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Dysfunctional B-cell activation in cirrhosis due to hepatitis C infection associated with disappearance of CD27+ B-cell population

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Abstract

Background—Chronic hepatitis C virus infection is a leading cause of cirrhosis and hepatocellular carcinoma. Both advanced solid tumors and hepatitis C have previously been associated with memory B-cell dysfunction. In this study we sought to dissect the impact of viral infection, cirrhosis and liver cancer on memory B-cell frequency and function in the spectrum of HCV disease.

Methods—Peripheral blood from healthy donors, HCV-infected patients with F1–F2 liver fibrosis, HCV-infected patients with cirrhosis, patients with HCV-related hepatocellular carcinoma and non-HCV-infected cirrhotics were assessed for B-cell phenotype by flow cytometry. Isolated B-cells were stimulated with anti-CD40 antibodies and TLR9 agonist for assessment of costimulation marker expression, cytokine production, immunoglobulin production and $CD4^+$ T-cell allostimulatory capacity.

Results—$CD27^+$ memory B-cells, and more specifically $CD27^+IgM^+$ B-cells, were markedly less frequent in cirrhotic patients independent of HCV infection. Circulating B-cells in cirrhotics were hyporesponsive to CD40/TLR9 activation as characterized by CD70 upregulation, TNFβ secretion, IgG production and T-cell allostimulation. Lastly, blockade of TLR4 and TLR9 signaling abrogated the activation of normal donor B-cells by cirrhotic plasma suggesting a role for bacterial translocation in driving B-cell changes in cirrhosis.

Conclusion—Profound abnormalities in B-cell phenotype and function occur in cirrhosis independent of hepatitis C viral infection. These B-cell defects may explain in part the vaccine hyporesponsiveness and susceptibility to bacterial infection in this population.

Keywords

hepatitis C; cirrhosis; hepatocellular carcinoma; human; B-cell; CD27; CD40; sCD14; TLR4; TLR9; bacterial translocation

INTRODUCTION

A complex interaction of hepatitis C viral (HCV) infection and B-cells evolves during the natural history of HCV infection. Upon initial infection, virus-specific neutralizing antibody

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responses develop weeks after initial viremia targeting hypervariable regions of the HCV envelope proteins continuously selecting antibody escape variants, an evolution that continues throughout the chronic phase of infection (1, 2). In addition to chronic stimulation of virus-specific B-cells, chronic HCV is often characterized by a non-specific polyclonal activation of B-cells (3), that has been attributed to interactions between the HCV E2 envelope protein and CD81, an activating tetraspannin coreceptor that colocalizes with the B-cell receptor complex (4). Despite the activation of virus-specific and non-virus-specific B-cells which could result in the proliferation and accumulation of memory B-cells, several studies have demonstrated that the frequency of $CD27^+$ memory B-cells is either unchanged (5) or modestly reduced in chronic HCV infection (6, 7). Controversy persists as to the fate of memory B-cells, with the reduced frequency attributed to 1) increased activation-induced apoptosis (6), a theory that has been contradicted by recent data showing relative resistance to apoptosis of memory B-cells in HCV (8, 9), 2) increased conversion of B-cells into short-lived plasmablasts (7), or 3) increased intrahepatic compartmentalization (7, 10).

Cirrhosis ultimately evolves in 20–30% of chronically HCV-infected patients. In cirrhotics, hepatic decompensation eventually develops as a result of progressive portal hypertension, hepatic synthetic insufficiency, and/or neoplastic transformation. Particularly after decompensation, cirrhotic patients are at high risk of invasive bacterial infections such as spontaneous bacterial peritonitis and bacteremia, likely mediated by reduced production or increased consumption of complement, altered neutrophil function (11), increased intestinal permeability (12), and bacterial translocation (13). B-cell dysregulation might also contribute to this immunocompromised state. Cirrhotic patients exhibit suboptimal seroconversion rates after vaccination with recombinant hepatitis B vaccine (14) and impaired IgG production after pneumococcal vaccination (15). Despite poor response to vaccination, cirrhosis has been associated with abnormally increased serum levels of pathogen-specific immunoglobulins (16–19). Despite these observations, the impact of cirrhosis on B-cells has not been thoroughly investigated.

We recently reported that advanced solid tumors such as melanoma and breast cancer were associated with marked reductions of peripheral memory B-cell populations and related B-cell hypofunction (20). Unpublished pilot data revealed a similar phenotype in HCV-infected cirrhotics with hepatocellular carcinoma (HCC). In this study, we sought to dissect the contribution of these three potential precipitating factors, HCV infection, cirrhosis, and cancer, to the observed phenotype and to further characterize the functional capacity of B-cells in early and advanced liver disease.

MATERIALS AND METHODS

Patients

Subjects and controls were recruited from the Gastroenterology Clinics at the Philadelphia VA Medical Center under an IRB-approved protocol. Patients were assessed for baseline demographics, hepatitis viral serologies, alcohol use history, and radiological findings. 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 (EF) 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 (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 (HCC) based on standard AASLD diagnostic guidelines (21).

Cells isolation

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Histopaque (Sigma, St. Louis, MO) density centrifugation. Surface phenotyping for CD27 expression was performed on freshly-thawed cryopreserved PBMC but the remainder of experiments were performed with fresh PBMC. $CD19^+$ B-cells were negatively selected using B-cell isolation kit II (Miltenyi Biotec, Auburn, CA) on an AutoMACS platform. Purity of isolated B-cells was greater than 95%. $CD4^+$ T-cells were isolated from cryopreserved PBMC via a negative selection bead cocktail (Miltenyi) with purity greater than 95%. Isolated lymphocytes were resuspended in RPMI 1640 with L-glutamine (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated human AB serum (Sigma Inc., St. Louis, MO), 1.5% HEPES (Invitrogen) and 1% penicillin/streptomycin (Invitrogen)(22).

Flow Cytometry

Surface phenotyping was performed using antibodies against CD3 (PerCP, SK7), CD14 (PerCP, MΦP9), CD19 (APC-H7, SJ25C1), CD21 (APC, B-ly4), CD27 (PE and V450, M-T271), CD38 (FITC, HIT2) and FcRL4 (PE, 413D12, BioLegend) with Live/Dead Aqua. A subset of fresh PBMC were also stained with IgD (AlexaFluor 700, IA6-2), IgG (V450, G18-145), IgM (FITC, G20-127). Isolated B-cells were stained with CD40 (FITC, LOB7/6), CD70 (PE, Ki-24), CD86 (V450, 2331(FUN-1)), and HLA-DR (APC, G46-6). Responder $CD4^+$ T-cells were CFSE-labeled (Invitrogen) and stained for CD3 (PerCP, UCHT-1) and CD4 (APC, RPA-T4). All monoclonal antibodies were purchased from Becton Dickinson (Franklin Lakes, NJ) except for anti-CD40 (AbD Serotec, Raleigh, NC), anti-FcRL4 (Biolegend, San Diego, CA) and fixable Live/Dead Aqua Staining kit (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 antibodies.

B-cell culture and Activation

B-cells were activated using anti-CD40 MAb and TLR9 ligation as previously described (23). Briefly, 2×10^5 freshly isolated B-cells were incubated with both CP-870,893 (kindly provided by Pfizer, New London, CT) plus CpG oligodeoxynucleotide (ODN) 2006 (Invivogen, San Diego, CA) or dual control (human IgG2κ (Chemicon International, Temecula, CA) and ODN2006 control (Invivogen)). After 48 hours, B-cells were washed, stained for activation markers, and utilized for mixed lymphocyte reaction (MLR) experiments.

