Supplementary Materials and Methods

Isolation of LIMCs

LIMCs were isolated from fresh human liver tissue. Tissue was washed in ice-cold phosphate-buffered saline to remove residuals of blood, cut into small pieces, mechanically digested in RPMI1640 using a Stomacher400 Circulator (Seward Stomacher400 Circulator [Cole-Parmer Instrument Co Ltd, London, United Kingdom]) for 330 seconds at 260 speed, filtered, and purified further by density gradient centrifugation using Lympholyte (Cedarlane Laboratories, Burlington, Canada) for 20 minutes at 550 × g. The LIMC population was extracted and further studied for their phenotype and function.

Isolation of Human Primary BECs

Human BECs were isolated from approximately 150 g of liver tissue. Tissue was digested enzymatically with collagenase type 1A (Sigma, Dorset, United Kingdom), filtered, and purified further via density gradient centrifugation over 33%/77% Percoll (Amersham Biosciences UK Ltd, Buckinghamshire, United Kingdom). BECs were extracted from the mixed nonparenchymal population via magnetic selection using antibodies against the cholangiocyte-specific receptor HEA-125 (50 μg/mL; Progen Biotechnik, Heidelberg, Germany). HEA-125–positive BECs were plated in rat-tail collagen-coated flasks and kept in culture and used at passage 4.

Flow Cytometry

Blood and liver-derived mononuclear cells were incubated with mouse anti-human or rat anti-human monoclonal fluorochrome-conjugated antibodies for 30 minutes in the dark before flow cytometry for surface marker phenotypic evaluation. Cells were washed and resuspended in phosphate-buffered saline supplemented with 1% fetal calf serum. Cells were fixed and permeabilized for immunofluorescent staining of intracytoplasmic cytokines and cytotoxic molecules.

Isolation of $CD28^{+/-}$ T-Cell Subsets and Effect of T-Cell–Conditioned Media on BECs

Fluorescence-activated, cell-sorted $CD3^{+}CD4^{+/-}CD28^{+/-}$ subsets were cultured overnight using 96-well, round-bottomed plates coated with anti-CD3 (OKT3; 5 μg/mL) and anti-CD28 (5 μg/mL) in media consisting of RPMI1640, Dulbecco's modified Eagle medium, and 10% heat-inactivated human AB serum (Life Technologies). Cells were cultured at a density of 50,000 cells/100 μL. Cell-free conditioned media then was transferred to primary BECs cultured (10,000 cells/well) as a monolayer in 96-well, flat-bottomed plates. After 4 days, the effect of conditioned media on BEC activation and death was monitored by staining with a live/dead cell discrimination dye (Biolegend, London, United Kingdom), according to the manufacturer's instructions, followed by antibody surface labeling for ICAM1, HLA-DR, CD40, and leukocyte function-associated antigen 3 markers of BEC activation, and analysis by flow cytometry.

Detection of Cytokine Expression Ex Vivo

Brefeldin A (1 μL/mL) was added 2 hours after initiation of stimulation of PSC PBMCs and LIMCs with plate-bound anti-CD3 (OKT3; 5 μg/mL) (at 10^6 cells/well) to allow intracellular cytokine accumulation. Cells were washed and stained with CD3, CD4, CD8, and CD28 antibodies before fixation, permeabilization, intracellular cytokine staining for TNFα and IFNγ, and detection with flow cytometry.

Immunohistochemical Analysis

Localization of $CD4^{+}$ and $CD8^{+}$ T cells as well as localization of T cells that had lost CD28 expression was tested using immunohistochemistry. Alcohol-fixed frozen tissue sections (7- to 10-μm thick) initially were blocked with BLOXALL endogenous peroxidase (Vector Laboratories Ltd, Peterborough, United Kingdom) and alkaline phosphatase solution for 10 minutes at room temperature. Sections then were washed with Tris-buffered saline–0.1% Tween20 and blocked with 2× casein block (Vector Labs) for 10 minutes before incubation with mouse anti-human CD8 (1:50; Vector Labs) for 30 minutes at room temperature. After washes, sections were incubated with the secondary antibody, ImmPRESS universal (Vector Labs), for 30 minutes, followed by developing using the Vector NovaRED Peroxidase Substrate kit (Vector Labs) for 5 minutes. Sections were blocked further with 2× casein for 5 minutes before incubation with mouse anti-human CD4 (1:25; Vector Labs) for 1 hour and the ImmPRESS-AP anti-mouse Ig (alkaline phosphatase) polymer detection kit (Vector Labs) for 30 minutes. The StayGreen/AP substrate chromogen (Abcam, Cambridge, United Kingdom) then was used for 20 minutes for green color development. Isotype-matched negative control antibodies also were used (IgG1, 1:25; Dako UK Ltd, Cambridgeshire, United Kingdom; and IgG2b, 1:50; eBioscience, Ltd, Hatfield, United Kingdom).

