Published in final edited form as:

Clin Immunol. 2014 February ; 150(2): 184–191. doi:10.1016/j.clim.2013.12.001.

Peripheral CD27⁺CD21⁺ B-cells represent an exhausted lymphocyte population in hepatitis C cirrhosis[☆]

Hiroyoshi Doi^b, Shiroh Tanoue^b, and David E. Kaplan^{a,b,*}

^aMedicine and Research Services, Philadelphia VA Medical Center, Philadelphia, PA, USA

^bDivision of Gastroenterology, Department of Medicine, University of Pennsylvania, USA

Abstract

Hepatitis C cirrhosis is associated with a profound disappearance of memory B-cells. We sought to determine if this loss is associated with the expansion of the CD27⁺CD21⁺ tissue-like memory B-cells with features of B-cell exhaustion. To this end, we quantified the frequency of CD27⁺CD21⁺ B-cells in healthy, non-cirrhotic HCV-infected, and cirrhotic patients. We examined the expression of putative inhibitory receptors, the proliferative and immunoglobulin-secreting capacity of CD27/CD21-defined B-cell subsets upon B-cell receptor and/or CD40 stimulation. We found that CD27⁺CD21⁺ B-cells are significantly increased in frequency relative to healthy donors in HCV-infected patients. CD27⁺CD21⁺ B-cells were hypoproliferative relative to naïve and resting memory B-cells upon agonistic stimulation, but retained similar capacity for antibody secretion. Conclusion: CD27⁺CD21⁺ tissue-like memory B-cells with exhausted proliferation circulate at increased frequency in cirrhotic and non-cirrhotic HCV-infected patients. This B-cell subset does not appear anergic, exhibiting immunoglobulin-secreting capacity on CD40 agonism indistinguishable from other CD27/CD21-defined B-cell subsets.

Keywords

Human; B-cell; Lymphocyte; Hepatitis C; Anergy; CD21

1. Introduction

An estimated 170 million individuals worldwide including 3 million persons in the United States are infected by the hepatitis C virus [1]. Over 70% of the persistently infected individuals develop chronic hepatic inflammation (hepatitis), which progresses to cirrhosis

[☆]Funding support: This work was supported by an unrestricted scientific grant from the Schering-Plough Research Institute affiliated with Merck Sharp & Dohme Corp (DEK). The authors would also like to thank the patients and volunteers who contributed samples. The content of this article does not reflect the views of the VA or of the US Government.

*Corresponding author at: Philadelphia VA Medical Center, Research Bldg 21, A402A, 3900 Woodland Avenue, Philadelphia, PA 19104, USA. Fax: +1 215 823 4289. dakaplan@mail.med.upenn.edu (D.E. Kaplan).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest

The authors declare no competing financial interests.

in approximately 20–30% of infected individuals usually over the course of 2–3 decades [2]. Hepatitis C infection is characterized by profound hyperglobulinemia consisting of non-virus-specific antibodies [3,4] produced by oligoclonally-activated B-cells [5,6]. Somewhat unexpectedly, chronic B-cell activation in chronic hepatitis C does not result in expansion of the memory B-cell pool in cohorts of mostly non-cirrhotic individuals [7–9]. Possible reasons cited for the lack of peripheral memory B-cell expansion include increased plasma cell differentiation [8], increased activation-induced B-cell apoptosis [8], and intrahepatic compartmentalization [10]. Among these explanations, activation-induced apoptosis has been contradicted by more recent data suggesting that B-cells in HCV-infected individuals are relatively resistant to apoptosis [11,12]. Rather than being expanded, we previously demonstrated that the circulating memory B-cell population disappears in cirrhotic but not non-cirrhotic HCV-infected patients [13]. The reduction in memory B-cells strongly correlated with multiple parameters of liver dysfunction and portal hypertension, also occurred in individuals with cirrhosis from other causes, and associated with a reduction in B-cell antigen-presenting cell function.

An alternative hypothesis to explain the disappearance of peripheral CD27⁺ memory B-cells is the conversion of activated memory B-cells into CD27[−]CD21[−] “tissue-like memory” B-cells that manifest evidence of B-cell anergy. A virus-specific anergic CD27[−]CD21[−] B-cell population has been described in HIV disease that may be identified by the expression of FcRL4 [14,15]. In common variable immunodeficiency, a tissue-homing peripheral CD21^{lo} B-cell population with impaired proliferation but exaggerated IgM secretory capacity with phenotypic similarities to the CD27[−]CD21^{lo} B-cell population in HIV has also been described [16]. Limited investigation in HCV disease has not identified an expansion of a similar CD27[−]CD21[−]FcRL4⁺ B-cell population [9,17] but did suggest that a hyporesponsive CD27[−]CD21^{lo} B-cell population does exist in HCV patients with cryoglobulinemia [17]. FcRL4 putatively mediates its inhibitory effect on B-cell activation via its cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM). Several other ITIM-containing receptors including CD22, CD72, CD300a, CD305 (LAIR-1), Fcγ7RIIB, and CD85j are expressed on B-cells [18–22,24], but the association of these ITIM-bearing receptor expression and B-cell activation in HCV disease remains largely unexamined.

