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Loss of CD28 Expression by Liver-Infiltrating T Cells Contributes to Pathogenesis of Primary Sclerosing Cholangitis

Evaggelia Liaskou[1], **Louisa E. Jeffery**[2], **Palak J. Trivedi**[1], **Gary M. Reynolds**[1], **Shankar Suresh**[1], **Tony Bruns**[1,3,4], **David H. Adams**[1], **David M. Sansom**[2,5], and **Gideon M. Hirschfield**[1]

[1]Centre for Liver Research and National Institute for Health Research Biomedical Research Unit in Liver Disease

[2]Medical Research Council Centre for Immune Regulation, School of Immunity and Infection, Institute of Biomedical Research, University of Birmingham, Birmingham, United Kingdom

[3]Department of Internal Medicine IV, Gastroenterology, Hepatology and Infectious Disease

[4]Center for Sepsis Control and Care, Jena University Hospital, Friedrich Schiller University of Jena, Jena, Germany

[5]Institute for Immunity and Transplantation, University College London, Royal Free Campus, London, United Kingdom

Abstract

Background & Aims—T-cell–mediated biliary injury is a feature of primary sclerosing cholangitis (PSC). We studied the roles of $CD28^-$ T cells in PSC and their regulation by vitamin D.

Methods—Peripheral and liver-infiltrating mononuclear cells were isolated from blood or fresh liver tissue. We analyzed numbers, phenotypes, functions, and localization patterns of $CD28^-$ T cells, along with their ability to activate biliary epithelial cells. We measured levels of tumor necrosis factor (TNF)α in liver tissues from patients with PSC and the effects of exposure to active vitamin D (1,25[OH]2D3) on expression of CD28.

Results—A significantly greater proportion of $CD4^+$ and $CD8^+$ T cells that infiltrated liver tissues of patients with PSC were $CD28^-$, compared with control liver tissue ($CD4^+$: 30.3% vs 2.5%, $P < .0001$; and $CD8^+$: 68.5% vs 31.9%, $P < .05$). The mean percentage of $CD4^+CD28^-$ T cells in liver tissues from patients with PSC was significantly higher than from patients with primary biliary cirrhosis or nonalcoholic steatohepatitis ($P < .05$). $CD28^-$ T cells were activated $CD69^+CD45RA^-$ C-C chemokine receptor (CCR)7^- effector memory and $perforin^+$ granzyme B^+ cytotoxic cells, which express CD11a, CX3CR1, C-X3-C motif receptor 6 (CXCR6), and CCR10—consistent with their infiltration of liver and localization around bile ducts. Compared with $CD28^+$ T cells, activated $CD28^-$ T cells produced significantly higher levels of interferon γ and

Address requests for reprints to: Gideon M. Hirschfield, MB BChir, PhD, FRCP, Centre for Liver Research, University of Birmingham, Wolfson Drive, Birmingham B15 2TT, United Kingdom. g.hirschfield@bham.ac.uk; fax: +44121 415 8701.

Author names in bold designate shared co-first authors.

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TNFα ($P < .05$), and induced up-regulation of intercellular cell adhesion molecule-1, HLA-DR, and CD40 by primary epithelial cells (3.6-fold, 1.5-fold, and 1.2-fold, respectively). Liver tissue from patients with PSC contained high levels of TNFα; TNFα down-regulated the expression of CD28 by T cells in vitro ($P < .01$); this effect was prevented by administration of 1,25(OH)2D3 ($P < .05$).

Conclusions—Inflammatory $CD28^-$ T cells accumulate in livers of patients with PSC and localize around bile ducts. The TNFα-rich microenvironment of this tissue promotes inflammation; these effects are reversed by vitamin D in vitro.

Keywords

Interferon; Autoimmunity; Biliary Epithelial Cells; Immune Regulation

Primary sclerosing cholangitis (PSC) is a poorly understood chronic immune-mediated biliary disease lacking effective treatment, as well as validated animal models. Despite features supporting a classic autoimmune etiology standard, immunosuppression is ineffective.1 As a result of progress in genetic studies, several risk loci have been identified for PSC; numerous loci for immunologically relevant proteins (eg, *HLA*, *CD28*, interleukin [*IL*]*2RA*, and *macrophage stimulating 1*).2 These findings parallel histologic observations that include a mixed inflammatory cell infiltrate consisting of lymphocytes, plasma cells, neutrophils, natural killer cells, Kupffer cells, and perisinusoidal macrophages.3 The majority of mononuclear cells in the portal infiltrates are T lymphocytes4 that produce high levels of tumor necrosis factor α (TNFα), supporting PSC as a predominantly T-helper 1-mediated disease.5

The cell surface molecule CD28 is a co-stimulatory molecule necessary for T-cell activation, survival, and proliferation,6 and the *CD28* locus is a newly recognized risk factor in PSC development. Prior work identified T cells lacking CD28 accumulating in various autoimmune diseases,7,8 and suggested that loss of CD28 occurs at chronic inflammatory sites as a consequence of continuous antigenic stimulation and TNFα exposure.9 $CD28^-$ T cells appear to be chronically activated immunopathogenic cells,10 less susceptible to regulation by $CD4^+CD25^+$ T regulatory cells (Tregs), thus making them potentially important drivers of persistent chronic inflammation.11 Although immunogenetic profiles underpin the risk of PSC, environmental factors are equally relevant. Vitamin D is an extrinsic factor repeatedly associated with autoimmunity, as well as cholestatic liver diseases.12,13 The local activation of vitamin D by immune cells suppresses the development of proinflammatory effector T cells while increasing the frequency and suppressive function of Tregs.14

We sought to elucidate the mechanisms of biliary injury in PSC using patient-derived samples, to study T-cell infiltration and CD28 expression, alongside intervention with vitamin D. Our data show expansion of $CD28^-$ T cells with an activated phenotype in human PSC liver, localization close to bile ducts, release of proinflammatory cytokines, and induction of epithelial cell activation. A TNFα-rich PSC microenvironment was evident and TNFα down-regulated T-cell CD28 expression in vitro, an effect prevented by vitamin D.

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Materials and Methods

Human Tissue and Blood

Fresh diseased liver tissue from our transplant program was available, as was nondiseased liver from surgical resections. Whole blood was obtained from healthy volunteers and PSC patients. All samples were collected after local research ethics committee approval and patient consent.

Isolation of peripheral blood and liver-infiltrating mononuclear cells—Peripheral blood mononuclear cells (PBMCs) and liver-infiltrating mononuclear cells (LIMCs) were isolated from peripheral blood and fresh human liver, respectively,15 as described in the Supplementary Materials and Methods section.

Isolation and culture of human primary biliary epithelial cells—Human biliary epithelial cells (BECs) were isolated from liver tissue, and cultured as previously reported16 and further described in the Supplementary Materials and Methods section.

Flow Cytometry

Flow cytometric analysis was performed on blood and liver-infiltrating T cells using a Cyan flow cytometer (Beckman Coulter, Bucks, United Kingdom), and analyzed using FlowJo (version 9; Treestar Inc, Ashland, OR) (see the Supplementary Materials and Methods section and Supplementary Table 1).

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

PBMCs from PSC patients were isolated and stained for CD3–fluorescein isothiocyanate, CD4-allophycocyanin, and CD28-phycoerythrin markers to allow isolation of $CD3^{+}CD4^{+/-}CD28^{+/-}$ subsets by fluorescence-activated cell sorting. Isolated cells were activated overnight and their cell-free conditioned media was used to culture primary BECs for 4 days (see the Supplementary Materials and Methods section and Supplementary Table 1).

Detection of Cytokine Expression Ex Vivo

PBMCs and LIMCs from PSC patients were isolated as described earlier, and stimulated in 96-well, round-bottomed tissue culture plates for 6 hours with plate-bound anti-CD3 (OKT3; 5 μg/mL) at 10^6 cells/well (see the Supplementary Materials and Methods section and Supplementary Table 1).

Long-term In Vitro Culture of $CD4^{+}$ T Cells

$CD4^{+}$ T cells were enriched to 94%–98% purity from PSC PBMCs using a negative selection antibody cocktail (StemCell Technologies, Manchester, United Kingdom) and stimulated with aCD3/aCD28 Dynabeads (Life Technologies, Paisley, United Kingdom), IL2 (50 U/mL; Peprotech, London, United Kingdom), in the presence/absence of TNFα (10 ng/mL; Peprotech), with or without $1,25(OH)_2D_3$ (10 nmol/L; ENZO Life Sciences, Exeter, United Kingdom). At 4 days, beads were removed using a magnet and cells were split to 0.5 × 10^6/mL. Cultures were assessed every 3–4 days and maintained at 0.5–1 × 10^6/mL.

