

Expansion of innate CD5^{pos} B cells expressing high levels of CD81 in hepatitis C virus infected liver

Michael P. Curry^{1,2}, Lucy Golden-Mason², Derek G. Doherty^{2,3}, Tina Deignan², Suzanne Norris⁴, Margaret Duffy⁵, Niamh Nolan⁶, William Hall^{5,7}, John E. Hegarty^{1,7,†}, Cliona O'Farrelly^{2,7,†,*}

¹The Liver Unit, St. Vincent's University Hospital, Elm Park, Dublin 4, Ireland

²Education and Research Centre, St. Vincent's University Hospital, Elm Park, Dublin 4, Ireland

³Institute of Immunology and Department of Biology, National University of Ireland, Maynooth, Ireland

⁴Institute of Liver Studies, King's College Hospital, London, UK

⁵The Virus Reference Laboratory, University College Dublin, Dublin 4, Ireland

⁶Department of Pathology, St. Vincent's University Hospital, Elm Park, Dublin 4, Ireland

⁷The Conway Institute, University College Dublin, Dublin 4, Ireland

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Background/Aims: Association of hepatitis C virus (HCV) with increased autoantibodies, mixed cryoglobulinaemia, non-Hodgkin's B-cell lymphoma and increased peripheral innate (CD5^{pos}) B cells suggests a role for B-lymphocytes in the pathogenesis of HCV-infection.

Methods: Flow cytometry was used to estimate CD5^{pos} B cell levels and CD81 co-expression in chronic HCV infection. Viral load was assessed using PCR.

Results: We demonstrate expansion of innate B cells in HCV-infected liver from patients with fibrosis score less than stage II (39%, % of total B cells, $P = 0.002$) and end stage HCV cirrhosis (20%, $P < 0.05$) compared with normal liver (8%). Expression of CD81, a signal transducing molecule and putative HCV receptor, was significantly increased on peripheral blood CD5^{pos} B cells compared with conventional B cells ($P = 0.0001$). Higher levels of CD81 on CD5^{pos} B cells were more dramatic in the liver of HCV-infected individuals. However, no significant difference was observed in the viral load of CD5^{pos}CD81^{High} B cells and CD5^{neg}CD81^{Low} B cells.

Conclusions: Increased expression of CD81 on innate B cells, a population that is expanded in the livers and peripheral blood of chronic HCV-infected patients, suggests a role in viral specific activation and clonal proliferation in chronic HCV infection.

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* Corresponding author. Tel.: +353-1-283-9444; fax: +353-1-283-8123.

E-mail address: cliona.ofarrelly@ucd.ie (C. O'Farrelly[†]).

[†] Contributed equally to the direction of this study.

Abbreviations: HCV, hepatitis C virus; ISH, in situ hybridisation; PBMC, peripheral blood mononuclear cells; *WaxId*, WA cross-reactive idiotype; IgG, immunoglobulin G; SEM, standard error of the mean; ELISA, enzyme linked immunosorbent assay; ALT, alanine aminotransaminase; RPMI 1640, Roswell Park Memorial Institute 1640 medium; FCS, foetal calf serum; HMC, hepatic mononuclear cells; MFI, median fluorescence intensity; PE, phycoerythrin; FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein; UTR, untranslated region.

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease with over 170 million people infected world-wide [1,2]. Almost one third of chronically infected individuals will develop cirrhosis and, of these, up to 2% per year will develop hepatocellular carcinoma [3,4]. Currently, end-stage liver disease secondary to HCV infection results in 8000–10 000 deaths per year and is a leading indication for liver transplantation in the United States [1,5]. HCV is predominantly hepatotropic with convincing immunohistochemical and in situ hybridisation data to show that hepa-

toocytes are a primary site of infection [6,7]. However, HCV can also infect and replicate within peripheral blood mononuclear cells (PBMCs), which may be important for viral dissemination and sequestration [8–16].