The purpose of this study was to determine if HCV-related cirrhosis is associated with expansion of the CD27[−]CD21[−] B-cell population and to determine if this population indeed represents an anergic B-cell population. We found that CD27[−]CD21[−] B-cells have an increased frequency relative to healthy donors both in cirrhotic and non-cirrhotic HCV-infected patients. We confirm that CD27[−]CD21[−] B-cells proliferate to a significantly lesser degree than naïve and resting memory B-cells after agonistic stimulation but retain similar capacity for antibody secretion. The expression of ITIM-containing CD305, CD22 and CD72 was lower in CD27[−]CD21[−] than naïve CD27[−]CD21⁺ B-cells. Overall these data suggest that proliferative exhaustion of CD27[−]CD21[−] B-cells does not infer functional anergy.

2. Methods

2.1. Patients

Subjects and controls were recruited from the Gastroenterology Clinics at the Philadelphia Veterans Affairs Medical Center following informed consent on an institutional review board-approved protocol. All patients were assessed for baseline demographics, hepatitis viral serologies, alcohol use history, and radiological findings. HIV-infected patients were excluded. Healthy donors (HD) had no evidence of liver disease or malignancy. Study subjects with HCV infection confirmed twice by commercial PCR assays were classified in this study as having: 1) early fibrosis (non-CIR HCV) based upon a liver biopsy within 3 years of the bleed date showing Metavir F2 fibrosis and/or Fibrotest F1–2 testing within 6 months; 2) cirrhosis (HCV CIR) based upon clinical decompensation (ascites, jaundice, encephalopathy, thrombocytopenia), radiological finding (splenomegaly, nodular liver, varices, ascites), liver biopsy within 5 years, and/or Fibrotest F4; or 3), hepatocellular carcinoma (HCV HCC) based on standard American Association for the Study of Liver Disease diagnostic guidelines [23]. Non-HCV infected cirrhotic patients (non-HCV CIR) were recruited as an additional control group.

2.2. Cells isolation

Peripheral blood mononuclear cells were isolated using Ficoll-Histopaque (Sigma, St. Louis, MO) density centrifugation and either cryopreserved in liquid nitrogen or used immediately. Functional assays were performed with freshly isolated PBMC. Surface phenotyping was performed on thawed cryopreserved PBMC. For some experiments, B-cells were isolated using an autoMACS platform with B-cell isolation kit II (Miltenyi Biotech).

2.3. Flow cytometry

Surface phenotyping of cryopreserved peripheral blood mononuclear cells was performed using antibodies against CD3 (PerCP, SK7), CD14 (PerCP, MΦP9), CD19 (APC-H7, SJ25C1), CD20 (2H7, PE-Cy7), CD21 (APC and V450, B-ly4), CD22 (APC, S-HCL-1), CD72 (FITC, J4-117) (24), CD305 (FITC, DX26), CD27 (PE and V450, M-T271), CD38 (FITC, HIT2), CD56 (PerCP, NCAM16.2), CD95 (APC, DX2), IgD (Alexa Fluor 700, IA6-2), IgG (V450, G18-145), IgM (FITC, G20-127), that were obtained from Becton Dickinson (Franklin Lakes, NJ). FcRL4 (PE-Cy7, 413D12) was obtained from BioLegend (San Diego, CA). Live/Dead Aqua was obtained from Invitrogen (San Diego, CA). All data were acquired on FACSCanto (BD) and analyzed using FlowJo (Tree Star Inc., Ashland OR) using cutoffs based on isotype antibody staining.

2.4. Proliferation assay

6×10^7 PBMC were flow-sorted based on B-cell marker expression (viable CD3⁺CD14[−]CD56[−]CD19⁺) into CD27[−]CD21[−] (tissue-like memory), CD27⁺CD21[−] (activated memory/marginal zone-like), CD27[−]CD21⁺ (naïve), and CD27⁺CD21⁺ (resting memory) populations using a BSL3 FACSARIA II sorter (BD San Jose CA) and suspended in RPMI 1640 media with L-glutamine (Invitrogen, San Diego, CA) supplemented with 10% heat inactivated fetal calf serum (Sigma Inc., St. Louis, MO), 1.5% HEPES (Invitrogen) and

1% penicillin/streptomycin (Invitrogen). Purity of each subset was confirmed at > 95% (data not shown). 0.5×10^4 cells in triplicate for each cell subset were stimulated with anti-IgG/A/M (Jackson Laboratories) and/or anti-hCD40 (clone HB14, BioLegend) plus rhIL-10 50 ng/ml plus rhIL-2 20 U/ml (R&D systems) $\times 72$ h with ^3H -thymidine added for the last 16 h of culture [25]. Matched pair analysis was used to measure differences in ^3H -thymidine incorporation (cpm) across subsets to control for modest inter-subject heterogeneity in absolute cpm. Differences in stimulation indices (cpm stimulated/cpm media control) were also compared across B-cell subsets by ANOVA.

2.5. Immunoglobulin secretion

50 μL of supernatant from wells stimulated with media alone, anti-IgG/A/M and/or anti-hCD40/IL-2/IL-10 proliferation wells was removed and stored for quantification of IgA, IgG₁, IgG₂, IgG₃, IgG₄, IgM using MILLIPLEX® MAP Human Immunoglobulin Isotyping Kit 96 Well Plate Assay (#HGAM-301K-06, Millipore, Billerica MA) on a Luminex 200 system (Luminex Corporation, Austin, TX) using Masterplex QT software (Hitachi/MiraiBio, South San Francisco, CA).

