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Direct-Acting Antiviral Therapy Restores Immune Tolerance to Patients With Hepatitis C Virus–Induced Cryoglobulinemia Vasculitis

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BACKGROUND & AIMS: Interferon-free direct-acting antiviral (DAA) therapies are effective in patients with hepatitis C virus–induced cryoglobulinemia vasculitis (HCV-CV). We analyzed blood samples from patients with HCV-CV before and after DAA therapy to determine mechanisms of these drugs and their effects on cellular immunity. **METHODS:** We performed a prospective study of 27 consecutive patients with HCV-CV (median age, 59 y) treated with DAA therapy (21 patients received sofosbuvir plus ribavirin for 24 weeks, 4 patients received sofosbuvir plus daclatasvir for 12 weeks, and 2 patients received sofosbuvir plus simeprevir for 12 weeks) in Paris, France. Blood samples were collected from these patients before and after DAA therapy, and also from 12 healthy donors and 12 individuals with HCV infection without CV. HCV load, cryoglobulins, and cytokines were quantified by flow cytometry, cytokine multiplex assays, and enzyme-linked immunosorbent assay. **RESULTS:** Twenty-four patients (88.9%) had a complete clinical response of CV to DAA therapy at week 24, defined by improvement of all the affected organs and the absence of relapse. Compared with healthy donors and patients with HCV infection without CV, patients with HCV-CV, before DAA therapy, had a lower percentage of CD4+CD25hiFoxP3+ regulatory T cells ($P < .01$), but higher proportions of IgM+CD21-/low memory B cells ($P < .05$), CD4+IFNγ+ cells ($P < .01$), CD4+IL17A+ cells ($P < .01$), and CD4+CXCR5+interleukin 21+ follicular T-helper (Tfh) cells ($P < .01$). In patients with HCV-CV, there was a negative correlation between numbers of IgM+CD21-/low memory B cells and T-regulatory cells ($P = .03$), and positive correlations with numbers of Tfh cells ($P = .03$) and serum levels of cryoglobulin ($P = .01$). DAA therapy increased patients' numbers of T-regulatory cells (1.5% ± 0.18% before therapy vs 2.1% ± 0.18% after therapy), decreased percentages of IgM+CD21-/low memory B cells (35.7% ± 6.1% before therapy vs 14.9% ± 3.8% after therapy), and decreased numbers of Tfh cells (12% ± 1.3% before therapy vs 8% ± 0.9% after therapy). Expression levels of B lymphocyte stimulator receptor 3 and programmed cell death 1 on B cells increased in patients with HCV-CV after DAA-based therapy (mean fluorescence units, 37 ± 2.4 before therapy vs 47 ± 2.6 after therapy, $P < .01$; and 29 ± 7.3 before therapy vs 48 ± 9.3 after therapy, $P < .05$, respectively). **CONCLUSIONS:** In a prospective clinical trial of patients with HCV-CV, DAA-based therapy restored disturbances in peripheral B- and T-cell homeostasis.

Keywords: PDCD1; Immune Regulation; Response To Treatment; Liver Disease.

Circulating mixed cryoglobulins are detected in 40%–60% of patients with chronic hepatitis C virus (HCV) infection, whereas overt cryoglobulinemia vasculitis (CV) is observed in only 5%–10% of cases.[1–4] It involves the activation of B cells, which generates pathogenic IgM and IgG

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Abbreviations used in this paper: **BAFF, B-cell–activating factor of the tumor necrosis factor family; BR3, B-lymphocyte stimulator receptor 3; CV, cryoglobulinemia vasculitis; DAA, direct-acting antiviral; EOT, end of treatment; HCV, hepatitis C virus; HD, healthy donor; IFN, interferon; IL, interleukin; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cell; PT, post-treatment; Tfh, follicular T-helper; TNF, tumor necrosis factor; Treg, regulatory T cell; VLP, virus-like particle; W0, week 0.**

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EDITOR'S NOTES

BACKGROUND AND CONTEXT

Antiviral treatment of patients with chronic hepatitis C and overt cryoglobulinemia vasculitis can lead to disappearance of symptoms and can induce an immunologic response.

NEW FINDINGS

In HCV-associated cryoglobulinemia vasculitis Tfh expansion is associated with Th1 and Th17 polarization, and marked expansion of IgM+CD21-/low memory B cells and Treg deficiency. Antiviral therapy improves abnormalities in B-cell homeostasis, with a decreased proportion of autoreactive memory B cells and decreased cryoglobulin levels after treatment. In addition, anti-HCV therapy improves T-cell homeostasis by restoring regulation/activation and Th1/Th17 imbalances.

LIMITATIONS

Long-term follow-up of patients is required.

IMPACT

Successful interferon-free therapy of patients with chronic hepatitis C and overt cryoglobulinemia vasculitis restores disturbances in peripheral B- and T-cell homeostasis.

with rheumatoid factor activity.[5,6] CV leads to clinical manifestations ranging from purpura, arthralgia, and fatigue, to more serious lesions with neurologic and renal involvement.[2] Despite success with combination antiviral treatment with or without immunosuppressive drugs, HCV-CV remains a serious disease, with an estimated 5-year survival rate of 75%.[4,7]

Treatment of HCV-CV remains difficult and requires targeting the downstream B-cell arm of autoimmunity and the viral trigger to obtain clinical resolution of symptoms.[8,9] Two prospective randomized controlled trials have shown the superiority of rituximab monotherapy compared with conventional immunosuppressive therapy in patients with HCV-CV in whom prior antiviral therapy failed to induce disease remission.[10,11] Rituximab was effective in 71.4%–83% of patients with CV.[10,11] However, in the absence of HCV clearance, frequent relapses occurred when B cells re-emerged in the peripheral blood. Direct-acting antivirals (DAAs) have proven to be very effective in patients with HCV-CV. A complete clinical response was achieved in 87.5% of HCV-CV patients treated with sofosbuvir plus ribavirin, and was correlated closely with virologic response.[12–14] We previously reported a quantitative defect in regulatory T cells (Tregs) and clonal expansion of $CD27^{+}IgM^{+}CD21^{-/low}$ memory B cells in patients with HCV-CV.[15–17] It has been shown that antiviral treatment can lead to the disappearance of symptoms and can induce an immunologic response (ie, a significant decrease in plasma cryoglobulin levels and increase in Tregs).[15,18] In addition, a decrease in B-lymphocyte stimulator receptor 3 (BR3) staining on B cells was associated with an increase in serum B-cell–activating factor of the tumor necrosis factor family (BAFF) after rituximab in HCV-CV.[19]

However, little evidence is available about immunologic dynamics after IFN-free DAA-based therapy. As a first examination of the immunologic effects of DAAs in HCV-CV, we evaluated both B- and T-cell subsets in patients at baseline (W0) and after DAA therapy at end of treatment (EOT) and post-treatment (PT). Our results indicate that in addition to their virologic effect, DAA-based therapy effectively normalizes many of the disturbances in peripheral B- and T-lymphocytes homeostasis.

Materials and Methods

Study Population

Twenty-seven consecutive patients with HCV-CV (median age, 59 y; range, 53–66 y) were included in the study and received interferon-free DAA therapy. All were positive for HCV RNA. The HCV viral load was quantified using the Abbott HCV RealTime assay (Abbott, Rungis, France), with a lower limit of detection of 12 IU/mL. To be eligible, the patient must have been at least 18 years of age or older (without any upper age limit), been informed of the study, and presented with active HCV vasculitis defined by clinically active vasculitis with skin, joint, renal, peripheral nerve, central neurologic, digestive, pulmonary, and/or cardiac involvement (no histologic evidence needed if patient had purpura), and chronic active HCV infection (positive HCV RNA). Exclusion criteria included nonactive cryoglobulinemia vasculitis, human immunodeficiency virus, or active hepatitis B virus infection, and current decompensated cirrhosis.

