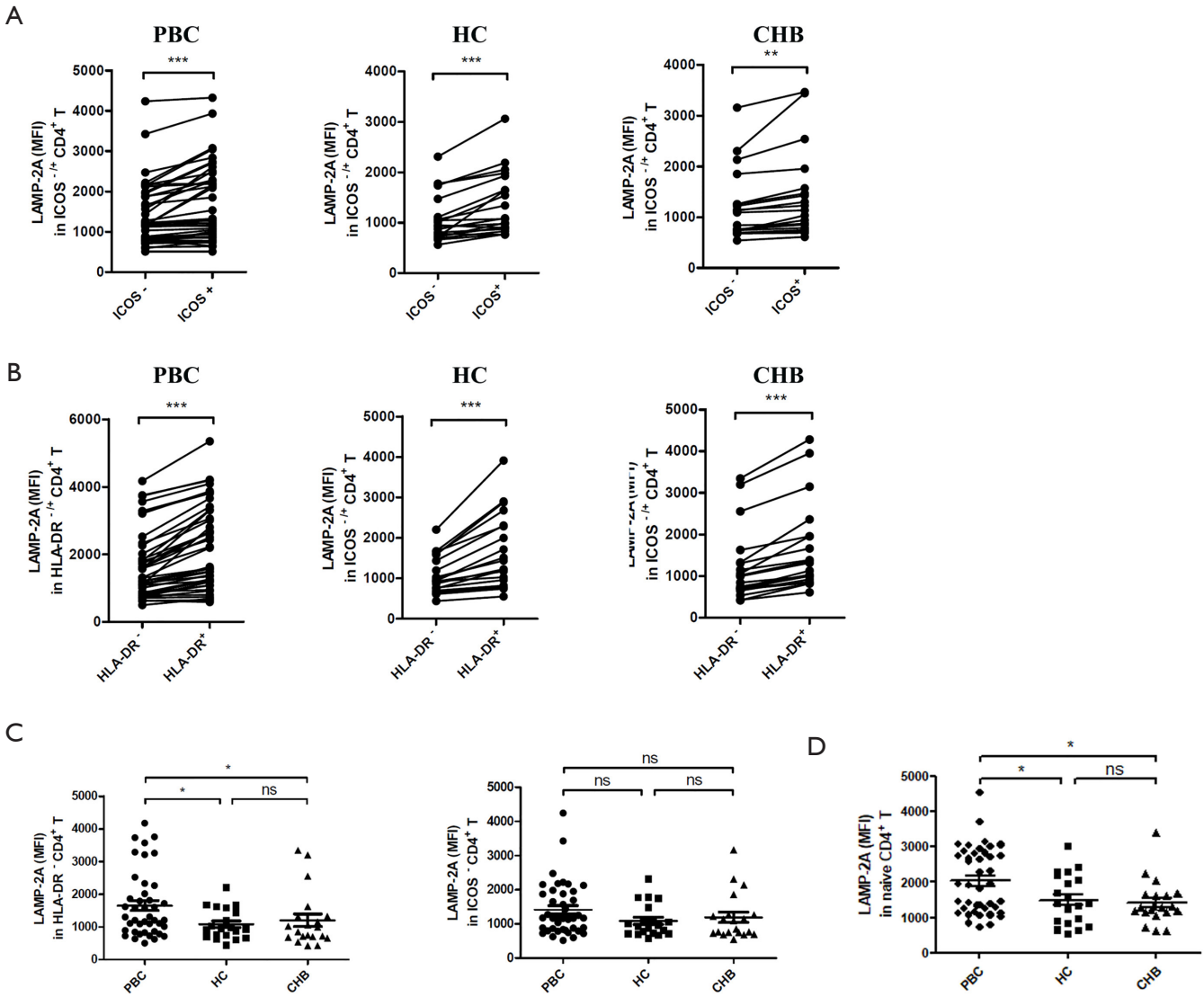

Figure 3 The increased expression of LAMP-2A in PBC non-activated CD4$^+$T Cells. The mean fluorescence intensity (MFI) of LAMP-2A in activated cells (A) (ICOS$^+$). (B) (HLA-DR$^+$) CD4$^+$T cells were significantly higher than non-activated cells (ICOS$^-$/HLA-DR$^-$) in all three groups. (C) The LAMP-2A expressions in non-activated (ICOS$^-$/HLA-DR$^-$) CD4$^+$T cells from PBC patients were higher than those in the HC and CHB groups. (D) The LAMP-2A expressions in naïve CD4$^+$T cells were higher in PBC patients than in the HC and CHB groups. *, P<0.05; ***, P<0.001; ns, P>0.05.

as shown in *Figures 5B,C*. Furthermore, the LAMP-2A expression levels in naïve CD4$^+$T cells were significantly lower in UDCA responders than in non-responders at 3 months [the Barcelona definition, 1,249.51 (1,038.82–1,425.91) *vs.* 2,284.57 (1,254.28–3,067.83), P=0.01], as shown in *Figure 5D*. There was a trend but no significant difference, however, when the Paris I definition was used [1,322.54 (1,031.22–2,818.46) *vs.* 1,862.12 (1,227.02–2,804.22), P=0.25], as shown in *Figure 5E*.

Discussion

Primary biliary cholangitis is considered to be a liver-specific autoimmune disease (21). The pathogenesis of the small bile duct destruction occurs through the interaction between the immune response and the biliary epithelial