2.6. Statistical analysis

The median values for clinical and immunologic parameters were compared using ANOVA (for normally-distributed values), the matched pair comparisons, nonparametric Kruskal–Wallis ANOVA, Wilcoxon Rank Sum, or Mann–Whitney U test as appropriate. The Spearman rank correlation was used for bivariate correlation of variables. Multivariate regression was performed using JMP 10 (SAS Institute Inc., Cary NC). A p-value < 0.05 was considered significant with the Bonferroni correction where required.

3. Results

3.1. Patient characteristics

Eight healthy donors, six non-cirrhotic HCV-infected and fifteen cirrhotic subjects were recruited. As shown in Table 1, the median age of the non-cirrhotic subjects was slightly higher than the other two groups although the age ranges greatly overlapped. The cirrhotic cohort exhibited well compensated liver disease with expected differences in median platelet count, serum albumin, total bilirubin and INR resulting from portal hypertension.

3.2. CD27⁺CD21⁺ B-cells are relatively overrepresented in B-cell pool in hepatitis C and hepatitis C-related cirrhosis

In our original cohort described in Doi et al. [13], we described a collapse of the peripheral CD27⁺ B-cell subset associated with cirrhosis. In our expanded cohort (Fig. 1B), we demonstrate that HCV-infected patients, both cirrhotic and non-cirrhotic, with and without HCC have a higher frequency of CD27⁺CD21⁺ B-cells in the peripheral blood (median 9.3% in healthy donors, 18.3% in non-cirrhotic HCV, 17.4% in HCV cirrhotics, 19.1% in HCV-infected cirrhotics with HCC, and 2.9% in non-HCV cirrhotics, $p < 0.0001$) compared to non-HCV-infected healthy donors and non-HCV-infected cirrhotic patients. Similar to Rakmanov et al. [16], we found that relative to naïve CD27⁺CD21⁺ B-cells, CD27⁺CD21⁺ B-cells manifest increased levels of expression of CD95 (Fig. 1C) but similar levels of

expression were identified in both resting and activated memory B-cells, and CD27⁺CD21⁺ B-cells were not CD21⁺CD95^{hi} in the majority of subjects (Fig. 1E). There was no difference in the level of expression of CD95 in CD27⁺CD21⁺ B-cells related to HCV infection or cirrhosis (Fig. 1D). Thus, expansion of a CD27⁺CD21⁺CD95^{int} B-cell population develops during the course chronic hepatitis C infection and dissimilar to the CD27⁺ B-cell populations persists after development of cirrhosis.

3.3. Inhibitor receptors possibly associated with B-cell exhaustion

We next sought to identify if certain markers previously associated with B-cell inhibition or anergy were preferentially expressed in peripheral CD27⁺CD21⁺ B-cells and/or preferentially overexpressed in cirrhotic patients relative to non-cirrhotic patients. As shown in Fig. 2A, LAIR-1 (CD305) expression was significantly higher in CD27⁺CD21⁺ B-cells from cirrhotic compared to non-cirrhotic subjects, but expression of CD305 in CD27⁺CD21⁺ B-cells was significantly lower than found in CD27⁺CD21⁺ naïve B-cells. FcRL4 was found in a significantly higher frequency of CD27⁺CD21⁺ B-cells than naïve, resting memory or activated memory B-cells, did not differ across patient groups, but was significantly lower than the expression found in the numerically small plasmablast population (Fig. 2B). CD22 expression correlated with the expression of CD21 and did not differ between patient groups (Fig. 2C). CD72 expression on CD27⁺CD21⁺ B-cells did not vary across groups (Fig. 2D); CD72 expression was greatest on naïve cells, intermediate on CD27⁺CD21⁺ B-cells, and lowest among CD27⁺ resting memory, activated memory and plasmablast. Thus, we found none of these previously described inhibitory markers that clearly differentiated the CD27⁺CD21⁺ B-cell population from the other B-cell subsets.

3.4. CD27⁺CD21⁺ B-cells are hypoproliferative to B-cell receptor and non-BCR stimulation

To test the proliferative capacity of CD27⁺CD21⁺ B-cells relative to other CD27/CD21-defined subsets, we flow-sorted B-cells from each quadrant in 8 cirrhotic subjects, then stimulated the cells via the BCR with anti-IgG/A/M, via CD40 with cytokine support, or both ("combined"). As shown in Fig. 3A, CD27⁺CD21⁺ B-cells proliferated approximately 1 log-fold less than naïve or activated memory B-cells and approximately 1.5 log-fold less than resting memory B-cells after stimulation via the B-cell receptors. Similar hyporesponsiveness was identified upon stimulation via CD40 in the presence of IL-2 and IL-10 (Fig. 3B). Combining these stimuli partially overcame the hyporesponsiveness of CD27⁺CD21⁺ B-cells, but these cells still proliferated 1.3 log-fold less than CD27⁺CD21⁺ and 1.7 log-fold less than naïve CD27⁺CD21⁺ B-cells.