Cytokines and $1,25(OH)_2D_3$ also were re-supplemented at these times, the concentration of IL2 being increased to 100 U/mL after 2 weeks.

Immunohistochemical Analysis

Dual-color immunohistochemistry was used to localize $CD4^+$ and $CD8^+$ T-cell subsets, and lymphocytes that lacked CD28 expression in human liver tissue (see the Supplementary Materials and Methods section and Supplementary Tables 1 and 2).

RNA Analysis

Total RNA was extracted from normal, PSC, and primary biliary cirrhosis (PBC) liver samples, using the RNeasy mini Kit (Qiagen, Manchester, United Kingdom), and analyzed as described in the Supplementary Materials and Methods section, for expression of TNFα, interferon γ (IFNγ), and IL17A by quantitative polymerase chain reaction analysis.

Cytokine Secretion Assays

The secretion of cytokines and chemokines from PSC LIMCs was investigated using the cytokine array panel A kit (R&D Systems, Abingdon, United Kingdom) (see the Supplementary Materials and Methods section).

Vitamin D Supplementation in PSC Patients

Patients were classified as 25(OH)D sufficient (>50 nmol/L), 25(OH)D insufficient (30–49 nmol/L), or severely 25(OH)D deficient (<30 nmol/L). Routine supplementation is advised for individuals with insufficient vitamin D levels (400 U/day colecalciferol). Those with severe deficiency are treated with high-dose replacement therapy (ergocalciferol capsules 50,000 U/wk for 5 weeks). Serum 25(OH)D levels were correlated with $CD28^-$ T-cell frequency in peripheral blood, before and after vitamin D therapy.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA) and SPSS software (SPSS, Inc, Chicago, IL). Data not normally distributed were evaluated using an unpaired Mann–Whitney, Wilcoxon matched-pairs signed rank, and Kruskal–Wallis tests. For correlations of $CD28^-$ T-cell frequency and clinical characteristics, Kolmogorov-Smirnov testing and the Spearman rank correlation coefficient were used. P values less than .05 were considered significant.

Results

$CD28^-$ T Cells Are More Frequent in Human PSC Liver Tissue

In PSC liver a reversal of the CD4:CD8 ratio (1:1.4) was detected compared with peripheral blood (2.3:1) (data not shown). $CD4^+$ T cells were found localized mainly to the portal tracts, and $CD8^+$ T cells were found localized mainly in the portal areas and in the parenchyma. Both $CD4^+$ and $CD8^+$ T cells were found in proximity to bile ducts (Figure 1*A*).

We studied the proportion of $CD4^+$ and $CD8^+$ T cells that were $CD28^-$ (Figure 1*B*). In the blood of PSC patients, 3.3% of $CD4^+$ T cells were $CD28^-$, whereas in PSC liver tissue this frequency was increased approximately 10-fold to 30.3% ($P < .0001$). By comparison, the frequencies of $CD4^+CD28^-$ T cells in normal blood and liver were only 0.96% and 2.5%, respectively. Higher frequencies of $CD28^-$ T cells were found in the $CD8^+$ compartment in both healthy controls and PSC patients. In healthy controls the frequencies of $CD8^+CD28^-$ cells were not significantly different in blood and liver (27.05% and 31.9%, respectively), whereas in PSC, $CD8^+CD28^-$ cells were increased in the blood (46.35%), and were significantly higher in the PSC liver (68.5%) when compared with uninflamed tissue (31.9%) ($P < .05$) (Figure 1*C*). We further studied the frequencies of $CD28^-$ T cells in control groups consisting of individuals with PBC and nonalcoholic steatohepatitis (NASH) liver diseases (Figure 1*D*). The proportion of $CD4^+$ T cells that were $CD28^-$ was significantly higher in PSC livers (30.3%) compared with PBC (15.0%; $P < .05$) and NASH livers (14.2%; $P < .05$). No significant difference was detected for $CD8^+CD28^-$ T cells.

$CD28^-$ T Cells Are Activated Memory Cells With Intracellular Stores of Cytotoxic Molecules

CD45RA and CCR7 expression was evaluated to classify $CD28^-$ T cells, in PSC and normal control livers, into naive ($CD45RA^+CCR7^+$; T naive), central memory ($CD45RA^-CCR7^+$; T central memory), effector memory ($CD45RA^-CCR7^-$; T effector memory), and terminally differentiated effector memory RA ($CD45RA^+CCR7^-$; terminally differentiated effector memory RA) populations (Figure 2*A*). Our data showed that $CD4^+CD28^-$ T cells in both blood and liver of PSC patients were mainly effector memory cells. PSC liver-infiltrating $CD8^+CD28^-$ T cells also were effector memory cells; however, in peripheral circulation they showed a terminally differentiated effector memory RA phenotype (Figure 2*A*). The expression of activation and exhaustion markers was examined further on $CD28^-$ T cells from PSC liver and blood, as shown in the representative contour plots and histograms (Figure 2*B*). The expression levels between $CD28^-$ and $CD28^+$ populations were not significantly different (Supplementary Figure 1*A*), but differences were seen between $CD28^-$ cells isolated from liver vs blood (Figure 2*C*). A higher proportion of $CD4^+CD28^-$ T cells from liver expressed the activation marker CD69 (42.8% vs 17.81%; $P = .05$) (Figure 2*C*), and more liver-infiltrating $CD8^+CD28^-$ T cells compared with circulating $CD8^+CD28^-$ T cells (49.38% vs 10.62%, respectively; $P < .0001$) (Figure 2*C*). Expression of the IL2-Rα chain (CD25) was generally low; only 11.7% and 4.8% of peripheral blood $CD4^+CD28^-$ and $CD8^+CD28^-$ T cells, respectively, expressed CD25, and expression by liver-infiltrating $CD28^-$ cells was less than 2% (Figure 2*C*). $CD8^+CD28^-$ T cells from circulation and liver tissue expressed T-cell immunoglobulin domain and mucin domain 3 (TIM3) at very low to undetectable levels (<1%). However, PSC liver-infiltrating $CD4^+CD28^-$ T cells showed 8.1% TIM3 expression, which was 4 times higher compared with circulating cells (2.0%; $P < .05$). Circulating $CD4^+$ and $CD8^+CD28^-$ T cells (27.2% and 18.7%, respectively) expressed programmed cell-death 1, whereas liver-infiltrating cells expressed slightly lower levels (24.6% and 13.7%, respectively) (Figure 2*C*). Freshly isolated peripheral blood $CD4^+$ and $CD8^+CD28^-$ T cells contained intracellular stores of granzyme B and perforin, but these cytotoxic molecules were absent from their $CD28^+$ counterparts ($P < .01$, $P < .001$) (Figure 2*D* and *E*). $CD4^+$ and $CD8^+CD28^-$ T cells in PSC liver and disease control (DC) (PBC and

NASH) groups were phenotypically similar. In the DC group, however, significantly higher CD25 expression was detected ($P < .05$) (Supplementary Figure 1*B*).

CD28$^-$ T Cells Are Equipped With Adhesion Molecules and Chemokine Receptors That Promote Tissue Infiltration and Localization Close to Bile Ducts

We investigated the mechanisms of CD28$^-$ T-cell infiltration into liver tissue by studying the expression of adhesion molecules and chemokine receptors previously associated with migration into and retention within the liver. Higher frequencies of CX3CR1$^+$ CD4$^+$ and CD8$^+$CD28$^-$ T cells were found in circulation than in liver (66.9% vs 43.5% for CD4$^+$ and 72.5% vs 20.8% for CD8$^+$ in peripheral blood vs liver, respectively). Frequencies of CX_3CR1^+ CD28$^+$ cells were significantly lower ($P < .05$) in the blood (Figure 3*A*). Of all liver-infiltrating CD4$^+$CD28$^-$ and CD28+ T cells in the PSC liver, 54.1% and 40.3% expressed CXCR6, respectively, levels much higher than their circulating counterparts. The expression of CXCR6 by CD8$^+$CD28$^-$ and CD28$^+$ cells was less than 38%, whereas more than 40% of CD4$^+$ T cells expressed CXCR6. PSC liver-infiltrating CD4$^+$ and CD8$^+$CD28$^-$ T cells expressed higher levels of CCR10 compared with their counterparts in the circulation. Moreover, 26.6% and 22.7% of PSC liver-infiltrating CD4$^+$CD28$^-$ and CD28$^+$ cells, respectively, expressed CCR9, at levels higher than circulating cells (6.9% and 5.2%, respectively). CCR9 expression was observed in less than 6.5% of total CD8$^+$ T cells in both peripheral circulation and liver of PSC patients. CD11a was expressed at high levels on both CD28$^-$ and CD28$^+$ T cells of both lineages in both blood and liver, whereas CD62L (L-selectin) was barely expressed (<6%) by liver-infiltrating CD28$^-$ and CD28$^+$ T cells of both lineages, consistent with them being memory/effector T cells. Although CD62L expression was significantly higher in CD4$^+$ and CD8$^+$ T cells in circulation, CD28$^-$ T cells from both subsets expressed significantly lower levels (44.8% and 40.6%, respectively) than their CD28$^+$ counterparts (93.3% and 80.7%, respectively) ($P < .01$, $P < .001$) (Figure 3*A*). We further observed that CD28$^-$ T cells localize around bile ducts in human PSC liver tissue (Figure 3*B*).