Mixed Lymphocyte Reaction

Mixed lymphocyte reaction (MLR) was performed as described previously (23). Briefly, after 48h stimulation, 6×10^4 dual-activated or dual-control B-cells were irradiated (3000 rad) and co-cultured with CFSE-labeled $CD4^+$ T-cells (B: T ratio = 1: 2) from a normal donor. CFSE-labeled $CD4^+$ T-cells were also coincubated with media alone or with anti-CD3/CD28 beads (kindly provided by Dr. Carl June). After 5 days, $CD4^+$ T-cell proliferation was assessed by flow cytometry. To compare the B-cell allostimulatory capacity across dates, we normalized CFSE dilution results according to the positive and negative control in each experiment. The %maximal CFSE dilution for each test condition was thus obtained by the following formula: [($\log_{10}$ geometric MFI of media exposed $CD4^+$ T-cells) - ($\log_{10}$ geometric MFI of dual activated or dual control B-cell-exposed $CD4^+$ T-cells)/($\log_{10}$ geometric MFI of media exposed $CD4^+$ T-cells) - ($\log_{10}$ geometric MFI of anti-CD3/CD28-stimulated $CD4^+$ T-cells)] controlling for background in dual control conditions. Simple comparisons of geometric mean MFI of dual activated cells divided by geometric mean MFI of dual control cells yielded similar results.

Cytokine/Immunoglobulin detection

Undiluted culture supernatant from 48-hour B-cell activation and 5-day T-cell co-cultures were collected and stored at −80°c. Cytokine (IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, TNFα, TNFβ, IL-21) or immunoglobulin levels (IgG1, IgG2, IgG3, IgG4, IgA, IgM) were quantified using Milliplex MAP Kit (Millipore, Billerica, MA) on a Luminex 200 system (Luminex Corporation, Austin, TX) using Masterplex QT software (Hitachi/MiraiBio, South San Francisco, CA).

ELISA

Freshly isolated plasma from whole blood were aliquoted and stored at −80°c for ELISA analysis of soluble CD14 (sCD14) (R&D systems, Minneapolis, MN) according to manufacturer's instructions.

TLR4 and TLR9-blockade

B-cells from healthy donors were negatively isolated as above. 5×10^4 B-cells were cultured in 50% plasma from cirrhotic donors, 50% plasma from normal donors, 10% human AB serum alone or supplemented with IgG/A/M (Jackson Immunoresearch, West Grove, PA), 1 μg/ml LPS (Sigma), or 1 μg/ml CpG ODN 2006 (Invivogen). Plasma wells were cultured in the presence or absence of the TLR4 antagonist R. sphaeroides-LPS (LPS-RS, Invivogen), anti-CD14 mAb (61D3, eBioscience), anti-TLR4 mAb (HTA125, Thermo Fisher, Rockford IL) or the TLR9 antagonist TTAGGG (Invivogen). After 72 hours, B-cells were stained for Live/Dead Aqua, HLA-DR, and CD38 and acquired on a FACSCanto.

Statistical Analysis

The median values for clinical and immunologic parameters were compared using ANOVA (for normally-distributed values), matched pair comparisons, nonparametric Kruskal-Wallis ANOVA, Wilcoxon Rank Sum, or Mann-Whitney U test as appropriate. Spearman rank correlation was used for bivariate correlation of variables. Multivariate regression was performed using JMP 9 (SAS Institute Inc, Cary NC). A p-value < 0.05 was considered significant with Bonferroni correction where required.

RESULTS

Patient Characteristics

Samples from 18 healthy donors (HD), 25 HCV-infected patients with F1–F2 fibrosis (EF), 19 with cirrhosis (CIR), 30 hepatocellular carcinoma (HCC) patients, and 5 non-HCV cirrhotics were studied (Table 1). The median age for HCC patients was approximately 6 years older than cirrhotic subjects, consistent with the natural history of HCC in HCV-related cirrhosis, but there were no other significant demographic differences among these groups. Expected differences in total bilirubin, serum albumin, platelet count, and INR were observed in patients with cirrhosis. Absolute lymphocyte counts were slightly reduced in patients with cirrhosis or HCC ($p = 0.039$), and therefore phenotypic differences were evaluated both as percentage per lymphoid population and as absolute population numbers (24).

Cirrhosis is associated with relative and absolute reduction of $CD27^+$ memory B-cells independent of hepatitis C viral infection

B-lymphocytes were defined using lymphoid gating, excluding non-viable cells, $CD3^+$ T-cells, $CD14^+$ monocytes, and then gating on $CD19^+$ cells (Figure 1A). Across the four patient groups, no significant differences were observed in the relative frequency and absolute number of $CD19^+$ B-cells among lymphoid cells (Figure 1C). While the frequency

of $CD27^+$ memory B-cells among $CD19^+$ cells was not significantly altered in HCV-infected patients with F1-2 fibrosis, there were strongly significant reductions in relative and absolute $CD27^+$ memory B-cell frequency in cirrhotic patients with or without HCC (Figure 1D). The frequency of CD27+ B-cells among CD19+ B-cells was not significantly different between fresh and cryopreserved samples (Supplemental Figure 1), and the intragroup differences remained significant when limiting analysis to cryopreserved samples (data not shown). Reduced $CD27^+$ B-cells frequency was also found in patients with non-HCV-related cirrhosis (alcohol, HBV, NASH) (Figure 1E). The reduction of CD27 expression was B-cell specific and the expression of CD27 on T-cells was not different across the patients groups (data not shown). Unlike $CD27^+IgG^+$ B-cell frequency that was preserved in cirrhotics, $CD27^+IgM^+$ B-cells were strikingly reduced (cirrhotic 16.3% vs. non-cirrhotic 32.4%, p=0.021, Figure 1F). A significant increase in $CD27^+CD38^{hi}$ plasmablasts among cirrhotic patients was also observed (Supplemental Figure 2). FcRL4, an inhibitory co-receptor on B-cells potentially identifying “exhausted” B-cells, was not found to be expressed in $CD27^+$, $CD27^-CD21^+$, or $CD27^-CD21^-$B-cell subsets in any patient group (data not shown). The frequency of $CD27^+/CD19^+$ B-cells was strongly correlated with several parameters related to progressive liver disease including total bilirubin, hypoalbuminemia, thrombocytopenia, and INR ((Figure 2A–D, all $p \leq 0.0001$). In summary, reductions in $CD27^+$ memory B-cell frequency, particularly $CD27^+IgM^+$ B-cells, are associated with cirrhosis independent of HCV infection, possibly due to increased peripheral conversion to short-lived plasmablasts.