Immunological studies of HCV infection classically focus on T lymphocytes. However, the association of HCV infection with mixed cryoglobulinaemia, the presence of HCV antigen in the cryoprecipitate, clonal B cell proliferation in peripheral blood, bone marrow and liver and the high prevalence of non-Hodgkin's lymphoma suggest a major role for B cells in the pathogenesis of HCV infection [17–25]. Moreover, B cells are more frequently infected than T cells, monocytes and neutrophils and limiting dilution studies indicate higher HCV titres in B-lymphocytes than T cells or monocytes [26,27]. We, and others, have reported an expansion of peripheral blood innate (CD5^{pos}) B cells, the human equivalent of murine B-1 cells, in HCV infection [28,29]. B-1 cells have restricted receptor gene segment usage [30], are a primary source of autoantibodies [31], can be activated by T-independent antigens [32] and are thus thought to comprise part of the functional and evolutionary bridge between the innate and adaptive immune systems. In this respect, B-1 cells are considered the B cell equivalent of $\gamma\delta$ T cells [33]. CD5^{pos} B cells have been shown to produce the monoclonal WA cross reactive idiotype rheumatoid factor in HCV infection [34–36] and may be responsible for the production of other auto-antibodies associated with HCV infection. Significant populations of innate-type T Lymphocytes including NT (CD3^{pos}CD56^{pos}) and $\gamma\delta$ -TCR^{pos} T-cells have been detected in normal human liver [37], however, little is known about the equivalent populations of hepatic innate B cells (B-1/CD5^{pos}). A primary aim of this study was to examine CD5^{pos} B cell populations in normal and HCV infected liver.

CD81, a widely expressed tetraspanin molecule involved in the regulation of T cell homeostasis, is expressed on hepatocytes and lymphocytes including B-lymphocytes. Recently, CD81 has been shown to bind HCV RNA in association with the E2 region of HCV envelope protein [38]. The role for CD81 as a viral co-receptor remains unclear although CD81 is capable of binding recombinant E2. It has been demonstrated that combination Interferon- α and Ribavirin anti-viral therapy down-regulates CD81 expression both in vitro and in vivo [39], suggesting a role for CD81 in HCV pathogenesis. In addition, a potential role for CD81:HCV interactions is suggested through inhibition of NK cell activity [40,41]. In this study, we explore differential surface expression of CD81 on hepatic and peripheral blood CD5^{pos} B cells and CD5^{neg} B cells. We also investigate the presence of HCV RNA and quantitate the intra-cellular viral load in purified peripheral B cell subpopulations.

Table 1
Demographic data of study populations^a

	Group 1 ELISA + /HCV RNA + (n = 34)	Group 2 ELISA + /HCV RNA – (n = 17)	Group 3 ELISA – /HCV RNA – (n = 6)	Group 4 Mild chronic HCV (n = 10)	Group 5 Severe HCV (n = 11)	Group 6 Normal donors (n = 9)
Sample type	Peripheral blood (viremic)	Peripheral blood (non-viremic)	Peripheral blood (HCV-naive)	Liver needle biopsy	Liver explant wedge biopsy	Liver donor wedge biopsy
Sex (M/F)	6/28	1/16	3/3	3/7	7/4	3/6
Age (mean \pm SEM years)	47.9 \pm 1.35	44.1 \pm 1.5	42 \pm 3.5	42.9 \pm 4.32	50.2 \pm 2.67	44 \pm 6.31
ELISA (+/–)	34/0	17/0	0/6	10/0	11/0	0/9
HCV genotype 1	30	N/A	N/A	7	6	N/A
2	0	N/A	N/A	1	1	N/A
3	4	N/A	N/A	2	2	N/A
Unknown	0	N/A	N/A	0	2	N/A
Aetiology anti-D Ig	25	15	N/A	4	1	N/A
Blood Tx	4	2	N/A	1	4	N/A
IVDU	5	0	N/A	4	2	N/A
Sporadic	N/A	N/A	N/A	1	4	N/A

^a Abbreviations: SEM = standard error of mean; Ig = Immunoglobulin; Tx = transfusion; IVDU = intravenous drug use, N/A not applicable.