The chemokine receptor expression was studied further on PBC patients. PSC liver-infiltrating CD4$^+$CD28$^-$ T cells expressed higher levels of CCR9 when compared with PBC (26.6% vs 12.5%) (Supplementary Figure 2*B*). Similarly, a higher proportion of CD4$^+$CD28$^+$ T cells in PSC liver expressed CCR9 when compared with PBC (22.7% vs 7.1%) (Supplementary Figure 2*B*). The absolute frequency of CCR9$^+$ T cells in peripheral blood of PSC patients was low, but remained higher compared with PBC patients (CD4$^+$CD28$^-$: 6.9% vs 4.3% and CD4$^+$CD28$^+$: 5.2% vs 3.3%) (Supplementary Figure 2*A*). In PBC peripheral blood, significantly higher levels of CD8$^+$CD28$^+$ T cells expressed CX_3CR1 (50%) when compared with PSC blood (12.1%; $P < .05$). Higher levels of PBC liver-infiltrating CD8$^+$CD28$^{+/-}$ T cells also expressed CX_3CR1 when compared with PSC. A higher proportion of PSC peripheral blood and liver-infiltrating CD4$^+$ and CD8$^+$ CD28$^-$ T cells expressed CCR10 compared with PBC (Supplementary Figure 2).

CD28$^-$ T Cells Release Proinflammatory Cytokines and Induce Activation of BECs

To further examine the role of CD28$^-$ T cells in PSC pathology, we studied their ability to produce proinflammatory cytokines immediately after isolation. More than 40% of

peripheral blood $CD4^+CD28^-$ T cells produced IFNγ after 6 hours of stimulation with anti-CD3, which was significantly higher compared with their $CD28^+$ counterparts ($P < .05$). Peripheral blood $CD8^+CD28^-$ T cells produced significantly more IFNγ (28%) when compared with $CD8^+CD28^+$ T cells ($P < .05$) (Figure 4*A*). Both $CD4^+$ and $CD8^+CD28^-$ T cells secreted more TNFα (14.5% and 7.2%, respectively) than $CD28^+$ T cells (Figure 4*A*). PSC liver-infiltrating $CD28^-$ T cells from both $CD4^+$ and $CD8^+$ subsets produced IFNγ (16.6% and 11.0%, respectively), but levels were not significantly different from their $CD28^+$ counterparts (Figure 4*B*). Liver-infiltrating cells also produced TNFα but at a lower level than IFNγ. In contrast to blood, liver-infiltrating $CD28^+CD4^+$ and $CD8^+$ T cells produced more TNFα when compared with $CD28^-$ T cells (Figure 4*B*).

Studies have reported that Tregs can inhibit cytokine production by $CD28^-$ T cells.11 In PSC liver tissue we detected low frequencies of $CD3^+CD4^+CD25^{hi}CD127^{low}$ Tregs at levels similar to those seen in normal tissue (Figure 4*C* and *D*).

Because $CD28^-$ and $CD28^+$ T cells showed different proinflammatory cytokine profiles, we were interested to observe the effect of their secretomes on BEC activation and survival. Conditioned media from activated $CD4^+CD28^-$ and $CD8^+CD28^-$ T cells induced the expression of intercellular cell adhesion molecule-1 (ICAM1), HLA-DR, and CD40 (Figure 5*A*-*C*) on primary BECs. No significant differences, however, were detected on the effects between $CD28^-$ and $CD28^+$ T cells. Neither $CD28^-$ nor $CD28^+$ cells induced leukocyte function-associated antigen 3 expression (data not shown). Only 8% of BECs were viable after 4 days of culture with activated $CD4^+CD28^-$ T-cell–conditioned media, whereas 29%–41% of BECs were alive when cultured with the media of all other subsets (Figure 5*D*).

TNFα Induces the Differentiation of $CD28^-$ T Cells and Vitamin D Overcomes This Effect

We detected significantly increased levels of TNFα and IFNγ messenger RNA in the PSC liver microenvironment compared with normal tissue (4.8 vs 1.7, $P < .05$; and 17.5 vs 3.2, $P < .01$, respectively) (Figure 6*A*) and increased levels of IL17A messenger RNA relative to PBC (Supplementary Figure 3*A*). PSC liver-infiltrating mononuclear cells were able to release TNFα and IFNγ after 24 hours of culture even without stimulation. IL2, IL4, and IL10 were low to undetectable (Figure 6*B*). PSC LIMCs also were producers of IL17, IL23, IL6, IL8, CCL2, CCL3, and CCL4 (Supplementary Figure 3*B*).

We cultured PSC $CD4^+$ T cells with recombinant (r)TNFα in vitro and found a significant increase in $CD4^+CD28^-$ T cells after 7–21 days. After 21 days, 22.9% of $CD4^+$ T cells spontaneously lost CD28 expression whereas this expression increased to 37.6% in the presence of exogenous rTNFα. Given the immunomodulatory effects of vitamin D we tested further whether the active form of vitamin D $1,25(OH)_2D_3$ had an effect on CD28 expression in the presence and/or absence of TNFα. Our data showed that $1,25(OH)_2D_3$ markedly suppressed the development of $CD28^-$ T cells (2%; $P < .05$) in vitro, even in cultures in which exogenous rTNFα was present (3.7%; $P < .05$) (Figure 6*C* and *D*).

Frequencies of $CD4^+CD28^-$ T Cells in PSC Patients Supplemented With Vitamin D

To examine whether vitamin D status influences $CD28^-$ T-cell frequencies in PSC patients we measured their peripheral blood $CD28^-$ T-cell frequencies and serum vitamin D

(25[OH]D) concentration. A trend toward a negative correlation with serum vitamin D was seen (nonparametric Spearman rank correlation test: $P = .1699$; and Spearman ρ(r): −0.1451) (Figure 7*A*). No significant associations were found between peripheral $CD28^-$ T-cell frequency and patient age, ethnicity, severity/activity of liver disease, or inflammatory bowel disease status (data not shown). In 14 PSC patients with severe vitamin D deficiency who returned for a routine clinical review, we repeated $CD4^+CD28^-$ T-cell frequency and vitamin D measurements. Our data showed that supplementation of vitamin D increased levels sufficiently (Figure 7*B*) and in 7 of these patients the $CD4^+CD28^-$ T-cell frequency was reduced by 5.8-fold (Figure 7*C*).

Discussion

We provide evidence that a significant proportion of PSC liver-infiltrating T cells lack CD28, an important immunoregulatory protein implicated in PSC pathogenesis by genetic association studies. We showed that $CD28^-$ T cells are recently activated memory/effector cells, equipped with a combination of adhesion molecules and chemokine receptors that allow infiltration into liver tissue and specific localization around bile ducts. Upon re-activation, $CD28^-$ memory/effector T cells release high levels of TNFα and IFNγ proinflammatory cytokines, which can activate neighboring BECs to express adhesion and costimulatory molecules. The important role of local TNFα is suggested by the finding of high intrahepatic levels of TNFα in PSC and its ability to induce CD28 loss in cultured T cells. The ability of vitamin D to prevent this effect suggests a novel therapeutic action of this vitamin in PSC. However, more extensive studies are required to evaluate this effect, particularly in larger cohorts of PSC patients, with vitamin D supplementation remaining a speculative approach to intervention at this time.