For localization of CD4+ and CD8+ T cells that had lost CD28 expression, alcohol-fixed frozen tissue sections (7- to 10-μm thick) also were used. Endogenous peroxidase was blocked using peroxidase blocking solution (Dako) for 10 minutes at room temperature. After washes in Tris-buffered saline–0.1% Tween20, sections were blocked with 2× casein block (Vector Labs) for 10 minutes followed by incubation with mouse anti-human CD28 (20 μg/mL) for 30 minutes at room temperature. After washes, ImmPRESS anti-mouse kit (Vector Labs) was used for 30 minutes, followed by washes and developing using the Vector DAB/nickel substrate (Vector Labs) for 5 minutes. The mouse on mouse elite peroxidase kit (Vector Labs) then was used according to the manufacturer's instructions, to continue the staining with mouse anti-human CD4 (1:15; Vector Labs) or mouse anti-human CD8 (1:40; Vector Labs) for 1 hour at room temperature. The ImmPRESS anti-mouse kit then was

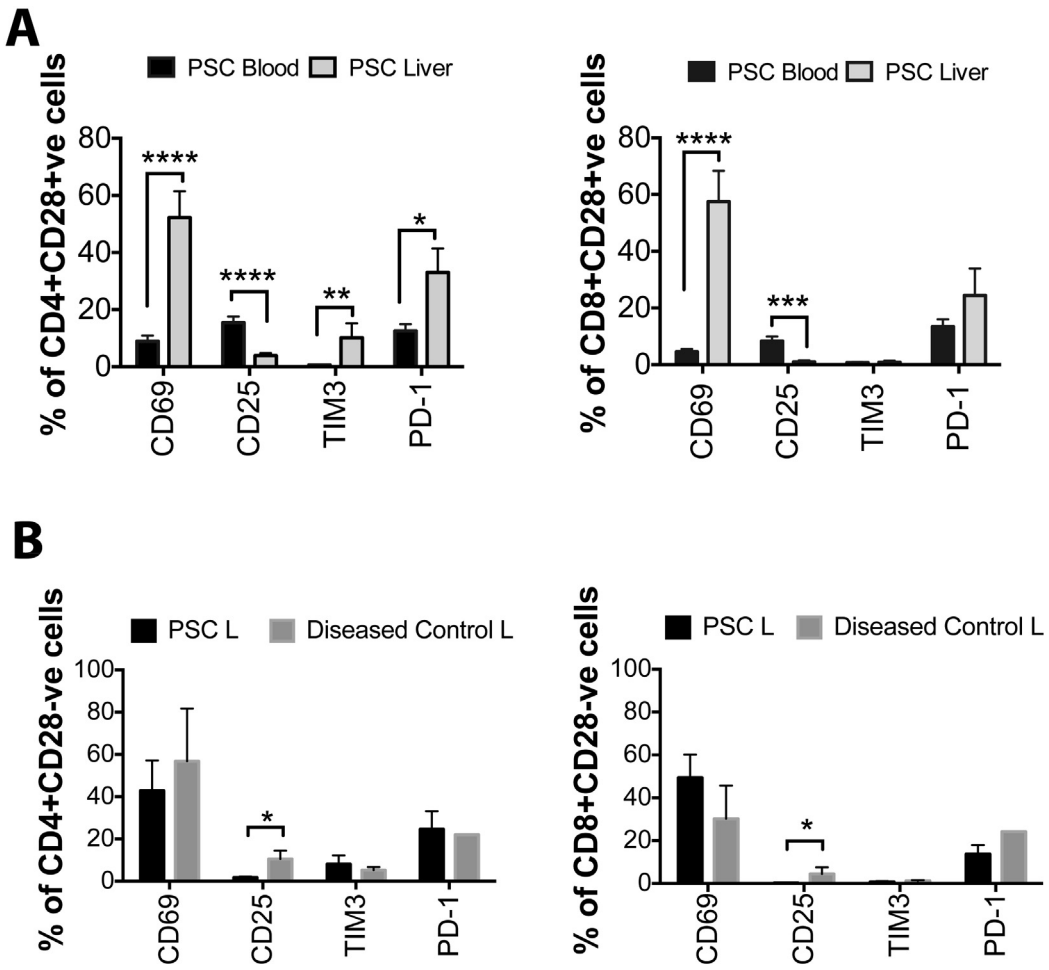
used for 30 minutes followed by developing using the 3-amino-9-ethylcarbazole peroxidase substrate kit for 30 minutes, counterstained with hematoxylin for 30 seconds, and mounted in aqueous mounting media. Isotype-matched negative control antibodies also were used (IgG1, 20 μg/mL for CD28 and at 1:15 for CD14 and IgG2b, 1:50; eBioscience).