2. Materials and methods

2.1. Subjects

Six groups of patients and sero-negative normal controls were analysed. Subjects have been divided based on sample type (liver/peripheral blood), viremia (PCR^{+/−}) and severity of HCV-associated liver disease (cirrhosis) to facilitate comparison of possible compounding variables between these groups (Table 1). All subjects gave written informed consent to participate in this study, which was approved by the Ethics Committee of St. Vincent's University Hospital. The first group included 34 patients with chronic HCV infection as evidenced by a positive second generation enzyme linked immunosorbent assay (ELISA) anti-HCV antibody test (Ortho Diagnostics) and a positive HCV RNA by reverse-transcriptase polymerase chain reaction (RT-PCR) with histological evidence of chronic hepatitis consistent with HCV disease. Twenty-five individuals had been exposed to HCV contaminated anti-D immunoglobulin (Ig) [42]. No patient had received anti-viral therapy prior to enrolling in the study. The second group included 17 patients with spontaneous resolved HCV infection as evidenced by a positive second generation ELISA and repeatedly negative HCV RNA by RT-PCR. Fifteen of these individuals had received contaminated anti-D Ig. Group 3 included six normal controls with no history of exposure to HCV (negative by ELISA).

Liver biopsy specimens were obtained from ten patients (group 4) with chronic HCV infection at the time of routine percutaneous needle biopsy for histological evaluation. All ten patients were non-cirrhotic with fibrosis score less than Stage II (Metavir fibrosis scale) [43]. None of these patients had received anti-viral therapy prior to enrolling in the study. A further 11 liver biopsy specimens (group 5) were obtained at time of liver transplantation for end stage HCV cirrhosis. The HCV genotype was not determined

for some of the patients in group 5. Normal liver biopsy tissue obtained from nine donor livers at the time of liver transplantation was included in group 6. All normal donors had normal serum alanine aminotransaminase levels and were sero-negative for HCV.

2.2. Isolation of peripheral blood lymphocytes

PBMCs were isolated by standard density gradient centrifugation. The final pellet was resuspended in Roswell Park Memorial Institute 1640 medium (RPMI) 1640 medium supplemented with 2% Hepses buffer and 10% foetal calf serum (complete RPMI).

2.3. Isolation of hepatic lymphocytes from liver biopsies

Liver biopsy specimens were immediately placed in complete RPMI for transport. Donor organs were extensively perfused with University of Wisconsin solution prior to obtaining the biopsy. Percutaneous needle and explant biopsies were washed three times in 50 ml salt free Hanks Balanced Salts Solution (Gibco BRL, Paisley, Scotland) to remove residual blood. Immunohistochemical studies performed in our laboratory have demonstrated that, the washing procedure used for non-perfused samples is as effective as perfusion for the removal of intravascular non-resident leukocytes (CD45^{pos}, COF, unpublished data). Hepatic mononuclear cells (HMCs) were isolated from donor and HCV explant liver biopsies as described previously [44]. A modification of this procedure was used to extract HMCs from needle biopsy specimens. In brief, the concentration of collagenase IV used in the disruption enzyme mix was half (0.025%) that used for donor/explant biopsy material (497 U/ml, Sigma-Aldrich, Ireland). Mechanical disruption was carried out using a glass pestle. The hepatocyte removal step was omitted and the final cell