Cryoglobulins were measured and classified as previously described.[20,21] Twelve healthy donors (HDs) and 12 HCV patients, age- and sex-matched controls, with positive viremia and without circulating cryoglobulins, also were included. The study was approved by the institutional ethics committee of Pitié-Salpêtrière Hospital and was performed in accordance with the Declaration of Helsinki. All participants provided informed consent.

Treatment

Twenty-seven consecutive patients with HCV-CV were treated with DAAs according to current guidelines. Twenty-one patients received antiviral therapy with sofosbuvir 400 mg/day plus ribavirin (200–1400 mg/day orally) for 24 weeks. Four patients received sofosbuvir plus daclatasvir for 12 weeks. Two patients received sofosbuvir plus simeprevir for 12 weeks. None of the patients received corticosteroids, immunosuppressants, or rituximab in the 6 months before inclusion. Twenty-four patients (88.9%) had a complete clinical response of their CV at week 24. The complete clinical response of CV was defined by improvement of all the affected organs involved at baseline and the absence of clinical relapse. The skin and articular improvement were evaluated clinically (ie, disappearance of purpura and/or ulcers and/or skin necrosis, disappearance of arthralgia and/or arthritis). Renal improvement was evaluated biologically (ie, proteinuria <0.3 g/24 h, disappearance of hematuria, and improvement of glomerular filtration rate $>$ 20% at week 24 if glomerular filtration rate $<$ 60 mL/min/1.73 m^2 at diagnosis). Peripheral neurologic improvement was evaluated clinically (ie, improvement of pains and paresthesia by visual analogue scales, improvement

of muscular testing in case of motor impairment at baseline) and/or electrophysiologically (ie, improvement of electromyogram abnormalities at week 24 compared with baseline). Twenty-two patients (81.5%) had a sustained virologic response (HCV-RNA negative) at week 12 PT.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation. PBMCs were stained with the following monoclonal antibodies (at predetermined optimal dilutions) for 30 minutes at 4°C: fluorescein isothiocyanate–conjugated CD21, Phycoerythrin-conjugated CD27 and BR3 and chemokine receptor 5, Alexa Fluor 450-conjugated CD3, allophycocyanin-conjugated IgM, energy-coupled dye–conjugated CD4 and CD19, PerCP–Cy7–conjugated CD25 and programmed death-ligand 1, VioBlue-conjugated IgD, and Brilliant Violet 510–conjugated CD127 (BioLegend, San Diego, CA). Memory B cells IgM^+ $CD21^{-/low}$ were identified as IgM+CD21-/low cells in CD19+ B lymphocytes. Detection of intracellular FoxP3 was accomplished using fixed and permeabilized cells according to the manufacturer's instructions (eBioscience, Paris, France), and then incubated with allophycocyanin-conjugated FoxP3. Tregs were identified as Foxp3+CD25+ cells in CD4+ T lymphocytes. Fluorescence-activated cell sorter analyses were performed on a Navios flow cytometer using Kaluza analysis software (Beckman Coulter, Villepinte, France).

Analysis of Cytokine Production

PBMCs from HD, HCV without cryoglobulinemia, and HCV-CV patients were stimulated for 4 hours with 50 ng/mL phorbol myristate acetate and 1 mmol/L ionomycin (Sigma-Aldrich, Saint-Louis, MO) in the presence of brefeldin A (BD PharMingen, Le Pont de Claix, France). Cells cultured in the presence of Brefeldin A were stained for cell surface markers and then permeabilized with Cytofix/Cytoperm buffer (BD PharMingen) and stained with fluorescein isothiocyanate–conjugated interferon (IFN)γ (BD PharMingen), Alexa Fluor 647-conjugated interleukin (IL)17A (eBioscience), and Alexa Fluor 647-conjugated IL21 (BioLegend). Th1 were identified as IFNγ+CD4+ cells in CD3+ T lymphocytes. Th17 were identified as IL17A+CD4+ cells in CD3+ T lymphocytes. Follicular T-helper (Tfh) cells were identified as CD4+CXCR5+IL21+ cells in CD3+ T lymphocytes.

The levels of the following serum cytokines were measured using Human Cytokine MILLIPLEX according to the recommendations of the manufacturer (Merck Millipore, Billerica, MA): IFNγ, tumor necrosis factor (TNF)α, IL12p70, IL4, IL5, IL10, IL13, IL17A, and IL21. The levels of BAFF (R&D Systems, Abingdon, United Kingdom) were determined by enzyme-linked immunosorbent assay.

Antigen-Specific Stimulation With Virus-Like Particles

PBMCs from HCV-CV patients were stimulated during 48 hours with peptide pools (15-mers overlapping by 10 amino acids; Eurogentec, Liège, Belgique), spanning the whole sequence of the H77 strains E1 and E2, as previously described.[22] During the last 4 hours, brefeldin A was added. Tfh, Th1, and Th17 were identified by flow cytometry as previously described.

Data Analysis and Statistics

Analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). Except when otherwise indicated, values are expressed as medians (interquartile ranges). Categoric variables were compared using the Fisher exact test or the chi-squared test, and continuous variables were compared using the t test or the Mann–Whitney U test when appropriate. Correlation significance was calculated with the Pearson equation. All tests were 2-tailed with a significance level of 0.05.

Results

Main Characteristics of HCV-CV Patients

Patient characteristics are detailed in Table 1. Twenty-seven patients with HCV-CV, with a median age of 59 years (range, 53–66 y) were included. The main clinical features of cryoglobulinemia vasculitis included purpura (89%), peripheral neuropathy (48%), glomerulonephritis (26%), and skin ulcers (15%). The median cryoglobulin level was 0.35 g/L (IQR, 0.17–0.81 g/L). Twenty-four patients (88.9%) had a complete clinical response of their CV. Twenty-two patients (81.5%) had a sustained virologic response (HCV-RNA negative) at week 12 PT. Ten (27%) patients presented negativation of cryoglobulinemia and 17

Table 1. Baseline Characteristics of the 27 Patients With HCV-CV

Characteristics	N = 27
Age, *y*	59 [53–66]
Male sex, n (%)	14 (52)
HCV genotype, n (%)	
1a	9 (33)
1b	8 (30)
2	2 (7.5)
3	2 (7.5)
4	4 (15)
5	2 (7.5)
Metavir liver fibrosis score, n (%)	
Stage 0	2 (7.5)
Stage 1	2 (7.5)
Stage 2	6 (22)
Stage 3	6 (22)
Stage 4	11 (41)
Baseline HCV-RNA level, log_{10} IU/mL	5.7 [4.9–6.4]
ALT level, *IU/L*	59 [37–77]
Serum cryoglobulin level, *g/L*	0.35 [0.17–0.81]
Serum C4 level, *g/L*	0.09 [0.04–0.16]
Serum rheumatoid factor level, *IU/mL*	27 [7–78]
Purpura, n (%)	24 (89)
Skin ulcer, n (%)	4 (15)
Polyneuropathy, n (%)	13 (48)
Kidney involvement, n (%)	7 (26)

NOTE. Values are expressed as medians [interquartile range].
ALT, alanine aminotransferase.

(63%) patients had persistent cryoglobulinemia after DAA-based therapy.