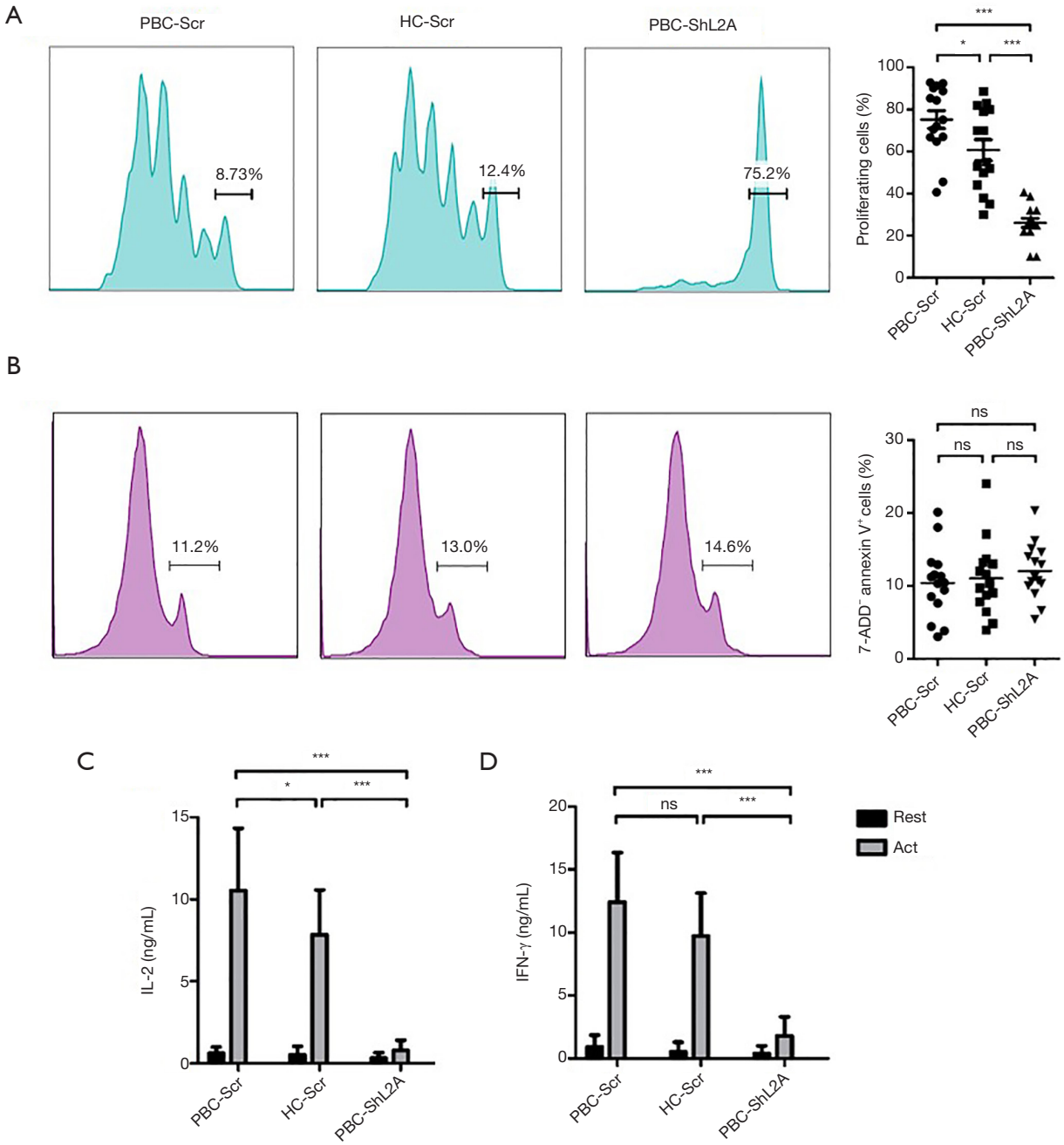

Figure 4 The excessive activation responses of naïve CD4⁺T cells from PBC patients. (A) The comparison of the proliferation ability of naïve CD4⁺T cells from PBC patients transfected with control shRNA (PBC-Scr), HC transfected with control shRNA (HC-Scr) groups, and PBC-naïve CD4⁺T cells transfected with LAMP-2A-specific shRNA (PBC-L2A) after stimulation by CD3/CD28 activator *in vitro*. The representative histograms of flow cytometry for each group and column chart are shown. (B) The comparison of the apoptosis of naïve CD4⁺T cells in PBC-Scr, HC-Scr, and PBC-L2A groups. (C,D) The IFN-γ and IL-2 levels in the cell supernatant of naïve CD4⁺T cells in the PBC, HC, and PBC-L2A groups were quantified by enzyme-linked immunosorbent assay (ELISA), including the resting naïve CD4⁺T cells (Rest) and cells following activation by CD3/CD28 activator (Act). *, P<0.05; ***, P<0.001; ns, P>0.05.

