Original Article
CD19+CD24hiCD38hi regulatory B cells: a potential immune predictive marker of severity and therapeutic responsiveness of hepatitis C

Qiannan Fang[1,3*], Yanan Deng[1*], Rongzhen Liang[1], Yongyu Mei[2], Zhaoxia Hu[2], Julie Wang[3], Jianbo Sun[4], Xiaohong Zhang[2], Joseph A Bellanti[5], Song Guo Zheng[3]

Departments of [1]Clinical Immunology, [2]Infectious Diseases, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510060, Guangdong, China; [3]Department of Internal Medicine, Ohio State University College of Medicine and Wexner Medical Center, Columbus, OH 43210, United States; [4]Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-Sen University, Guangzhou 510060, Guangdong, China; [5]Department of Pediatrics and Microbiology-Immunology, Georgetown University Medical Center, Washington, DC, United States. *Equal contributors.

Abstract: Objectives: Hepatitis C virus (HCV) infection is associated with abnormal immune responses. Since regulatory T (Tregs) and B (Bregs) cells modulate the progression of infectious diseases, this study aimed at examining how these cells are involved with the development of HCV infection. Methods: The frequencies of circulating Bregs and Tregs were characterized using flow cytometry. Both the association and dynamic changes of these cells with related clinical parameters were analyzed after Direct-Acting Antiviral (DAA) agent treatments. Additionally, both regulatory B and T and naïve B and T cells were sorted and stimulated with healthy or HCV sera in vitro. Results: Bregs frequency in HCV-infected patients increased significantly and were positively correlated with levels of sera HCV RNA load, Alanine aminotransferase (AST) and total bilirubin (TBILI). Additionally, the increased Bregs returned to normal levels after DAA treatment. However, Tregs increased markedly in patients with HCV-cirrhosis and were significantly associated with Aspartate aminotransferase to Platelet Ratio Index (APRI) and Fibrosis 4 (FIB-4) scores. Furthermore, HCV sera doesn't expand either Tregs or Bregs, however, it does induce the IL-10 expression in B cells although it fails to induce FOXP3 expression in CD4+ T cells. Conclusions: Increased Bregs not only may be associated with poor viral eradication and liver injury but also may provide a predictive marker of HCV disease therapeutic efficacy following DAA-treatment. HCV sera may selectively induce Bregs. Tregs probably do not control disease status in the early stages but may contribute to the progression of liver fibrosis in the late stages of HCV infection.

Keywords: Regulatory B cells, HCV infection, DAA, regulatory T cells

Introduction

Hepatitis C virus (HCV) infection still remains a major global health problem. Persistent infection with HCV causes chronic hepatitis C (CHC) and many patients progressively and eventually develop liver cirrhosis, hepatocellular carcinoma (HCC) and end-stage liver disease [1].

During chronic viral infection, effector T cells become progressively exhausted and their protective antiviral immune activity is reduced [2]. A growing body of studies emphasized the role of regulatory T cells in chronic viral infectious diseases [3]. It is well known that Tregs are critical for the suppression of immune responses and maintain homeostasis of immune function [4-6]. Many studies have shown Tregs contribute to the impairment of virus-specific T cell responses involved in the pathogenesis of chronic HCV infection [7].

Similar to Tregs, regulatory B cells (Breg), a newly defined B cell subgroup with the capacity of inhibiting immune responses, play an important role in maintenance of peripheral tolerance [8, 9]. Breg cells mainly produce regulatory cytokines, such as IL-10, and are comprised of

many phenotypic subsets. CD1d^{hi}CD5$^+$ cells, have been identified to be responsible for IL-10 production by B cells [10], while in the CD24^{hi}CD27$^+$ B cell subset, IL-10 producing cells are not only highly concentrated but also have a higher proliferative capacity in response to mitogenic stimulation [11]. Moreover, in the human, a subset of Bregs that exhibits a CD19$^+$CD24^{hi}CD38^{hi} phenotype [12], has been identified within immature transitional B cells and plays a crucial role in regulating T-cell responses by releasing IL-10 [11]. IL-10 serves as the most important functional molecule for Bregs, its regulation is controlled by many transcription factors, such as c-Maf, AhR [13, 14]. To date, the role of Breg cells has been identified in the regulation of several immune-mediated processes of different disease types, such as autoimmune diseases [12], infectious diseases [15], and cancers [16].

It is now well-established that the antiviral immune response determines the outcome of virus infection. The direct-acting antiviral (DAA) regimen is one of the major safe and effective antiviral therapeutic procedures [17]. DAA-induced sustained virologic responses (SVR) reduce the risk of cirrhosis and hepatocellular carcinoma as well as many other extra-hepatic manifestations of HCV infection [18].

In the present study, we directed a major investigative effort at not only identifying regulatory T and B cells in peripheral blood of HCV-infected patients but also correlating the frequencies and dynamic changes of these immune cell subpopulations prior to and after DAA drug treatment. To our knowledge, this is the first study that investigates the frequency and correlation of CD19$^+$CD24^{hi}CD38^{hi} B regulatory cells in HCV-infected patients.

Materials and methods

Human subjects

A total of 109 patients with chronic HCV infection and 49 healthy control subjects from the Third Affiliated Hospital of Sun Yat-Sen University and Guangzhou blood center were enrolled in the study (**Table 1**). The diagnosis of chronic HCV infection was based on the detection of serum HCV RNA in the sera for more than 6 months. Subjects infected with the hepatitis A, B, D, or E viruses, those co-infected with human immunodeficiency virus (HIV), and those with other liver diseases were excluded. Peripheral blood samples were obtained from all control subjects, and patients with HCV infection prior to, during and after DAA drug treatment. All human studies were approved by the Research Ethical Committee from The Third Affiliated Hospital of Sun Yat-Sen University and signed the informed consent.

Clinical index assay

The clinical data of individual subjects enrolled in the study were collected from hospital records. Moreover, the APRI and Fibrosis-4 indices were non-invasive scoring systems that are usually used to estimate the degree of liver fibrosis based on several laboratory tests. The APRI values were calculated using the following formula: APRI = (AST[U/L]/platelets [109/L]) × 100. An APRI index ≤0.5 signified the absence of significant fibrosis; APRI index ≤1.0 signified the absence of cirrhosis; APRI >1.5 signified the presence of significant fibrosis; and APRI >2 signified the presence of cirrhosis. APRI scores between 1.0 and 1.5 signified the progressive stages of fibrosis [19, 20]. FIB-4 values were determined using the following formula: age (years) × AST [U/L]/(Platelets [109/L] × (ALT [U/L])1/2). A Fibrosis 4 score (FIB-4) <1.45 and >3.25 enables the correct identification of patients who have moderate or significant fibrosis [20, 21].

Flow cytometry analysis

Peripheral venous blood samples (5 ml) were collected from all subjects and analyzed immediately. Freshly peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll (Axis-Shield) and stained using the following anti-human monoclonal antibodies: anti-CD4, anti-CD25, anti-CD127, anti-CD226, anti-TIGIT, anti-CD19, anti-CD38 and anti-CD24. These samples were incubated at 4°C for 30 min and protected from light. The cells were analyzed with BD LSR Fortessa flow cytometry (BD Biosciences) after washed once with staining buffer. The collected data were analyzed with FlowJo software (version V10).

Cells isolation and culture

All CD19$^+$CD24^{hi}CD38^{hi} regulatory B cells, CD19$^+$CD24^{int}CD38^{int} naïve B cells, CD4$^+$CD25$^+$

Table 1. Baseline characteristics of HCV-infected patients and healthy controls

Characteristic	Patients (109) (mean ± SEM)	HC[§] (49) (mean ± SEM)
Age (years)	46.99 ± 1.405	41.93 ± 2.163
Gender (M/F)	62/47	27/20
HCV genotype (1/no 1)	51/58	NA[§]
Baseline HCV RNA (log10 U/L)	6.07 (3.44-8.05)	NA[§]
ALT[§] level (U/L)	74.7 (14-344)	≤40
AST[§] level (U/L)	64.6 (17-277)	≤40

Abbreviations: HC[§]: healthy control; ALT[§]: alanine aminotransferase; AST[§]: aspartate aminotransferase; NA[§]: not applicable. Date are shown as medium (range).

