Enhanced B-cell differentiation driven by advanced cirrhosis resulting in hyperglobulinemia

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Abstract

Background and Aim: The mechanism underlying hyperglobulinemia in cirrhosis, a long appreciated phenomenon, has never been clearly understood. The aim of this study is to investigate the basis for changes in humoral immunity observed in cirrhosis.

Methods: We retrospectively reviewed our medical record to analyze serum immunoglobulin (Ig) levels in patients with liver disease. We also prospectively analyzed peripheral blood mononuclear cells and sera from liver disease patients. Peripheral blood mononuclear cell surface marker expressions were measured by flow cytometry and serum B-cell-activating factor was measured by enzyme-linked immunosorbent assay. Expression of specific gene expression in magnetically separated B cells was also analyzed by real-time polymerase chain reaction.

Results: In retrospective analysis, we found that advancing cirrhosis, irrespective of underlying etiology or hepatocellular carcinoma, resulted in progressively increasing levels of serum IgG and IgA. In prospective analysis using clinical samples, we demonstrated that advancing cirrhosis stage was associated with increased toll-like-receptor (TLR)9 expression in CD27⁺ B cell and serum B-cell-activating factor levels but decreased CD27⁺ memory B-cell frequency. The remaining CD27⁺ B cells in peripheral blood exhibited increased activation-induced cytidine deaminase mRNA expression. Finally, we also demonstrated isolated B cells from advanced cirrhosis were more reactive to TLR9 stimulation that drove antibody secreting cells differentiation leading to hyperimmunoglobulinemia *in vitro*.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Conclusions: Enhanced TLR9-induced differentiation into antibody secreting cell may explain peripheral reductions of circulating CD27⁺ memory B cells as well as increased serum Ig levels in cirrhosis.

Keywords

AID; B cell; BAFF; cirrhosis; immunoglobulin; TLR9

Introduction

Patients with advanced liver disease have diminished innate and adoptive immune function,¹ referred to as cirrhosis-associated immune dysfunction syndrome. While diverse underlying mechanisms of cirrhosis-associated immune dysfunction syndrome have been reported,² we recently demonstrated that the B-cell compartment is highly impacted by cirrhosis.³ Similar to aberrant B-cell memory phenotypes observed in other immunocompromised states such as human immunodeficiency virus infection,⁴ cirrhotic patients have decreased and dysfunctional peripheral CD27⁺ memory B cells.³ The decreased frequency of peripheral CD27⁺ memory B cells may partially be explained by enhanced apoptosis induced by serum endotoxin and Fas ligand,⁵ factors associated with gut microbial translocation, and monocyte activation in cirrhosis. The impact of abnormalities in the B-cell memory compartment observed *ex vivo* on immune function *in vivo* remains speculative. Some data suggest that cirrhotic patients have impaired vaccine-induced immunoglobulin (Ig)G responses to peptide and glycoprotein antigens.^{6,7} Paradoxically, hyperglobulinemia has long been a phenomenon associated with cirrhosis.⁸ For example, elevated IgG levels observed in chronic hepatitis C virus (HCV) infection have been shown to become more evident as fibrosis progresses.⁹ By contrast, elevated IgA levels have been observed in alcoholic liver disease.¹⁰ Serum Ig levels are maintained by memory B cells and antibody secreting cells (ASCs) such as plasmablasts or plasma cells in a highly regulated manner that includes regulation of expression of activation-induced deaminase (AID),¹¹ critically involved in somatic hypermutation (SHM), class switch recombination,¹² and B lymphocyte-induced maturation protein-1 (Blimp-1).¹³ Toll-like-receptors (TLRs), ligation of the B-cell receptor, various cytokines, and B-cell-activating factor (BAFF/Blys) also control B-cell activation, differentiation, and functions.¹⁴

We hypothesized that the cirrhotic state would be found to induce B-cell gene differentiation programs that would be expected to result in enhanced Ig production. We confirmed that Ig elevations in hepatic disease become progressively more apparent as cirrhosis clinical stage worsens, largely independent of the underlying disease etiology. Peripheral memory B cells in cirrhosis indeed exhibit enhanced expression of AID, suggesting a relatively increased frequency of early stage of ASCs.¹⁵ We further observed enhanced response to TLR9 signal in cirrhotic B cells *in vitro* resulting in ASC differentiation and increased Ig production that could explain the observed hyperimmunoglobulinemia associated with advanced cirrhosis.