Cirrhotic B-cells are hyporesponsive to activation by ligation of CD40 plus TLR9

In our prior work, peripheral B-cells CD27 expression was directly related to the capacity of B-cells to be activated by CD40 plus TLR9 ligation (23). To determine the impact of $CD27^+$ B-cell reduction and B-cell function in cirrhosis, we stimulated isolated B-cells with anti-CD40 MAb combined with CpG ODN or appropriate controls for 48h then assessed the expression of the activation markers CD40, CD70, CD86 and HLA-DR. As shown in Figure 3A–C, we detected a slight increase in the upregulation of the activation/costimulation markers CD86 and HLA-DR among CIR relative to EF patients, but no difference in CD40 upregulation. By contrast, upregulation of CD70 was significantly reduced in cirrhotic patients (with and without HCC) relative to normal donors (Figure 3D). The upregulation of CD70 was strongly associated with baseline CD27 expression ($R^2=0.36$, $p<0.001$, Figure 3E). We noted no significant intragroup differences in the production of IL-4, IL-6, IL-8, IL-10, IL-12, or TNFα by activated B-cells (Table 2A). However, cirrhotic B-cells tended to secrete less TNFβ relative to healthy donor B-cells (70.2 vs. 27.9 pg/ml, p=0.01, Table 2A and Figure 4A). TNFβ production was strongly correlated with baseline CD27 expression ($R^2=0.34$, p=0.005, Figure 4B). Interestingly, strong associations were also observed between baseline CD27 expression and IL-6, IL-12, and TNFα production although no significant intragroup differences were observed (Figure 4C–D). Furthermore, cirrhotic B-cells also produced less total IgG (but not IgA or IgM) than normal donor B-cells (Figure 4E). Thus, cirrhotic B-cells are hyporesponsive to strong activating stimuli as manifested by impaired upregulation of CD70, TNFβ and IgG production.

Cirrhotic B-cell antigen-presentation capacity is impaired relative to normal donor B-cells

To test the allostimulatory capacity of cirrhotic B-cells relative to normal donor B-cells, we performed a mixed lymphocyte reaction using 48h-activated and control B-cells to stimulate normal donor $CD4^+$ T-cells. Cirrhotic B-cells (with or without HCC) were less capable of stimulating alloreactive $CD4^+$ T-cell proliferation than non-cirrhotic HCV patient or healthy donor B-cells (Figure 5A). Interestingly, B-cell allostimulatory capacity was not correlated with memory B-cell frequency, CD86 or HLA-DR (Figure 5B–E) but did correlate strongly with the degree of upregulation of CD40 upon activated B-cells ($R^2=0.37$, p=0.002, Figure

5F). B-cell allostimulatory capacity did not significantly correlate with B-cell cytokine production (data not shown). T-cells stimulated by cirrhotic B-cells were impaired in their capacity to produce TNFα and TNFβ (Table 2B). In multivariable logistic regression analysis, only CD40 expression and %$CD70^+$ B-cells were the only independent predictors of B-cell allostimulatory capacity (data not shown). Thus, cirrhotic B-cells are impaired in their capacity to stimulate $CD4^+$ T-cells, an effect that appears to correlate with impaired upregulation of costimulation markers after CD40/TLR9 activation.

Circulating factors in cirrhotic plasma hyperactivate B-cells

By ELISA, levels of sCD14, a soluble LPS adaptor protein produced and shed by monocytes after LPS exposure (25), were significantly increased in cirrhotic plasma (Figure 6A). sCD14 concentrations were strongly inversely associated with $CD27^+$ B-cell frequencies (R^2=0.40, p< 0.001, Figure 6B). B-cells do not express mCD14, but sCD14 can directly transfer LPS to myeloid-differentiation-2 (MD-2) activating the TLR4 pathway (26). It has also previously been shown that bacterial DNA, a potential TLR9 ligand, can often be detected in cirrhotic plasma (27). We therefore investigated the potential role of TLR4 and TLR9 ligands in cirrhotic plasma in activating B-cells. Normal donor B-cells were cultured with 50% plasma from non-cirrhotic (Non-CIR, n=8) or cirrhotic (CIR, n=8) patients for 72 hours for measurement of activation (HLA-DR, CD38, CD27, CD19). Cirrhotic plasma induced a significant up-regulation of the expression of HLA-DR, up-regulation of CD38, and down-regulation of CD19 (Figure 6C). HLA-DR expression was associated with serum sCD14 levels (R^2=0.29, p=0.033) (data not shown). Up-regulation of HLA-DR expression by cirrhotic plasma, which could also be induced by exposure to LPS or CpG, was abrogated by antagonism of either TLR4 or TLR9 (Figure 6D). Abrogation of HLA-DR upregulation was detected with three different approaches to TLR4 blockade: LPS-RS, which inhibits LPS binding to LPS-binding protein, neutralization of sCD14, and direct blockade of TLR4 (Figure 6E). Lastly, similar to LPS and CpG, cirrhotic plasma protected B-cells from apoptosis in 72 hour culture, an effect that was abrogated by TLR4 and/or TLR9 blockade (Figure 6F). Thus, soluble factors associated with bacterial translocation such as LPS and CpG motifs that are elevated in cirrhotic plasma are capable of activating B-cells *in vitro*. While the long-term effects of such activation cannot be modeled *ex vivo*, these data suggest a possible mechanism underlying the phenotypic and functional perturbations of peripheral blood B-cells in cirrhosis.

DISCUSSION

In our study, we have uniquely found that among patients with chronic hepatitis C, only those that have progressed to cirrhosis display a loss of $CD27^+$ memory B-cells with associated functional abnormalities. The non-cirrhotic and cirrhotic HCV-infected patients we studied were similar in age, gender, ethnicity, viral genotype and duration of infection making viral or demographic factors very unlikely to explain the observed differences. Furthermore, this phenotype was also identified in patients with non-HCV-related cirrhosis strongly implicating hepatic fibrosis and/or portal hypertension in the development of this phenotype. The loss of $CD27^+$ memory B-cells appears to be a phenomenon common to several immunocompromised states such as advanced solid tumors (23), HIV infection (28), and common variable immunodeficiency (CVID)(29). While HIV and cirrhosis both are associated with bacterial translocation, a common underlying pathophysiology with CVID and advanced malignancy is not immediately obvious but perhaps may be related to splenic dysfunction.

The loss of $CD27^+$ memory B-cells in cirrhosis was associated with several functional consequences including impaired activation, impaired TNFβ and IgG production, and impaired allostimulatory capacity. This impaired activation and reduced capacity to recruit

T-cell help may explain the observed vaccine hyporesponsiveness in cirrhotic patients (14, 15). Paradoxically, overall immunoglobulin levels are elevated in cirrhotics due to increased levels of pathogen-specific immunoglobulins such as antibodies against *Saccharomyces cerevisiae* (ASCA) and against Galα1–3Galβ1–3GlcNAc (alpha-Gal), a glycan epitope found in bacterial cell walls (16, 17). Quite strikingly, we have shown that cirrhosis is associated with profound reductions of $CD27^{+}IgM^{+}$ B-cells, a subset of memory B-cells thought to be generated in response to T-independent antigens (30). Based on these observations, further investigation is warranted to determine the specific impact of cirrhosis on T-dependent and T-independent antigen responses as well as on optimal adjuvants that may improve vaccine efficacy in cirrhotics.

Our findings indicate that TLR ligands associated with bacterial translocation circulating in cirrhotic patients directly activate B-cells *in vitro*, an effect that can be attenuated with TLR4 and/or TLR9 blockade. TLR9 is constitutively expressed on B-cells (31) and it has been suggested that TLR9 agonists might impact the nature of B-cell immunoglobulin responses in cirrhosis (18). Human B-cells express minimal basal levels of TLR4 but upregulate TLR4 expression on exposure to various stimuli (32). LPS-LBP bound to sCD14 can directly bind MD-2 on mCD14-negative cells (26). Consistent with prior studies, we found that sCD14 levels were elevated in cirrhotic plasma (33) and that sCD14 levels correlated with *in vitro* B-cell activation. Elevated sCD14 levels have previously been found in systemic lupus erythematosus (34) and HIV infection (35), both of which are also associated with $CD27^{+}$ B-cell reductions. In particular, HIV, which infects gastrointestinal lymphoid tissue early in infection and compromises intestinal integrity, leads to increased bacterial translocation, non-specific immune activation (36), and ultimately is associated with memory B-cell loss (37–39). Our data suggests a similar pathogenesis of memory B-cell loss in cirrhosis albeit within the limitations of what can be demonstrated in *ex vivo* human B-cells. *In vivo* animal studies will be critical to determine the complex interaction of portal hypertension, bacterial translocation, hypersplenism and hepatic microenvironmental factors on B-cell memory generation and maintenance.