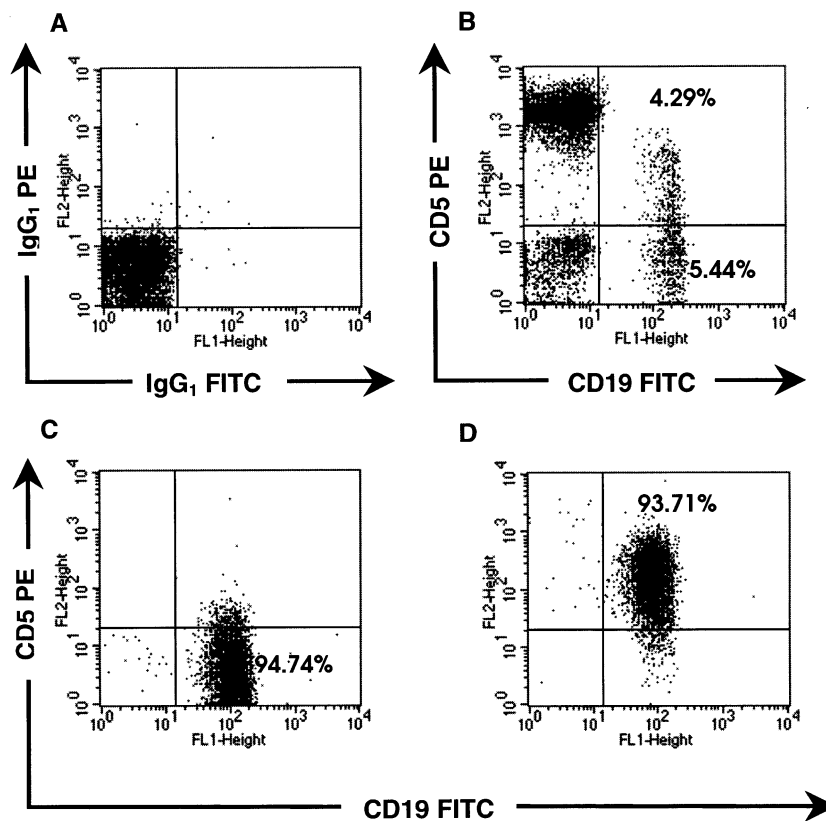


Fig. 1. Magnetic bead separation generates highly purified B cell sub-populations. Flow cytometric dot plot demonstrating cells stained with isotype matched controls (A); unseparated sample stained with anti-CD19-FITC and anti-CD5-PE (B); enriched CD19^{pos}CD5^{neg} cells (C); and enriched CD19^{pos}CD5^{pos} cells (D). This is representative of five samples.

pellet was resuspended in a smaller volume (300–500 μ l RPMI, Gibco-BRL, Paisley, Scotland). These modifications were found to produce the best yield from the small amount of tissue available from needle biopsy material (data not shown).

2.4. Phenotypic characterisation of B cell subpopulations and expression of CD81

B cell populations in blood/liver were phenotypically characterised by surface expression of CD81, CD19 and CD5 using fluorescent-labelled monoclonal antibodies. Briefly, 1×10^5 PBMCs/HMCs were labelled with: fluorescein isothiocyanate (FITC-conjugated) anti-CD5 (Pharmingen, Oxford, UK), phycoerythrin (PE) anti CD81 (Pharmingen) and peridinin chlorophyll protein anti-CD19 (Becton-Dickinson, Oxford, UK) or isotype matched controls. The cells were incubated in the dark at room temperature for 10 min and washed twice with 1 ml of phosphate buffered saline, 1% bovine serum albumin and 0.02% sodium azide (Sigma, Poole, UK). The cells were fixed with paraformaldehyde (1%) and analysed by flow cytometry. Lymphocyte gating was performed using forward scatter: side scatter parameters and 20 000 cells from this gate were acquired for analysis. Acquisition and analysis were performed using a FACscan flow cytometer and Cell Quest software (Becton-Dickinson). CD19^{pos} cells were expressed as a percentage of the lymphogate and CD19^{pos}CD5^{pos}/CD19^{pos}CD5^{neg} cells were expressed as percentage of total CD19^{pos} cells. The median fluorescence intensity (MFI), which correlates directly with the number of molecules expressed on a per cell basis, of CD81 was used to quantitate the surface expression of CD81 on B cell sub-populations.