3.5. CD27⁺CD21⁺ B-cells in cirrhotic patients have intact capacity to produce immunoglobulin

We hypothesized that reduced proliferation of CD27⁺CD21⁺ B-cells upon BCR and CD40 ligation would be associated with reduced immunoglobulin production. However, as shown in Table 2, there was no difference in production of IgM, IgA, or any IgG subclass after CD40/IL-2/IL-10 stimulation among CD27⁺CD21⁺, CD27⁺CD21⁺ naïve, CD27⁺CD21⁺ activated memory and CD27⁺CD21⁺ resting memory B-cells. BCR stimulation via anti-IgG/A/M induced very modest Ig production (for IgG1, mean difference in CD27⁺CD21⁺ 28.5 mg/dl, CD27⁺CD21⁺ naïve 33.0 mg/dl, CD27⁺CD21⁺ 0 mg/dl, CD27⁺CD21⁺ 27.24

mg/dl, $p = 0.0017$ for difference between media and anti-IgG/A/M, $p = 0.07$ for difference of CD27⁺CD21⁻ from other groups, data not shown). By contrast, CD40 ligation induced 3 log increases of IgG1 (for IgG1, mean difference in CD27⁻CD21⁻ B-cells 1324 mg/dl, CD27⁻CD21⁺ naïve 2102 mg/dl, CD27⁺CD21⁻ 1358 mg/dl, CD27⁺CD21⁺ 1192 mg/dl, $p < 0.0001$ for difference between media and CD40, $p = 0.45$ for differences between subsets). Similar patterns were seen for IgM, IgA, IgG3 and IgG4. Thus, while hypoproliferative, CD27⁻CD21⁻ B-cells are not distinguished from other CD27/CD21-defined subsets in terms of Ig secretion capacity.

4. Discussion

Understanding the fate of memory B-cells is critical for vaccine development in cirrhosis. It is well recognized that cirrhosis is associated with a marked increased risk of invasive bacterial infections such as spontaneous bacterial peritonitis, gram-negative bacteremia and gram-positive sepsis, likely mediated by reduced production of complement and altered neutrophil functions [26,27]. It is also clinically well recognized that cirrhotic patients have suboptimal responses to vaccines such as the hepatitis B vaccine. In several series, HBV vaccinations in cirrhotic patients yielded response rates from 13 to 54% [28,29] with minimal improvement by doubling the dose [30], much poorer than the results in non-cirrhotic patients. In a study of conjugated pneumococcal vaccine in liver disease, cirrhotic patients showed transient exaggerated early IgM and IgA production but impaired total IgG response suggesting defective IgG class-switching among cirrhotic patients relative to healthy donors [31]. To date, the observed vaccine hyporesponsiveness in cirrhotic patients has not been associated with any specific B-cell functional impairment.

An exhausted B-cell phenotype has been previously associated with a CD27⁻CD21^{lo} “tissue-like memory” B-cell subset in HIV-infected subjects [15], a subset that has been variably associated with viral load and treatment status [32,33]. This B-cell subset has been found to exhibit lesser proliferation with BCR engagement plus CD40 ligation or BCR engagement plus TLR9 ligation, but the hypoproliferative “exhausted” phenotype could be partially overcome by combining all three stimuli [15,34]. We similarly found impaired proliferation in the CD27⁻CD21⁻ B-cell subset with BCR stimulation alone and with CD40 stimulation, but partially recovered with combined BCR/CD40 stimulation. Contrasting with this study in HIV infection, and concurring with findings from Charles et al. [17], we found no significant association of the CD27⁻CD21⁻ with the expression of the putative inhibitory receptor FcRL4 in HCV infected patients. Interestingly, FcRL4 was highly expressed on a small population of CD27^{hi}CD21⁻ that are likely plasmablasts [35], the significance of which needs further exploration. One possible mechanism of induction of an exhausted state could be chronic viral antigenic stimulation, as suggested in by the enrichment of HIV-specific B-cells in the CD27⁻CD21^{lo} population in HIV-infected patients [15]. A second possible common mechanism between HIV infection and HCV infection and HCV-induced cirrhosis is chronic B-cell agonism by bacterial DNA or endotoxin associated with gut bacterial translocation [36,37]. Prospective association of chronic viral antigenic stimulation (or chronic CpG or LPS exposure) leading to expansion of CD27⁻CD21⁻ B-cells has not been established in humans. However, successful viral control under ART therapy of HIV disease has been associated with reduction of CD27⁻CD21^{lo} B-cells in one study [32]. In

HCV, CD27⁺CD21^{lo} B-cells have been associated with virus-induced clonally expanded B-cells [17,38]. A similar expansion of clonally expanded, autoreactive, hypoproliferative CD27⁺CD21^{lo} B-cells in autoimmune processes [39] provides additional evidence for the importance of chronic antigen exposure in the induction of this subpopulation. In unpublished observations, we have identified statistically significant increases of CD27⁺CD21⁺ B-cells in a cohort of acute HCV patients studies within 6 months of infection relative to frequencies observed in uninfected healthy donors suggesting that induction of the CD27⁺CD21⁺ may occur rapidly in the setting of high level viremia.