CD127^{low} regulatory T cells, CD4+CD45RA+ naïve T cells were sorted with flow cytometry sorter (purify >95%). Regulatory or naïve B cells were stimulated with Lipopolysaccharides (LPS) 1 ug/ml and CD40L 1 ug/ml in the presence of HCV patients or healthy control sera. Placenta blue count at day 0, day 3, and day 6. IL-10 expression was detected by flow cytometry. Regulatory T cells were stimulated with anti-CD3/CD38 dynabeads (beads: cells = 1:3) and rhIL-2 (300 U/ml), rhTGF-β (5 ng/ml) under HCV or healthy sera, Placenta blue count at day0, day3 and day6. Naïve T cells were stimulated with anti-CD3/CD38 dynabeads (beads: cells = 1:5) and rhIL-2 (100 U/ml), rhTGF-β (5 ng/ml) during different serum stimulation for 3 days. FOXP3 expression was tested Flow cytometry. For intracellular staining of cytokine, cells were stimulated with PMA (0.25 mg/ml), ionomycin (0.25 mg/ml) and Brefeldin A (5 mg/ml) at the last five hours. Then cells fixed, permeabilized and stained (Invitrogen) with anti-IL-10 antibody.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from naïve B cells stimulated with different sera using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions; 1 µg RNA was used to synthesize the first strand of cDNA using the PrimeScript RT reagent Kit (Takara). PCR was performed on a 7500 fast (ABI) RT-PCR machine using SYBR Premix Ex Taq (Takara). Primers sequences for the following mRNA were used: IL-10 (Forward: GGCGCTGTCAT-CGATTTCTTC Reverse: GCCACCCTGATGTCTCA-GTT), c-Maf (Forward: AGCAAGTCGACCACC-TCAAG Reverse: CTGGAATCGCGTGTCAGACT),

GATA3 (Forward: GCCCCTCATTAAGCC-CAAG Reverse: TTGTGGTGGTCTGACA-GTTCG), TGF-β (Forward: CGACTCGC-CAGAGTGGTTAT Reverse: AGTGAACC-CGTTGATGTCCA), AhR (Forward: AC-ATCACCTACGCCAGTCG Reverse: CG-CTTGAAGGATTTGACTTGA). The amplified conditions included a three-step schedule as follows: 95°C for 10 min, 95°C for 15 s, and 60°C for 60 s for 40 cycles. The expression levels of the mRNAs were reported as fold change *vs* healthy control serum.

Statistical analysis

All data comparisons and the correlated figures were made by GraphPad Prism version 7.0 software (GraphPad Software). All data were presented as mean values ± the standard error of the mean (SEM). The Student's t test was used for two-group comparisons. A one-way ANOVA test was used for assessment of the multiple groups. Correlation analyses were carried out using linear regression analysis. *P* values less than 0.05 were considered statistically significant.

Results

Characteristics of HCV-infected patients

A total 109 of HCV patients (including chronic HCV patients 89 and HCV patients with cirrhosis 20) and 49 healthy controls were recruited. As expected, all patients had disease duration more than 6 months and were consistent with their chronic state. In comparison with that in the HC, abnormally higher levels of sera ALT, AST, positive HCV RNA load were identified (**Table 1**).

High frequency of CD19+CD24hiCD38hi regulatory B cells but not CD4+CD25+CD127low regulatory T cells in the peripheral blood of HCV-infected patients compared to healthy controls

We first characterized the frequency of CD19+CD24hiCD38hi Bregs and CD4+CD25+ CD127low Tregs in these subjects (**Figure 1A-C**). The proportion of CD19+CD24hiCD38hi Bregs in peripheral blood CD19+ B cells of HCV-infected patients was significantly greater than HC (*P*<0.001) (**Figure 1B**). In addition, Tregs are characterized by high expression of CD4, CD25
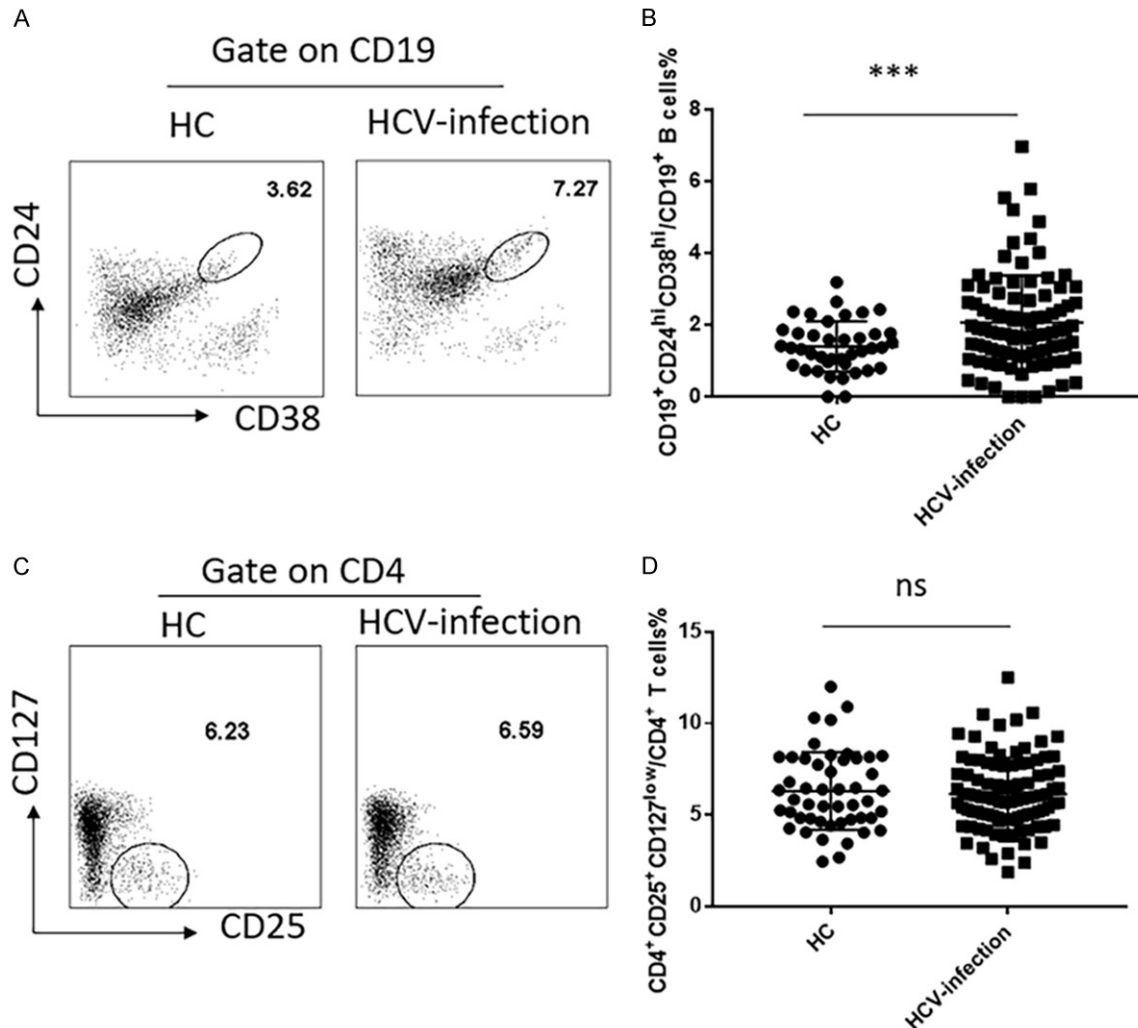
Figure 1. Increased frequency of CD19+CD24hiCD38hi regulatory B cells but not CD4+CD25+CD127low regulatory T cells in HCV-infected patients and healthy controls. A. CD19+CD24hiCD38hi regulatory B cells were detected by flow cytometry. B. The proportion of CD19+CD24hiCD38hi regulatory B cells was compared between HCV-infected patients (2.069 ± 0.1344) (N = 95) and Healthy controls (1.401 ± 0.1115) (N = 39). C. Gating strategy of CD4+CD25+CD127low regulatory T cells on flow cytometry. D. The proportion of CD4+CD25+CD127low regulatory T cells was compared between HCV-infected patients (6.15 ± 0.1975) (N = 99) and Healthy controls (6.308 ± 0.3033) (N = 49). Values were Mean ± SEM. Statistical analyses were performed using t test. ***$P<0.001$, ns, not significant.

and low expression of CD127 (IL-7 receptor α) in the peripheral blood [22]. However, there were no significant differences in the percentage of CD25+CD127low cells within CD4+ T cells among these two groups of subjects ($P>0.05$) (**Figure 1D**). Bregs may play a pivotal role in the persistent infection of HCV virus.