2.5. Magnetic bead separation of peripheral blood CD5⁺ and CD5⁻ B cells

Peripheral blood mononuclear cells from five HCV RNA positive patients were separated into CD19^{pos}, CD19^{pos}CD5^{pos} and CD19^{pos}CD5^{neg} fractions using a combination of the Minimacs CD19 Multisort kit and anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Separation was performed according to the manufacturers instructions with minor modifications. Briefly, 1×10^7 PBMCs were incubated with 20 μ l of CD19 Multisort Microbeads for 15 min at 4°C. These cells were passed through a Minimacs positive-selection column. After passing through a second positive-selection column, this positive fraction was then incubated with 40 μ l of MACS Multisort Release Reagent for 10 min at 4°C. These cells were incubated with 30 μ l of MACS Multisort Stop Reagent and passed through a Minimacs column. Cells were further labelled with anti-CD5PE in the dark for 10 min at 4°C, followed by 40 μ l MACS anti-PE for 15 min at 4°C and separated using two Minimacs positive-selection columns. Positive and negative fractions were examined for purity by 2-colour flow cytometry using anti-19FITC and anti-CD5PE (Becton-Dickinson). Cells were washed three times in Minimacs buffer solution to ensure removal of any contaminating serum. All samples were stored at -80°C until use. Separation of peripheral blood lymphocytes from five HCV RNA-positive patients yielded three distinct populations of cells (CD19^{pos}, CD5^{pos}CD19^{pos} and CD5^{neg}CD19^{pos} cells) with greater than 92% purity (Fig. 1). An average of 4.6×10^6 CD19^{pos} cells, 3.4×10^6 CD19^{pos}CD5^{neg} cells and 6.32×10^5 CD19^{pos}CD5^{pos} cells were obtained following bead isolation.

2.6. Genotyping of HCV

HCV genotypes were determined by restriction fragment length polymorphism of the 5' non-coding region as described by Davidson et al. and classified according to the nomenclature proposed by Simmonds et al. [45,46].

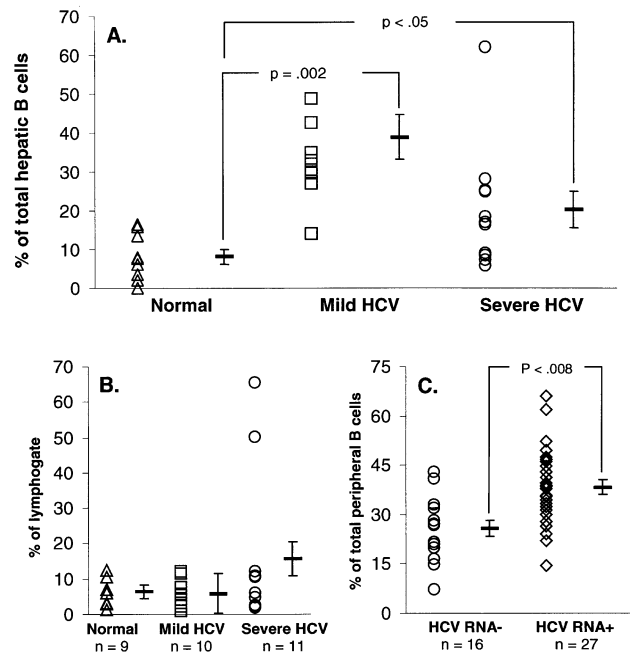


Fig. 2. CD5^{pos} B cells are specifically expanded in the peripheral blood and the liver of patients with chronic HCV infection. Hepatic B cells from ten individuals with mild, and 11 individuals with severe (end-stage) chronic HCV infection, show an increase in co-expression of CD5 when compared with nine normal controls (A). No overall expansion in the total B cell population (relative to other lymphocyte populations) was observed in HCV infected liver tissue when compared with normal liver tissue (B). The percentage of B cells that co-express the CD5 molecule in the peripheral blood is significantly increased in 27 HCV RNA positive patients compared with 16 individual with resolved HCV infection (C). Error bars represent the mean \pm SEM.

2.7. Detection of intracellular HCV RNA

RNA was extracted from the serum and from the sorted populations of B lymphocytes of five patients with chronic HCV infection by the guanidinium thiocyanate-phenol-chloroform method. The entire volume of purified RNA was subjected to reverse transcription with 100 ng of random primers (500 μ g/ml) (Promega), 200 μ M of dNTP (Promega), 20 units of RNAsin (40 u/ μ l) (Promega) and 100 units of MMLV RT (200 u/ μ l, Promega). The reaction was performed at 37°C for 90 min followed by 95°C for 5 min. The cDNA obtained was amplified by nested RT-PCR using two pairs of primers recognising the HCV 5' untranslated region as previously described [47]. The amplification product was revealed by ethidium bromide staining after agarose gel-electrophoresis.

2.8. Measurement of intracellular HCV RNA

Quantitation of intracellular HCV RNA from CD19^{pos}CD5^{pos} and CD19^{pos}CD5^{neg} sorted cell populations was performed using the Cobas Amplicor HCV Monitor kit according to the manufacturers instructions (Roche Diagnostics, NJ, USA).