The fate of "lost" $CD27^{+}$ B-cells in cirrhosis remains incompletely defined. One potential fate is the evolution of an "exhausted" phenotype similar to that described in HIV disease, in which an increased frequency of hypoproliferative $CD27^{-}CD21^{-}$ B-cells with elevated expression of an inhibitory Fc-receptor like molecule (FcRL4) and other inhibitory molecules disproportionately consisting of HIV-specific B-cells has been identified (39). While we did identify an increase in $CD27^{-}CD21^{-}$ B-cells in cirrhotic patients with HCC, we did not identify an increase of FcRL4 expressing cells in any group of patients or cell subset (data not shown). An alternative explanation for the reduction of $CD27^{+}$ B-cells in chronic HCV patients is an increased conversion of activated $CD27^{+}$ B-cells to short-lived plasmablasts (6, 7). Our data showing an increase in $CD27^{+}CD38^{hi}$ in cirrhotics provides modest support for this hypothesis for the cirrhotic patient subset. HCV E2-CD81 interactions (40) also have been postulated to drive activation-induced apoptosis in chronic HCV. *In vitro* studies support an activating role of CD81 ligation in B-cells from chronic HCV patients (4, 41). However, E2-CD81 interactions cannot explain the loss of $CD27^{+}$ memory B-cells we identified in non-HCV cirrhotics or alterations of B-cell memory that have been identified in some HBV patients (7). Further challenging the activation-induced apoptosis hypothesis are data from Sugalski et al. and Mizuochi et al. which demonstrate that that HCV-infected patient B-cells manifest increased survival *in vitro* relative to normal donor B-cells (8, 9). Our *in vitro* data do suggest that soluble factors in plasma from cirrhotic patients promote B-cell survival.

A third explanation for peripheral memory B-cell loss could be compartmentalization of activated $CD27^{+}$ memory B-cells to the intrahepatic or lymphoid compartments due to

upregulation of homing markers such as CXCR3 (8, 10, 42), a possible mechanism that was not explored in this study. In the intrahepatic compartment, a pro-fibrotic role of B-cells has been suggested by work in the B-cell deficient mice treated with CCl_4 (43), by association of plasma cells and activated stellate cells in autoimmune liver disease (44), and by anecdotal regression of cirrhosis associated with rituximab in case reports (45). The intrahepatic compartment in cirrhotics does appear to be enriched for $CD27^+$ memory B-cells (Supplemental Figure 3) but study of animal models will be critical to precisely define the fate of $CD27^+$ memory B-cells in cirrhosis and will be helpful in determining whether or not intrahepatic B-cells may play a pathological role in chronic liver injury/fibrosis.

CONCLUSION

Independent of chronic hepatitis C infection, memory $CD27^+$ and more specifically $CD27^+IgM^+$ B-cells are profoundly reduced in the peripheral blood of patients with cirrhosis with or without hepatocellular carcinoma in direct relationship with parameters associated with hepatic metabolic dysfunction and portal hypertension. The remaining B-cells are hyporesponsive to activation via CD40 and TLR9 with impaired upregulation of costimulation markers, production of TNFβ, and production of IgG. The remaining B-cells upon activation are also less effective at stimulating $CD4^+$ T-cell responses. The presence of elevated levels of sCD14 and attenuation of B-cell activation by TLR4 and TLR9 blockade *in vitro* suggest that the loss of peripheral memory B-cells may be a consequence of chronic B-cell activation as a result of increased gut permeability due to portal hypertension. These findings shed light on vaccine hyporesponsiveness and increased susceptibility to bacterial infection in cirrhotic patients which might be ameliorated by therapies designed to reduce microbial translocation or block chronic pathogen-induced B-cell activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CIR	cirrhotic group
EF	early fibrosis
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HD	healthy donor
PBMC	peripheral blood mononuclear cells

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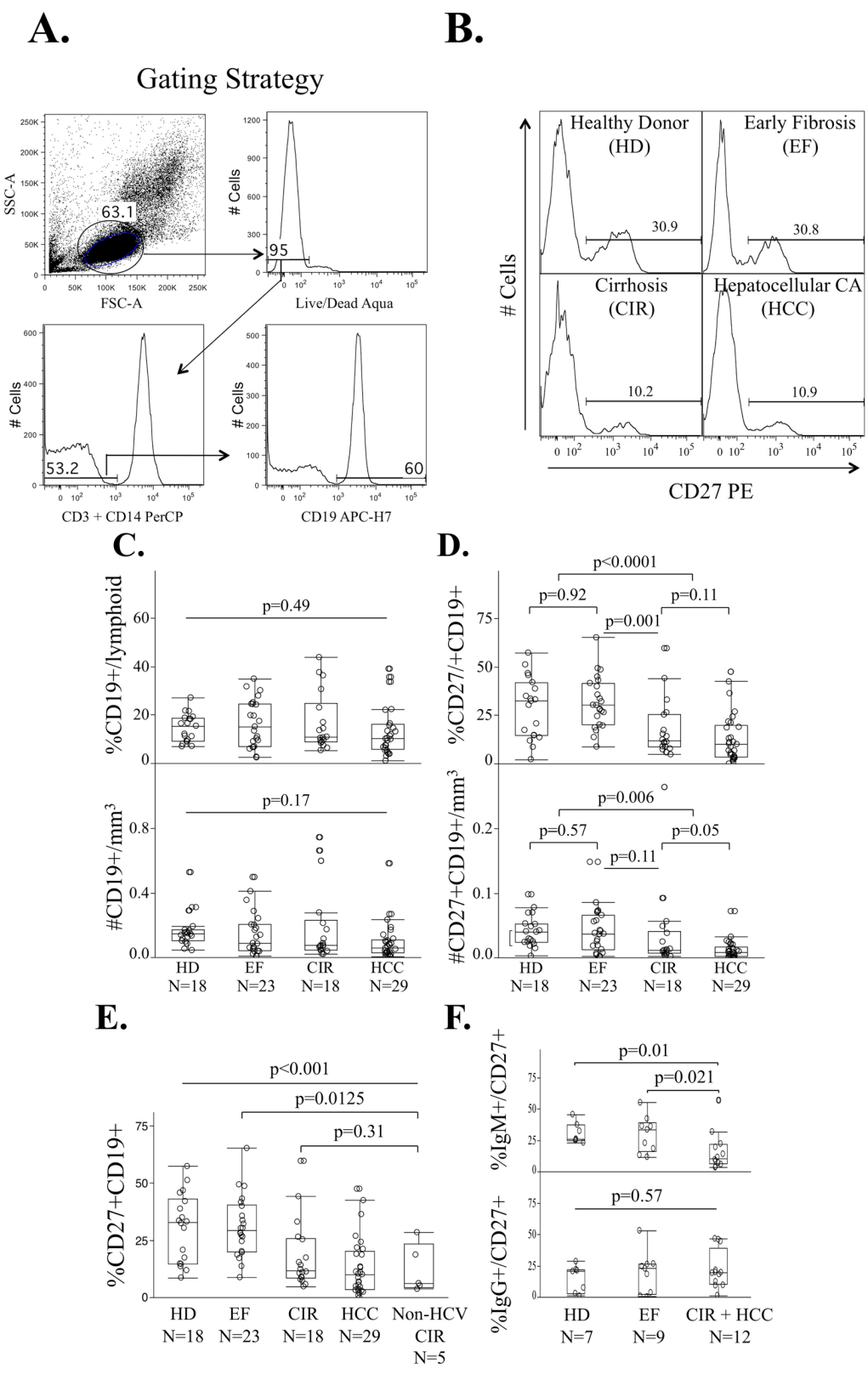