We show that $CD28^-$ T cells express high levels of CD69 and no CD45RA, suggesting an active effector/memory phenotype. Both $CD4^+$ and $CD8^+CD28^-$ T cells expressed programmed cell-death 1, a marker associated with both activated and exhausted T cells.17,18 PSC liver-infiltrating $CD4^+CD28^-$ T cells expressed low levels of TIM3, which generally is considered a marker of T-cell exhaustion, suggesting that $CD28^-$ cells in PSC liver are activated effector cells.19 TIM3 levels were higher on $CD28^-$ than $CD28^+$ cells, further suggesting that $CD28^-$ cells have undergone many cycles of activation. The higher CD25 expression on $CD28^-$ cells of the DC group agrees with our data showing the presence of more $CD28^-$ cells in PSC because CD25 expression is dependent on CD28-mediated signals. Both $CD4^+$ and $CD8^+$ $CD28^-$ cells readily produced proinflammatory cytokines upon activation and contained perforin and granzyme B.

Our data suggest mechanisms to explain the accumulation of $CD28^-$ T cells in human PSC liver tissue by migration from peripheral blood. We found that $CD28^-$ T cells are equipped with CD11a and chemokine receptors CX_3CR1, CXCR6, and CCR10, which allow their infiltration into tissue and migration toward BECs, which strongly express the chemokine ligands CX_3CL1, CXCL16, and CCL28.20–22 This recruitment may be amplified in a paracrine fashion because $CD28^-$ T cells could release TNFα and IFNγ proinflammatory cytokines, which increase adhesion molecule expression and chemokine secretion from BECs. The ability of $CD28^-$ T cells to up-regulate ICAM1, HLA-DR, and CD40 on BECs

could promote BECs as T-cell targets in PSC. Increased ICAM1 expression on bile ducts is a characteristic feature of liver diseases in which bile ducts are the major targets of immune-mediated destruction, such as PSC and PBC.23 Increased binding of effector cells to cholangiocyte ICAM1 not only promotes accumulation but also allows recognition of major histocompatibility complex antigens and the activation of cytolytic mechanisms that induce cholangiocyte apoptosis.24

Previous studies showed the ability of Tregs to inhibit cytokine production by $CD28^-$ T cells but not their proliferation.11 In PSC liver tissue we detected low frequencies of $CD4^+CD25^{hi}CD127^{low}$ Tregs at levels similar to those seen in normal tissue, and lower than the frequencies previously reported for other autoimmune and chronic inflammatory conditions.25 A reduction in the frequency of peripheral blood Tregs and in $Foxp3^+$ T cells in PSC liver, with an apparent impaired suppressive capacity, recently was reported.26 Reduced numbers of Tregs in PSC liver therefore may lead to uncontrolled cytokine production by $CD28^-$ T cells. Other investigators have shown that TNFα can modulate the expression of CD28 at a transcriptional level.9 Our data showed high levels of TNFα in human PSC liver, suggesting that the local cytokine microenvironment may promote the differentiation and accumulation of $CD28^-$ T cells. We also showed that activated liver-infiltrating $CD28^+$ T cells produce high levels of TNFα, which may act in an autocrine manner to down-regulate their own CD28 expression. CD28 signaling is required for IL2 production, which in turn is needed for induction of Tregs. Therefore, in PSC liver, in which 30.3% of $CD4^+$ T cells are $CD28^-$ T cells, IL2 production likely is defective and this might be a contributing factor for the reduced frequency of Tregs in PSC liver tissue. Because both $CD28^-$ and $CD28^+$ T cells localize close to bile ducts and produce TNFα, we suggest that such local cytokine production can induce the emergence of $CD28^-$ T cells and the amplification of the inflammatory response as indicated by their ability to activate BECs. TNFα also can activate BECs to express receptors such as tumor necrosis factor receptor superfamily members involved in apoptosis and cholangiocyte death,6 further implicating TNFα in the pathogenesis of biliary destruction in PSC. Correspondingly, in our coculture system, we observed that peripheral blood $CD4^+CD28^-$, which produced the highest levels of TNFα, promoted the greatest death of BECs. $CD28^-$ T cells also are cytotoxic cells, expressing perforin and granzyme B, and other investigators have shown the synergistic effect of them in cholangiocyte injury.27

We also show that $1,25(OH)_2D_3$ can increase the absolute expression of CD28 on in vitro stimulated cells and prevent emergence of the $CD28^-$ population during long-term culture, even in the presence of exogenous rTNFα. This finding is consistent with recent data showing that vitamin D could increase the median fluorescence of CD28 in $CD4^+$ T cells from healthy controls and multiple sclerosis patients during in vitro stimulation.28 We observed that in a subset of PSC patients with profound deficiency in vitamin D, the frequency of $CD28^-$ T cells in the circulation was reduced after supplementation and correction of hypovitaminosis. However, in as many patients an increase or stability of $CD28^-$ T-cell frequency was observed. No significant clinical differences were detected between the cohorts experiencing a reduction in $CD28^-$ frequency vs those in whom $CD28^-$ frequency remained stable/increased (Supplementary Table 3). We suggest that, in vivo, a more complex set of immunoregulatory interactions is likely to participate and other factors

in the disease background of the patients also might affect results. Moreover, we speculate that in patients in whom supplementation of vitamin D had no obvious effect, it actually may have prevented the further expansion of $CD28^-$ proinflammatory cells. We therefore highlight a potential therapeutic use of vitamin D in PSC that merits further in-depth evaluation.

Our findings are important in the context of a disease that lacks therapy and has poorly refined animal models in which to test new therapies. We recognize that the increased frequency of $CD28^-$ T cells is a feature of chronic inflammatory and autoimmune disease. Notably, our data show a significant difference in the frequency of $CD4^+CD28^-$ T cells in PSC liver, compared with PBC and NASH, with PSC liver-infiltrating T cells also showing high CCR9 expression, further suggesting the specificity of these cells for PSC. It is important, however, to highlight the inherent difficulty of comparing and contrasting liver diseases that are fundamentally distinct because of the different patterns of liver injury and response to injury over time. Although $CD28^-$ T cells were detected in all 3 chronic liver diseases studied, we believe specificity lies in the antigen specificity of these cells. Genetic associations at the *CD28* locus have been identified in several autoimmune diseases (eg, PSC, celiac disease, type 1 diabetes, rheumatoid arthritis, alopecia areata, and autoimmune thyroid disease), but notably not in well-powered assessments in inflammatory bowel disease or PBC. The basis of the profound pleiotropy observed for risk genes in immune-mediated conditions is poorly understood, and our findings provide the contextual data for a PSC-centered evaluation of *CD28* risk variants.

The frequent, and often profound, deficiency of vitamin D associated with liver disease may make the mechanism particularly applicable to cholestatic liver disease; indeed, increased numbers of $CD4^+CD28^-$ T cells have been reported in association with bile duct damage in PBC.29 Our vitamin D data are provocative and interesting, albeit presently still are a hypothesis-generating concept at this time.

In summary, in PSC patients we show the significance of $CD28^-$ cells, which release proinflammatory cytokines and cytotoxic mediators and express the necessary receptors to allow their localization close to the bile ducts and induce their activation. We provide evidence that TNFα, which is enriched in the PSC liver microenvironment, can enhance the accumulation of $CD28^-$ T cells and show that this effect can be overcome with vitamin D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

BEC	biliary epithelial cell
CCR	C-C chemokine receptor
CXCR	C-X3-C motif receptor
DC	disease control
ICAM1	intercellular cell adhesion molecule-1
IFNγ	interferon γ
IL	interleukin
LIMC	liver infiltrating mononuclear cell
NASH	nonalcoholic steatohepatitis
PBC	primary biliary cirrhosis
PBMC	peripheral blood mononuclear cell
PSC	primary sclerosing cholangitis
rTNFα	recombinant tumor necrosis factor α
TIM3	T-cell immunoglobulin domain and mucin domain 3
TNFα	tumor necrosis factor α
Treg	T regulatory cell