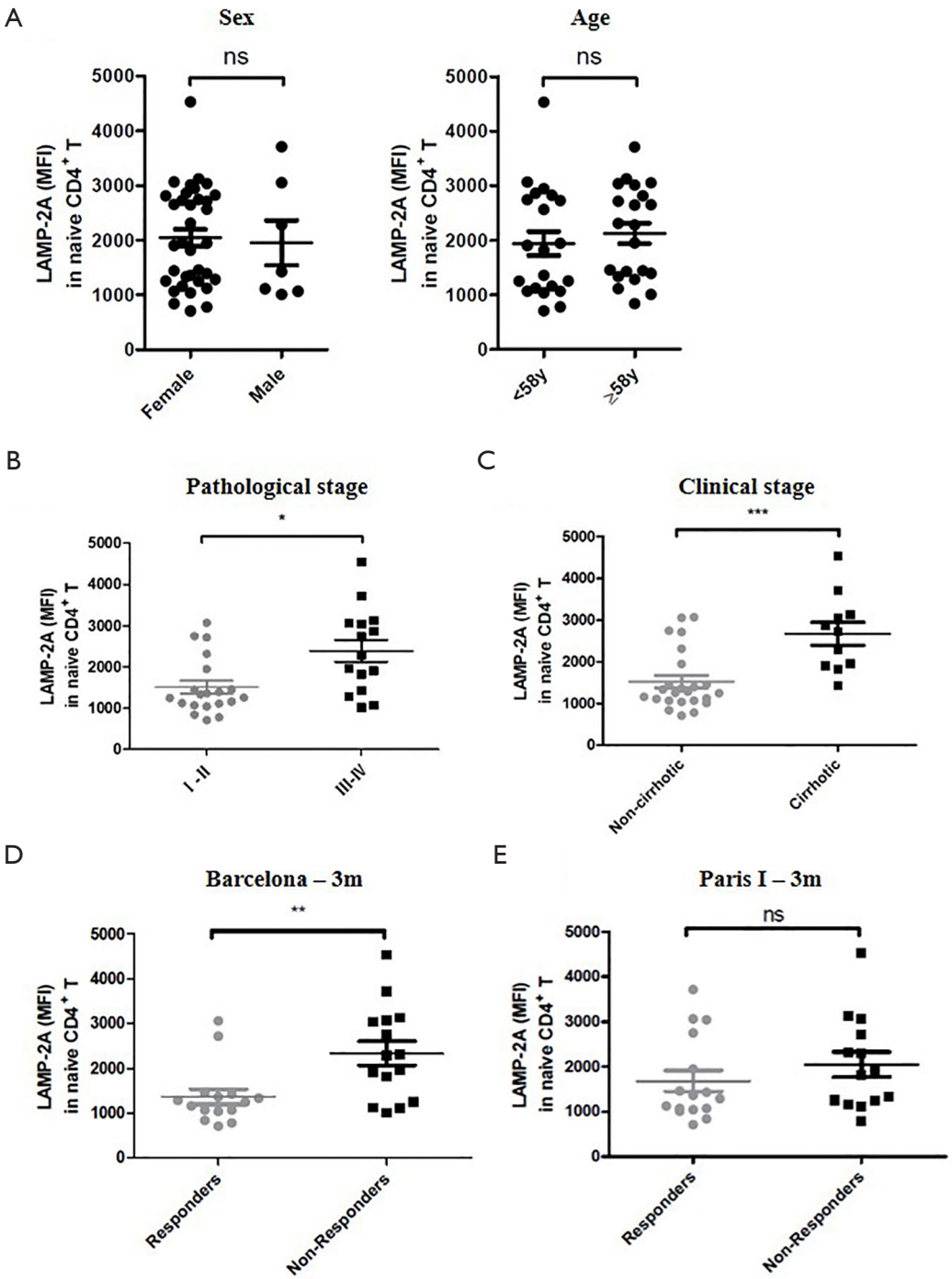

Figure 5 LAMP-2A expressions in naïve CD4$^+$T cells from PBC patients were related to disease severity and UDCA response. (A) The LAMP-2A expressions in naïve CD4$^+$T cells from PBC patients were not related to sex or age. (B) The LAMP-2A expressions in naïve CD4$^+$T cells from PBC patients with an advanced clinical stage were significantly increased in terms of pathological stage and (C) the degree of liver fibrosis. (D) The LAMP-2A expressions in naïve CD4$^+$T cells from UDCA responders were dramatically lower than those from non-responders according to the Barcelona definition and (E) the Paris I definition after 3 months of treatment. *, P<0.05; **, P<0.01; ***, P<0.001; ns, P>0.05.

cells (22). The autoimmune nature of PBC involves the loss of tolerance to a series of mitochondrial autoantigens, including the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) (23,24). This response contributes to lymphocytic cholangitis, which progresses to bile duct injury followed by chronic cholestasis and liver fibrosis.