Abnormalities in Peripheral Cell Homeostasis of HCV-CV

Compared with HDs and HCV controls, pretreatment abnormalities in HCV-CV patients included a decreased percentage of $CD4^+CD25^{hi}FoxP3^+$ regulatory T cells (1.5% ± 0.2% vs 3.7% ± 0.2% for HDs, $P < .01$; vs 2% ± 0.4% for HCV controls, $P = .6$) (Figure 1*A* and *B*). We also found increases in $IgM^+CD21^{-/low}$ memory B cells (35.7% ± 6.2% vs 7% ± 1.2% for HDs, $P = .0004$; vs 23.1% ± 4.6% for HCV controls, $P = .23$) (Figure 1*C* and *D*); $CD4^+IFN\gamma^+$ (21.6% ± 1.9% vs 10.8% ± 0.8% for HD, $P < .0001$; vs 16.2% ± 1.7% for HCV controls, $P = .15$); $CD4^+IL17A^+$ (2.4% ± 0.4% vs 0.85% ± 0.12% for HDs, $P = .007$; vs 0.88% ± 0.16% for HCV controls, $P = .002$); and $CD4^+CXCR5^+IL21^+$ Tfh cells (12.2% ± 1.4% vs 4.5% ± 0.75% for HDs, $P = .0001$; vs 6% ± 1.6% for HCV controls, $P = .003$) (Figure 2).

We have evaluated the effect of antiviral therapy in PBMCs of HCV-CV patients after antigen-dependent stimulation by virus-like particles (VLPs). We observed an expansion in Tfh, Th1, and Th17 at baseline (week 0 [W0]) that decreased after antiviral therapy (EOT and PT) (Supplementary Figure 1). Proportions of $CD4^+CXCR5^+IL21^+$ after VLP stimulation were 3.9% ± 0.1% at W0, 2.7% ± 0.4% at EOT, and 2.5% ± 0.3% at PT. Proportions of CD4+IL21+ after VLP stimulation were 33.5% ± 0.1% at W0, 8.3% ± 0.9% at EOT, and 5.8% ± 0.4% at PT. Proportions of CD4+IFNγ+ after VLP stimulation were 16.3% ± 2.9% at W0, 2.8% ± 1.4% at EOT, and 3.6% ± 0.9% at PT. Proportions of CD4+IL17A+ after VLP stimulation were 4.2% ± 0.8% at W0, 0.9% ± 0.4% at EOT, and 1.% ± 0.3% at PT.

We also examined the expression of CD27, IgD, and CD38 in B cells to differentiate naive (IgD^+CD27^-), switched memory (IgD^-CD27^+), unswitched memory (IgD^+CD27^+) cells, and plasmablasts ($IgD^-CD27^{high}CD38^{high}$). We did not observe statistically significant differences in naive B cells (41.2% ± 7.3% in HCV-CV vs 52% ± 5.8% for HD, $P = .47$; and 50.3% ± 7.3% for HCV controls, $P = .63$), switched memory B cells (37% ± 6.3% in HCV-CV vs 24.3% ± 3.3% for HD, $P = .52$; and 21.6% ± 4.3% for HCV controls, $P = .23$), unswitched memory B cells (16.6% ± 4.7% in HCV-CV vs 14% ± 2.5% for HD, $P = .37$; and 15.2% ± 3.4% for HCV controls, $P = .75$), and plasmablasts (2.3% ± 0.39% in HCV-CV vs 1% ± 0.19% for HD, $P = .09$; and 1.7% ± 0.44% for HCV controls, $P = .59$) (data not shown).

Correlation Between IgM^+ $CD27^+$ $CD21^{-/low}$ Memory B Cells With Tregs, Tfh, and Cryoglobulin Levels

We next asked whether the expansion of $IgM^+CD21^{-/low}$ memory B cells was correlated with Tregs and Tfh frequency, cryoglobulin levels, and HCV viral load. $IgM^+CD21^{-/low}$ memory B cells were correlated negatively with Tregs (r = -0.46; $P = .03$), and correlated positively with Tfh (r = 0.44; $P = .03$) and cryoglobulin levels (r = 0.59; $P = .01$). There was no correlation with the baseline HCV viral load ($P = .88$) (Figure 3).

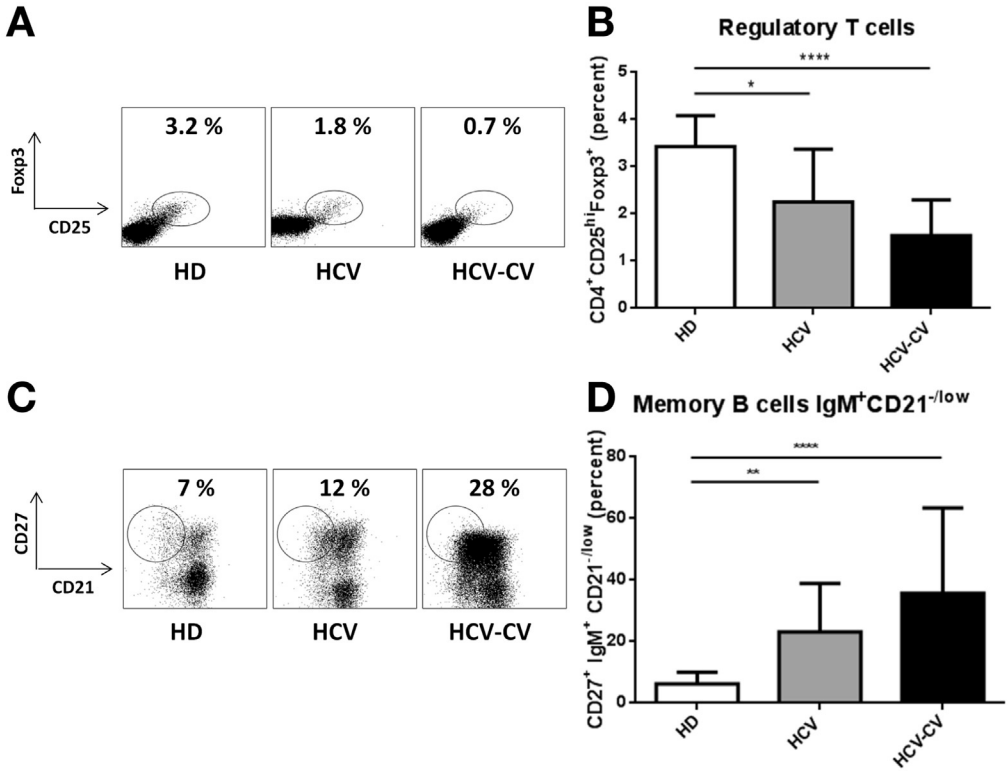

Figure 1. Quantitative deficiency of regulatory T cells and expansion of memory B cells $IgM^+CD21^{-/low}$ in HCV-CV. (*A*, *C*) Flow cytometry figures of a healthy control, a patient with HCV without cryoglobulinemia and a patient with HCV-CV demonstrating $CD4^+CD25^{hi}$ $FoxP3^+$ regulatory T cells and IgM^+ $CD21^{-/low}$ memory B cells frequencies. (*B*, *D*) Histograms representing the percentage of $CD4^+D25^{hi}$ $FoxP3^+$ regulatory T cells and $IgM^+CD21^{-/low}$ memory B cells (mean ± SEM) in HD, HCV controls and patients with HCV-CV at baseline. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