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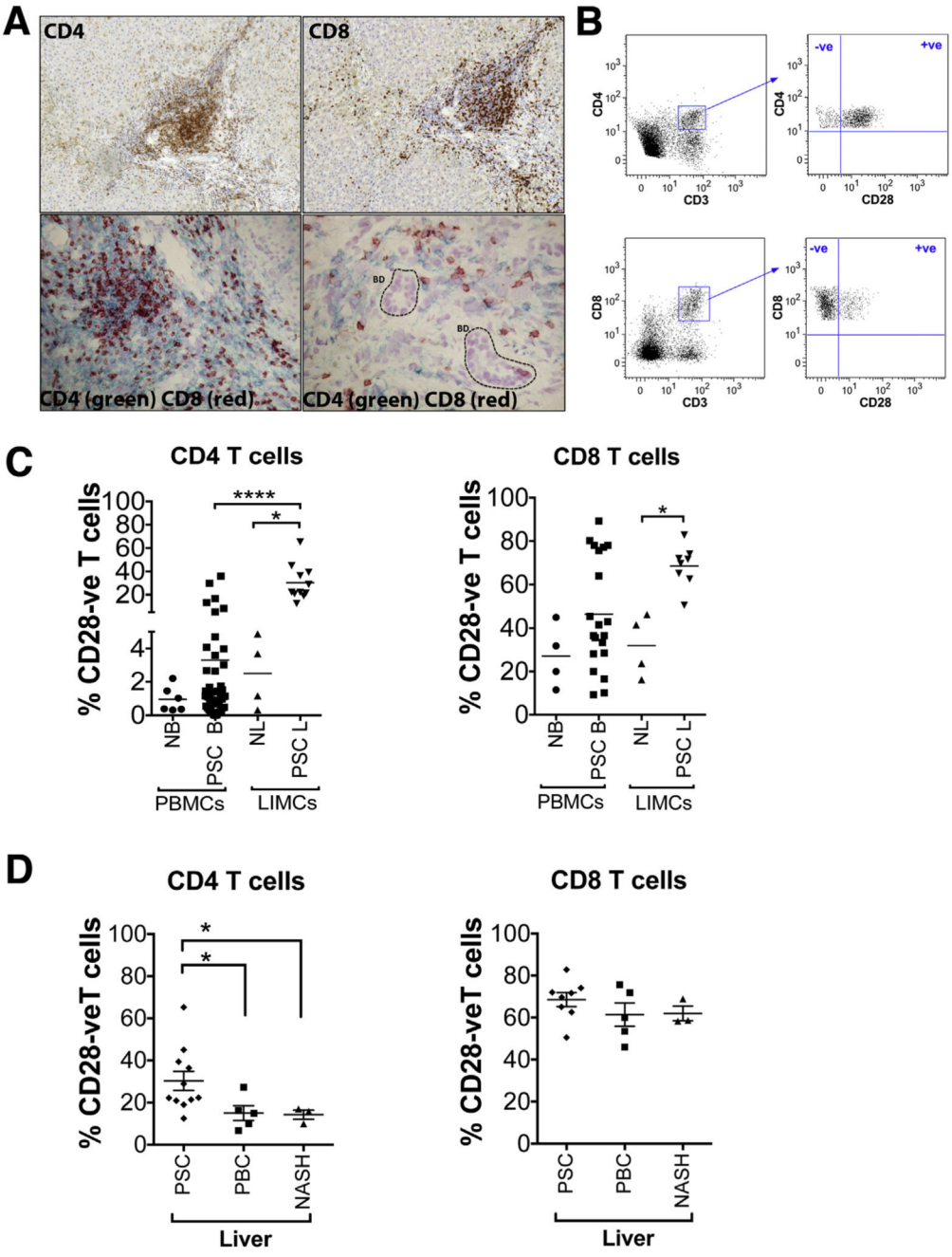

Figure 1.

CD28$^-$ T cells are more frequent in human PSC liver. (*A*) Single-color (magnification, 200×) and dual-color immunohistochemistry (CD4 [*green*] and CD8 [*red*]; 200× and 400×, respectively) showing localization of CD4$^+$ and CD8$^+$ T cells in human PSC liver tissue. BD, bile duct. (*B*) Representative flow cytometry dot plots showing the gating strategy defining CD28$^-$ T cells. (*C*) The frequency of CD3$^+$CD4$^+$CD28$^-$ and CD3$^+$CD8$^+$CD28$^-$ T cells in human PSC blood (n = 50 and n = 20, respectively) and liver (n = 11 and n = 8, respectively) was analyzed by flow cytometry and compared with blood (n = 6 and n = 4,

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respectively) and liver of healthy controls (n = 4, both). *$P < .05$, ****$P < .0001$. The ratio of $CD4^+CD28^-$ T cells in PSC LIMCs:PBMCs was 9:1 and in normal LIMCs:PBMCs was 3:1. The ratio of $CD8^+CD28^-$ T cells in PSC LIMCs:PBMCs was 1.4:1 and in normal LIMCs:PBMCs was 1:1. (*D*) The frequency of $CD3^+CD4^+CD28^-$ and $CD3^+CD8^+CD28^-$ T cells was analyzed in human PSC liver (n = 11 and n = 8, respectively) and compared with PBC (n = 5) and NASH (n = 3) human livers. *$P < .05$. NB, normal blood; NL, normal liver; PSC B, PSC blood; PSC L, PSC liver.

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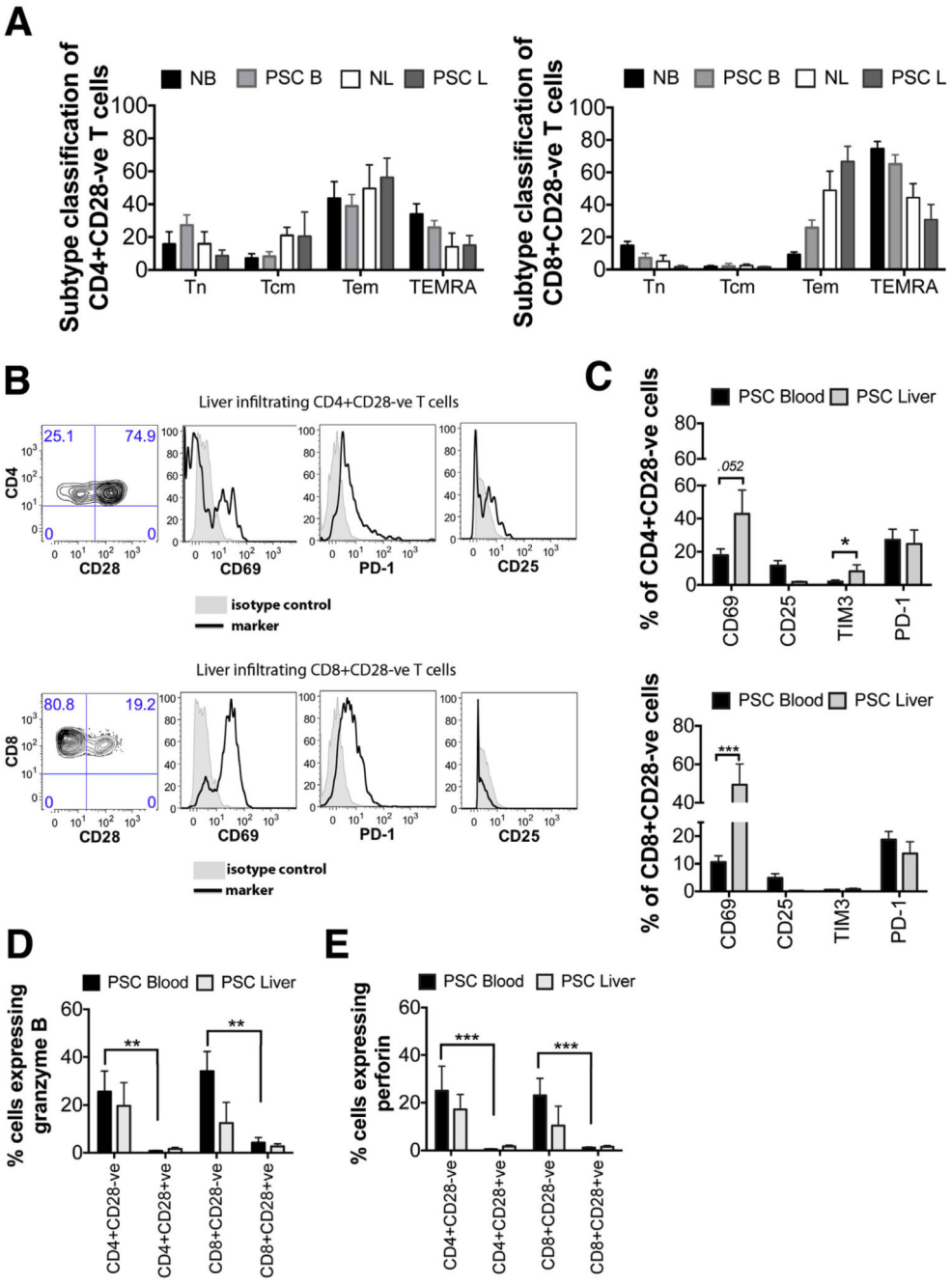

Figure 2. Phenotypic characterization of CD28⁻ T cells in blood and liver of PSC patients.