PBC has diverse abnormalities in both immunological responses and regulations, which include the presence of autoreactive T cells (6,7), B cells (25), and antimitochondrial auto-antibodies (AMAs) (26). Many autoreactive T cells are distributed around the damaged bile ducts in PBC patients (27) and secrete multiple cytokines and chemokines. $CD4^+$T cells play a pivotal role in PBC pathogenesis (28). Recent studies have demonstrated that the IL-12/Th1 signaling pathway is a central mechanism in the etiopathogenesis of PBC. For PBC patients, IL-12A and IL-12RB2 variants were highly correlated with PBC in three independent genome-wide association studies (29-31). For murine models of PBC, namely dnTGFbRII mice, IL-12p40 KO-dnTGFbRII mice showed a significant improvement in histological cholangitis and significantly decreased levels of pro-inflammatory cytokines (32).

In the present study, we provided evidence of an excessive $CD4^+$T cell activation in the peripheral blood of PBC patients. The HLA-DR antigen indicates chronic T cell activation (33). Costimulatory protein ICOS is important for intact immune pathways (34), Th2 responses (35), and Th17 development and regulation (36). Our results suggested that the peripheral $CD4^+$T cells of PBC patients showed a relatively high HLA-DR/ICOS activation. In accordance with previous literature showing increased levels of IFN-γ, a Th1 cytokine promoting cytotoxic T cell activity; IL-4, a Th2 cytokine promoting B cell activation and antibody production; and IL-17, a Th17 cytokine promoting T cell activation and inflammation (37), our results showed profoundly increased frequencies of Th1, Th2, and Th17 cells in PBC patients.

Some studies have reported that LAMP-2A plays a crucial role in immunocyte responses, including immunological recognition and intracellular antigen presentation (11), and $CD4^+$T cell activation. It is reported that LAMP-2A may degrade two negative regulators of T cell activation, the ubiquitin ligase, Itch, and the calcineurin inhibitor, RCAN1, by chaperon-mediated autophagy (13).

Our earlier research revealed an increase of LAMP-2 in PBC patients' serum, and it showed a gradually decrease along with the therapy by UDCA, It suggested that this reduction of LAMP-2 might assist in predicting responses to UDCA treatment (38). These previous findings regarding the important role of LAMP-2 in PBC and in the functioning of T cells prompted the design of the current study.

Interestingly, we found that LAMP-2A expression was upregulated in non-activated $CD4^+$T cells of PBC patients compared to those of the HCs. Due to the crucial role of LAMP-2A in the process of $CD4^+$T cell activation, we hypothesized that the naïve $CD4^+$T cells of PBC patients were more liable to be activated. To test this idea, we isolated and stimulated naïve $CD4^+$T cells *in vitro* that had been collected from PBC patients and HC. As expected, the naïve $CD4^+$T cells of the PBC patients showed a higher capability for proliferation and activation-induced cytokine production. By deleting the gene encoding LAMP-2A in the naïve $CD4^+$T cells from the PBC patients, these excessive activation responses were reversed.

Additionally, there is a high expression level of LAMP-2A in newly diagnosed patients who exhibit a high pathological grade and an insufficient UDCA response after 3 months of treatment. In general, the diagnosis of PBC requires a pathological liver biopsy which is often an uncomfortable procedure for the patient and carries the risk of complications Additionally, PBC patients' responses to UDCA are typically determined after 1 year of treatment, which may cause a delay in medical opinion and deterioration of the disease. Our study has potentially identified a novel biomarker—levels of LAMP-2A in naïve $CD4^+$T cells—for monitoring PBC activity and the response to UDCA treatment in a more timely and convenient manner. However, further studies with larger sample sizes and longer follow-up times are required to validate the current findings.