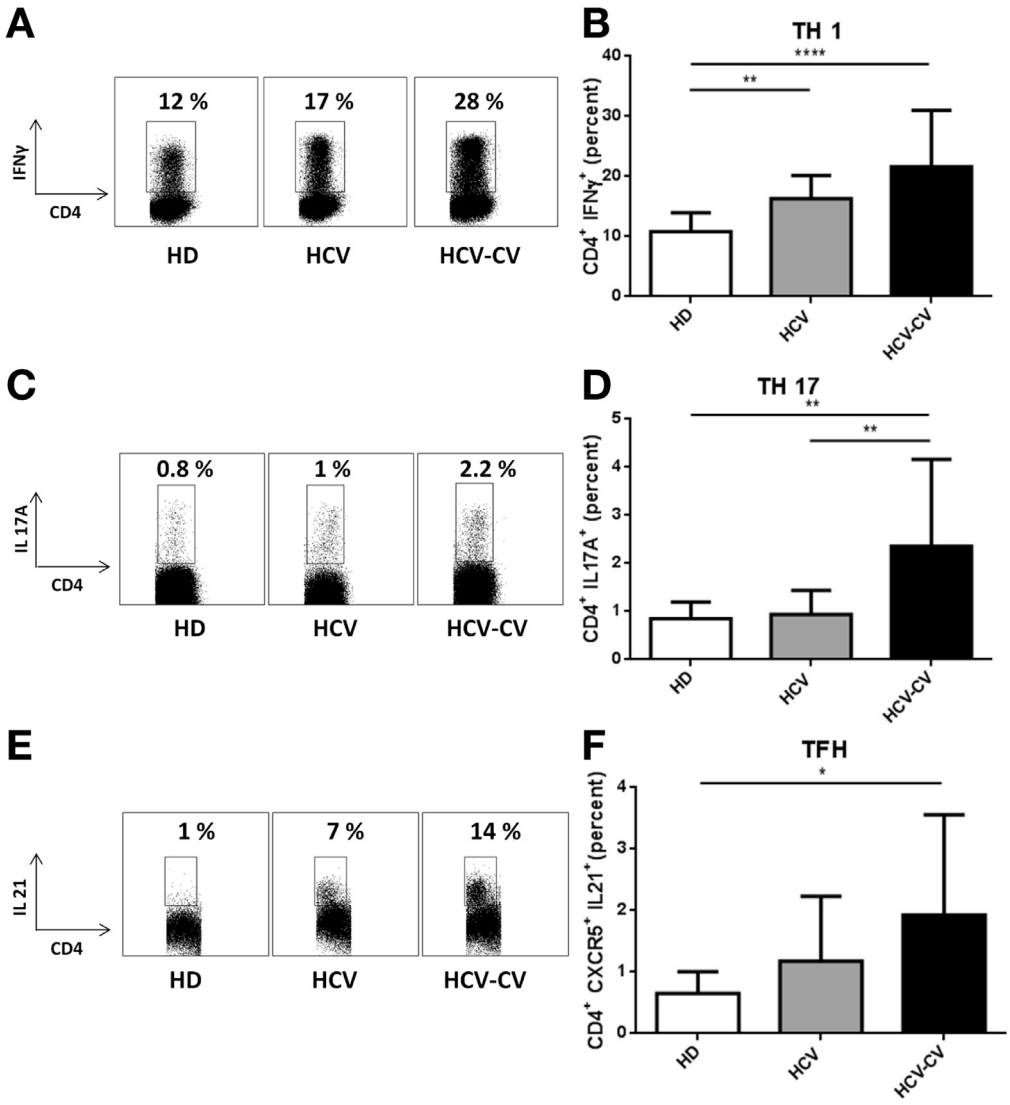

Figure 2. Th1, Th17 polarization and expansion of Tfh in HCV-CV. (*A, C, E*) Flow cytometry figures of a HD, a patient with chronic HCV infection without cryoglobulinemia vasculitis and a patient with HCV-CV demonstrating $CD4^+IFN\gamma^+$ and $CD4^+IL\text{-}17A^+$ and Tfh percentages among $CD4^+$. (*B, D, F*) Histograms representing the percentage of $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}17A^+$ and Tfh (mean ± SEM) in HD, HCV control and patients with HCV-CV at baseline. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

IFN-Free DAA-Based Therapy Improves Peripheral Cell Homeostasis in HCV-CV

Regulatory T-cell deficiency, $IgM^+CD21^{-/low}$ memory B-cell expansion, and Tfh ($CD4^+CXCR5^+IL21^+$) expansion significantly reversed after DAA therapy (1.5% ± 0.18% at W0 vs 2.1% ± 0.18% at EOT, and vs 2.7% ± 0.3% at PT, 35.7% ± 6.1% at W0 vs 14.9% ± 3.8% at EOT, and vs 10.3% ± 2.9% PT, 12% ± 1.3% at W0 vs 8% ± 0.9% at EOT, and vs 5.6% ± 1.2% PT, respectively) (Figure 4). The cryoglobulin level decreased from 0.35 (IQR, 0.17–0.81) at baseline (W0) to 0.22 (IQR, 0.06–0.52) g/L at EOT ($P = .009$) (Figure 4*G*) to 0.13 (IQR, 0–0.33) g/L at PT ($P < .001$) (Figure 4*G*). We did not observe a significant difference in frequency of $CD4^+IFN\gamma^+$ (21.6% ± 1.9% vs 21.2% ± 2.1%; $P = .85$) or $CD4^+IL17A^+$ (2.4% ± 0.4% vs 1.6% ± 0.3%; $P = .1$) before and after DAA. Comparison between HCV-CV virologic responders (n = 22) and non-responders (n = 5) to DAA-based therapy did not show a significant difference in terms of proportion of Tregs, $IgM^+CD21^{-/low}$ memory B cells, and Tfh, Th1, and Th17 cells before and after DAA.

DAA-based therapy did not impact T- and B-cell homeostasis in HCV patients without cryoglobulinemia (Supplementary Figure 2).

Serum levels of IFNγ, IL12 p70, IL17A, and IL21 decreased significantly in HCV-CV patients after DAA therapy (26 ± 4.6 vs 22.9 ± 4.7 pg/mL; 13.1 ± 2.4 vs 11.5 ± 3.2 pg/mL; 20.7 ± 7.1 vs 19.8 ± 7.9 pg/mL; and 20.4 ± 7.6 vs 17.3 ± 6.9 pg/mL, respectively; $P < .05$) (Table 2). Serum levels of TNFα, IL4, IL5, IL10, and IL13 did not differ significantly before and after DAA therapy. We did not observe significant changes in serum levels of IFNγ, IL12 p70, IL17A, IL21, and TNFα in HCV patients without cryoglobulinemia between W0, EOT, and PT (Table 2).

Serum levels of BAFF tended to decrease after DAA therapy but did not reach statistical significance (1898 ± 371 vs 1491 ± 194 pg/mL; $P = .58$).