Figure 1. Reduction of CD27$^+$ memory B-cells in cirrhosis

A. Gating strategy for identification of $CD19^+$ B-cells. B. Representative histograms of CD27 expression of CD19+ B-cells in healthy donors (HD), HCV with F1–F2 (early) fibrosis (EF), HCV cirrhotics (CIR) and HCV cirrhotics with hepatocellular carcinoma (HCC). **C.** Relative and absolute number of $CD19^+$ B-cells in HD, HCV, EF, CIR and HCC.**D.** Relative and absolute number of $CD27^+$ B-cells in HD, HCV, EF, CIR and HCC. **E.** Frequency of $CD27^+CD19^+$ B-cells in non-HCV cirrhotic patients relative to other patient groups. **F.** Distribution of $CD27^+IgM^+$ and $CD27^+IgG^+$ B-cells across patient groups. All statistical comparisons made using Kruskal-Wallis and pairwise Wilcoxon Rank Sum tests.

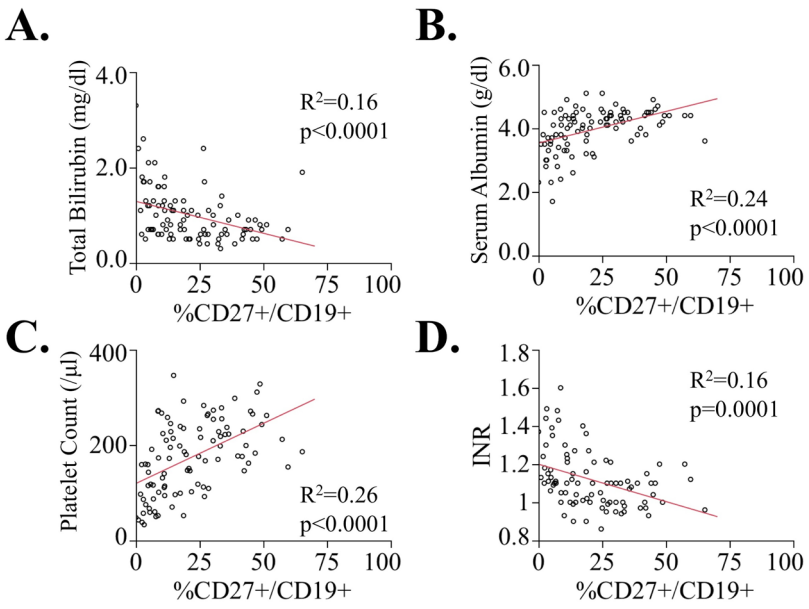


Figure 2. Correlation of CD27+ B-cell frequency and hepatic dysfunction

Spearman Rank correlation of $CD27^+$ memory B-cell frequency and **A.** total bilirubin, **B.** serum albumin, **C.** platelet count, and **D.** international normalized ratio (INR).

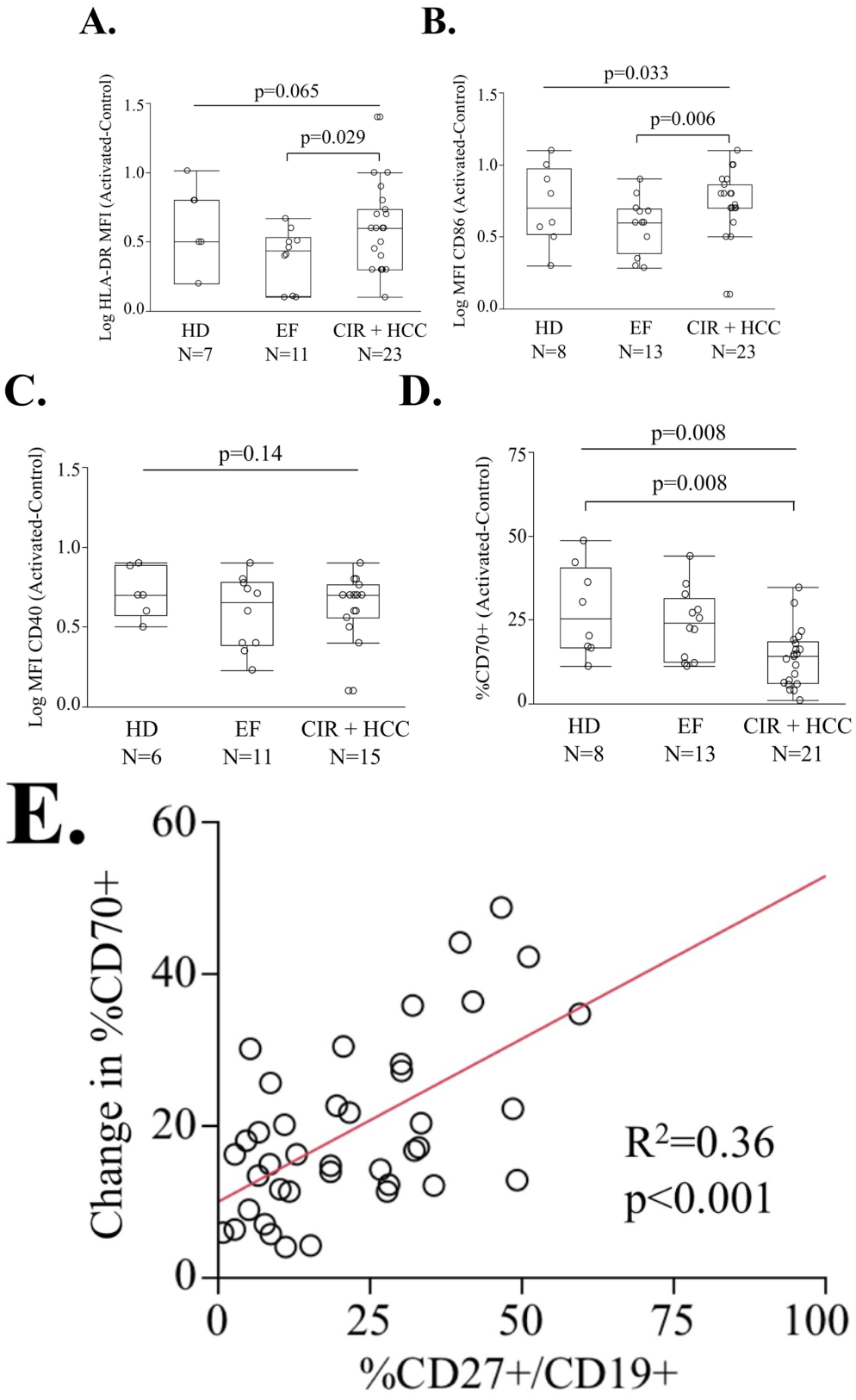


Figure 3. Impaired activation of cirrhotic B-cells by CD40/TLR9 ligation

Difference in geometric mean fluorescence intensity of **A.** HLA-DR, **B.** CD86, **C.** CD40, and **D.** and frequency of $CD70^+$ B-cells in HD, EF and CIR/HCC patients compared by Kruskal-Wallis and pairwise Wilcoxon Rank Sum tests. **E.** Spearman rank correlation between upregulation of CD70 expression upon CD40/TLR9 stimulation and baseline $CD27^+$ B-cell frequency.