A striking finding of our study was that B-cell proliferative responses correlated poorly with immunoglobulin secretion. Specifically, CD27⁺CD21⁺ B-cells proliferated 2-log-fold less than CD27⁺CD21⁺ and CD27⁺CD21⁺ B-cells yet produced similar quantities of IgG1 and IgM similar to a CD21^{lo}CD95^{hi} population identified in common variable immunodeficiency [16]. Contrasting with this study, we did not find that CD21⁺ B-cells were predominantly CD95^{hi}, exhibiting CD95 expression similar to CD27⁺ resting and activated memory B-cells in healthy and HCV-infected participants. Furthermore, we found that BCR agonism potentiated CD40-induced proliferation of CD27⁺CD21⁺ B-cells but attenuated CD40-induced Ig production. BCR and CD40 ligation in T-dependent antibody responses classically are associated with synergistic effects on B-cell activation and antigen secretion [40], however it has been shown in mice that BCR stimulation in combination with CD40 ligation and either TLR4 or TLR9 agonism prevents the conversion of B-cells into antibody secreting cells by regulating BLIMP-1[41]. Our data further support that in humans, “exhausted” proliferation responses to BCR or CD40 agonism in CD21⁺ B-cells, do not necessarily reflect a completely anergic functional state. It has been postulated that the preservation of Ig secretion by CD21⁺ B-cells might be associated with increased autoimmunity [16], a theory further supported by the association of CD21^{lo} B-cells with systemic lupus erythematosus [42], rheumatoid arthritis [43], Sjögren’s syndrome [39] and HCV-related cryoglobulinemia [17]. While our subjects did not manifest overt autoimmune phenomenon, the increased frequency of CD27⁺CD21⁺ B-cells in chronic HCV and HCV-related cirrhosis might further explain the strong association of HCV and various autoimmune diseases [44].

5. Conclusion

Peripheral CD27⁺CD21⁺ B-cells with an exhausted proliferative response to B-cell receptor and CD40/TLR9 agonism circulate at a higher frequency in patients with chronic HCV and chronic HCV-induced cirrhosis. Inhibitory receptors such as FcRL4, LAIR-1, CD22 and CD72 do not appear phenotypically specific to the CD27⁺CD21⁺ B-cell subset. While hypoproliferative, CD27⁺CD21⁺ retain similar capacity to become antibody secreting cells after CD40 agonism, but produce little IgM or IgG1 in the presence of B-cell receptor ligation. Future studies should elucidate the impact of CD27⁺CD21⁺ B-cell frequency on clinical responsiveness to antigen-induced T-dependent memory in chronic HCV infection and related advanced liver disease.

Abbreviations

HCV	hepatitis C virus
HIV	human immunodeficiency virus
TLR	toll-like receptor
BCR	B-cell receptor
LAIR-1	leukocyte-associated immunoglobulin-like receptor 1
FcRL4	Fc-receptor like protein 4

References

1. Alter MJ. Epidemiology of hepatitis C virus infection. *World J Gastroenterol.* 2007; 13:2436–2441. [PubMed: 17552026]
2. Seeff LB. Natural history of chronic hepatitis C. *Hepatology.* 2002; 36:S35–S46. [PubMed: 12407575]
3. Musset L, Lunel F, Cacoub P, Mannant PR, Silvain C, Lacombe C, Opolon P, et al. Increased serum immunoglobulin G1 levels in hepatitis C virus infection. *Hepatology.* 1995; 21:1755–1757. [PubMed: 7768523]
4. Gonzalez-Quintela A, Alende MR, Gamallo R, Gonzalez-Gil P, Lopez-Ben S, Tome S, Otero E, et al. Serum immunoglobulins (IgG, IgA, IgM) in chronic hepatitis C. A comparison with non-cirrhotic alcoholic liver disease. *Hepatogastroenterology.* 2003; 50:2121–2126. [PubMed: 14696478]
5. Carbonari M, Caprini E, Tedesco T, Mazzetta F, Tocco V, Casato M, Russo G, et al. Hepatitis C virus drives the unconstrained monoclonal expansion of VH1-69-expressing memory B cells in type II cryoglobulinemia: a model of infection-driven lymphomagenesis. *J Immunol.* 2005; 174:6532–6539. [PubMed: 15879157]
6. Charles ED, Green RM, Marukian S, Talal AH, Lake-Bakaar GV, Jacobson IM, Rice CM, et al. Clonal expansion of immunoglobulin M+CD27+ B cells in HCV-associated mixed cryoglobulinemia. *Blood.* 2008; 111:1344–1356. [PubMed: 17942751]
7. Ni J, Hembrador E, Di Bisceglie AM, Jacobson IM, Talal AH, Butera D, Rice CM, et al. Accumulation of B lymphocytes with a naive, resting phenotype in a subset of hepatitis C patients. *J Immunol.* 2003; 170:3429–3439. [PubMed: 12626604]
8. Racanelli V, Frassanito MA, Leone P, Galiano M, De Re V, Silvestris F, Dammacco F. Antibody production and in vitro behavior of CD27-defined B-cell subsets: persistent hepatitis C virus infection changes the rules. *J Virol.* 2006; 80:3923–3934. [PubMed: 16571809]
9. Oliviero B, Cerino A, Varchetta S, Paudice E, Pai S, Ludovisi S, Zaramella M, et al. Enhanced B cell differentiation and reduced proliferative capacity in chronic hepatitis C and chronic hepatitis B virus infections. *J Hepatol.* 2011; 55(1):53–60. [PubMed: 21145853]
10. Mizuochi T, Ito M, Saito K, Kasai M, Kunitura T, Morohoshi T, Momose H, et al. Possible recruitment of peripheral blood CXCR3+ CD27+ CD19+ B cells to the liver of chronic hepatitis C patients. *J Interferon Cytokine Res.* 2010; 30:243–252. [PubMed: 20377416]
11. Mizuochi T, Ito M, Takai K, Yamaguchi K. Peripheral blood memory B cells are resistant to apoptosis in chronic hepatitis C patients. *Virus Res.* 2011; 155:349–351. [PubMed: 20875472]
12. Sugalski JM, Rodriguez B, Moir S, Anthony DD. Peripheral blood B cell subset skewing is associated with altered cell cycling and intrinsic resistance to apoptosis and reflects a state of immune activation in chronic hepatitis C virus infection. *J Immunol.* 2010; 185:3019–3027. [PubMed: 20656924]
13. Doi H, Iyer TK, Carpenter E, Li H, Chang KM, Vonderheide RH, Kaplan DE. Dysfunctional B-cell activation in cirrhosis resulting from hepatitis C infection associated with disappearance of CD27-positive B-cell population. *Hepatology.* 2012; 55:709–719. [PubMed: 21932384]