2.9. Statistical analysis

A two-tailed paired/unpaired *t* test was used where appropriate to compare differences between cell populations and patient/control groups. A *P* value of <0.05 was taken as significant.

Table 2
Expression of CD81 is significantly higher on peripheral blood CD5^{pos} B cells than on CD5^{neg} B cells in chronic/resolved HCV infection^a

HCV RNA ^{pos}				HCV RNA ^{neg}			
CD5 ^{neg}	B cells	CD5 ^{pos}	B cells	CD5 ^{neg}	B cells	CD5 ^{pos}	B cells
254		212		340		461	
469		437		255		328	
66		96		562		673	
89		130		294		352	
352		453		305		333	
280		449		542		626	
239		249		133		160	
152		198		160		138	
132		139		380		531	
138		245		180		257	
254		392		305		429	
305		378		195		226	
107		153		108		121	
305		327		229		302	
220		316		130		194	
151		259		305	Mean	505	Mean
205		259		264	275.71*	284	348.24*
264		273					
148		264					
100		143					
93		111					
111		148					
79		189					
111		168					
393		414					
143		154					
453		352					
70		202					
421	Mean	487	Mean				
254	211.93*	264	262.03*				

^a * $P \leq 0.0001$ two-tailed paired t test.

3. Results

3.1. Expression of CD5 by peripheral blood and hepatic B cells

Small proportions of B cells found in normal liver tissue express the CD5 molecule (Mean \pm standard error of the mean, SEM, $8.16 \pm 1.98\%$, % of total CD19^{pos} B cells, $n = 9$). However, this population was significantly expanded in livers of individuals with HCV cirrhosis ($20.32 \pm 4.79\%$, $n = 11$, $P < 0.05$). There is an even greater expansion of these cells in the livers of non-cirrhotic HCV-infected individuals ($38.95 \pm 5.64\%$; $n = 10$, $P < 0.002$, Fig. 2a). Interestingly, there was no overall expansion in the total B cell population, relative to other lymphocyte populations, in HCV infected liver tissue when compared with normal liver tissue (Fig. 2b). However, as HCV-infected liver is characterised by an inflammatory infiltrate, it is likely that overall B cell numbers are increased. Increased levels of CD5^{pos} B cells were also observed in the peripheral blood of HCV infected individuals ($38.23 \pm 2.22\%$) when compared with those with resolved infection ($25.89 \pm 2.36\%$, $P < 0.008$, Fig. 2c).

3.2. Expression of CD81 by B cells and B cell subsets

Significant CD81 expression was seen on all B cell subsets tested. Expression of CD81 by peripheral blood B cells was not increased in viremic patients (MFI \pm SEM; 258 ± 21), when compared with individuals who had resolved their infection (284 ± 32.5) and normal controls (270 ± 39 ; $P = 0.43$) (data not shown). However, CD81 expression was consistently higher on CD5^{pos} B cells when compared with CD5^{neg} B cells in all but three of 30 individuals with chronic HCV infection (262.03 ± 20.9 versus 211.93 ± 21.75 ; $P = 0.00006$, Table 2). Increased levels of CD81 were also observed on CD5^{pos} B cells in all but one of 17 individuals with resolved infection (348.24 ± 40.45 versus 275.71 ± 31.64 ; $P = 0.00007$, Table 2). Normal controls also had higher expression of CD81 on CD5^{pos} B cells (307 ± 31.8 versus 246 ± 29.7 ; $P = 0.0004$, data not shown). Higher levels of CD81 expression on CD5^{pos} B cells were even more dramatic in the liver of HCV infected individuals when compared with conventional CD5^{neg} B cells (657.32 ± 194.53 versus 304.56 ± 99.38 , $P < 0.03$, $n = 6$, Fig. 3).