Methods

Subjects and samples.

Patients and controls were recruited from the Gastroenterology Clinic at Showa University Hospital and obtained written informed consents under Institutional Review Board-approved protocols in accordance with Declaration of Helsinki. Cirrhosis was defined based upon clinical parameters, radiological findings, or liver biopsy. Patients were classified as follows: (i) chronic liver disease without cirrhosis (PreCIR), (ii) compensated cirrhosis (CIR_{comp}), and (iii) decompensated cirrhosis (CIR_{decomp}) substratified by Child–Turcotte–Pugh score. Hepatocellular carcinoma (HCC) was diagnosed based on radiological finding by computed tomography and/or magnetic resonance imaging or pathological diagnosis in the setting of underlying cirrhosis. Age-matched healthy donors (HDs) without any significant comorbidities were also recruited. All samples from patients with viral hepatitis B or C were obtained prior to any antiviral therapy. The diagnosis of alcoholic cirrhosis was based upon the absence of viral hepatitis and the presence of a history of alcohol abuse or dependence. The diagnosis of non-alcoholic fatty liver disease was based on radiological evidence of steatosis, the absence of significant alcohol use and viral hepatitis, and evidence of metabolic syndrome as de-fined by the existence of at least two of dyslipidemia, high fasting glucose, or hypertension on top of the visceral fat accumulation. Patients with causes of chronic liver disease other than viral hepatitis, alcohol, or non-alcoholic fatty liver disease were classified as “Others.” We excluded patients with hepatitis B virus/HCV co-infection, virus-infected alcoholic abuse patients, autoimmune hepatitis, and primary biliary cirrhosis. In an associated prospective study, samples were collected from 2012 to 2016. Retrospectively, we reviewed all our medical record of patients registered as liver disease patients between 2006 and 2016. The serum Igs measured during standard clinical care in a large cohort of patients with chronic liver disease were evaluated.

Cell isolation.

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO, USA) density centrifugation. PBMCs for phenotyping were cryopreserved prior to analysis in Cellbanker R (Amsbio, Abingdon, OX, UK). CD19⁺ B cells, CD27[−] (naïve) B cells, and CD27⁺ memory B cells were isolated using B-cell isolation kit II or memory B-cell isolation kit on an AutoMACS platform (Miltenyi-Biotec, Bergisch Gladbach, Germany). Purity of isolated B-cell subsets was greater than 95%.

Flow cytometric analysis.

The antibodies used for pheno-typing included CD3 (PerCP, SK7), CD14 (PerCP, HCD14), and CD19 (APC-Cy7, HIB19) from BioLegend (San Diego, CA, USA). CD27 (PE, M-T271) was from BD Biosciences (San Jose, CA, USA), Live/Dead Fixable Dead Cell Stain Kits from Invitrogen (Eugene, OR, USA), and TLR4 and TLR9 from eBioscience (San Diego, CA, USA). Surface phenotyping and intracellular staining for TLR9 were performed as per manufacturer’s instructions. All flow cytometric data were acquired on LSR Fortessa (BD Biosciences) and analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Quantification of mRNA expression.

mRNA levels of activation markers (CD69, CD80, and CD86), AID, and Blimp-1 in isolated CD27⁺ and CD27⁻ B cells from eight HDs and cirrhotic patients were measured. Each B-cell subset was separated from PBMC, and immediately, the RNA was extracted using RNeasy Mini kit (Qiagen, Venlo, Netherlands). cDNA was transcribed using transcriptor universal cDNA master (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's protocol. The expression levels of mRNA were measured by quantitative reverse transcription–polymerase chain reaction using SYBR Green (Kapa Biosystems, Wilmington, MA, USA) on CFX96 Real-Time System (Bio-Rad, Irvine, CA, USA). Delta–delta Cts (Cts) were calculated using beta-actin as a housekeeping gene and converted to fold change relative to a single HD control.