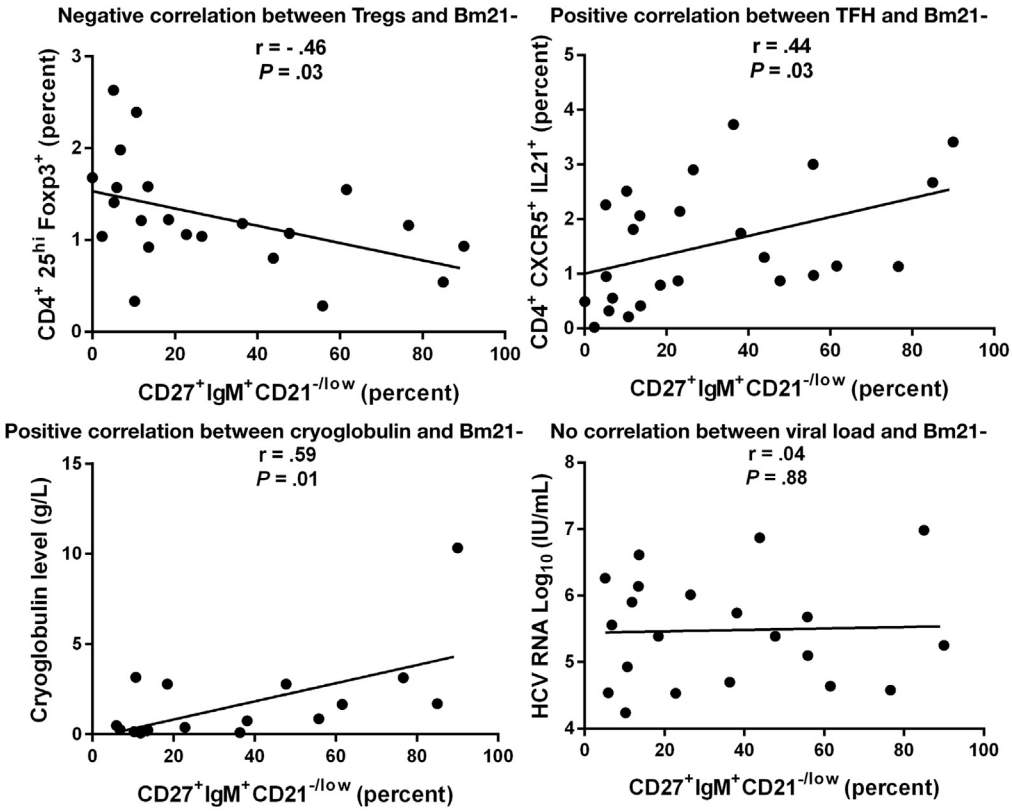

Figure 3. Correlation of memory B cells $IgM^+CD21^{-/low}$ with regulatory T cells, Tfh and cryoglobulin serum levels. Correlations between $IgM^+CD21^{-/low}$ memory B cells and $CD4^+CD25^{hi}$ $FoxP3^+$ regulatory T cells (*A*), between IgM^+ $CD21^{-/low}$ memory B cells and $CD4^+CXCR5^+IL21^+$ Tfh cells (*B*), between $IgM^+CD21^{-/low}$ memory B cells and cryoglobulin level (*C*) and between $IgM^+CD21^{-/low}$ memory B cells and HCV viral load (*D*) in patients with HCV-CV. $IgM^+CD21^{-/low}$ memory B cells were negatively correlated with Tregs and positively correlated with Tfh and cryoglobulin serum levels. No correlation was observed between IgM^+ $CD21^{-/low}$ memory B and HCV viremia.

BR3 and PD-L1 Expression on B Cells Are Modulated by DAA Therapy, and Expression of BR3 Was Correlated Negatively With BAFF in Serum

BR3 staining levels on B cells were increased in HCV-CV patients when compared before and after DAA therapy (mean fluorescence intensity [MFI], 37 ± 2.4 vs 47 ± 2.6; $P < .01$) (Figure 5*A* and *B*). Similarly, PD-L1 staining levels on B cells were increased in HCV-CV patients when compared before and after DAA therapy (MFI, 29 ± 7.3 vs 48 ± 9.3; $P < .05$) (Figure 5*C* and *D*). MFI of BR3 was correlated negatively with BAFF in serum (r = -0.46; $P = .039$) (Figure 5*E*).

We analyzed PD-1 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) expression on Tregs before and after DAA-based therapy. We did not observe a difference relative to PD-1 expression (MFI, 1.9 ± 0.7 at W0 vs 1.7 ± 0.5 at EOT), but an increase in CTLA-4 expression (MFI, 9.3 ± 2.6 at W0 vs 11.7 ± 3.8 at EOT) after DAA-based therapy.

Discussion

Mixed cryoglobulinemia vasculitis is a serious autoimmune condition that is induced predominantly by chronic HCV infection. It is an example of infection-associated tolerance failure, which leads to an immune response directed against host tissues. As such, it affords a particularly interesting insight into the delicate interrelation between invading infection, host tissue, and the host immune system. The pathogenesis of mixed cryoglobulinemia vasculitis traditionally has been considered to be immune complex–mediated. However, a number of T-cell abnormalities have been described, including polarization toward Th1 subsets[23] and quantitative deficiency of circulating regulatory T cells. In this study we identified significant T- and B-cell abnormalities in patients with HCV-CV compared with healthy donors and HCV controls. We observed Tfh expansion associated with Th1 and Th17 polarization in HCV-CV. As previously reported,[16,17] HCV-CV patients had Treg deficiency and marked expansion of autoreactive $IgM^+CD21^{-/low}$ memory B cells. We also observed a negative correlation between regulatory T-cell deficiency and $IgM^+CD21^{-/low}$ memory B-cell expansion, as well as a positive correlation between Tfh expansion and $IgM^+CD21^{-/low}$ memory B-cell expansion in HCV-CV. HCV has been shown to lower B- and T-cell activation thresholds,[24,25] which may promote immune injury to self. Therefore, the finding that enhanced frequency and function of $CD4^+CD25^+$ cells was associated with a lower degree of HCV-associated liver inflammation,[26,27] as well as the finding that activated Tregs in patients with chronic HCV infection suppress effector cell activity in a non–antigen-specific manner,[28] both may suggest a beneficial role for Tregs in reducing immune-mediated bystander injury in chronic HCV infection. However, the exact role and function of Tregs in HCV-infected patients without cryoglobulinemia still is debated.[29]

IL21 is known to drive Th1 and Th17 differentiation, and to decrease Treg cell frequency.[30] IL21 also drives