14. Moir S, Malaspina A, Pickeral OK, Donoghue ET, Vasquez J, Miller NJ, Krishnan SR, et al. Decreased survival of B cells of HIV-viremic patients mediated by altered expression of receptors of the TNF superfamily. *J Exp Med*. 2004; 200:587–599.
15. Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA, Roby G, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med*. 2008; 205:1797–1805. [PubMed: 18625747]
16. Rakhmanov M, Keller B, Gutenberger S, Foerster C, Hoenig M, Driessen G, van der Burg M, et al. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. *Proc Natl Acad Sci U S A*. 2009; 106:13451–13456. [PubMed: 19666505]
17. Charles ED, Brunetti C, Marukian S, Ritola KD, Talal AH, Marks K, Jacobson IM, et al. Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B cell subset. *Blood*. 2011; 117:5425–5437. [PubMed: 21421840]
18. Rudge EU, Cutler AJ, Pritchard NR, Smith KG. Interleukin 4 reduces expression of inhibitory receptors on B cells and abolishes CD22 and Fc gamma RII-mediated B cell suppression. *J Exp Med*. 2002; 195:1079–1085. [PubMed: 11956299]
19. van der Vuurst de Vries AR, Clevers H, Logtenberg T, Meyaard L. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur J Immunol*. 1999; 29:3160–3167. [PubMed: 10540327]
20. Meyaard L. The inhibitory collagen receptor LAIR-1 (CD305). *J Leukoc Biol*. 2008; 83:799–803. [PubMed: 18063695]
21. Wu HJ, Bondada S. CD72, a coreceptor with both positive and negative effects on B lymphocyte development and function. *J Clin Immunol*. 2009; 29:12–21. [PubMed: 19067131]
22. Silva R, Moir S, Kardava L, Debell K, Simhadri VR, Ferrando-Martinez S, Leal M, et al. CD300a is expressed on human B cells, modulates BCR mediated signaling and its expression is down-regulated in HIV infection. *Blood*. 2011; 117(22):5870–5880. [PubMed: 21482706]
23. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology*. 2011; 53:1020–1022. [PubMed: 21374666]
24. Nitschke L, Tsubata T. Molecular interactions regulate BCR signal inhibition by CD22 and CD72. *Trends Immunol*. 2004; 25:543–550. [PubMed: 15364057]
25. Kaplan DE, Sugimoto K, Ikeda F, Stadanlick J, Valiga M, Shetty K, Reddy KR, et al. T-cell response relative to genotype and ethnicity during antiviral therapy for chronic hepatitis C. *Hepatology*. 2005; 41:1365–1375. [PubMed: 15915458]
26. Propst-Graham KL, Preheim LC, Vander Top EA, Snitily MU, Gentry-Nielsen MJ, et al. Cirrhosis-induced defects in innate pulmonary defenses against *Streptococcus pneumoniae*. *BMC Microbiol*. 2007; 7:94. [PubMed: 17956621]
27. Baumann M, Witzke O, Canbay A, Patschan S, Treichel U, Gerken G, Philipp T, et al. Serum C3 complement concentrations correlate with liver function in patients with liver cirrhosis. *Hepatogastroenterology*. 2004; 51:1451–1453. [PubMed: 15362774]
28. Carey W, Pimentel R, Westveer MK, Vogt D, Broughan T. Failure of hepatitis B immunization in liver transplant recipients: results of a prospective trial. *Am J Gastroenterol*. 1990; 85:1590–1592. [PubMed: 2252022]
29. Chalasani N, Smallwood G, Halcomb J, Fried MW, Boyer TD. Is vaccination against hepatitis B infection indicated in patients waiting for or after orthotopic liver transplantation? *Liver Transplant Surg*. 1998; 4:128–132.
30. Horlander JC, Boyle N, Manam R, Schenk M, Herring S, Kwo PY, Lumeng L, et al. Vaccination against hepatitis B in patients with chronic liver disease awaiting liver transplantation. *Am J Med Sci*. 1999; 318:304–307. [PubMed: 10555092]
31. McCashland TM, Preheim LC, Gentry MJ. Pneumococcal vaccine response in cirrhosis and liver transplantation. *J Infect Dis*. 2000; 181:757–760. [PubMed: 10669371]
32. Fogli M, Torti C, Malacarne F, Fiorentini S, Albani M, Izzo I, Giagulli C, et al. Emergence of exhausted B cells in asymptomatic HIV-1-infected patients naive for HAART is related to reduced immune surveillance. *Clin Dev Immunol*. 2012; 2012:829584. [PubMed: 22474482]