Immunoglobulin isotyping and enzyme-linked immunosorbent assay.

Sera from HD and patients were freshly isolated and cryopreserved prior to analyses. Ig isotypes were measured with Bio-Plex Pro Human Isotyping Assays (Bio-Rad) on BioPlex 3D Suspension Array System. Enzyme-linked immunosorbent assay for BAFF (R&D Systems, Minneapolis, MN, USA) and A proliferation-inducing ligand (APRIL) (eBioscience, San Diego, CA, USA) were performed as per manufacturer's instructions.

B-cell stimulation assay.

CD19⁺ B cells were isolated from PBMC and resuspended in Roswell Park Memorial Institute 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (GE Healthcare Sciences, Salt Lake City, UT, USA), 1.5% HEPES (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). The 1×10^5 B cells were activated with 0.5 µg/mL BAFF (ProSpec, Vineland, NJ, USA) and/or 1 µg/mL CpG oligodeoxynucleotide (ODN) 2006 (InvivoGen, San Diego, CA, USA). After 48 h, the ASC differentiations were analyzed by flow cytometry, and the Ig levels in the undiluted culture supernatants were measured using Bio-Plex Pro Human Isotyping Assays (Bio-Rad).

Statistical analysis.

The values for clinical and immuno-logic parameters were compared using analysis of variance, non-parametric Kruskal–Wallis analysis of variance, Wilcoxon rank sum test, and matched-pair analysis, as appropriate using JMP Pro13 (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered significant.

Results

Patients with advanced liver disease are prone to have increased immunoglobulins.

Patient characteristics of both retrospective cohorts and prospective study were shown in Tables 1 and 2, respectively. We first retrospectively analyzed clinically obtained serum Ig levels in patients with liver disease. As shown in Figure 1a, patients with advanced liver disease across disease etiologies manifested increased serum IgG, IgA, and IgM levels relative to age-matched HDs. Particularly among patients with chronic HCV infection and alcoholic liver disease, advancing cirrhotic status was also associated with increasing serum

IgG levels (PreCIR vs CIR_{comp}, $P < 0.0001$, and CIR_{comp} vs CIR_{decomp}, $P < 0.0001$, in HCV and alcoholic liver disease). Specifically, among patients with alcoholic liver disease, serum IgA levels were significantly higher in advanced stage (PreCIR vs CIR_{comp}, $P = 0.0002$, and CIR_{comp} vs CIR_{decomp}, $P < 0.0001$). Consistent with the previous reports,⁹ chronic HCV infection without cirrhosis was specifically associated with serum IgG elevation compared with HD ($P < 0.0001$) compatible with a virus-induced hyperglobulinemia. The development of HCC in patients with cirrhosis had no impact on serum IgG (Fig. S1), IgA, and IgM levels (data not shown). Thus, more advancing liver disease is associated with progressively increasing serum Ig levels with modest etiology-specific variations.

In a subset of HD, PreCIR, and CIR patients, we evaluated IgG isotypes finding that cirrhotic patients had increased IgG1 (HD vs CIR, $P = 0.0370$, and PreCIR vs CIR, $P = 0.0627$) and a trend towards increased IgG2 (HD vs CIR, $P = 0.0772$). IgG3 and IgG4 did not differ among the three groups (Fig. 1b). When analyzed by underlying etiology, IgG1 increase was more evident in CIR compared with PreCIR (Fig. 1c) consistent with clinical finding. Among PreCIR patients, only in HCV-infected persons showed significant increase compared with HD ($P = 0.0022$) suggesting a possible role of viral infection status in driving Ig subtype differentiation.

Peripheral CD27⁺ B cells decline in frequency during advancing cirrhosis of all etiologies.