RNA Extraction, Complementary DNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction

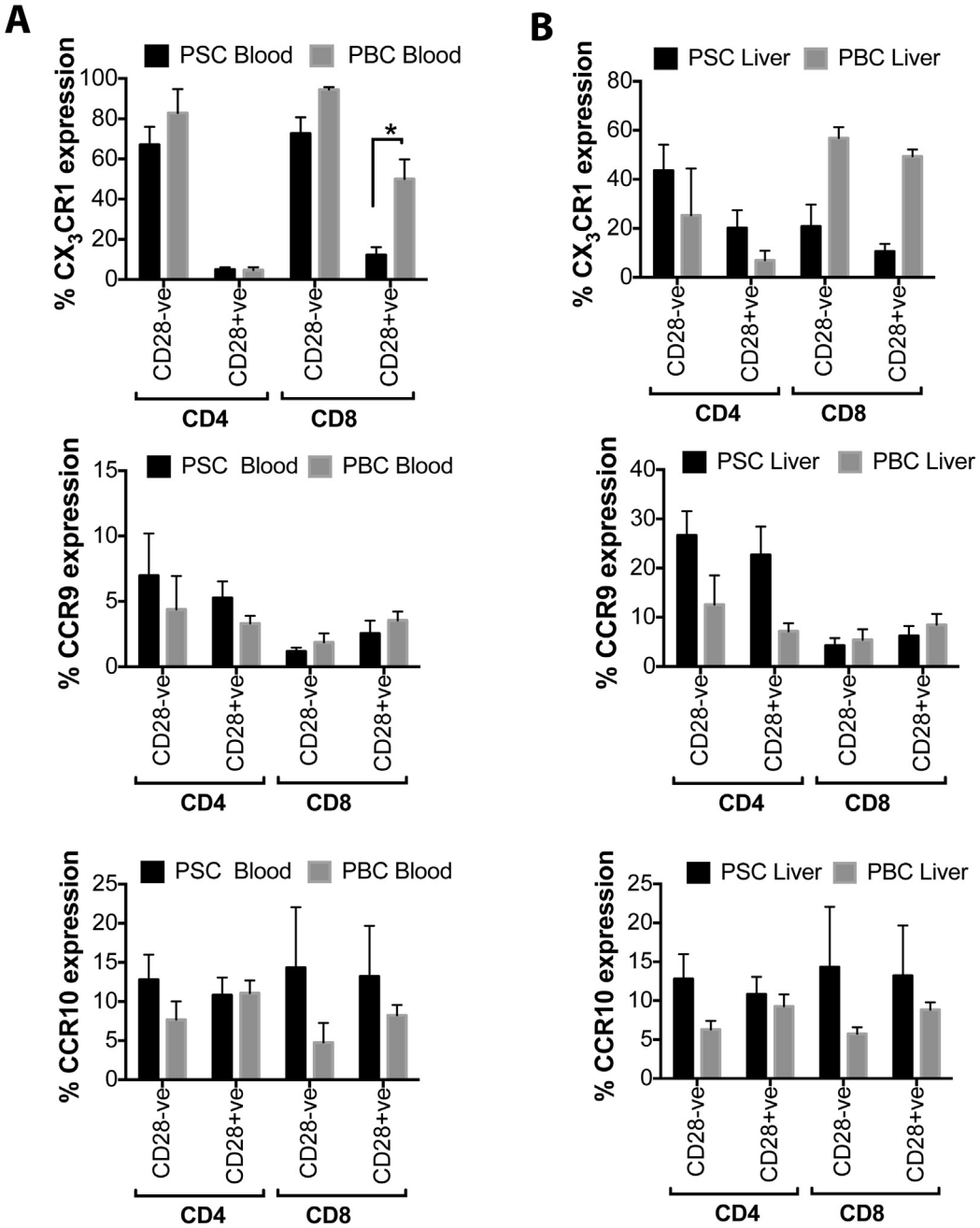
Total RNA was extracted from normal (n = 6) and diseased PBC (n = 5), and PSC (n = 9) human liver tissues to test TNFα, IFNγ, and IL17A messenger RNA expression. The eluted RNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Extracted RNA (50 μg) was transcribed into complementary DNA using the iScript complementary DNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative analyses were performed using TaqMan Fluorogenic 5' nuclease assays using gene-specific 5'FAM-labeled probes (Life Technologies) run on an ABI Prism 7700 sequencer (Life Technologies) with β-actin used as an internal control. Differential expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method.