33. Sciaranghella G, Tong N, Mahan AE, Suscovich TJ, Alter G. Decoupling activation and exhaustion of B cells in spontaneous controllers of HIV infection. *AIDS*. 2013; 27:175–180. [PubMed: 23135171]
34. Visentini M, Cagliuso M, Conti V, Carbonari M, Cibati M, Siciliano G, Cristofolletti C, et al. Clonal B cells of HCV-associated mixed cryoglobulinemia patients contain exhausted marginal zone-like and CD21 low cells overexpressing Stra13. *Eur J Immunol*. 2012; 42:1468–1476. [PubMed: 22678901]
35. Holz LE, Yoon JC, Raghuraman S, Moir S, Sneller MC, Rehermann B. B cell homeostasis in chronic hepatitis C virus-related mixed cryoglobulinemia is maintained through naive B cell apoptosis. *Hepatology*. 2012; 56:1602–1610. [PubMed: 22556016]
36. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, Kazzaz Z, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006; 12:1365–1371. [PubMed: 17115046]
37. Balagopal A, Philp FH, Astemborski J, Block TM, Mehta A, Long R, Kirk GD, et al. Human immunodeficiency virus-related microbial translocation and progression of hepatitis C. *Gastroenterology*. 2008; 135:226–233. [PubMed: 18457674]
38. Terrier B, Joly F, Vazquez T, Benech P, Rosenzweig M, Carpentier W, Garrido M, et al. Expansion of functionally anergic CD21⁻/low marginal zone-like B cell clones in hepatitis C virus infection-related autoimmunity. *J Immunol*. 2011; 187:6550–6563. [PubMed: 22084433]
39. Saadoun D, Terrier B, Bannock J, Vazquez T, Massad C, Kang I, Joly F, et al. Expansion of autoreactive unresponsive CD21⁻/low B cells in Sjogren's syndrome-associated lymphoproliferation. *Arthritis Rheum*. 2013; 65:1085–1096. [PubMed: 23279883]
40. Haxhinasto SA, Bishop GA. Synergistic B cell activation by CD40 and the B cell antigen receptor: role of B lymphocyte antigen receptor-mediated kinase activation and tumor necrosis factor receptor-associated factor regulation. *J Biol Chem*. 2004; 279:2575–2582. [PubMed: 14604983]
41. Boeglin E, Smulski CR, Brun S, Milosevic S, Schneider P, Fournel S. Toll-like receptor agonists synergize with CD40L to induce either proliferation or plasma cell differentiation of mouse B cells. *PLoS ONE*. 2011; 6:e25542. [PubMed: 21991317]
42. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, Warnatz K. A new CD21^{low} B cell population in the peripheral blood of patients with SLE. *Clin Immunol*. 2004; 113:161–171. [PubMed: 15451473]
43. Kremlitzka M, Polgar A, Fulop L, Kiss E, Poor G, Erdei A. Complement receptor type 1 (CR1, CD35) is a potent inhibitor of B-cell functions in rheumatoid arthritis patients. *Int Immunol*. 2013; 25:25–33. [PubMed: 22962438]
44. Vergani D, Gregorio GV, Choudhuri K, Ma Y, Vegnente A, Mieli-Vergani G. Hepatitis virus induced autoimmunity. *Acta Gastroenterol Belg*. 1998; 61:217–218. [PubMed: 9658613]

Figure 1.

CD27⁻CD21⁻ B-cells are relatively overrepresented in B-cell pool in hepatitis C and hepatitis C-related cirrhosis. A. Gating strategy used to quantify CD27⁻CD21⁻ B-cells. B. Frequency of CD27⁻CD21⁻ B-cells in healthy donors (HD), non-cirrhotic HCV-infected patients (non-CIR HCV), HCV cirrhotic (HCV CIR), hepatocellular carcinoma (HCC) and non-HCV cirrhotic (non-HCV CIR). C. Geometric mean fluorescence intensity of CD95 in CD27/CD21-defined B-cell subsets in all patient cohorts. D. Geometric mean fluorescence intensity of CD95 in CD27⁻CD21⁻ B-cell across patient cohorts. E. Frequency of CD21⁻CD95^{hi} B-cells across patient cohorts. p-Values by the Wilcoxon tests.

Figure 3.

CD27⁻CD21⁻ B-cells are hyporesponsive to BCR and non-BCR stimulation. ^3H incorporation (cpm) of CD27/CD21-defined B-cell subsets (average of 3 wells with 1×10^5 per well) after stimulation with anti-IgG/A/M (A), anti-CD40 + CpG + IL-2 + IL-10 (B), and combined stimulation (C). Data from cells isolated from 8 cirrhotic patients. * $p < 0.05$, ** $p < 0.01$ relative to CD27⁻CD21⁻ population.

Table 1

Baseline patient characteristics.