We next explored the relationship between various stages of liver disease and B-cell phenotype by flowcytometry (Fig. S2a). Consistent with our previous report in HCV-infected patients,³ cirrhotic patients had decreased peripheral CD27⁺ memory B cells, especially in advanced stage in frequency (mean: HD 27.1%, PreCIR 24.1%, CIR_{comp} 17.0%, and CIR_{decomp} 11.2%) (Fig. 2a) and in number (Fig. 2b) but unaffected by the presence of HCC. This reduction is observed in HCV-uninfected patients (mean: PreCIR 22.2%, CIR_{comp} 17.4%, and CIR_{decomp} 10.3%) (Fig. S2b). Thus, progressive declines of peripheral CD27⁺ memory B-cell frequency were observed in advancing stages of cirrhosis irrespective of underlying disease etiology. Moreover, peripheral CD27⁺ B-cell number tended to be associated inversely with IgG and IgA but not with IgM (Fig. S2c). Thus, memory B-cell frequency declines observed in cirrhosis were paradoxically associated with increasing hyperglobulinemia suggesting that ASCs in cirrhosis may lose memory B-cell phenotypic markers and/or compartmentalize into secondary lymphoid organs.

CD27⁺ B cell–toll-like-receptor 9 expression and serum B-cell-activating factor increase in advanced cirrhosis.

We next focused on potential activators of B cells such as TLRs, BAFF, and APRIL. Ligands for TLR4 (lipopolysaccharide) and TLR9 (CpG-DNA) are known to increase in peripheral blood in cirrhosis.¹⁶ These ligands have previously been shown to activate B cells³ and to induce differentiation into plasma cells.¹⁷ We found that TLR9, but not TLR4, expression increased in CD27⁺ B cells in decompensated cirrhosis (CIR_{comp} vs CIR_{decomp}, $P = 0.0036$) (Figs 2c,S3). BAFF and APRIL are other critical activating factors for B cell that affect class switching and plasma cell differentiation.¹⁸ As shown in Figure 3a, BAFF levels were increased in cirrhosis (PreCIR vs CIR_{decomp}, $P = 0.0005$, and CIR_{comp} vs CIR_{decomp}, $P = 0.0548$) while APRIL did not differ across patient groups ($P = 0.69$) (not shown). BAFF

levels correlated with serum IgG ($P = 0.0041$, $R^2 = 0.1972$) and IgA ($P = 0.0031$, $R^2 = 0.2075$) but not IgM (Fig. 3b). BAFF levels correlated strongly with peripheral monocyte frequency ($P < 0.0001$, $R^2 = 0.4790$) (Fig. 3c), the primary source of BAFF production.¹⁹ Thus, both exogenous signals and endogenous receptors that favor ASC differentiation are markedly increased in cirrhosis and correlate with hyperglobulinemia.

Circulating peripheral CD27⁺ B cells in cirrhosis are programmed for somatic hypermutation and class switch.

Immunoglobulin G and IgA are typically produced by class switched CD27⁺ B cells.^{18,20} Counterintuitively, IgG and IgA are increased in cirrhosis in the setting of a paucity of CD27⁺ B cells. To investigate this apparent paradox, we analyzed differences in mRNA expression of activation markers including CD86,²¹ SHM/class switch recombination (AID), and ASC differentiation (Blimp-1) on CD27⁺ and CD27⁻ B cells in HD and CIR patients by reverse transcription–polymerase chain reaction. No significant differences of activation markers such as CD69, CD80, and CD86 were identified between HD and CIR patients in both CD27⁺ (Fig. 4a) and CD27⁻ B cells. However, we found significantly increased expression of AID on CD27⁺ B cells from cirrhotic patients compared with those from HD ($P = 0.0038$) (Fig. 4b). Furthermore, the AID expression was strongly associated with activation as evidenced by CD86 expression ($R^2 = 0.3781$, $P = 0.0112$). Contrary to our expectation, another transcription factor required for ASC differentiation, Blimp-1 mRNA expression in CD27⁺ was decreased in CIR compared with HD ($P = 0.0238$) and inversely associated with AID ($R^2 = 0.2212$, $P = 0.0660$) (Fig. 4c). We speculate that this unexpected finding could reflect rapid loss of CD27 and homing to secondary lymphoid organs of Blimp-1^{high} B cells *in vivo*. No significant differences in AID and Blimp-1 were observed in CD27⁻ B cells across groups. Overall, these data suggest that memory B cells in cirrhosis are primed for class switching, SHM, and differentiation.