Cytokine Secretion Assays

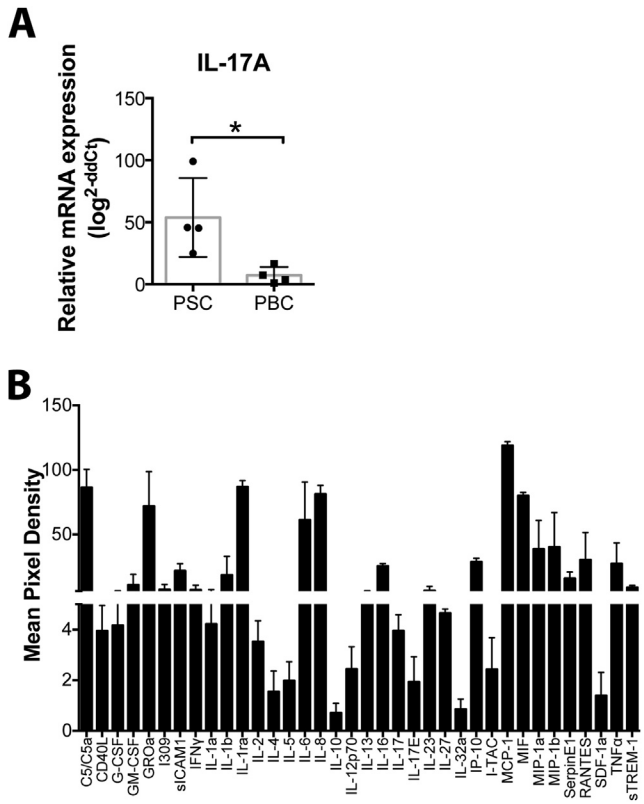
PSC liver-infiltrating mononuclear cells were cultured at 10^6 cells/mL for 24 hours in RPMI1640 supplemented with 10% fetal calf serum. Cell-free supernatant was tested for cytokine and chemokine presence using the cytokine array panel A kit (R&D Systems).