In conclusion, the results of the present study support the concept that the abnormally increased LAMP-2A expression in the naïve $CD4^+$T cells of PBC patients might be related to a tendency for excessive activation. The LAMP-2A expression level of PBC-naïve $CD4^+$T cells could potentially be a useful marker to assess disease severity and drug response without the need for a liver biopsy and offers a faster mode of assessment than the current standard treatment protocols. LAMP-2A could also be a novel therapeutic target for the treatment of PBC by reversing excessive responses and consequently reducing biliary injury.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The Institutional Research Ethics Committee of Xijing Hospital of Digestive Diseases approved this study (KY20173316-1). The study conformed to the provisions of the Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from each participant.

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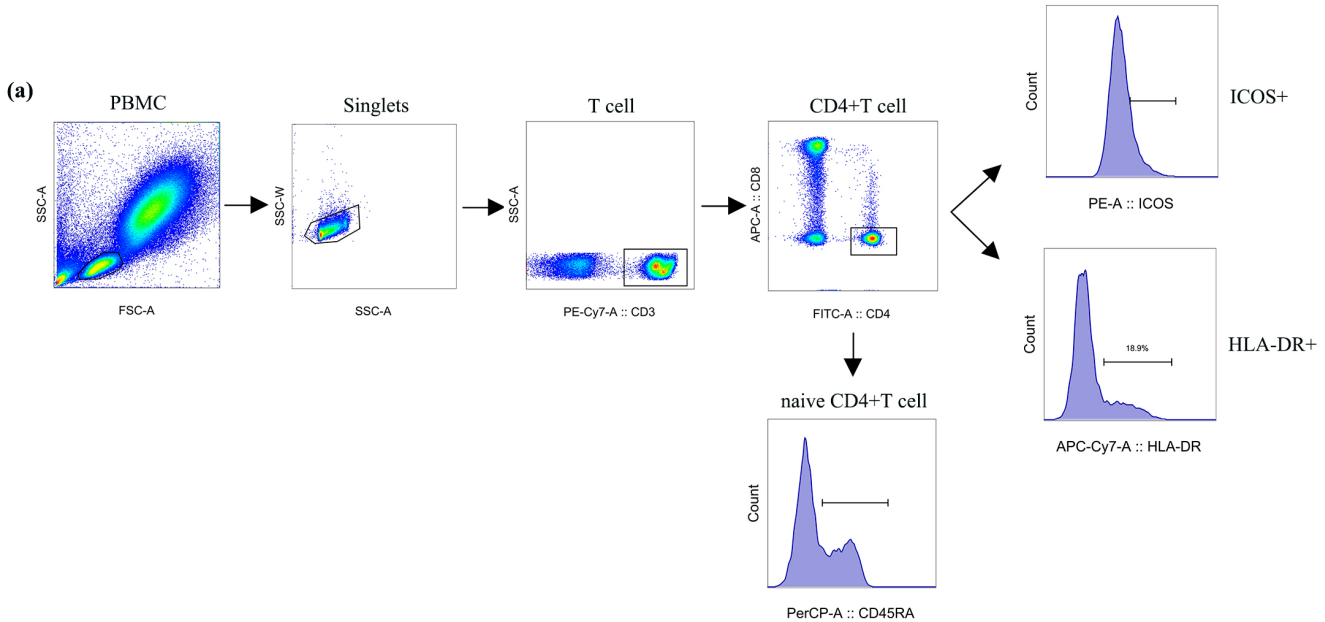

Figure S1 The gating strategy for ICOS$^+$, HLA-DR$^+$, and naïve CD4$^+$T cells. The threshold for HLA-DR and ICOS is defined via the method of fluorescence minus one (FMO).