Toll-like-receptor 9 signals induce antibody secreting cell differentiation especially in advanced cirrhotic patients resulting in hyper-immunoglobulinemia.

To investigate the impact of BAFF and TLR9 signaling, isolated B cells from four HD and four patients from each group (PreCIR, CIR_{comp}, and CIR_{decomp}) were stimulated. While BAFF stimulation alone did increase the frequency of plasma cell differentiation (%CD138⁺, $P = 0.04$), TLR9 stimulation not only more strongly increased plasmablast differentiation but also strongly induce Ig production: unstimulated *versus* CpG, IgG1 and IgG2, $P < 0.0001$; IgA, $P = 0.0009$; and IgM, $P < 0.0001$ (Fig. 5a,b). Nonsignificant trends in levels of secreted Igs by B cells were observed related to progressing liver disease severity (Fig. 5c). TLR9 signaling accompanied by modest BAFF help could induce ASC differentiation resulting in increased Ig productions.

Discussion

We demonstrate that serum Igs progressively increase in advancing cirrhosis independent of liver disease etiology. We postulate the mechanism underlying this observation is enhanced differentiation of B cells into plasmablasts driven by increased TLR9 and BAFF signaling resulting in elevated IgG and IgA levels in cirrhosis.

Consistent with our previous report,³ CD27⁺ memory B-cell frequency decreases progressively in advancing stages of cirrhosis. We confirmed here that this phenomenon is not specific to viral hepatitis-related cirrhosis. The increased expression of AID and reduced Blimp-1 in the remaining CD27⁺ B cells, and enhanced CD138 upregulation *in vitro*, suggests that B cells that have undergone SHM are highly primed to differentiate into ASC in cirrhosis during which memory B-cell markers are lost. One driver of enhanced differentiation may be BAFF, levels of which are increased in patients with cirrhosis consistent with prior reports.²² Cirrhosis is also associated with increased circulating TLR ligands such as CpG and endotoxin, both strong inducers of AID activity.^{23,24} While we postulate that reduced Blimp-1 levels in cirrhotic memory B cells reflect rapid differentiation and loss of memory B-cell markers,¹⁵ the finding might be explained by altered kinetics of Blimp-1 expression,^{25,26} enhanced Blimp-1-mediated trafficking to secondary lymphoid organs,²⁷ or enhanced apoptosis of Blimp-1-expressing B cells.^{3,28}

Increased circulating Ig levels were predominantly etiology independent and confirm earlier findings.^{9,29} Similar to prior observations, we found that among pre-cirrhotic patients, only HCV-infected patients exhibit IgG elevations, which have previously been observed and attributed to antigen-specific and antigen-non-specific (HCV E2-cellular CD81) stimulation.³⁰ Decompensated cirrhotic patients exhibited increased IgG levels in all etiologies, but alcoholic cirrhotic patients specifically exhibited a more profound elevation of IgA (HCV vs ALC, CIR_{comp}, $P = 0.0006$, and CIR_{decomp}, $P = 0.0005$). We postulate that TLR9 signaling may be the dominant driver of IgA class switching as previously reported.¹⁰ It is unlikely that alcohol itself drives Ig production or IgA class switching because patients with advanced non-cirrhotic alcoholics, chronic pancreatitis did not manifest IgG or IgA elevations (IgG: 1176 ± 256.5 , IgA: 238.1 ± 75.1 , and IgM: 107.1 ± 28 , $n = 10$). As the increases of Igs in advancing cirrhosis were independent of the underlying etiology, we postulate that increasing gut microbial translocation during cirrhosis progression augments B-cell exposure to bacterially derived TLR ligands, previously shown to activate B cells *in vitro*.³ In fact, other groups reported lipopolysaccharide³¹ and CpG-ODN¹⁰ increase and are significant in cirrhotic patients and murine models.³² Increased TLR9 expression of memory B cells could enhance sensitivity to CpG-ODN, a well-described B-cell stimulant previously shown to induce CD27⁺ B-cell differentiation into plasmablasts³³ after B-cell receptor cross-linking or cytokine activation.¹⁴