Supplementary Figure 1. Phenotypic characterization of $CD28^+$ T cells in blood and liver of PSC patients. The expression of CD69, CD25, TIM3, and programmed cell-death 1 (PD-1) on $CD28^+$ T cells isolated straight ex vivo also was analyzed using flow cytometry. (*A*) Data show the percentage of $CD4^+CD28^+$ and $CD8^+CD28^+$ T cells expressing CD69 (n = 23 [blood] and n = 6 [liver]), CD25 (n = 22 [blood] and n = 7 [liver]), TIM-3 (n = 14 [blood] and n = 5 [liver]), and PD-1 (n = 14 [blood] and n = 6 [liver]). *Bars* indicate the mean ± SEM. $^*P < .05$, $^{**}P < .01$, $^{****}P < .001$ as compared with expression levels in PSC liver samples. (*B*) Phenotypic characteristics of $CD28^-$ T cells in the PSC liver and disease control group consisted of PBC and NASH livers. Data show the percentage of $CD4^+CD28^-$ and $CD8^+CD28^-$ T cells expressing CD69 (n = 6 [PSC liver] and n = 3 [disease control]), TIM3 (n = 5 [PSC liver] and n = 2 [disease control]), and PD-1 (n = 6 [PSC liver] and n = 1 [disease control]). *Bars* indicate the mean ± SEM. $^*P < .05$.



Supplementary Figure 2. $CD28^-$ T cells show a differential expression of chemokine receptors between PSC and PBC. The expression of chemokine receptors on $CD28^-$ and $CD28^+$ T cells of CD4 and CD8 T cells from PSC and PBC patients was analyzed using flow cytometry. (*A, B*) Data show the percentage of $CD28^-$ and $CD28^+$ T cells that express the chemokine receptors CX_3CR1 (n = 7 [PSC blood] vs n = 3 [PBC blood], and n = 6 [PSC livers] vs n = 2 [PBC livers]), CCR9 (n = 4 [PSC blood] vs n = 4 [PBC blood], and n = 5 [PSC livers] vs n = 2 [PBC livers]), and CCR10 (n = 6 [PSC blood] vs n = 3 [PBC blood], and n = 6 [PSC livers] vs n = 2 [PBC livers]). *$P < .05$.



Supplementary Figure 3. Cytokine profile of PSC liver microenvironment. (*A*) IL17A messenger RNA (mRNA) expression in 4 PSC and 4 PBC livers. Scatter dot plots show relative mRNA levels in PSC livers with respect to PBC livers (mean ± SEM). *$P < .05$. (*B*) Liver-infiltrating mononuclear cells from 3 PSC liver samples were cultured for 24 hours before collection of their cell-free conditioned media. Cytokines and chemokines in the media were measured using a Human Cytokine Array kit. Expression levels are reported as the mean pixel density in arbitrary units.