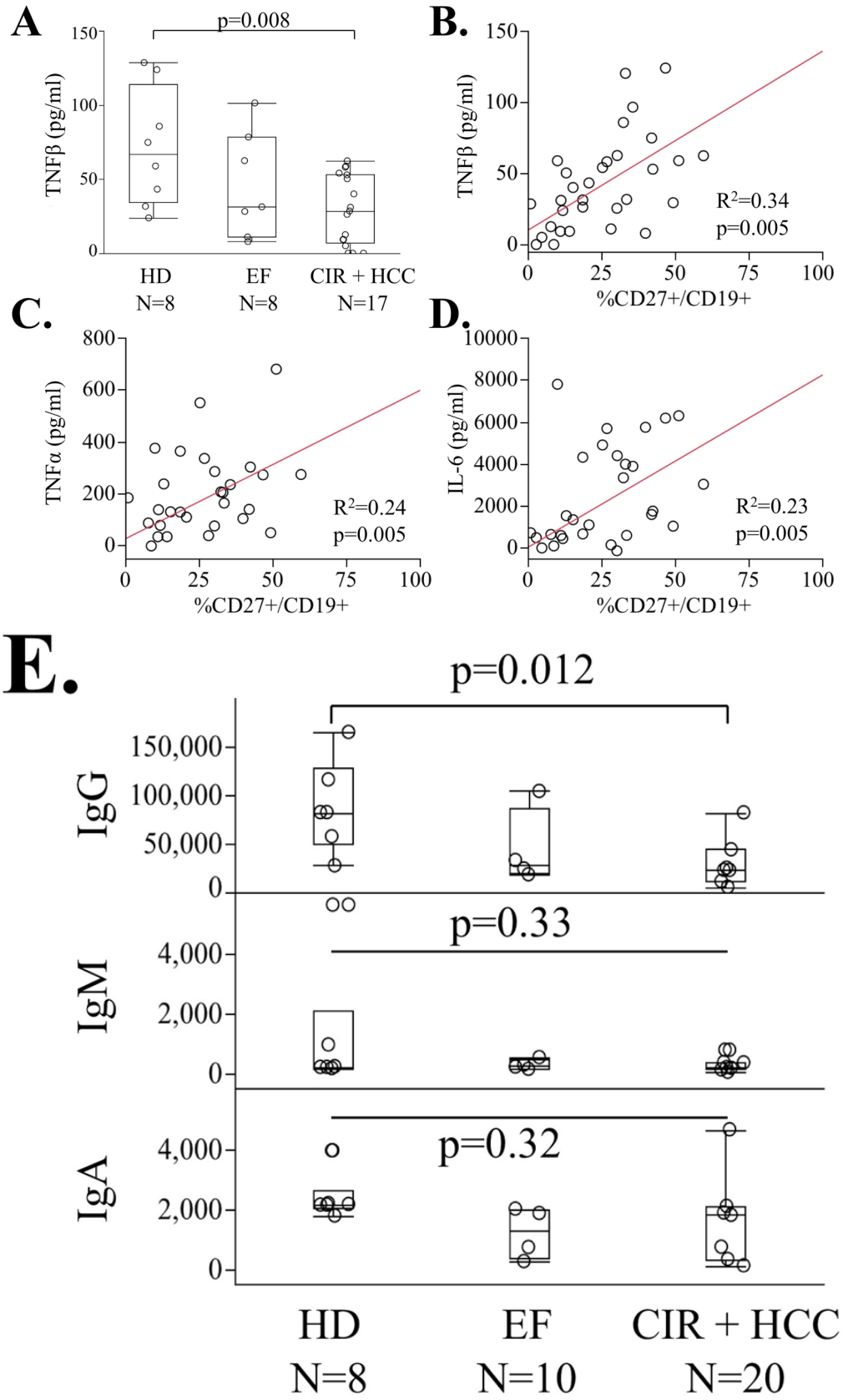


Figure 4. Impaired cytokine production by cirrhotic B-cells after CD40/TLR9 activation

A. TNFβ secretion upon CD40/TLR9 stimulation in HD, EF and CIR/HCC patients. Spearman rank correlations of **B.** TNFβ, **C.** TNFα, and **D.** IL-6 and $CD27^+$ B-cell frequency. **E**. IgG, IgM, and IgA titers in CD40/TLR9 across patient groups compared by Kruskal-Wallis and pairwise Wilcoxon Rank Sum tests.