(*A*) The expression of CD45RA and CCR7 on $CD4^+CD28^-$ and $CD8^+CD28^-$ T cells was analyzed by flow cytometry for normal blood (NB) PBMCs (n = 4), PSC blood (PSC B) PBMCs (n = 14), normal liver (NL) LIMCs (n = 4), and PSC liver (PSC L) LIMCs (n = 4). Cells were classified into naive ($CD45RA^+CCR7^+$; T naive [Tn]), central memory ($CD45RA^-CCR7^+$; T central memory [Tcm]), effector memory ($CD45RA^-CCR7^-$; T effector memory [Tem]), and terminally differentiated effector memory RA ($CD45RA^+CCR7^-$; terminally differentiated effector memory RA [TEMRA]) populations.

(*B*) $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were selected and $CD28^-$ T cells gated as shown in the representative contour plots were analyzed for CD69, programmed cell-death 1 (PD-1), CD25, and TIM3 expression. Representative histograms for the marker (*solid line*) and its isotype control (*shaded area*) are shown. (*C*) Data show the percentage (mean ± SEM) 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]), TIM3 (n = 14 [blood] and n = 5 [liver]), and PD-1 (n = 14 [blood] and n = 6 [liver]). $*P < .05$, $***P < .001$. (*D* and *E*) $CD28^-$ and $CD28^+$ T lymphocytes from PSC PBMCs (n = 9) and LIMCs (n = 5) were analyzed by flow cytometry for the presence of intracellular deposits of granzyme B and perforin. $**P < .01$, $***P < .001$.

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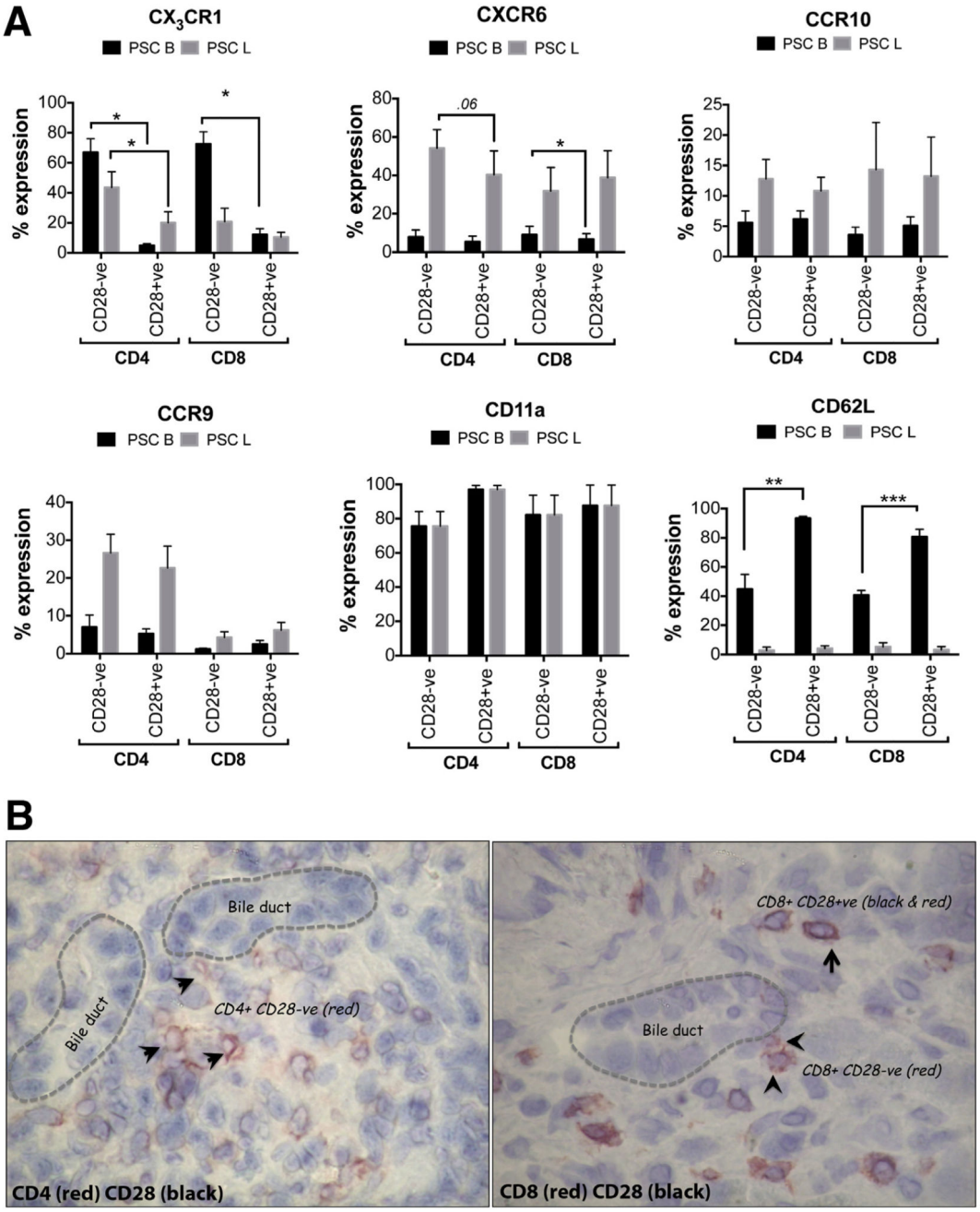

Figure 3.

CD28$^-$ T cells are equipped with adhesion molecules and chemokine receptors that promote tissue infiltration and localization close to the bile ducts. (*A*) The expression of chemokine receptors CX_3CR1 (n = 7 [blood] and n = 6 [liver]), CXCR6 (n = 7 [blood] and n = 6 [liver]), CCR9 (n = 4 [blood] and n = 5 [liver]), and CCR10 (n = 9 [blood] and n = 5 [liver]), and adhesion molecules CD11a (n = 4 [blood] and n = 4 [liver]), and CD62L (n = 7 [blood] and n = 3 [liver]) on CD28$^-$ and CD28$^+$ T cells of CD4$^+$ and CD8$^+$ T cells from blood (PSC B) and liver (PSC L) of PSC patients was analyzed using flow cytometry. Data show the

percentages of $CD28^-$ and $CD28^+$ T cells that express the chemokine receptors. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. (*B*) Representative dual-color immunohistochemistry image showing the localization of $CD4^+CD28^-$ and $CD8^+CD28^-$ T cells in human PSC liver tissue (magnification, 400×). *Arrowheads* point to CD28- ve T cells in red and *arrows* point to CD28+ T cells in black and red.