Increased numbers of Bregs are positively correlated with the values of clinical measures in the HCV-infected patients

To further understand the significance of increased numbers of Breg cells in the patho-genesis of HCV infection, we analyzed the potential relationship between the numbers of Bregs and the values of clinical parameters. As shown in **Figure 2A**, the proportion of circulating Breg cells was correlated positively with the levels of sera HCV RNA loads, AST and total bilirubin levels, though the correlation was not very strong. Moreover, the percentage of Bregs was significantly higher in ALT abnormal patients than that in patients with normal sera ALT levels (Supplementary Figure 1A). Nonetheless, the genotype seems to not affect the proportion of peripheral Bregs (Supplementary Figure 1B). As for Treg cells, no significant cor-
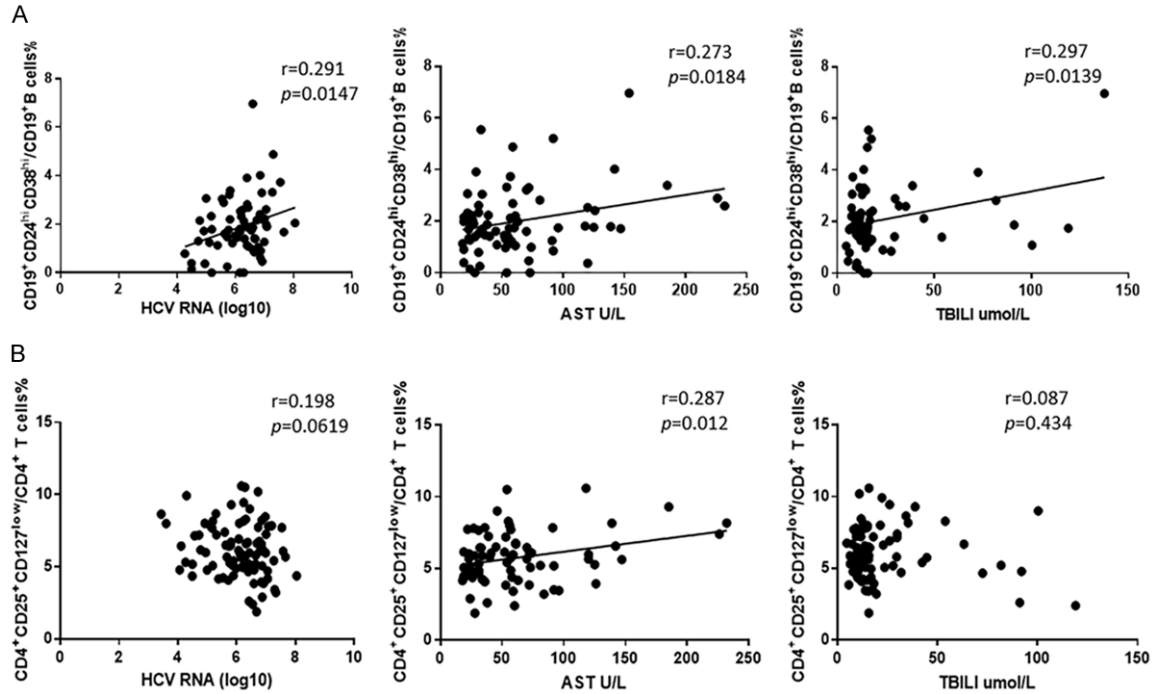
Figure 2. Correlation analysis between CD19+CD24hiCD38hi regulatory B cells or CD4+CD25+CD127low regulatory T cells and clinical parameters. A. The proportion of CD19+CD24hiCD38hi regulatory B cells was positively correlated with HCV RNA loads (r = 0.291, *P* = 0.0147, N = 83) (left) and sera AST levels (r = 0.273, *P* = 0.0184, N = 87) (middle) and sera total bilirubin levels (r = 0.297, *P* = 0.0139, N = 70) (right). B. The proportion of CD4+CD25+CD127low regulatory T cells was not associated with HCV RNA loads (r = 0.198, *P* = 0.0619, N = 90) (left) and sera total bilirubin levels (r = 0.087, *P* = 0.434, N = 83) (right), but positively correlated with sera AST levels (r = 0.287, *P* = 0.0012, N = 91) (middle). Statistical analyses were performed using linear regression analysis.

relation was found between the proportion and HCV RNA loads, total bilirubin levels, but low positively correlated with sera AST levels (**Figure 2B**). Therefore, increased numbers of Breg but not Treg cells are associated with the virus replication and liver injury in HCV patients in this population.
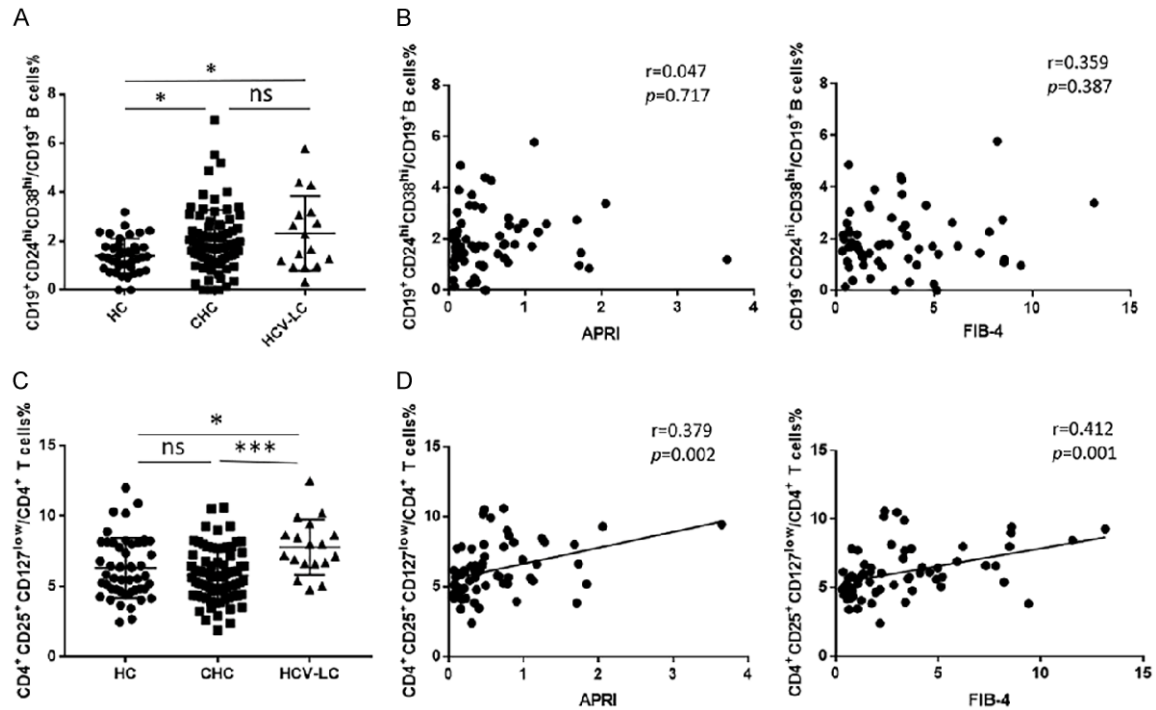
CD4+CD25+CD127low regulatory T cells increase in patients with hepatitis C cirrhosis and have the positive correlation with APRI and FIB-4 scores than CD19+CD24hiCD38hi regulatory B cells

Since previous studies reported that Tregs can activate fibrogenesis in hepatic stellate cells, and promote the progression the chronic HCV induced liver fibrosis [23], we next investigated whether the frequency of peripheral circulating Tregs and Bregs could relate to liver fibrosis and be associated with disease involvement. As shown in **Figure 3A**, chronic hepatitis C patients (CHC) and patients with hepatitis C cirrhosis (HCV-LC) had a higher percentage in

Bregs. However, there were no statistical differences between the two groups. Unlike Bregs, the patients with hepatitis C cirrhosis had a significantly higher percentage of Tregs compared with HC and CHC (**Figure 3C**). In addition, since APRI index and Fibrosis-4 score were usually used for estimating the degree of liver fibrosis [20]. We studied the correlation and observed that Tregs were significantly positively associated with the APRI index and Fibrosis-4 score (**Figure 3D**). Moreover, patients with higher APRI or FIB-4 indexes had significantly higher percentages of Tregs than those patients with lower APRI or FIB-4 indexes (P<0.05) (Supplementary Figure 2A, 2B). These results suggest that the circulating Tregs but not Bregs play a more important role in the progression of cirrhosis.