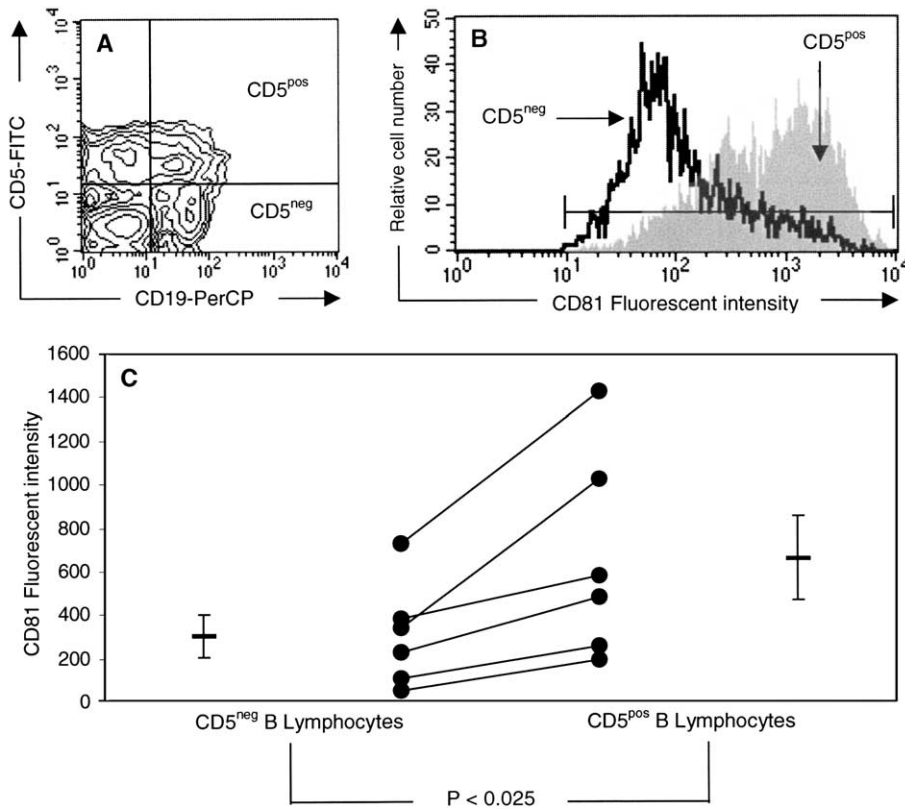


Fig. 3. CD5^{pos} B lymphocytes express higher levels of CD81 than CD5^{neg} B cells. Representative flow cytometric contour plot of hepatic B lymphocytes showing CD5^{pos} and CD5^{neg} B cells in the upper right and lower right quadrants, respectively (A). The histogram shows the relatively low intensity of staining for CD81 (Log₁₀ fluorescence) on CD5^{neg} and the much higher level on CD5^{pos} cell populations from one individual (B). For each of the individuals tested hepatic CD5^{pos} B cells expressed higher levels of CD81 than their CD5^{neg} counterparts (C). Error bars represent the mean ± SEM.

3.3. HCV infection of B cells subsets

Intracellular HCV RNA was detected in the B-lymphocyte subsets of five chronically infected patients (all genotype 1b). Qualitative nested RT-PCR detected HCV RNA in the serum, CD19^{pos} cells, CD19^{pos}CD5^{pos} cells and CD19^{pos}CD5^{neg} cells (Fig. 4). Quantitative analysis for intracellular HCV infection in three patients indicated no significant difference ($P = 0.48$) in viral load of CD19^{pos}CD5^{pos}CD81^{Hi} cells and CD19^{pos}CD5^{neg}CD81^{Lo} cells and no correlation between the surface expression of CD81 and the intracellular viral load (Table 3).

4. Discussion

Innate lymphocytes recognise conserved structures that signal viral infection. Failure to eliminate HCV may result from a deficiency of such innate lymphocytes. Indeed, depletion of NK cells and CD56⁺ T cells have been reported to occur in the livers of chronically HCV-infected individuals [48,49]. In this study we focus on another innate lymphocyte population, the CD5^{pos} B cells, in chronic HCV infection. The most frequent and well-characterised extrahepatic manifestations of chronic HCV infection are cryoglobulinaemia and rheumatoid factor activity [19,29,

Table 3
Comparison of median fluorescence intensity of cell surface CD81 and intracellular hepatitis C viral load in CD5^{neg} and CD5^{pos} B cells from three patients with chronic HCV genotype 1b infection

	Median fluorescence intensity ^a		Viral load (copies/million cells)	
	CD5 ⁻ B-cells	CD5 ⁺ B-cells	CD5 ⁻ B-cells	CD5 ⁺ B-cells
Sample 1	297	357	27 666	33 166
Sample 2	324	432	32 833	21 000
Sample 3	283	449	99 666	26 000
Mean value	301	413	53 388	26 722
P value	0.02		0.32	

^a CD81 Log₁₀ fluorescence.