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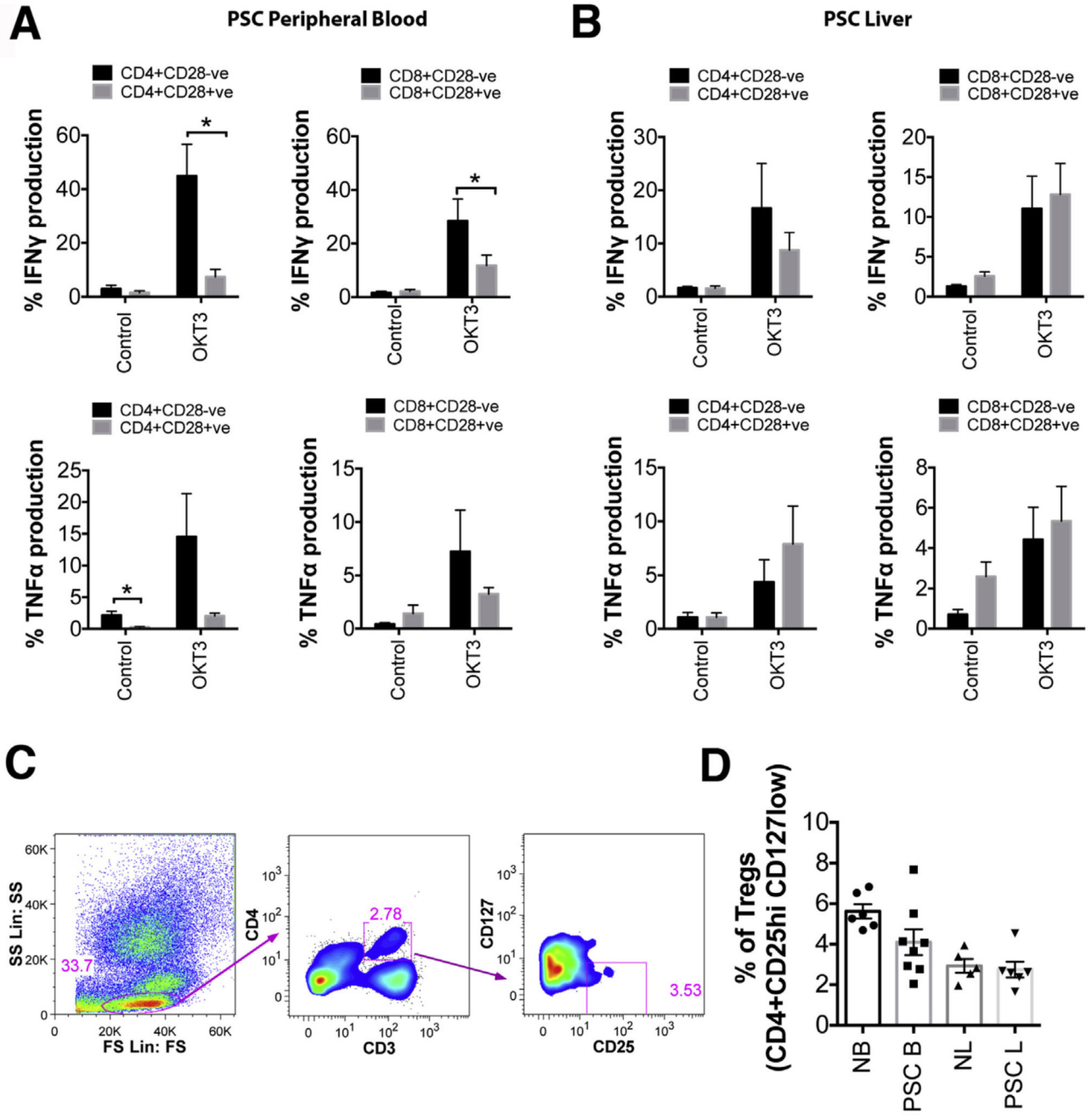

Figure 4.

CD28^{-} T cells release proinflammatory cytokines and their supernatants are able to activate human primary BECs in vitro. Data from (*A*) 6 PSC peripheral blood samples and (*B*) 6 PSC liver samples showing TNFα and IFNγ production by CD4^{+}CD28$^{+/-}$ and CD8^{+}CD28$^{+/-}$. *$P < .05$. (*C*) Representative flow cytometry plots showing the gating strategy for defining T-regulatory cells. (*D*) Data show the percentage of CD3^{+}CD4^{+} T cells that are CD25^{hi} CD127^{low}, in blood and liver of normal and PSC patients (normal blood [NB], n = 6; PSC blood [PSC B], n = 8; normal liver [NL], n = 5; PSC liver [PSC L], n = 6).

Each *shape* represents the value from each individual, and the *line* represents the mean value ± SEM.

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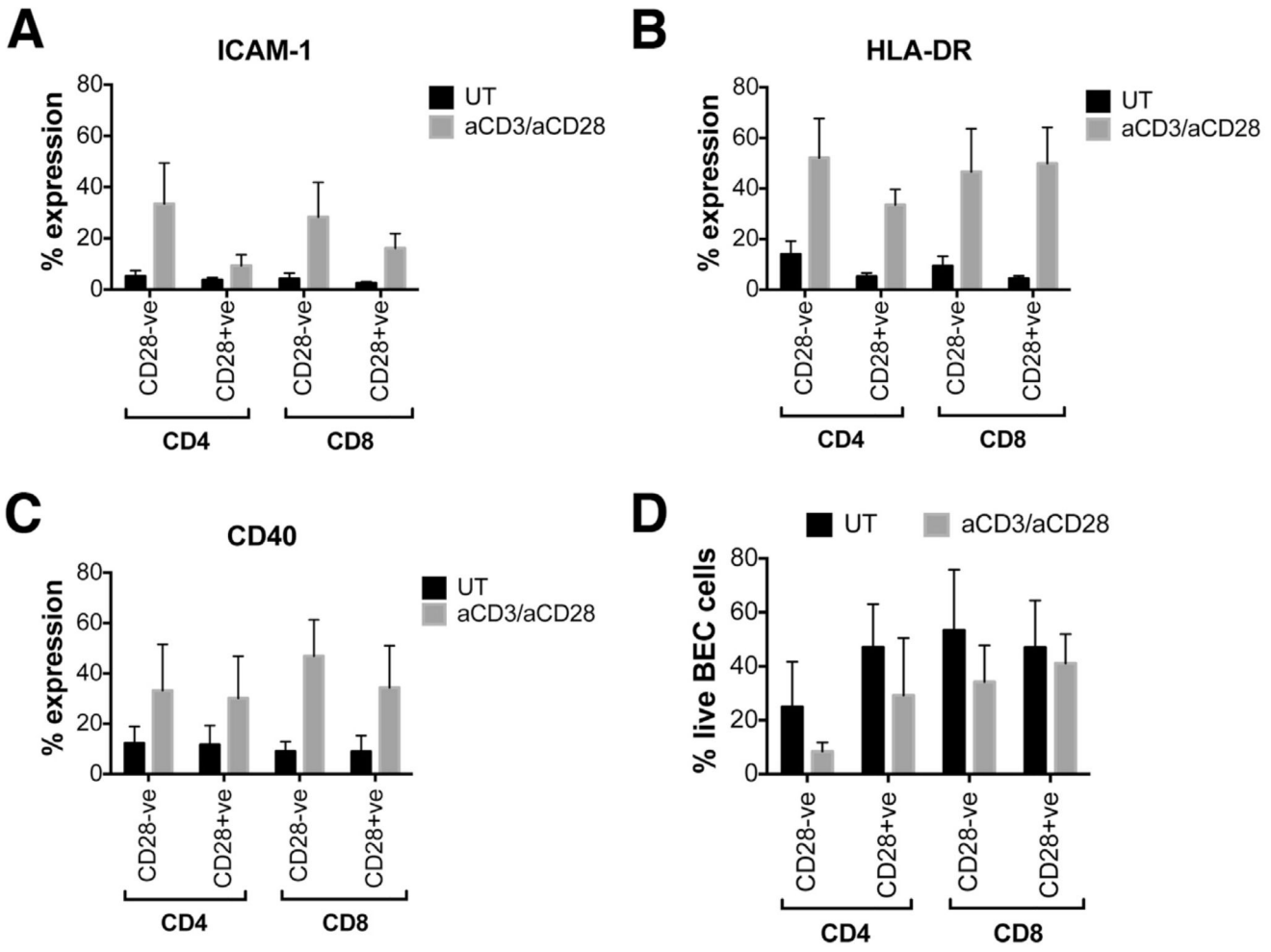

Figure 5.

Supernatants from CD28$^-$ T cells are able to activate human primary BECs in vitro. (*A*–*C*) Data show the indirect effects of untreated (UT) and aCD3-/aCD28-treated, cell-sorted CD28$^-$ and CD28$^+$ T cells on the percentage expression of ICAM1, HLA-DR, and CD40 (n = 5) on BECs. (*D*) Data show the percentage of live BECs after 4 days of coculture with the T-cell–conditioned media (n = 3).