Previous study revealed that the expression of CD226 and TIGIT on human Tregs might be involved in the stability and immunosuppressive function of Treg [24]. In our data, the expression of TIGIT on Treg in HCV-infected

Figure 3. CD4+CD25+CD127low regulatory T cells increase in patients with hepatitis C cirrhosis and have the positive correlation with APRI index and Fibrosis-4 scores than CD19+CD24hiCD38hi regulatory B cells. A. The proportion of CD19+CD24hiCD38hi regulatory B cells were compared between chronic HCV patients (N = 89) and HCV liver cirrhosis patients (N = 16) and healthy controls (N = 47). C. CD4+CD25+CD127low regulatory T cells were compared between chronic HCV patients (N = 89) and HCV liver cirrhosis patients (N = 18) and healthy controls (N = 49). Values were Mean ± SEM. Statistical analyses were performed using one-way ANOVA. (*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$). B. The correlation between CD19+CD24hiCD38hi regulatory B cells and APRI index (r = 0.047, P = 0.717, N = 64) (left) and Fibrosis-4 score (r = 0.359, P = 0.387, N = 60) (right). D. The correlation between CD4+CD25+CD127low regulatory T cells and the APRI index (r = 0.379, P = 0.002, N = 67) (left), Fibrosis-4 score (r = 0.412, P = 0.001, N = 62) (right). Statistical analyses were performed using linear regression analysis.

patients was significantly elevated, primarily in chronic hepatitis C patients (Supplementary Figure 3C). Data indicated that the expression of TIGIT on Tregs was positively correlated with the percentages of Tregs in the peripheral blood and sera AST levels (Supplementary Figure 4A, 4B). TIGIT+ Tregs may be the crucial subpopulation of Tregs that contributes to the development of HCV infection.
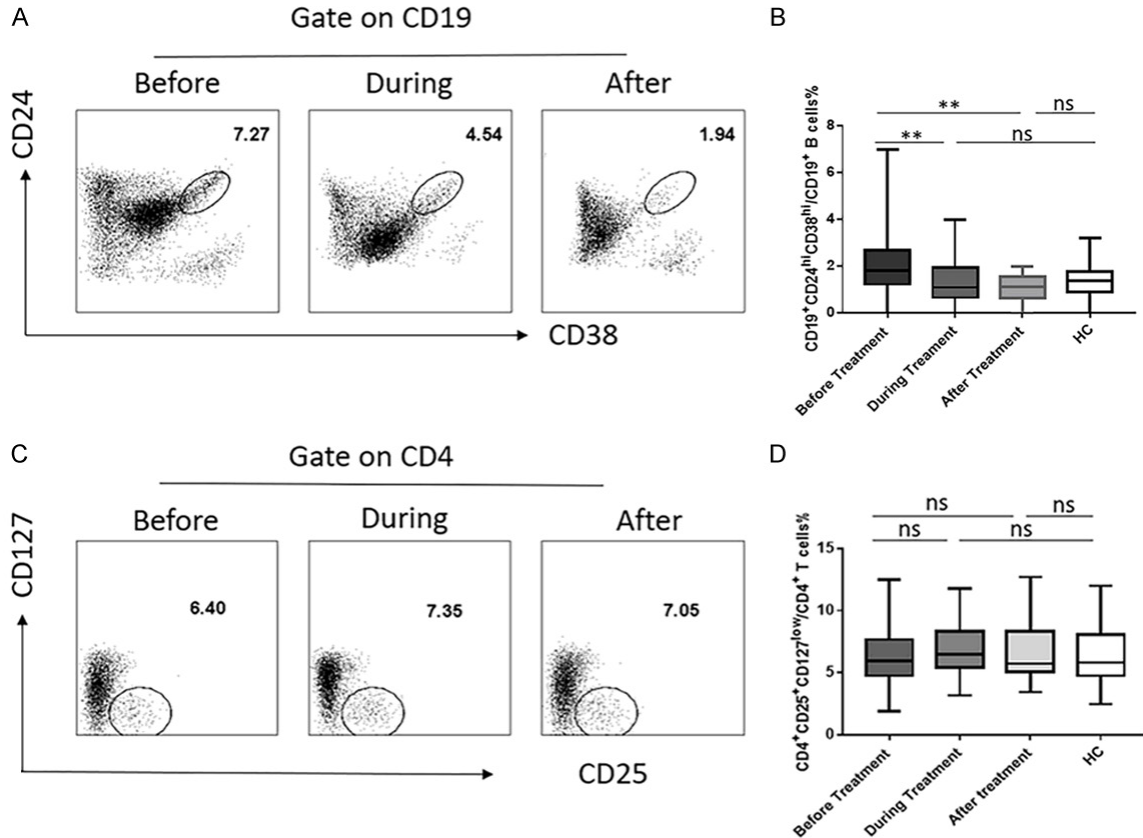
The dynamic changes of the CD19+CD24hiCD38hi regulatory B cells and CD4+CD25+CD127low regulatory T cells in HCV-infected patients with DAA treatment

In recent years, although the development of DAA treatment has dramatically improved HCV treatment options [25]. A statistically significant decrease in the proportion of Bregs was observed during and after DAA treatment compared to prior treatment. Usually patients take DAA for 12 weeks, 0-12 weeks were named

during DAA treatment and 12 weeks was named after DAA treatment, when patients achieve a sustained virologic response. Additionally, there were no significant differences of Bregs among the during after treatment and healthy controls (**Figure 4B**). Interestingly, the frequency of Tregs did not show significant changes after antiviral treatment (**Figure 4D**). It is likely that the important indicator of successful therapy in HCV patients is the viral load; Bregs but not Tregs had a positive correlation with HCV RNA load. After treatment, the amount of virus in patients was significantly reduced and diminished eventually; Bregs then gradually returned to normal levels.

The sera from HCV-infected patients could induce more IL-10 production in B cells but not more FOXP3 expression in T cells

To further understand why the numbers of regulatory B cells increased in HCV-infected

Figure 4. The dynamic changes of the CD19+CD24hiCD38hi regulatory B cells and CD4+CD25+CD127low regulatory cells in HCV-infected patients with DAA treatment. (A) Change of the CD19+CD24hiCD38hi regulatory B cells before (N = 97), during (N = 34), and after DAA (N = 16) treatment and (B) statistical chart. (C) Change of the CD4+CD25+CD127low regulatory cells before (N = 99), during (N = 67) and after DAA (N = 22) treatment and (D) statistical chart. Values were Mean ± SEM. Statistical analyses were performed using one-way ANOVA. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, ns, not significant.

patients, we conducted a series of experiments *in vitro* to determine whether the increase of regulatory B cells was due to the amplification of Breg cells or the induction from non-Breg cells. First, we observed that the healthy PBMC stimulated by the sera from HCV-infected patients produced a significant higher proportion of the CD19+CD24hiCD38hi regulatory B cells when compared with healthy control sera (**Figure 5A**). Then, we sorted the CD19+ CD24hiCD38hi regulatory B cells for the amplification or induction of Breg cells from CD19+CD24intCD38int naïve B cells that had been stimulated with LPS and CD40L. Previous study has demonstrated this protocol can induce IL-10+ B cells [11].