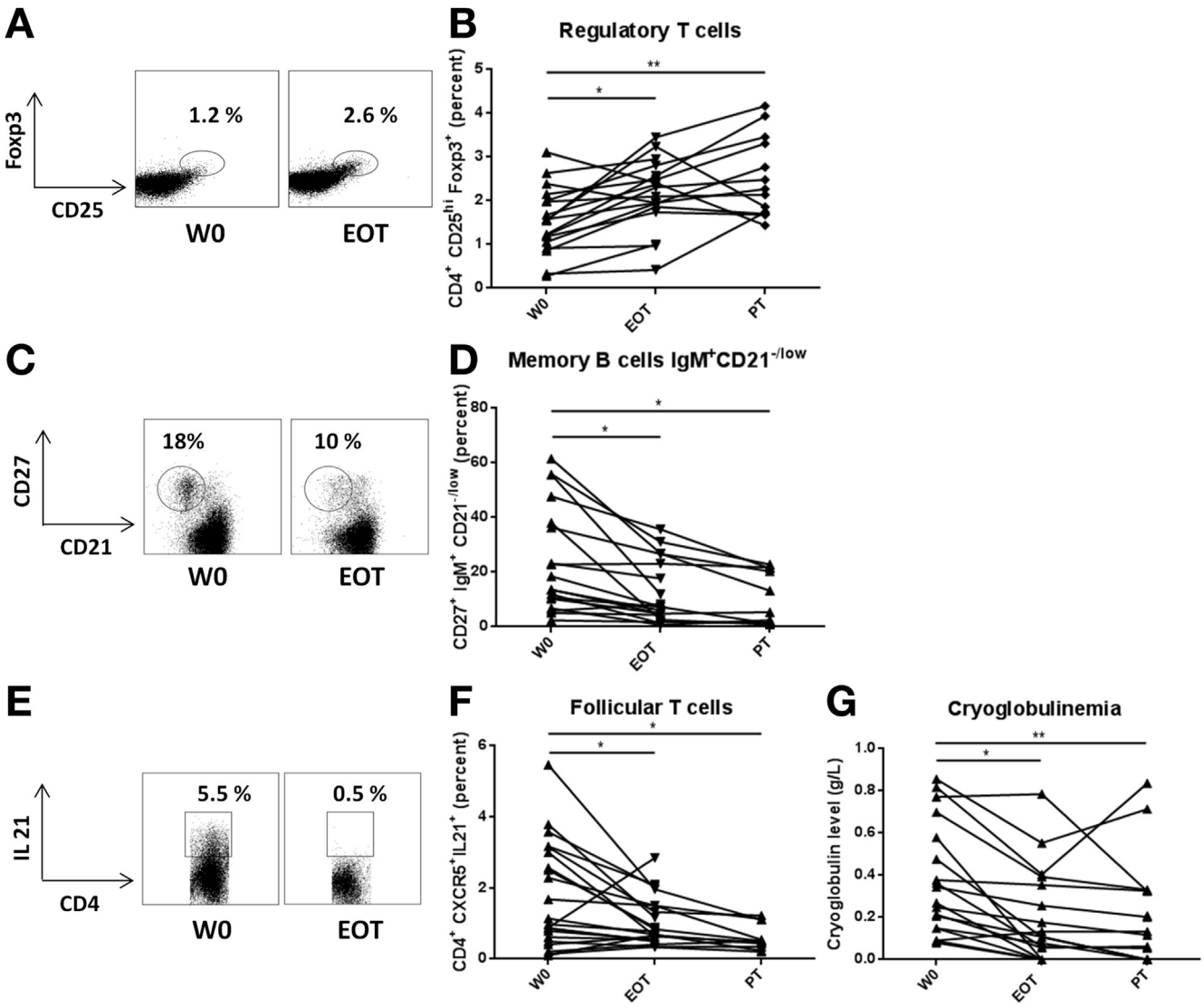

Figure 4. DAA-based therapy reverts regulatory T cell deficiency, and expansion of $IgM^+CD21^{-/low}$ memory B cells and Tfh. Flow cytometry figures showing the increase in $CD4^+CD25^{hi}FoxP3^+$ regulatory T cells (*A*), the decrease in $IgM^+CD21^{-/low}$ memory B cells (*C*) and $CD4^+CXCR5^+IL21^+$ Tfh cells (*E*) following DAA-based therapy in a representative patient with HCV-CV. Individual changes in Tregs (*B*), $IgM^+CD21^{-/low}$ memory B cells (*D*) and Tfh (*F*) percentages and in cryoglobulin serum levels (*G*), before DAA therapy (W0), at end of DAA therapy (EOT) and between 12 and 24 weeks post treatment (PT). $^*P < .05$, $^{**}P < .01$.

Table 2. DAA-Based Therapy Decreases Th1, Th17 Polarization, and IL21 Production in HCV-CV Patients

	W0		EOT		$PT_{12–24}$	
Cytokines in serum	HCV-CV	HCV	HCV-CV	HCV	HCV-CV	HCV
IFNγ	26 ± 4.6	17.4 ± 3.4	22.9 ± 4.7[a]	18.3 ± 5.3	14.5 ± 3.5[a]	13.3 ± 5.9
IL12 p70	13.1 ± 2.4	12.3 ± 8.2	11.5 ± 3.2[a]	10.2 ± 5.3	10.5 ± 4.2[a]	11.4 ± 8.7
IL17A	20.7 ± 7.1	12.9 ± 5.9	19.8 ± 7.9[a]	7.5 ± 3.7	11.5 ± 8.8[a]	4.2 ± 4.2
IL21	20.4 ± 7.6	9.6 ± 1.5	17.3 ± 6.9[a]	9.3 ± 1.7	14.5 ± 3.7[a]	9.6 ± 3.8
TNFα	11.4 ± 0.9	14.3 ± 9.7	14.4 ± 2.9	14.1 ± 13.1	14.5 ± 2.4	14.8 ± 4.5

NOTE. Data are expressed as means ± SEM.
[a]Statistically significant ($P < .05$) difference for the comparison between HCV-CV patients at W0 (before DAA), end of DAA therapy (EOT), and between 12 and 24 weeks PT.