We demonstrated that serum BAFF levels are increased in cirrhosis of all etiologies supporting that findings of other.^{34,35} HCV infection itself has been reported to increase BAFF,³⁶ but we could not reproduce such a finding in pre-cirrhotic individuals. BAFF as a driver of IgG secretion has previously been reported in autoimmune diseases such as systemic sclerosis³⁷ and Sjogren's syndrome.³⁸ Some data suggest that BAFF and TLR9 pathways act synergistically to drive IgG secretion.³⁹ It is possible that the peripheral monocytois that we observed could partially explain elevated BAFF levels in advanced cirrhosis, because monocytes are the primary source of BAFF.^{40,41} Although BAFF stimulation significantly increased IgG1 production *in vitro*, the impact was less powerful than TLR9 stimulation. In addition to TLR ligands or BAFF,⁴² cirrhosis is also associated with increases of many other cytokines in sera, including IL-6²² that could drive B-cell activation and IgG1 class switching.

When B cells were stimulated by CpG *in vitro*, ASC frequency and Ig production were significantly increased compared with unstimulated B cells in patients with cirrhosis, an effect that was even more pronounced in B cells from decompensated cirrhotic patients. These findings are consistent with clinical observations. It is not clear if this reflects different assays used in the clinic *versus* research laboratory or differences in signaling that occur when B cells are stimulated after isolation *in vitro* rather than *in vivo*. *In vivo* studies in rodent models of cirrhosis would be necessary to study this issue further as well as overcome other inherent limitations of these observational human studies to more precisely understand the kinetics of the interaction of TLR9 and BAFF on B-cell activation, differentiation, Ig secretion, and compartmentalization associated with progressive liver disease.

In conclusion, increased antigen-non-specific stimuli such as TLR9 and BAFF observed in cirrhosis drive enhanced Ig production, especially of IgG1 and IgA. The interaction of these factors and B-cell differentiation genes such as AID provides strong circumstantial evidence for the mechanism of hyperglobulinemia observed in cirrhosis. Despite elevated serum Ig levels, patients with cirrhosis remain highly susceptible to gut-derived bacterial infections. Better understanding of this apparent contradiction may foster development of interventions to lessen the clinical impact of cirrhosis-associated immunodeficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Retrospective analysis of serum immunoglobulin (Ig) levels in liver disease patients. (a) Using our medical record, we retrospectively analyzed Ig levels classified by liver function in addition to underlying hepatic etiology. IgG, IgA, and IgM levels were demonstrated in healthy donors (HDs) (leftmost) and five etiology patient groups. In each group, patients were further classified depending on three hepatic function levels; precirrhosis (PreCIR), compensated cirrhosis (CIR_{comp}), and decompensated cirrhosis (CIR_{decomp}). Dotted line in each Ig indicates upper normal limit in our hospital. (b) With cryopreserved sera, we performed Ig isotyping using Bio-Plex system. IgG isotyping. In cirrhotic patients, IgG1 was significantly increased. IgG2, IgG3, and IgG4 were not different across the groups (sample number [n]: HD, n = 15; PreCIR, n = 21; and CIR, n = 21). (c) IgG1 concentration was increased in cirrhosis. Hepatitis C virus (HCV) infection without cirrhosis showed increased compared with HD (HD, n = 15; HCV-PreCIR, n = 8; CIR, n = 8; alcohol-PreCIR, n = 8; CIR, n = 8; non-alcoholic fatty liver disease (NAFLD)-PreCIR, n = 5; and CIR, n = 5). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. HBV, hepatitis B virus; NS, not significant.

Figure 2.