Interestingly, there was no significant difference in the total numbers of Breg amplified during six days culture under different conditions (Supplementary Figure 5). Since IL-10 is the most important functional molecule for Bregs, we selected this cytokine as the differentiation indicator. As we expected, we noted that sera from HCV patients indeed induced naïve B cells to produce more IL-10 including mRNA and protein levels (**Figure 5B-D**). We also did a series of similar experiments on CD4+CD25+CD127low regulatory T cells, but no significant differences were found (Supplementary Figure 6A-E), suggesting HCV-sera neither expands nor induces Treg cells. Given many transcription factors, such as c-Maf, GATA3, AhR, play a crucial role in regulating the secretion of IL-10 by regulatory B cells, and AhR could directly bind to and regulate the expression of IL-10 in Bregs [13, 14], we also detected the expression of those transcription factors and found that, compared with c-Maf and GATA3, AhR increased significantly after HCV serum stimulation (**Figure 5E**). It is possible that the increasing of IL-10 secreting in B cells under HCV-infected patients is mediated by the HCV-sera-AhR signaling.
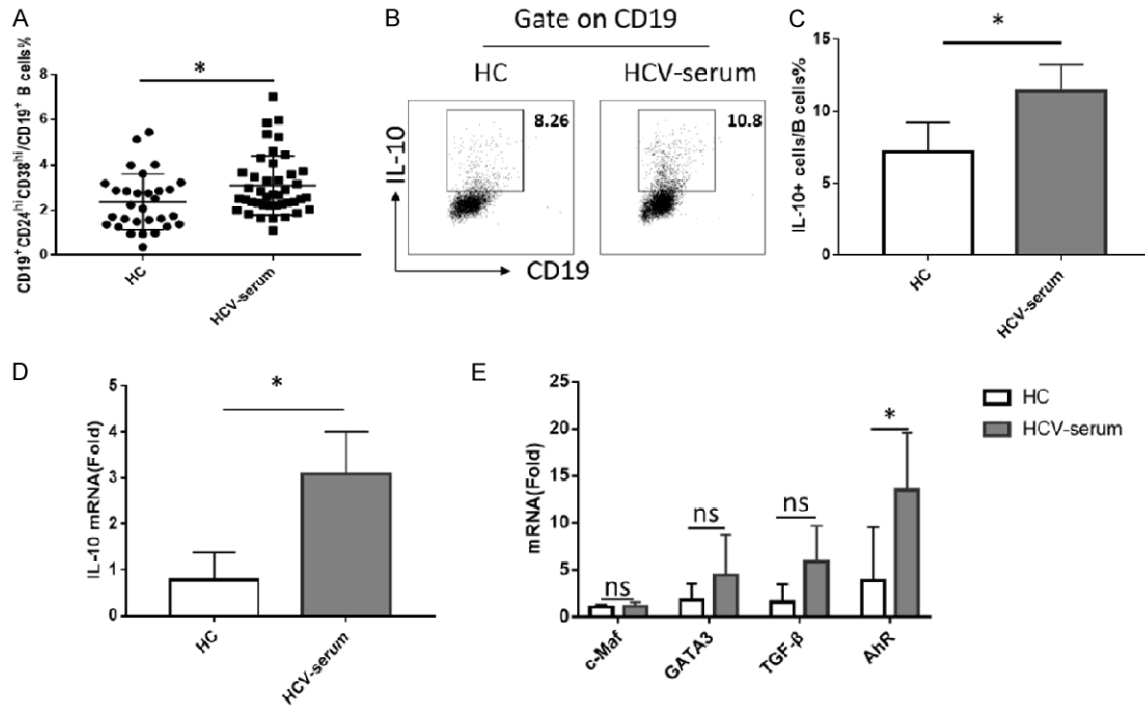
Figure 5. The sera from HCV-infected patients could induce more IL-10 produce in B cells but not more FOXP3 expression in T cells. (A) Healthy PBMC stimulated with LPS (1 ug/ml) for 3 days under HCV-sera (N = 42), produced high percentages of CD19+CD24hiCD38hi regulatory B cells compared with healthy control (N = 30). (B) Sorted CD19+CD24intCD38int naïve B cells from healthy control stimulated with LPS (1 ug/ml) and CD40L (1 ug/ml) under different sera for 3 days. HCV sera induced B cell produce more IL-10, including mRNA (D) and protein level (C). (E) The mRNA levels of relative transcription factors were detected in B cells stimulated with different serum conditions. Values were Mean ± SEM of three independent experiments. Statistical analyses were performed using t test. *: P<0.05, ns, not significant.

Discussion

Approximately 70% of HCV infected individuals may develop chronic infection [16, 20]. A complex and mutual interaction between innate and adaptive immune responses plays a major role in the clearance of HCV infection [26]. Many patients develop immunodeficiency with impaired T cells responses and dysfunctional B cell activation [27]. Meanwhile, regulatory cells take part in the viral evasion and the persistence of HCV infection [28].

Among immune suppressors including T, B, DC, macrophage and other subsets, Tregs play a dominate role in immune tolerance [28-31]. Treg cells comprise a collection of innate or natural Treg subsets (tTreg), induced in the periphery (pTreg) and differentiated ex vivo (iTreg) [32, 33]. Treg cells regulate immune response through direct cell to cell contact or through the elaboration of cytokines [34, 35]. In addition, B regulatory (Breg) cells, which

mainly secret IL-10 suppressive cytokines, have been rigorously studied in the last decade [10, 36]. A subset of Bregs in human peripheral blood exhibit a CD19+CD24hiCD38hi phenotype and are the IL-10 producer which expresses both CD1dhi and immunoglobulin D [35, 37]. These cells represent about 1% of peripheral blood mononuclear cells and decrease in patients with systemic lupus erythematosus (SLE), fail to suppress tumor necrosis factor (TNF) and IFN production by Th1 cells [12, 37]. Many studies have begun to focus on the relationship between this group of Bregs and immune-related disorders [12, 38] and hold a great promise for their clinical application.

In the present study, we found that the frequencies of circulating CD19+CD24hiCD38hi regulatory B cells were significantly increased in HCV patients. This finding could implicate that Bregs may have been activated after HCV antigenic stimulation in helping the virus escape from attacking via their immunosup-

pressive role. In addition, we found significant percentages of circulating Bregs were positively correlated with the levels of sera AST, TBILI and HCV virus load which reflect to liver damage [39]. Other similar studies have reported that circulating regulatory B cells may have an impact on the pathogenesis of various diseases such as chronic hepatitis B virus [40], rheumatoid arthritis [37], even in cancers [38].

We previously reported that Treg cells are effective in preventing liver damage [41, 42]. Our results were also different from previous studies reporting data that CD4^{+}CD25^{+}Foxp3^{+} Tregs were increased in patients with chronic hepatitis C and may mediate the sustained suppression of HCV-specific T-cell responses [3, 43-45]. Those differences may derive from phenotypic differences in patients recruited, disease duration, and methods used for Treg cells evaluation. Additionally, race, disease activity and treatment also have been shown to have an impact on the frequencies of Treg [4, 42].

Nonetheless, major findings of the present study are that Tregs but not Bregs were significantly positively associated with the APRI index and Fibrosis-4 scores. These results indicate that Tregs may have a pivotal role in the disease progression stage and promote the occurrence and development of liver cirrhosis in the late stage, which is consistent with other studies [23, 46]. The liver fibrosis process is mainly caused by the imbalance of synthesis and degradation of extracellular matrix produced by activated hepatitis stellate cells [46, 47], they could promote the activation of hepatic stellate through the secretion of cytokines, such as TGF-β [48]. It is known that TGF-β promotes the liver fibrosis [49]. Thus, Treg cells have multiple roles in the pathogenesis of liver inflammation, damage and fibrosis. In the early stage, Treg can suppress inflammation and damage, nonetheless, Treg cells may promote liver fibrosis through TGF-β. TGF-β is crucial for Treg development and differentiation, forming a feedback circulation of Treg, TGF-β, Treg and fibrosis.

With regard to the treatment of HCV, sustained virologic response is the primary purpose because the clinical remission is associated closely with viral clearance. The successful effectiveness and tolerance of direct-acting antivirals (DAAs) in eradicating HCV virus is confirmedly established and become the greatest triumphs of medical therapeutics in the last 20 years [17, 50]. There was a significant decrease of Bregs during and after treatment compared to baseline levels. As this immune mediator was activated by antigenic stimulation at baseline, along with the dramatic viral decline and the reduced liver inflammation, effective therapy aiming at HCV virus plays a strong role in immune balance recovery. After treatment, the amounts of virus in the patients were significantly reduced and diminished eventually, Bregs then gradually return to normal levels, which suggest that the higher frequencies of Bregs in patients with chronic HCV infection may play a relevant role in DAA-induced antiviral responses and can be an important predictor of the achievement of virologic response following DAA drugs treatment in HCV-infected patients. Nevertheless, Tregs may modify multiple immune responses and thus determine the further course of liver disease after HCV clearance, and play a profound impact on the pathogenesis of HCV associated liver cirrhosis.