Supplementary Table 1. Antibodies Used for Flow Cytometry

Antibody	Clone	Final concentration	Source
CD3-Pacific Blue	UCHT1	2 μL/10^6 cells	BD Pharmingen
Mouse IgG1, κ–Pacific Blue	X40	2 μL/10^6 cells	BD Pharmingen
CD3-FITC	UCHT1	2 μL/10^6 cells	BD Pharmingen
CD4-V500	RPA-T4	2 μL/10^6 cells	BD Pharmingen
Mouse IgG1, κ–V500	X40	2 μL/10^6 cells	BD Pharmingen
CD4-APC	RPA-T4	2 μL/10^6 cells	BD Pharmingen
Mouse IgG1, κ–APC	MOPC-21	2 μL/10^6 cells	BD Pharmingen
CD8-PeCy7	RPA-T8	2 μL/10^6 cells	BD Pharmingen
Mouse IgG1, κ–PE/CY7	MOPC-21	2 μL/10^6 cells	BD Pharmingen
CD8-FITC	RPA-T8	2 μL/10^6 cells	BD Pharmingen
CD28-PE	CD28.2	2 μL/10^6 cells	BD Pharmingen
Mouse IgG1 κ–PE	MOPC-21	2 μL/10^6 cells	BD Pharmingen
CD28-APC	CD28.2	2 μL/10^6 cells	BD Pharmingen
CD69-FITC	FN50	2 μL/10^6 cells	BD Pharmingen
CD25-APC	M-A251	5 μL/10^6 cells	BD Pharmingen
PD-1-APC	MIH4	5 μL/10^6 cells	BD Pharmingen
Mouse IgG1, κ–APC	MOPC-21	5 μL/10^6 cells	BD Pharmingen
TIM3-PeCy7	F38-2E2	5 μL/10^6 cells	eBioscience
CD45RA-V450	HI100	5 μL/10^6 cells	BD Pharmingen
Mouse IgG2b, κ–V450	27-35	5 μL/10^6 cells	BD Pharmingen
CD45RO-PE	UCHL-1	5 μL/10^6 cells	BD Pharmingen
Mouse IgG2a, κ–PE	G155-178	5 μL/10^6 cells	BD Pharmingen
CCR7-PE/Cy7	3D12	5 μL/10^6 cells	BD Pharmingen
CD62L-APC	DREG-56	5 μL/10^6 cells	BD Pharmingen
CX_3CR1- PE/Cy7	2A9-1	2 μL/10^6 cells	Biolegend
Rat IgG2b, κ-PE/Cy7	RTK4530	2 μg/mL	Biolegend
CXCR6-APC	K041E5	4 μL/10^6 cells	Biolegend
Mouse IgG2a-APC	X39	4 μL/10^6 cells	BD Pharmingen
CCR10-PE	314305	2 μg/mL	R&D Systems
Rat IgG2a-PE	54447	2 μg/mL	R&D Systems
CD11a-PE	HI111	2 μL/10^6 cells	BD Pharmingen
Granzyme B- FITC	GB11	5 μL/10^6 cells	BD Pharmingen
Perforin -PE	δG9	5 μL/10^6 cells	BD Pharmingen
Mouse IgG2b, κ-PE	27-35	5 μL/10^6 cells	BD Pharmingen
IFNγ-APC	B27	0.5 μL/10^6 cells	BD Pharmingen
TNFα-FITC	MAb11	3 μL/10^6 cells	BD Pharmingen
ICAM1-FITC	RR1/1	3 μL/10^6 cells	eBioscience
Mouse IgG1-FITC	MOPC-21	3 μL/10^6 cells	BD Pharmingen
CD40-APC	HI40a	5 μL/10^6 cells	Immunostep
LFA-3-PE	L300.4	5 μL/10^6 cells	Becton Dickinson
HLA-DR-PE/Cy7	L243 (G46-6)	2.5 μg/mL	BD Pharmingen
Mouse IgG2a-PE/Cy7	MOPC-173	2.5 μg/mL	BD Pharmingen

APC, allophycocyanin; FITC, fluorescein isothiocyanate; LFA-3, leukocyte function-associated antigen 3; PD-1, programmed cell-death 1; PE, phycoerythrin.

Supplementary Table 2. Antibodies Used for Immunohistochemical Analysis

Antibody	Clone	Final concentration	Source
CD4	1F6	1:25	Vector Laboratories
Mouse IgG1		1:25	Dako
CD8	4B11	1:50	Vector Laboratories
Mouse IgG2b		1:40	eBioscience
CD28	CD28.2	20 μg/mL	eBioscience

Supplementary Table 3. Clinical Characteristics of PSC Patients Supplemented With Vitamin D

	Responders	Nonresponders
Interval between sampling, *d*[a]	92 (78–105)	91 (42–189)
Age at time of sample, *y*	42 (20–61)	55 (35–69)
UDCA exposure	5/7	6/7
With IBD	5/7	6/7
Active IBD, %[b]	0/5	2/6
On immunosuppression	0/7	1/7
Liver disease status		
Precirrhotic	2/7	3/7
Cirrhotic: compensated	5/7	3/7
Cirrhotic: decompensated	0/7	1/7
Dominant strictures	0/7	1/7
Ascending cholangitis[b]	1/7	3/7
Increase in vitamin D level, *nmol/L*[a]	57 (31–80)	43 (30–67)
Laboratory parameters at baseline[a]		
Bilirubin level, μ*mol/L*	22 (12–39)	43 (13–75)
ALT level, *IU/L*	61 (24–123)	74 (35–87)
AST level, *IU/L*	71.5 (33–90)	72 (45–115)
ALP level, *IU/L*	346 (115–393)	296 (172–430)
Albumin level, *g/dL*	46 (42–50)	43 (33–44)
Platelets, $\times 10^3/mm^3$	204 (130–298)	244 (204–327)

IBD, inflammatory bowel disease; UDCA, ursodeoxycholic acid.
[a]Values for continuous variables represent median (interquartile range).
[b]Represents clinical events within the preceding 2 months.