Decreased peripheral CD27⁺ B cell with toll-like receptor 9 (TLR9) expression in advanced cirrhosis. The surface marker and TLR9 expressions on B cell were analyzed by flowcytometry using cryopreserved peripheral blood mononuclear cell samples from patients and controls. (a) The frequency of CD27⁺ cells among CD19⁺ B cells in healthy donor (HD), PreCIR, compensated (CIR_{comp}), and decompensated (CIR_{decomp}) cirrhosis are demonstrated. Cirrhotic patients had decreased peripheral CD27⁺ B-cell frequency even compared with PreCIR. Patients with more advanced cirrhosis tend to have less CD27⁺ B cell. (b) The significant reduction of CD27⁺ cells among CD19⁺ B cells in cirrhosis was observed in number as well. (c) The representative data of TLR9 expression on CD27⁺ B cell from (■) HD, (...) PreCIR, (—) CIR_{comp}, and (—) CIR_{decomp} are shown in overlay histograms. Hepatocellular carcinoma existence did not affect the expression (data not shown). (d) Geometric mean fluorescence intensity (gMFI) of TLR9 expression in CD27⁺ B cell was increased in decompensated cirrhosis (HD, *n* = 19; PreCIR, *n* = 16; CIR_{comp}, *n* = 25; and CIR_{decomp}, *n* = 34). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. NS, not significant.

Figure 3.

B-cell-activating factor (BAFF) was increased in the sera from more advanced cirrhotic patients. BAFF in sera from healthy donor (HD) and patients were measured by enzyme-linked immunosorbent assay (HD, $n = 10$; PreCIR, $n = 10$; CIR_{comp}, $n = 5$; and CIR_{decomp}, $n = 15$). $*P < 0.05$ and $**P < 0.01$. (a) BAFF was increased in cirrhosis, especially in advanced stage ($***P < 0.001$). (b) BAFF concentrations were associated with serum immunoglobulin (Ig)G and IgA but not IgM measured in clinic. (c) Monocyte number was calculated by white blood cell count, and monocyte frequency was strongly associated with BAFF concentrations in patients ($P < 0.0001$, $R^2 = 0.4790$). , HD; , PreCIR; , CIR_{comp}; , CIR_{decomp}.

Figure 4.

The differences of mRNA expression on CD27⁺ B cell between healthy donor (HD) and cirrhosis (CIR). mRNA expressions were measured by quantitative real-time polymerase chain reaction using SYBR Green. Cts were calculated and converted to fold change relative to a single HD control using beta-actin as housekeeping gene. The differences in CD27⁺ B cell between HD and CIR were demonstrated. No significant difference was observed on CD27⁻ B cells (data not shown) (HD, $n = 8$; CIR, $n = 8$). (a) mRNA expression of activation markers (CD69, CD80, and CD86) in CD27⁺ B cells was not different between HD and CIR. (b) Activation-induced deaminase (AID) mRNA expression in CD27⁺ B cell was significantly higher in cirrhotic patients and was associated with CD86 mRNA expression. (c) B lymphocyte-induced maturation protein-1 (Blimp-1) mRNA expression was lower in cirrhotic patients and tended to inversely associate with AID mRNA expression. * $P < 0.05$ and ** $P < 0.01$. Open diamonds are healthy donors and filled circles are cirrhosis.

Figure 5.

The differences of mRNA expression on CD27⁺ B cell between healthy donor (HD) and cirrhosis (CIR). B cells were isolated and stimulated with B-cell-activating factor (BAFF), CpG, or the combinations. After 48 h, CD138⁺ plasma cell, CD27⁺CD38⁺ plasmablast differentiation, and immunoglobulin (Ig) secretion were analyzed (HD, $n = 4$; PreCIR, $n = 4$; CIR_{comp}, $n = 4$; and CIR_{decomp}, $n = 4$). (a) Igs including IgG1 and IgA secreted in culture supernatants were significantly increased when stimulated with CpG when analyzed for all 16 samples in each group by matched-pair analysis. (b) BAFF and CpG stimulation induce significant differentiations into antibody secreting cell differentiation; CD138⁺ plasma cells and CD27⁺CD38⁺ plasmablast (matched-pair analysis). (c) Toll-like receptor 9 signal increase plasma cell differentiation in cirrhosis especially in decompensated stage. The values are expressed by the ratio to unstimulated samples. Unst, unstimulated. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. NS, not significant.