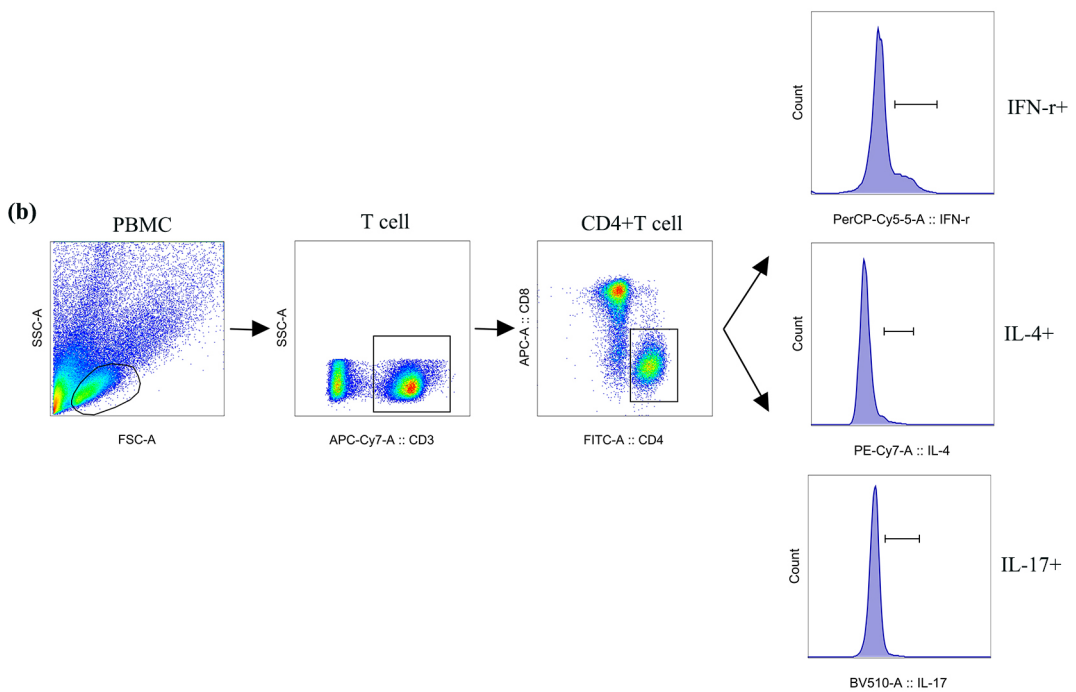

Figure S2 The gating strategy for IFN-γ$^+$, IL-4$^+$, and IL-17$^+$ CD4$^+$T cells after stimulation by Phorbol-12-myristate-13-acetate (PMA) *in vitro*.

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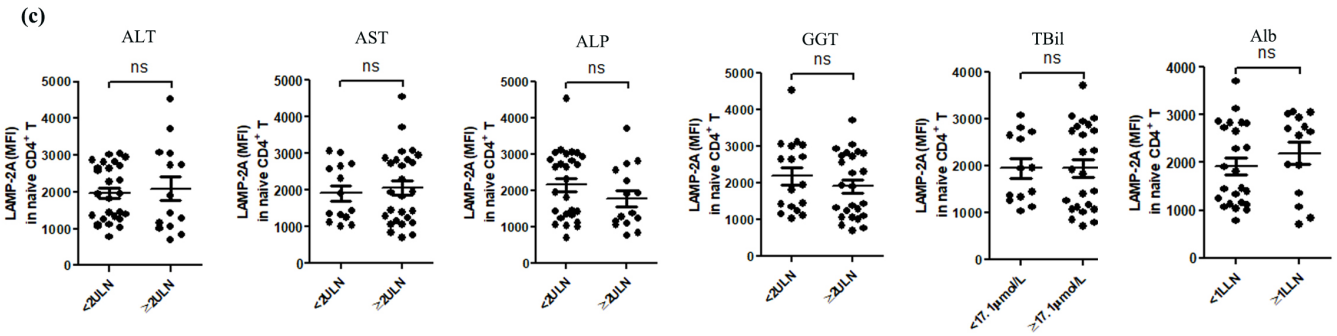

Figure S3 The LAMP-2A expression in naïve CD4$^+$T cells was independent of hepatic biochemical indices, including alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), total bilirubin (TBil), and albumin (Alb) [upper limit of normal (ULN), lower limits of normal (LLN)].

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