We also demonstrated that increased circulating Bregs may be due to the induced differentiation rather than amplification. It is likely that HCV-sera induces the differentiation of Breg cells and its function through mediating an AhR signaling. Indeed, AhR directly binds and regulates the expression of IL-10 in Bregs [13]. Nonetheless, HCV-serum does not significantly affect Treg cells. It is under so far why HCV-sera has a different role in Treg and Breg cells. Additionally, underlying mechanisms thereby HCV-sera promotes Breg cells warrant a further study in the future.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Song Guo Zheng, Department of Internal Medicine, Ohio State University College of Medicine and Wexner Medical Center, Columbus, OH 43210, United States. E-mail: SongGuo.Zheng@osumc.edu; Dr. Xiaohong Zhang, Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510060, Guangdong, China. E-mail: 1716511502@qq.com

References

[1] Wang L, Qiu J, Yu L, Hu X, Zhao P and Jiang Y. Increased numbers of CD5+CD19+CD1dhighIL-10+ Bregs, CD4+Foxp3+ Tregs, CD4+CXCR5+Foxp3+ follicular regulatory T (TFR) cells in CHB or CHC. J Transl Med 2014; 12: 251.

[2] Saeidi A, Zandi K, Cheok YY, Saeidi H, Wong WF, Lee CYQ, Cheong HC, Yong YK, Larsson M and Shankar EM. T-cell exhaustion in chronic infections: reversing the state of exhaustion and reinvigorating optimal protective immune responses. Front Immunol 2018; 9: 2569.

[3] Zhai N, Chi X, Li T, Song H, Li H, Jin X, Crispe IN, Su L, Niu J and Tu Z. Hepatitis C virus core protein triggers expansion and activation of CD4(+)CD25(+) regulatory T cells in chronic hepatitis C patients. Cell Mol Immunol 2015; 12: 743-749.

[4] Horwitz DA, Zheng SG and Gray JD. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD-25(+) regulatory T cells are not mirror images of each other. Trends Immunol 2008; 29: 429-435.

[5] Liu ZM, Wang KP, Ma J and Guo Zheng S. The role of all-trans retinoic acid in the biology of Foxp3+ regulatory T cells. Cell Mol Immunol 2015; 12: 553-557.

[6] Hu G, Liu Z, Zheng C and Zheng SG. Antigen-non-specific regulation centered on CD25+Foxp3+ Treg cells. Cell Mol Immunol 2010; 7: 414-418.

[7] Langhans B, Braunschweiger I, Arndt S, Schulte W, Satoguina J, Layland LE, Vidovic N, Hoerauf A, Oldenburg J, Sauerbruch T and Spengler U. Core-specific adaptive regulatory T-cells in different outcomes of hepatitis C. Clin Sci (Lond) 2010; 119: 97-109.

[8] Mauri C and Ehrenstein MR. The 'short' history of regulatory B cells. Trends Immunol 2008; 29: 34-40.

[9] Wang P and Zheng SG. Regulatory T cells and B cells implication on autoimmune diseases. Int J Clin Exp Pathol 2013; 6: 2668-2674.

[10] Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M and Tedder TF. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity 2008; 28: 639-650.

[11] Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, Hall RP, St Clair EW and Tedder TF. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood 2011; 117: 530-541.

[12] Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR and Mauri C. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. Immunity 2010; 32: 129-140.

[13] Piper CJM, Rosser EC, Oleinika K, Nistala K, Krausgruber T, Rendeiro AF, Banos A, Drozdov I, Villa M, Thomson S, Xanthou G, Bock C, Stockinger B and Mauri C. Aryl hydrocarbon receptor contributes to the transcriptional program of IL-10-producing regulatory B cells. Cell Rep 2019; 29: 1878-1892, e1877.

[14] Liu M, Zhao X, Ma Y, Zhou Y, Deng M and Ma Y. Transcription factor c-Maf is essential for IL-10 gene expression in B cells. Scand J Immunol 2018; 88: e12701.

[15] Majlessi L, Lo-Man R and Leclerc C. Regulatory B and T cells in infections. Microbes Infect 2008; 10: 1030-1035.

[16] Biragyn A and Lee-Chang C. A new paradigm for an old story: the role of regulatory B cells in cancer. Front Immunol 2012; 3: 206.

[17] Au JS and Pockros PJ. Novel therapeutic approaches for hepatitis C. Clin Pharmacol Ther 2014; 95: 78-88.

[18] Singer AW, Reddy KR, Telep LE, Osinusi AO, Brainard DM, Buti M and Chokkalingam AP. Direct-acting antiviral treatment for hepatitis C virus infection and risk of incident liver cancer: a retrospective cohort study. Aliment Pharmacol Ther 2018; 47: 1278-1287.

[19] Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS and Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology 2003; 38: 518-526.

[20] Zhang M, Zhang L, Li H, Chen Z, Luo A, Liu B, Chen M, Peng M, Ren H and Hu P. Circulating T follicular helper cells are associated with rapid virological response in chronic hepatitis C patients undergoing peginterferon therapy. Int Immunopharmacol 2016; 34: 235-243.

[21] Vallet-Pichard A, Mallet V, Nalpas B, Verkarre V, Nalpas A, Dhalluin-Venier V, Fontaine H and Pol S. FIB-4: an inexpensive and accurate

marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. Hepatology 2007; 46: 32-36.

[22] Khalili A, Ebrahimpour S, Maleki I, Abedi-ankenari S and Afrouzi MM. CD4+CD25+ CD127low FoxP3+ regulatory T cells in Crohn's disease. Rom J Intern Med 2018; 56: 158-166.

[23] Langhans B, Kramer B, Louis M, Nischalke HD, Huneburg R, Staratschek-Jox A, Odenthal M, Manekeller S, Schepke M, Kalff J, Fischer HP, Schultze JL and Spengler U. Intrahepatic IL-8 producing Foxp3(+)CD4(+) regulatory T cells and fibrogenesis in chronic hepatitis C. J Hepatol 2013; 59: 229-235.

[24] Elhai M, Chiocchia G, Marchiol C, Lager F, Renault G, Colonna M, Bernhardt G, Allanore Y and Avouac J. Targeting CD226/DNAX accessory molecule-1 (DNAM-1) in collagen-induced arthritis mouse models. J Inflamm (Lond) 2015; 12: 9.

[25] Langhans B, Nischalke HD, Krämer B, Hausen A, Dold L, van Heteren P, Hüneburg R, Nattermann J, Strassburg CP and Spengler U. Increased peripheral CD4+ regulatory T cells persist after successful direct-acting antiviral treatment of chronic hepatitis C. J Hepatol 2017; 66: 888-896.

[26] Burton JR Jr, Klarquist J, Im K, Smyk-Pearson S, Golden-Mason L, Castelblanco N, Terrault N and Rosen HR; Virahep-C Study Group. Prospective analysis of effector and regulatory CD4+ T cells in chronic HCV patients undergoing combination antiviral therapy. J Hepatol 2008; 49: 329-338.

[27] Bowen DG and Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 2005; 436: 946-952.

[28] Eiza N, Toubi E and Vadasz Z. Increased T and B regulatory cell function contributes to the persistence of HCV and other viral infections. Isr Med Assoc J 2016; 18: 159-61.

[29] Hayday A and Tigelaar R. Immunoregulation in the tissues by gammadelta T cells. Nat Rev Immunol 2003; 3: 233-242.

[30] Horwitz DA, Zheng SG, Gray JD, Wang JH, Ohtsuka K and Yamagiwa S. Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease. Semin Immunol 2004; 16: 135-143.

[31] Lan Q, Fan H, Quesniaux V, Ryffel B, Liu Z and Zheng SG. Induced Foxp3(+) regulatory T cells: a potential new weapon to treat autoimmune and inflammatory diseases? J Mol Cell Biol 2012; 4: 22-28.

[32] Zheng SG, Gray JD, Ohtsuka K, Yamagiwa S and Horwitz DA. Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. J Immunol 2002; 169: 4183-9.

[33] Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, Rudensky A, Sakaguchi S, Shevach EM, Vignali DA and Ziegler SF. Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol 2013; 14: 307-308.

[34] Zheng SG, Wang JH, Gray JD, Soucier H and Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. J Immunol 2004; 172: 5213-21.