Table 1

Patient characteristics of retrospective study

Etiology	HD	HCV	HBV	Alcohol	NAFLD	Others
Total number	25	178	107	104	85	49
Pre-cirrhosis	—	108	87	25	67	31
Cirrhosis	—	45	13	64	14	14
HCC	—	25	7	15	4	4
Compensated CIR	—	38	13	30	11	9
Decompensated CIR	—	32	7	49	7	9
Age	56.8 (24–79)	59.9 (22–80)	51.1 (24–79)	62.2 (34–81)	53.8 (22–79)	59.4 (35–80)
Gender (M/F)	16/9	99/79	60/47	83/21	45/40	27/22
Platelets (10 ⁴ per μ L)	24 (12.3–34.8)	16 (2.9–40.9)	18.6 (4.4–34.9)	15 (3–45.3)	21.2 (6.5–39.2)	17.1 (3.3–33.5)
PT-INR	1.06 (0.9–1.3)	1.15 (0.9–1.7)	1.1 (0.9–1.7)	1.2 (0.9–1.75)	1 (0.9–1.35)	1.1 (0.9–2.2)
Albumin (g/dL)	4.3 (3.0–5.0)	3.5 (1–4.6)	4.2 (2.1–5.3)	3.5 (1.2–5.1)	4.3 (2.0–5.4)	3.9 (2.3–5.1)
Totalbilirubin (mg/dL)	0.74 (0.3–1.4)	1.2 (0.3–6.3)	0.9 (0.3–3.0)	1.8 (0.2–9.8)	0.9 (0.4–1.4)	1.2 (0.3–3.6)
ALT (IU/L)	22.1 (9–42)	51.2 (8–154)	40 (8–180)	35.7 (6–142)	84.1 (7–275)	55 (15–245)

Data are shown as “mean (min–max).”, ALT, alanine aminotransferase; CIR, cirrhosis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HD, healthy donor; NAFLD, non-alcoholic fatty liver disease; PT-INR, prothrombin time–international normalized ratio.

Table 2

Patient characteristics of prospective study

Etiology	HD	HCV	HBV	Alcohol	NAFLD	Others
Total number	18	35	5	26	10	5
Pre-cirrhosis	—	10	1	4	5	1
Cirrhosis	—	12	3	13	3	3
HCC	—	13	1	9	2	1
Compensated CIR	—	12	3	8	2	1
Decompensated CIR	—	13	1	14	3	3
Age	60.4 (37–79)	65.3 (42–80)	64.8 (38–78)	63.1 (45–76)	58.2 (44–69)	72.1 (68–77)
Gender (M/F)	12/6	24/11	4/1	19/7	5/5	2/3
Platelets (10 ⁴ per μ L)	25.2 (18.7–34.8)	14.0 (2.9–29)	13.8 (8.6–21.4)	11.4 (3.1–34.2)	14.4 (6.5–23.7)	10.1 (9.8–10.8)
PT-INR	1.06 (0.99–1.09)	1.2 (0.96–1.79)	1.1 (1–1.2)	1.23 (1.03–1.57)	1.1 (0.98–1.2)	1.1 (1.09–1.23)
Albumin (g/dL)	4.2 (3.0–4.7)	3.7 (2–4.8)	3.8 (2.1–4.8)	3.2 (1.2–4.5)	3.9 (2.6–4.9)	3.5 (3–4.2)
Totalbilirubin (mg/dL)	0.74 (0.3–1.2)	1.1 (0.3–4.7)	0.9 (0.5–1.6)	1.9 (0.4–8.8)	1.1 (0.6–1.4)	1.3 (0.5–1.9)
ALT (IU/L)	22.7 (9–34)	48.5 (8–145)	28.3 (17–42)	40.7 (12–812)	65.2 (29–173)	29.3 (22–35)

Data are shown as “mean (min–max).”, ALT, alanine aminotransferase; CIR, cirrhosis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HD, healthy donor; NAFLD, non-alcoholic fatty liver disease; PT-INR, prothrombin time–international normalized ratio.