Characteristic	Healthy Donors (HD)	Non-cirrhotic HCV (Non-CIR HCV)	Cirrhotic HCV (HCV CIR)	HCV-related hepatocellular carcinoma (HCV HCC)	Non-HCV cirrhosis ^a (Non-HCV CIR)	p-Value (Wilcoxon)		
						HD vs Non-CIR HCV	HD vs HCV CIR	Non-CIR HCV vs HCV CIR
N	19	12	19	29	9			
Median age (range)	50 (24–67)	52 (44–66)	53 (41–63)	58 (48–65)	59 (53–69)	<0.0001 ^b	0.38	0.21
Ethnicity (W/B/H)	7/11/0	2/10/0	9/9/0	8/20/1	6/3/0	0.31		
Gender (M/F)	17/2	12/0	18/0	29/0	9/0	0.18		
Median ALT IU/l (IQR) ^c	22(19–27)	44 (30–72)	72 (52–95)	64 (45–90)	36(21–54)	<0.0001	0.0002	<0.0001
Median albumin g/dl (IQR)	4.5(4.3–4.6)	4.4 (4.0–4.5)	4.2 (3.8–4.4)	3.3(3.0–3.8)	3.9(3.7–4.5)	<0.0001	0.35	0.009
Median total bilirubin mg/dl (IQR)	0.6 (0.5–0.8)	0.7(0.6–1.0)	1.1 (0.8–1.4)	1.2(0.8–1.7)	0.7(0.6–1.1)	<0.0001	0.17	<0.0001
Median INR (IQR)	1.0(1.0–1.1)	1.0(0.9–1.0)	1.1 (1.0–1.2)	1.2(1.1–1.3)	1.1 (1.0–1.2)	<0.0001	0.024	0.056
Median platelets K/mm ³ (IQR)	239(216–272)	199(176–250)	115(98–179)	102(58–153)	175(128–202)	<0.0001	0.07	<0.0001
								0.00012

^a Causes of non-HCV CIR included alcoholic cirrhosis (N = 3), nonalcoholic steatohepatitis (N = 3), hemochromatosis (N = 2), and chronic hepatitis B (N = 1).^b Significant differences between HD and HCC (0.0009), HD and non-HCV cirrhosis (0.0027), and cirrhotic HCV and HCC (0.0001).^c Interquartile range.

Table 2

Immunoglobulin production by CD27 and CD21-defined cell subset among cirrhotic patients.

Ig Subset/stimulation	IgM mg/dl (mean $\pm$ SD)		IgG1 mg/dl (mean $\pm$ SD)		IgG2 mg/dl (mean $\pm$ SD)	
	Media	CD40/IL-2/IL-10	Media	CD40/IL-2/IL-10	Media	CD40/IL-2/IL-10
CD27 ⁻ CD21 ⁻	5.3 $\pm$ 0.5	77.7 $\pm$ 16.9	20.7 $\pm$ 11.0	1344.9 $\pm$ 271.2	60.8 $\pm$ 14.6	74.1 $\pm$ 22.2
CD27 ⁻ CD21 ⁺	6.0 $\pm$ 0.6	83.8 $\pm$ 8.7	9.1 $\pm$ 11.9	2111.5 $\pm$ 1704.7	64.0 $\pm$ 13.6	77.3 $\pm$ 19.0
CD27 ⁺ CD21 ⁻	6.0 $\pm$ 1.1	88.8 $\pm$ 18.4	36.4 $\pm$ 32.1	1394.3 $\pm$ 509.0	61.0 $\pm$ 7.7	77.6 $\pm$ 16.1
CD27 ⁺ CD21 ⁺	5.8 $\pm$ 0.5	75.1 $\pm$ 25.5	27.2 $\pm$ 16.8	1219.3 $\pm$ 513.3	60.7 $\pm$ 8.8	70.5 $\pm$ 12.5
Ig Subset/stimulation	IgG3 mg/dl (mean $\pm$ SD)		IgG4 mg/dl (mean $\pm$ SD)		IgA mg/dl (mean $\pm$ SD)	
	Media	CD40/IL-2/IL-10	Media	CD40/IL-2/IL-10	Media	CD40/IL-2/IL-10
CD27 ⁻ CD21 ⁻	0.2 $\pm$ 0.1	176.1 $\pm$ 70.3	0.5 $\pm$ 0.1	13.0 $\pm$ 2.4	3.0 $\pm$ 0.4	225.5 $\pm$ 33.9
CD27 ⁻ CD21 ⁺	0.3 $\pm$ 0.1	167.7 $\pm$ 89.3	0.5 $\pm$ 0.0	10.7 $\pm$ 5.1	2.8 $\pm$ 0.3	227.6 $\pm$ 16.3
CD27 ⁺ CD21 ⁻	0.6 $\pm$ 0.5	208.5 $\pm$ 107.9	0.6 $\pm$ 0.1	12.8 $\pm$ 3.7	23.4 $\pm$ 36.1 [*]	236.4 $\pm$ 178.6
CD27 ⁺ CD21 ⁺	0.3 $\pm$ 0.1	180.4 $\pm$ 112.1	0.5 $\pm$ 0.0	11.2 $\pm$ 3.4	4.1 $\pm$ 2.1	201.4 $\pm$ 59.6

^{*} p < 0.05 relative to CD27⁻CD21⁻ and CD27⁻CD21⁺.