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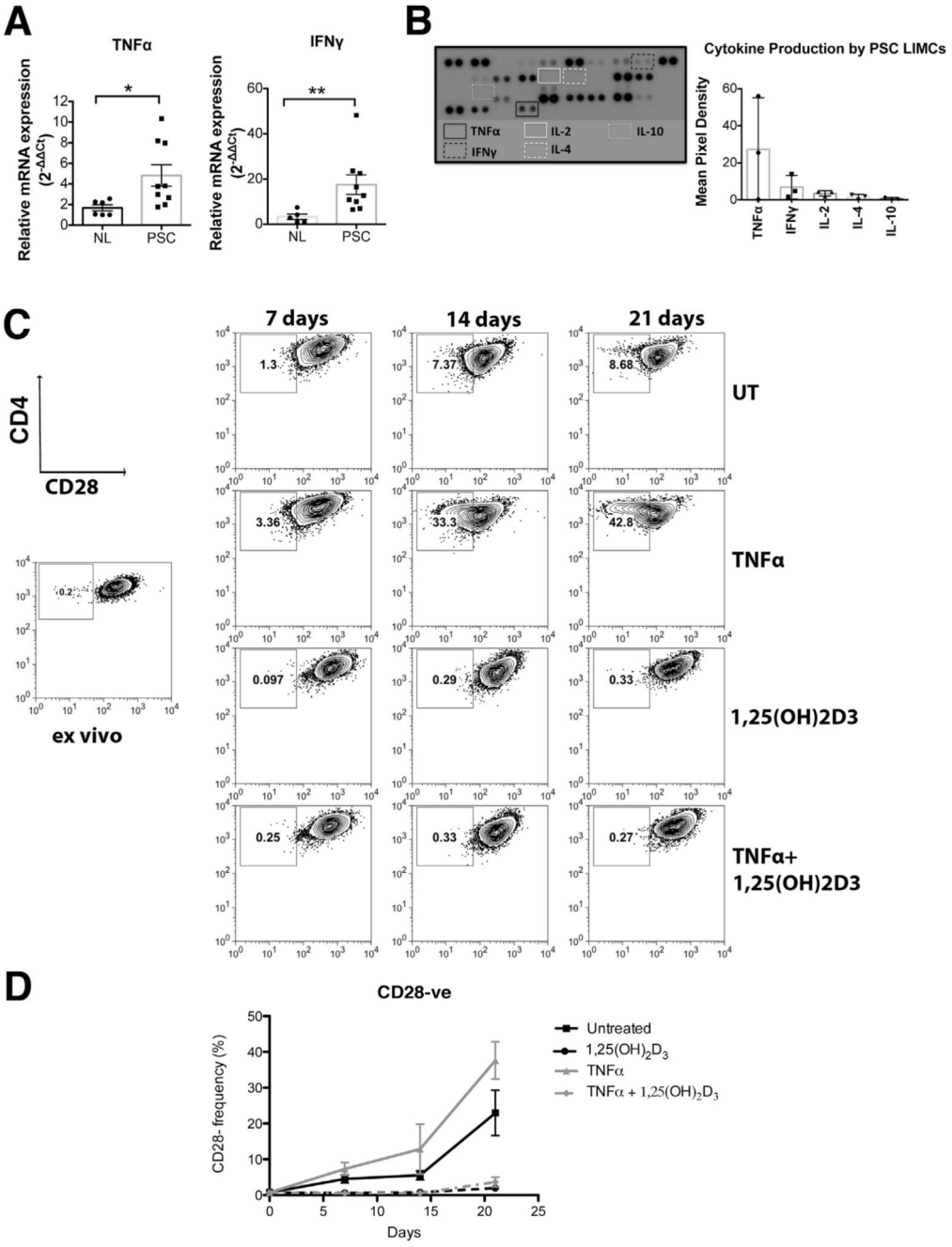

Figure 6.

TNFα enhances the emergence of CD28$^-$ T cells and 1,25(OH)$_2$D$_3$ overcomes this effect. (*A*) TNFα and IFNγ messenger RNA (mRNA) expression in 6 normal liver (NL) and 9 PSC liver tissues was measured by quantitative polymerase chain reaction. Scatter dot plots show relative mRNA levels in diseased livers with respect to 1 NL tissue (mean ± SEM). *$P < .05$, **$P < .01$. (*B*) Cytokines and chemokines released from 3 PSC LIMCs as analyzed with the Human Cytokine Array kit (R&D Systems, Abingdon, United Kingdom). Expression levels are reported as mean pixel density in arbitrary units. (*C*) CD4$^+$ T cells from blood of PSC

patients were stimulated with aCD3/aCD28 beads and cultured for 21 days in the presence or absence of TNFα, with or without 1,25$(OH)_2D_3$. The frequency of $CD28^-$ cells was measured at 0, 7, 14, and 21 days by flow cytometry as shown in the representative contour plots. (*D*) Data from 7 donors.

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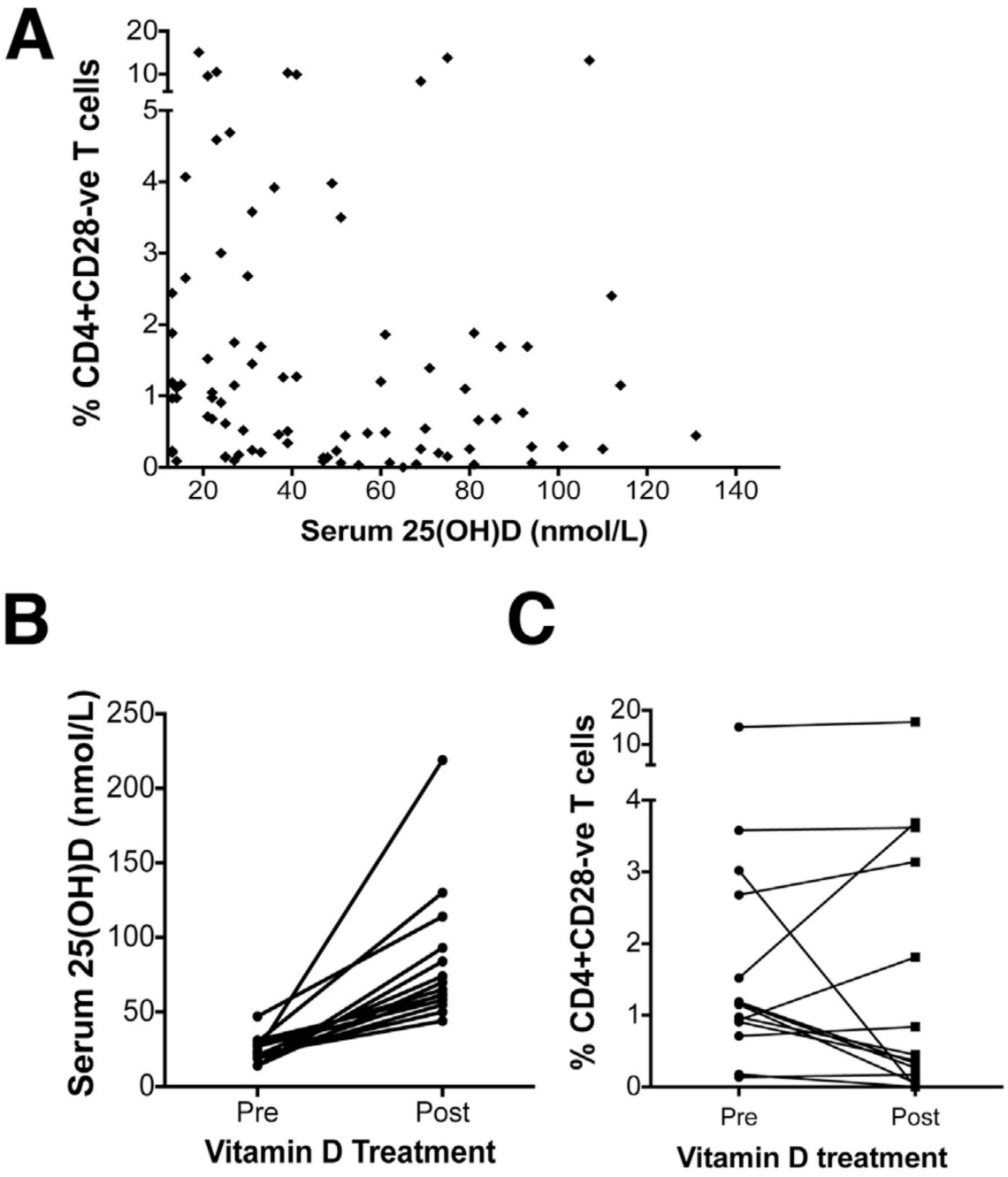

Figure 7.

$CD4^+CD28^-$ T cells in PSC patients supplemented with vitamin D. (*A*) Serum 25(OH)D levels of 92 PSC patients were correlated with the frequency of $CD4^+CD28^-$ T cells in circulation. Each dot represents the value from each individual. (*B*) PSC patients who had insufficient serum vitamin D levels were supplemented with vitamin D as part of their medical treatment. The levels of serum 25(OH) vitamin D before and after supplementation are shown. (*C*) Data show the percentage of $CD4^+CD28^-$ T cells after several weeks when

serum vitamin D levels reached sufficiency. Data were analyzed using the Wilcoxon matched-pairs signed rank test ($P = .669$).

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