[35] Zhong H, Liu Y, Xu Z, Liang P, Yang H, Zhang X, Zhao J, Chen J, Fu S, Tang Y, Lv J, Wang J, Olsen N, Xu A and Zheng SG. TGF-β-induced CD8+CD103+ regulatory T cells show potent therapeutic effect on chronic graft-versus-Host disease lupus by suppressing B cells. Front Immunol 2018; 9: 35.

[36] Mauri C and Bosma A. Immune regulatory function of B cells. Annu Rev Immunol 2012; 30: 221-241.

[37] Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA and Mauri C. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. Sci Transl Med 2013; 5: 173ra123.

[38] Gheybi MK, Farrokhi S, Ravanbod MR, Ostovar A, Mehrzad V and Nematollahi P. The correlation of CD19+CD24+CD38+ B cells and other clinicopathological variables with the proportion of circulating tregs in breast cancer patients. Breast Cancer 2017; 24: 756-764.

[39] Sun Y, Gu J, Liu R, Zhou H, Lu L, Dai X and Qian X. IL-2/IL-6 ratio correlates with liver function and recovery in acute liver injury patients. APMIS 2019; 127: 468-474.

[40] Liu Y, Cheng LS, Wu SD, Wang SQ, Li L, She WM, Li J, Wang JY and Jiang W. IL-10-producing regulatory B cells suppressed effector T but enhanced regulatory T cells in chronic HBV infection. Clin Sci (Lond) 2016; 130: 907-919.

[41] Billerbeck E, Bottler T and Thimme R. Regulatory T cells in viral hepatitis. World J Gastroenterol 2007; 13: 4858-4864.

[42] Lu L, Feng M, Gu J, Xia Z, Zhang H, Zheng S, Duan Z, Hu R, Wang J, Shi W, Ji C, Shen Y, Chen G, Zheng SG and Han YP. Restoration of intrahepatic regulatory T cells through MMP-9/13-dependent activation of TGF-beta is critical for immune homeostasis following acute liver injury. J Mol Cell Biol 2013; 5: 369-379.

[43] Boettler T, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von Weizsacker F and Thimme R. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific

CD8+ T cells during chronic hepatitis C virus infection. J Virol 2005; 79: 7860-7867.

[44] Cabrera R, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C and Nelson DR. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. Hepatology 2004; 40: 1062-1071.

[45] Ayers CL, Firan M, Pillai V, Lee WM and Karandikar NJ. Viral interactions with B-cells contribute to increased regulatory T-cells during chronic HCV infection. Viral Immunol 2011; 24: 119-129.

[46] Ward SM, Fox BC, Brown PJ, Worthington J, Fox SB, Chapman RW, Fleming KA, Banham AH and Klenerman P. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. J Hepatol 2007; 47: 316-324.

[47] Zhang X, Ouyang X, Xu Z, Chen J, Huang Q, Liu Y, Xu T, Wang J, Olsen N, Xu A and Zheng SG. CD8+CD103+ iTregs inhibit chronic graft-versus-host disease with lupus nephritis by the increased expression of CD39. Mol Ther 2019; 27: 1963-1973.

[48] Lu L, Ma J, Wang X, Wang J, Zhang F, Yu J, He G, Xu B, Brand DD, Horwitz DA, Shi W and Zheng SG. Synergistic effect of TGF-beta superfamily members on the induction of Foxp3+ Treg. Eur J Immunol 2010; 40: 142-152.

[49] Tu X, Zhang Y, Zheng X, Deng J, Li H, Kang Z, Cao Z, Huang Z, Ding Z, Dong L, Chen J, Zang Y and Zhang J. TGF-beta-induced hepatocyte lincRNA-p21 contributes to liver fibrosis in mice. Sci Rep 2017; 7: 2957.

[50] Ioannou GN and Feld JJ. What are the benefits of a sustained virologic response to direct-acting antiviral therapy for hepatitis C virus infection? Gastroenterology 2019; 156: 446-460, e442.
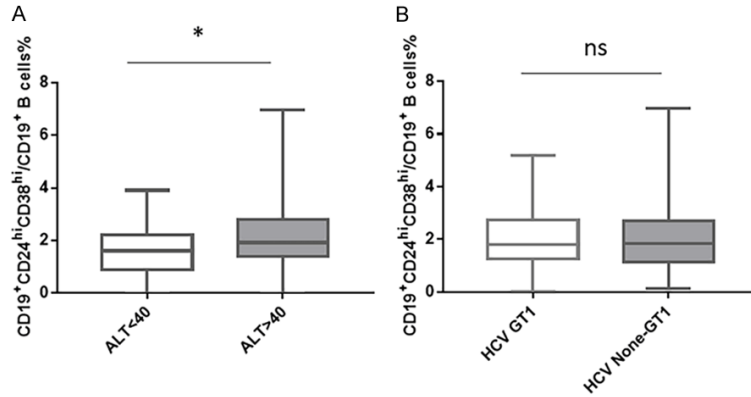
Supplementary Figure 1. A. The HCV genotype dose not influence the proportion CD19+CD24hiCD38hi regulatory B cells. The proportion of CD19+CD24hiCD38hi regulatory B cells between the ALT<40 U/L (1.603 ± 0.1931) and ALT>U/L (2.312 ± 0.1888) groups (N = 35 for ALT<40 and N = 64 for ALT>40). B. The proportion of CD19+CD24hiCD38hi regulatory B cells between HCV GT1 (2.012 ± 0.1841) and HCV none-GT1 (2.125 ± 0.2236) group (N = 52 for HCV GT1 and N = 47 for none-GT1). Statistical analyses were performed using t test. *: P<0.05, ns, not significant. (HCV GT1: HCV genotype 1, HCV None-GT1: HCV no genotype 1).
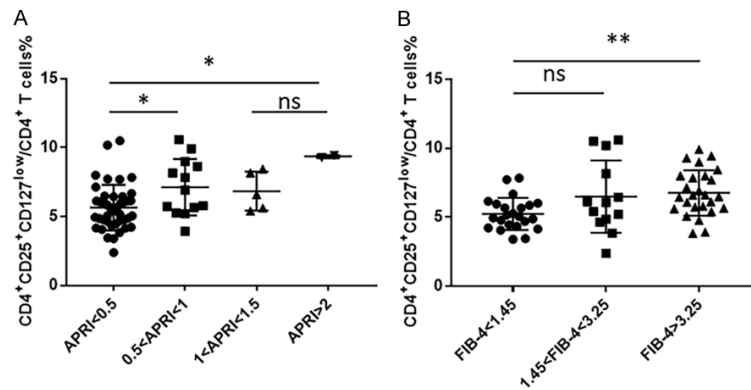