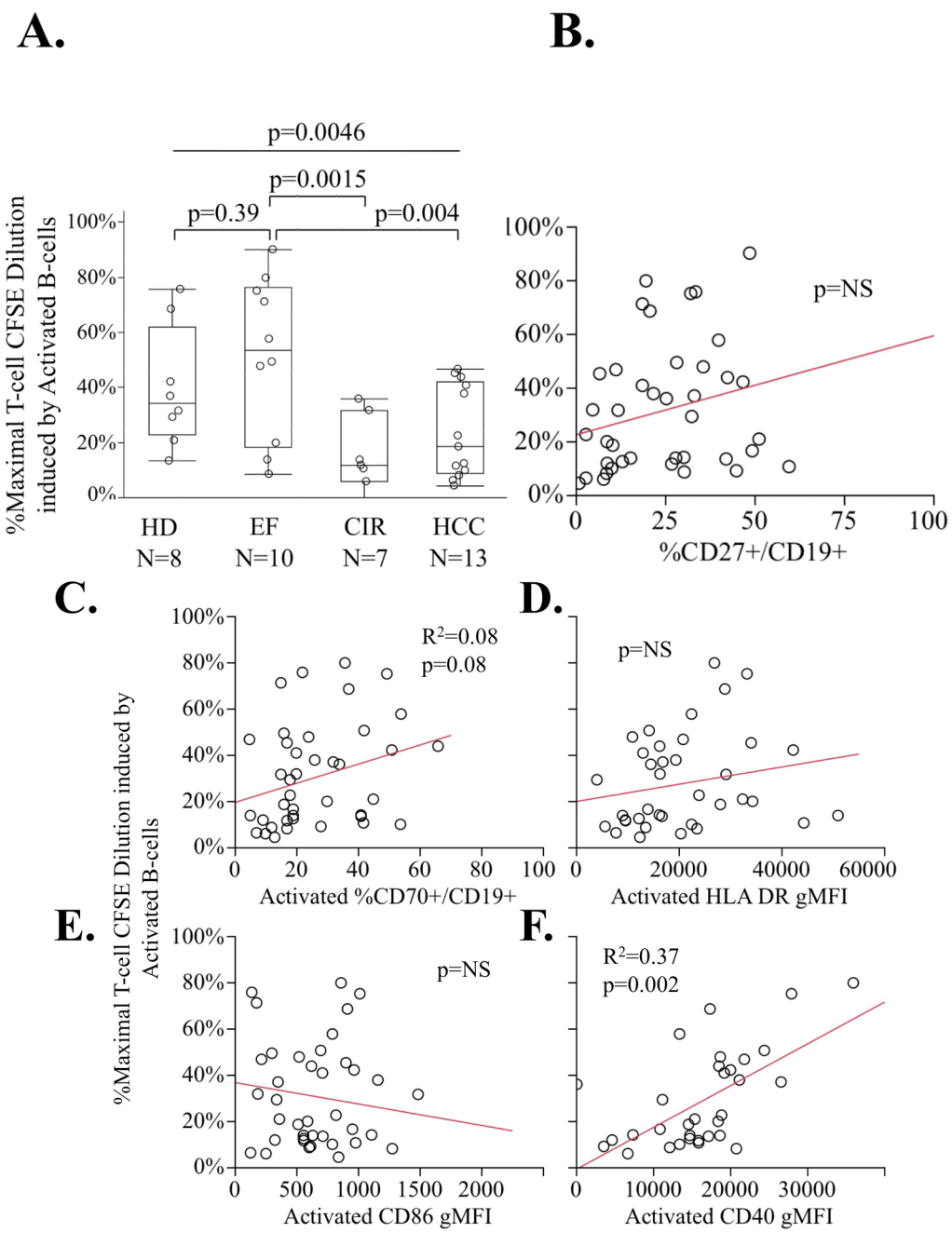
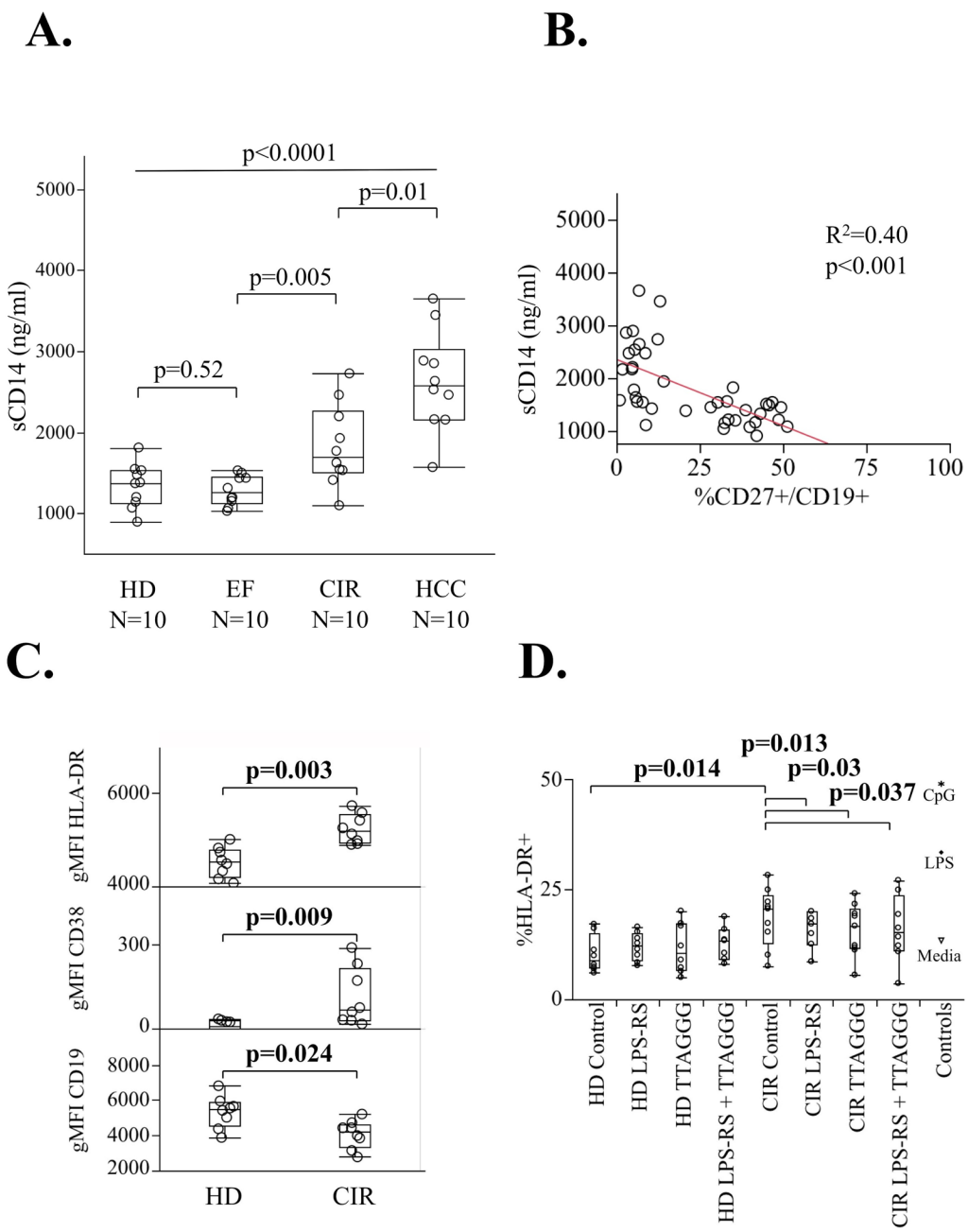


Figure 5. Reduced allostimulatory capacity of cirrhotic B-cells

A. Maximal CFSE dilution (calculated as gMFI of CFSE in T-cells co-cultured with activated B-cells minus gMFI of media-control T-cells divided by gMFI of anti-CD3/CD28-bead stimulated T-cells minus gMFI of media-control T- cells) across patient groups. Correlation of maximal CFSE dilution and **B.** baseline $CD27^+$ B-cell frequency, **C.** frequency of $CD70^+$ on post-CD40/TLR9 activation B-cells, **D.** post-activation B-cell HLA DR expression, **E.** post-activation B-cell CD86 expression, **F.** post-activation B-cell CD40 expression.