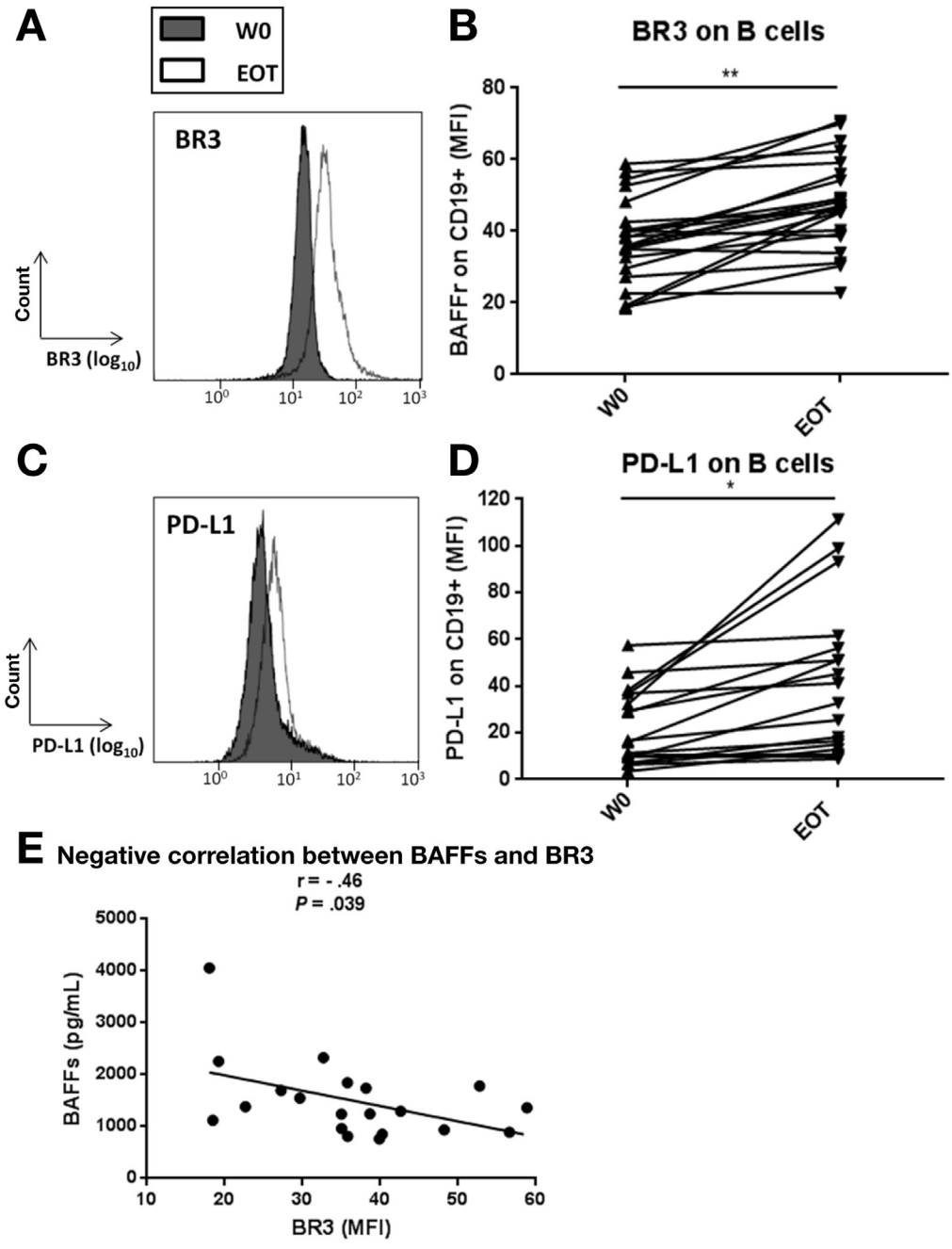

Figure 5. DAA-based therapy increases B lymphocyte stimulator receptor 3 (BR3) and PDL1 staining on $CD19^+$, and correlation between BAFFs and BR3. Representative histograms of BR3 (*A*) and PD-L1 (*C*) staining on B cells in a patient with HCV-CV, before and after DAA therapy. Individual changes in median fluorescent intensity (MFI) of BR3 (*B*) and PD-L1 (*D*), before (W0) and after DAA therapy (EOT). Negative correlation between BAFF in serum and MFI of BR3 (E). $^*P < .05$, $^{**}P < 0.01$.

autoreactive B-cell responses, promoting Tfh differentiation and germinal center reaction by skewing the follicular regulatory T cell to follicular helper T-cell balance.[31] Interestingly, in chronic HCV infection without cryoglobulinemia vasculitis, IL21 production by Tfh is impaired when compared with healthy controls.[32] The decreased frequency of IL21-producing $CXCR5^+CD4^+$ T cells and the lower serum IL21 levels in chronic HCV patients without CV did not lead to an altered Tfh–B-cell interaction.[21] Conversely, we observed Tfh (ie, $CXCR5^+CD4^+IL\text{-}21^+$) expansion in HCV-CV compared with healthy donors and HCV controls, which could contribute to aberrant B-cell activation, generating pathogenic IgM and IgG with rheumatoid factor activity.

In the present study we have shown further that after DAA use, complete remission in autoimmune manifestations as well as viral clearance are associated with normalization of the significant disturbances in peripheral T- and B-lymphocyte homeostasis. This is very relevant because, in the first place, immunologic changes are expected to be different according to the achievement of viral clearance or not. In addition, several studies have shown that despite HCV eradication, some patients still present immune activation (positive cryocrit) or clinical symptoms (active vasculitis).[14,33] The immune changes associated with these relevant clinical outcomes still remain to be understood. DAAs have been developed to target nonstructural viral

proteins involved in HCV viral replication (NS3, NS4A, NS4B, NS5A, and NS5B), thereby resulting in disruption of the viral life cycle. In addition to this direct action, they also may help in the recovery of innate immune processes via interferon production.[34] However, in our study only 2 patients received protease inhibitors. Although we did not observe any correlation between HCV viremia and $IgM^{+}CD21^{-/low}$ memory B-cell expansion before antiviral therapy, HCV clearance was associated closely with clinical and immunologic response.[12,15,33] After DAA-based therapy, a significant decrease in Th1 (IFNγ, IL12 p70) and Th17 (IL17A) cytokine serum levels was observed.

We previously showed that the use of low-dose IL2 leads to regulatory T-cell recovery and concomitant clinical improvement in patients with HCV-CV.[35] Tfh cells are central players in a number of autoimmune diseases because they facilitate both the aberrant generation of autoantibodies and the formation or maintenance of ectopic follicles.[36] Taken together, our results suggest critical cross-talk between Tregs, Tfh, and autoreactive IgM memory B cells for maintaining tolerance in HCV-CV patients. Of particular interest in this context is the 2-way interaction between B cells and T cells. B cells provide signals to T cells through antigen presentation, and T cells provide "help" to B cells through the delivery of cytokines and cell-surface ligands. These interactions create the potential for a positive feedback loop or vicious cycle.

It recently has emerged that the PD-1/PD-L1 pathway has a role in regulating lymphocyte activation, promoting regulatory T-cell development and function, the breakdown of tolerance, and the development of autoimmunity.[37] This pathway exerts critical inhibitory functions in the setting of persistent antigenic stimulation such as during encounters with self-antigens, chronic viral infections, and tumors.[38] Ligation of PD-1 by either PD-L1 or PD-L2 attenuates effector T-cell proliferation, cytokine secretion, and survival. In the presence of anti-CD3 and transforming growth factor-β, PD-L1 can induce a profound increase in the de novo generation of $CD4^{+}Foxp3^{+}$ Tregs from naive $CD4^{+}$ T cells.[39] Increased PD-L1 expression on B cells is an important regulator of Tfh cell activity.[40] PD-L1 directly modulates TH1 cell differentiation by promoting a tolerogenic regulatory T-cell phenotype.[41] The absence of PD-L1 on B cells leads to an increase in Tfh cells with abundant IgG production.[42] In our study, we found increased PD-L1 expression on B cells after DAA therapy. Together, these results suggest that the normalization of PD-L1 expression on B cells in HCV-CV after DAA therapy contributes to restoring immune tolerance, and controlling B-cell responses and B-regulatory T-cell interaction.

BAFF is a critical factor for B-cell survival and maturation. It has been well demonstrated that BAFF is involved in the pathogenesis of many autoimmune and B-cell lymphoproliferative disorders.[43–46] BR3 is the most abundant B-lymphocyte stimulator receptor. We previously showed a decrease in BR3 staining on B cells in patients with HCV-CV, especially in clonal autoreactive IgM memory B cells.[19] Studies have shown that BAFF/BAFF-R signaling plays an essential role in the survival and maintenance of both follicular and marginal zone B cells, but not germinal center, normal B cells.[47,48] BAFF also is involved in the regulation of Tfh cells. BAFF directly stimulates T-cell proliferation and cytokine production.[49]

In the present study, BR3 and PD-L1 expressions on B cells increased after DAA therapy. This is in sharp contrast to the results found after rituximab treatment (ie, a marked increase in serum BAFF concentration and a decrease in BR3 staining), a factor that may indicate an increase in BAFF-receptor–ligand activity.[19] It may be hypothesized that repopulation of the B-cell compartment in a BAFF-rich environment favors autoreactive clones, which may be of special importance in HCV-induced B-cell proliferative disorders. Along this line, it recently was shown that rituximab does not reset defective early B-cell tolerance checkpoints.[50] Taken together, these results indicate that BAFF-receptor–ligand system normalization is a characteristic of remission induced by IFN-free DAA-based therapy.

In conclusion, our study identified Tfh expansion associated with Th1 and Th17 polarization, and marked expansion of $IgM^{+}CD21^{-/low}$ memory B cells and Treg deficiency in HCV-CV. We also showed that IFN-free DAA-based therapy dramatically improves abnormalities in B-cell homeostasis, with a decreased proportion of autoreactive memory B cells and decreased cryoglobulin levels after treatment. In addition, the results reported herein indicate that DAA may be an efficient therapy in HCV-CV patients not only because it reduces or abolishes the production of cryoglobulin, but also because it improves T-cell homeostasis by restoring regulation/activation and Th1/Th17 imbalances.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2017.02.037.