Supplementary Figure 2. Comparison of the percentages of CD4+CD25+CD127low regulatory T cells among different APRI or FIB-4 groups. A. The proportion of CD4+CD25+CD127low regulatory T cells in different groups according to the APRI index (the numbers of the four groups are 36, 13, 5 and 2). B. The proportion of CD4+CD25+CD127low regulatory T cells in different groups according to the FIB-4 scores (the numbers of the three groups are 23, 13 and 25). Statistical analyses were performed using one-way ANOVA. *: P<0.05, **: P<0.01, ns, not significant.
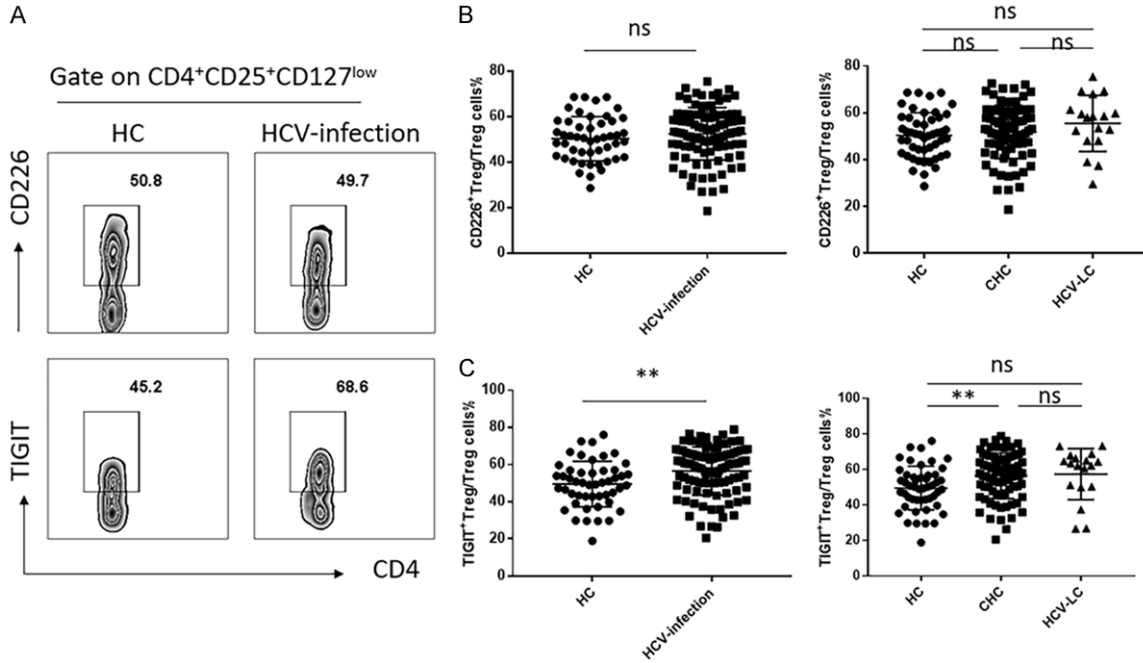
Supplementary Figure 3. TIGIT⁺ Tregs increase in chronic HCV-infected patients. A. CD226 and TIGIT expression on peripheral blood CD4⁺CD25⁺CD127^low regulatory T cells was detected by flow cytometry. B. The expression levels of CD226 on Tregs were compared and analyzed. (left) HCV-infected patients and healthy controls groups (N = 47 for healthy controls and N = 99 for patients with HCV-infection), (right) chronic HCV patients and HCV liver cirrhosis patients, as well as healthy controls groups (the numbers of three groups are 47, 89, and 18). C. The expression levels of TIGIT on Tregs were compared and analyzed. (left) HCV-infected patients and healthy controls (N = 47 for healthy controls and N = 99 for patients with HCV-infection), (right) chronic HCV patients and HCV liver cirrhosis patients and healthy controls (the numbers of three groups are 47, 89, and 18). Statistical analyses were performed using t test (HCV-infection *versus* HC) and one-way ANOVA (CHC *versus* HCV-LC and HC). *: $P<0.05$, **: $P<0.01$, ns, not significant.
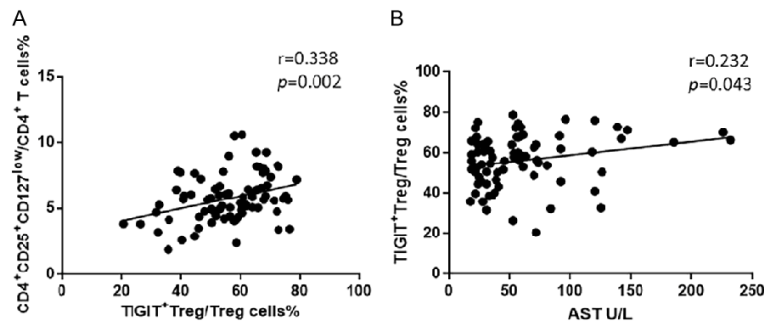

Supplementary Figure 4. TIGIT⁺ Tregs have a positive correlation with Tregs and sera AST levels. (A) The expression of TIGIT on Tregs was positively correlated with the proportion of Tregs (r = 0.338, P = 0.002) (N = 68) and (B) sera AST levels (r = 0.232, p = 0.043) (N = 68). Linear regression analysis was performed.
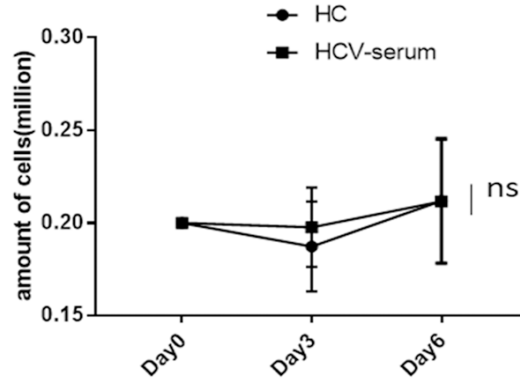
Supplementary Figure 5. HCV sera does not amplify CD19$^+$CD24^{hi}CD38^{hi} regulatory B cells *in vitro*. CD19$^+$CD24^{hi}CD38^{hi} regulatory B cells were sorted from healthy subjects and stimulated with LPS (1 ug/ml) and CD40L (1 ug/ml) in the presence of healthy and HCV sera for days indicated in *vitro*. Total numbers in each well were counted in each time point. Values were Mean ± SEM of three separate experiments. Statistical analyses were performed using one-way ANOVA. ns, not significant.
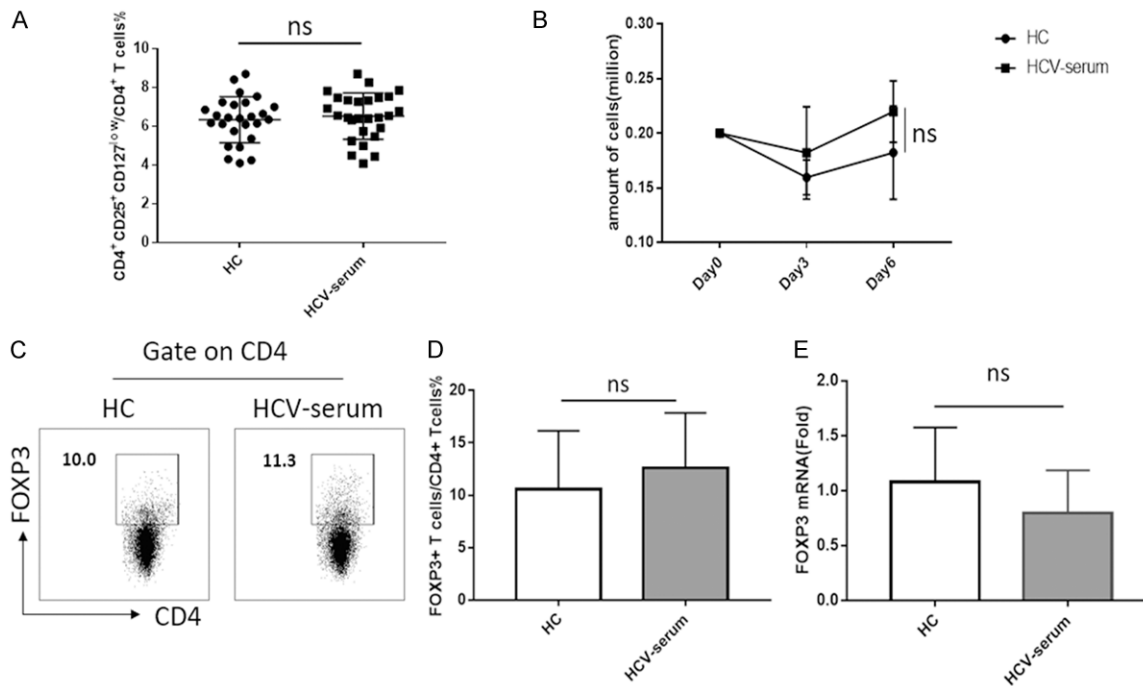
Supplementary Figure 6. HCV serum neither expands nor induces the regulatory T cells. (A) PBMC isolated from healthy controls stimulated with anti-CD3/CD28 antibodies for 3 days under the HCV (N = 26) and control sera (N = 27). The percentages of CD4$^+$CD25$^+$CD127^{low} cells were compared and analyzed. The t test was performed between two groups. (B) CD4$^+$CD25$^+$CD127^{low} cells isolated from healthy subjects were stimulated with anti-CD3/CD28 coating beads and rhIL-2, rhTGF-β with healthy (HC) and HCV sera (HCV-serum) for days indicated and total cell numbers were counted. Statistical analyses were performed using one-way ANOVA. (C-E) The expression of FOXP3 from naïve T cells stimulated with CD3/CD28 coating beads and rhIL-2, rhTGF-β in the presence of healthy and HCV sera for 3 days, (C) is representative of flow analysis. (D, E) indicate Foxp3 protein and mRNA levels. Values were Mean ± SEM of three independent experiments. Statistical analyses were performed using t test. ns, not significant.

3