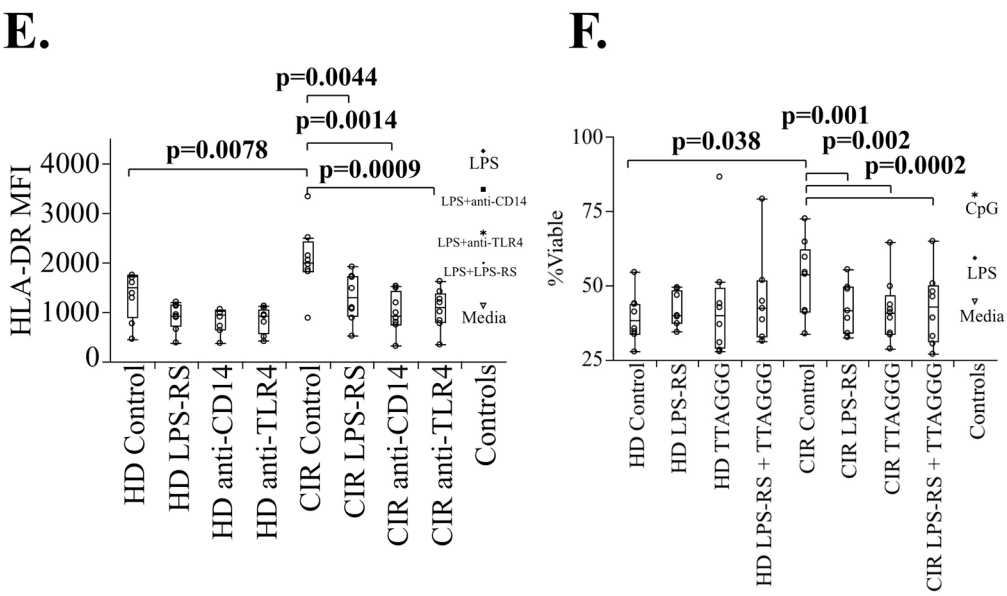


Figure 6. TLR4 and TLR9 activation of B-cells in cirrhosis

A. sCD14 plasma concentration by ELISA across patient groups. **B.** Inverse correlation of sCD14 concentrations and $CD27^{+}$ B-cell frequency. **C.** Effect of cirrhotic versus healthy donor plasma on expression of HLA-DR, CD38, and CD19 on normal donor B-cells after 72 hours culture. Representative data from three separate experiments with different B-cell donors are shown. **D.** Impact of TLR4 (LPS-R. sphaeroides) and/or TLR9 (TTAGGG) antagonism of HD and CIR plasma on normal donor B-cells after 72 hours culture. Representative data from three separate experiments with different B-cell donors shown. **E.** Impact of three approaches to block TLR4 activation (LPS-R. sphaeroides, anti-CD14 and anti-TLR4) on the expression of HLA-DR on normal donor B-cells co-cultured with HD and CIR plasma. **F..** Preservation of B-cell viability (lack of Live/Dead Aqua staining) by cirrhotic plasma and effect of TLR4 (LPS-R. sphaeroides) and/or TLR9 (TTAGGG) antagonism.

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Table 1

Baseline Patient Characteristics

		Healthy Donors	Early Fibrosis	Cirrhosis	HCC	Non HCV-CIR	Statistical analysis		
Patient number		**18**	**25**	**19**	**30**	**5**	**overall**	**CIR/HCC vs EF**	**CIR VS HCC**
Age	medium (min-max)	49	56	52	58	56	0.0003	0.9152	0.0004
Gender	M/F	17/1	25/0	19/0	30/0	5/0		1	1
Ethnicity	White/Black/As/H	5/12/1/0	5/20/0/0	10/9/0/0	9/20/0/1	2/3/0/0			
ALT (IU/dl)	Median (25th– 75th)	23.9 (20.3–26.3)	52.6 (36.0–57.0)	81.5 (55.0–92.5)	75.9 (45.3–87.5)	39.4 (23.0–53.0)	**0.0001**	**0.014**	0.37
Albumin (g/dl)	Median (25th– 75th)	4.4 (4.3–4.6)	4.3 (4.1–4.5)	4.1 (3.8–4.4)	3.3 (3.0–3.8)	4.0 (3.7–4.4)	**<0.0001**	**<0.0001**	**0.0001**
Total bilirubin (g/dl)	Median (25th– 75th)	0.6 (0.5–0.8)	0.8 (0.6–0.9)	1.2 (0.8–1.5)	1.3 (0.8–1.7)	0.9 (0.7–1.1)	**<0.0001**	**0.0003**	0.61
INR	Median (25th– 75th)	1.1 (1.0–1.1)	1.0 (1.0–1.0)	1.2 (1.1–1.2)	1.2 (1.1–1.3)	1.2 (1.1–1.2)	**<0.0001**	**<0.0001**	0.21
Platelets (K/mm3)	Median (25th– 75th)	241.3 (215.0–269.5)	228.0 (180.0–265.0)	136.7 (100.0–169.5)	118.0 (62.0–146.0)	163.8 (109.0–191.0)	**<0.0001**	**<0.0001**	0.18
WBC (K/mm3)	Median (25th– 75th)	6.4 (5.2–7.9)	6.8 (5.6–7.7)	5.7 (4.5–7.6)	6.0 (4.3–7.2)	6.4 (5.0–7.0)	0.52	0.12	0.98
ALC (K/mm3)	Median (25th– 75th)	2.2 (1.5–2.7)	2.0 (1.7–2.4)	1.8 (1.3–2.4)	1.5 (0.8–2.2)	2.3 (2.0–2.2)	**0.04**	0.12	0.19
AFP (ng/ml)	median (25th– 75th)			15.1 (2.6–14.0)	636.4 (23.1–409.5)	3.6 (2.5–4.2)			**0.0056**

INR: International Normalized Ratio

ALC: Absolute Lymphocyte Count

Table 2

Activated B-cell and T-cell Cytokine Production

A. B-cell (48h)

Cytokine		HD N=8	E N=9	CIR N=18	p-value ANOVA	p-value HD vs. CIR
IFNg (pg/ml)	Median (25th–75th)	3.7 (0.0–8.2)	0.0 (0.0–1.5)	0.0 (0.0–4.5)	0.16	
IL-4 (pg/ml)	Median (25th–75th)	0.0 (0.0–2.2)	15.2 (0.0–43.9)	1.0 (0.0–2.7)	0.10	
IL-6 (pg/ml)	Median (25th–75th)	2503.7 (733.3–5692.0)	1163.5 (595.1–5842.9)	2481.3 (637.8–5000.5)	0.99	
IL-8 (pg/ml)	Median (25th–75th)	767.2 (222.8–1694.6)	2545.7 (191.6–7746.3)	2990.9 (288.5–6425.0)	0.42	
IL-10(pg/ml)	Median (25th–75th)	324.1 (58.6–487.1)	190.7 (44.7–566.4)	67.3 (17.1–444.0)	0.49	
IL-12p40 (pg/ml)	Median (25th–75th)	45.5 (28.5–106.9)	21.7 (6.1–100.4)	19.0 (7.6–82.0)	0.39	
IL-17 (pg/ml)	Median (25th–75th)	0.0 (0.0–.0)	0.0 (0.0–.0)	0.0 (0.0–.0)	0.99	
TNFa (pg/ml)	Median (25th–75th)	187.7 (123.8–275.2)	133.3 (112.8–294.9)	243.9 (142.0–399.2)	0.47	
TNFb (pg/ml)	Median (25th–75th)	66.8 (34.5–114.3)	31.3 (19.6–70.4)	27.4 (3.8–53.1)	**0.019**	**0.0082**

B. T-cell (120h)

Cytokine		HD N=8	EF N=9	CIR N=18	p-value ANOVA	p-value HD vs. CIR
IFNg (pg/ml)	Median (25th–75th)	470.0 (252.3–1505.9)	398.8 (74.0–838.8)	249.2 (15.5–814.5)	0.39	
IL-2 (pg/ml)	Median (25th–75th)	194.2 (153.4–289.9)	186.9 (84.7–238.8)	138.1 (29.7–357.5)	0.56	
IL-4 (pg/ml)	Median (25th–75th)	30.5 (25.3–35.6)	26.9 (14.7–29.7)	16.8 (3.2–35.1)	0.42	
IL-6 (pg/ml)	Median (25th–75th)	112.8 (78.1–206.2)	82.0 (53.9–167.5)	126.0 (60.0–182.0)	0.89	
IL-8 (pg/ml)	Median (25th–75th)	215.9 (155.7–332.9)	636.5 (136.5–2284.3)	306.8 (134.7–671.3)	0.21	
IL-10 (pg/ml)	Median (25th–75th)	0 (0–0)	0 (0–5.5)	0 (0–9.3)	0.34	
IL-17 (pg/ml)	Median (25th–75th)	0 (0–0)	0 (0–0)	0 (0–0)	0.99	
TNFa (pg/ml)	Median (25th–75th)	214.7 (172.6–233.1)	265.5 (58.1–437.9)	65.8 (26.9–91.8)	**0.0074**	**0.003**
TNFb (pg/ml)	Median (25th–75th)	163.4 (96.0–199.5)	168.9 (9.4–259.4)	70.5 (9.1–95.0)	**0.05**	**0.013**