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Conflicts of interest
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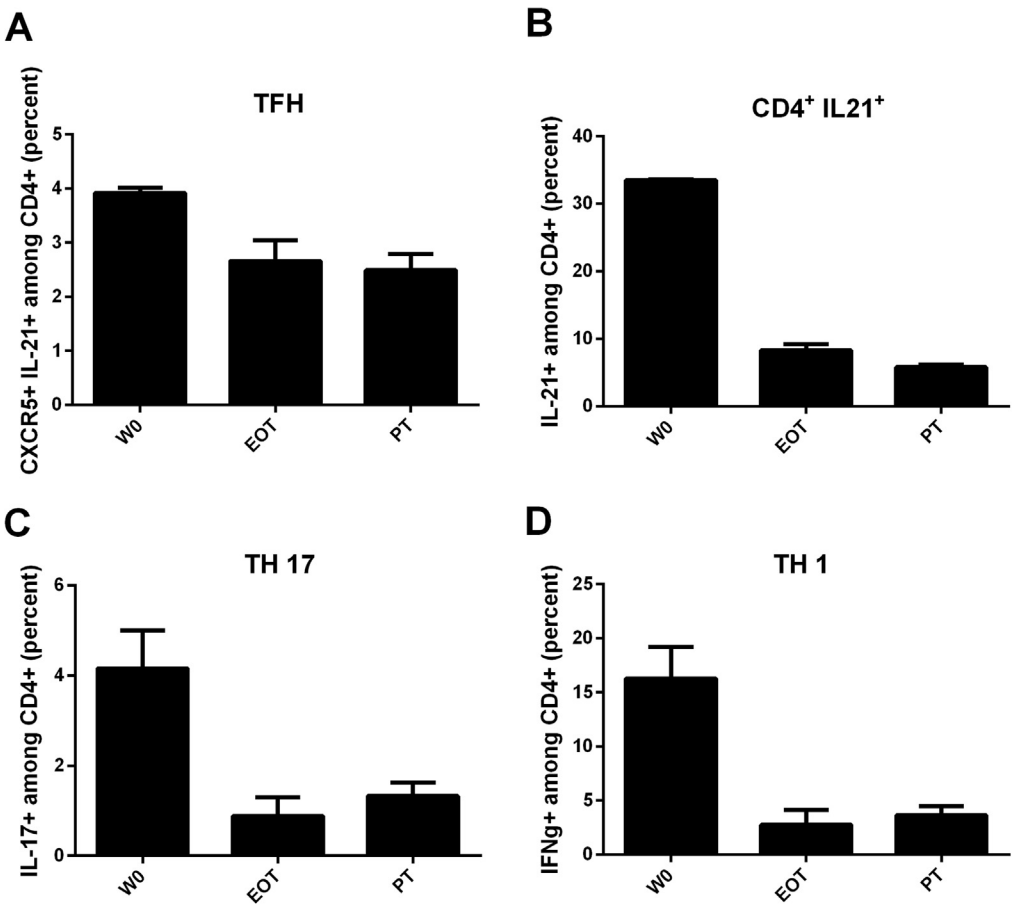
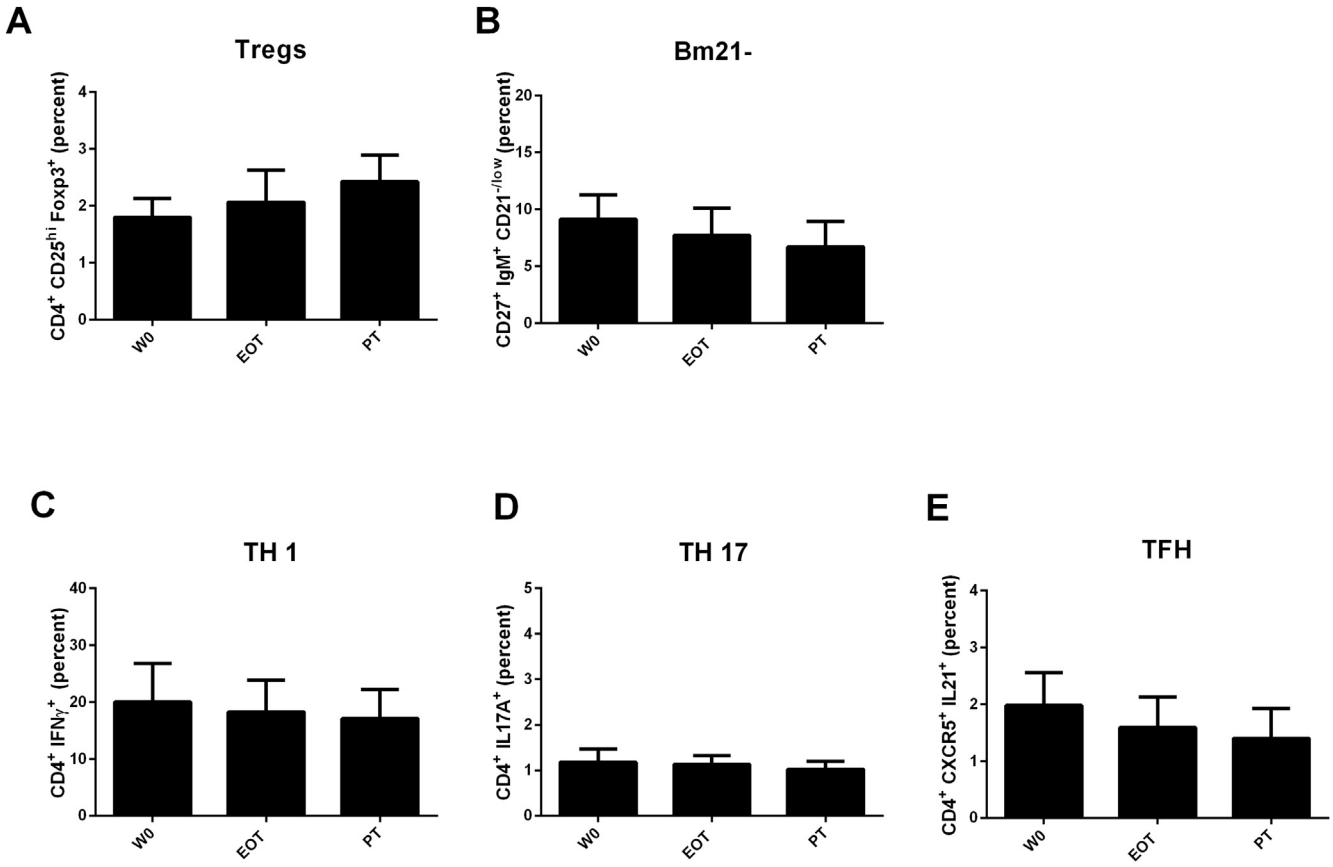


Supplementary Figure 1. DAA-based therapy reverts Tfh, Th1 and Th17 polarization after antigen-specific stimulation. Changes in $CD4^+$ $CXCR5^+IL21^+$ (*A*), $CD4^+$ $IL21^+$ (*B*), $CD4^+IFN\gamma$ (*C*), and $CD4^+IL17A^+$ (*D*) percentages, before DAA therapy (W0), at end of DAA therapy (EOT) and between 12 and 24 weeks post treatment (PT).



Supplementary Figure 2. DAA-based therapy did not impact on T- and B-cells homeostasis in HCV patients without cryoglobulinemia. Changes in Tregs (*A*), $IgM^{+}CD21^{-/low}$ memory B cells (*B*), Th1 (*C*), Th17 (*D*) and Tfh (*E*) percentages, before DAA therapy (W0), at end of DAA therapy (EOT) and between 12 and 24 weeks post treatment (PT).