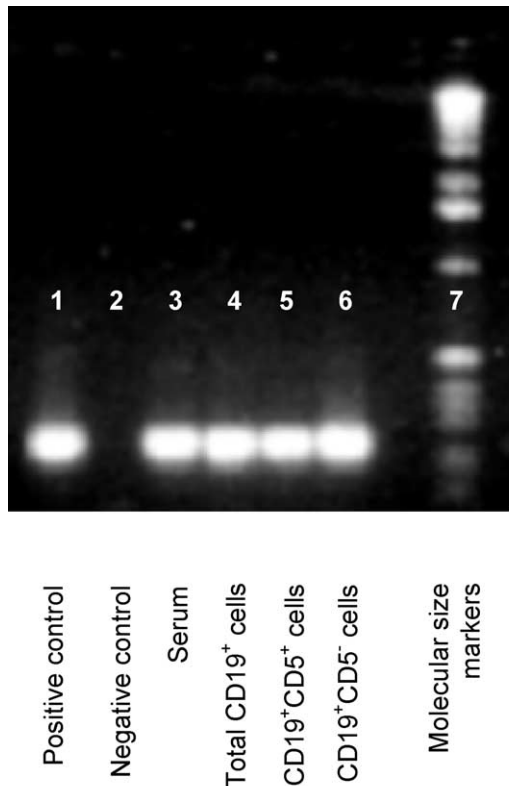


Fig. 4. HCV is detectable in CD5^{pos} and CD5^{neg} B cell populations. RT-PCR analysis of serum (lane 3), total CD19^{pos} cells (lane 4), CD19^{pos}CD5^{pos} cells (lane 5), and CD19^{pos}CD5^{neg} cells (lane 6) for HCV RNA. Positive and negative controls and molecular size markers are present in lane 1, 2, and 7, respectively.

50,51]. The presence of immune complexes in HCV disease coupled with the increased incidence of non-Hodgkin's lymphoma and the demonstration of monoclonal proliferation of B cells in the peripheral blood, bone marrow and liver strongly suggest that B cells are important in the immunopathogenesis of HCV infection [21,23,24,51]. Furthermore, HCV infection has been associated with the development of lymphoproliferative diseases in the post liver transplant setting [52]. We have previously shown expansion of peripheral blood CD5^{pos} B cells in viremic HCV infected individuals which is accompanied by the appearance of rheumatoid factor in the serum of these individuals [28]. In this study, we demonstrate, in contrast to other innate lymphocyte populations [49], a specific expansion of CD5^{pos} B cells, but not overall B cell levels, in the liver of HCV infected individuals. We also show high levels of CD81 expression by all CD5^{pos} B cells not just in chronic HCV-infection. These results suggest that HCV viremia may induce proliferation of or favour the survival of innate B cells.

CD81, a cell surface molecule expressed on hepatocytes and lymphocytes, is known to interact with the E2 protein of HCV suggesting that it could be a cellular receptor for HCV [38]. Binding of the HCV envelope protein to human CD81

has been clearly demonstrated. There was no increase in viral load in cells with high levels of CD81 expression suggesting that CD81 is not directly involved in HCV entry into cells. However, the sample number used in this study is too small to draw a definitive conclusion. In this study, we have demonstrated that surface expression of CD81 is increased on expanded subpopulations of CD19^{pos} B cells that co-express CD5. However, despite the differential expression of CD81 on B cell subpopulations, we were unable to demonstrate a correlation between the HCV load in purified populations of CD5^{pos}CD81^{Hi} B cells and CD5^{neg}CD81^{Lo} B cells and the surface expression of CD81. This indicates that neither increased expression of surface CD81, reflecting more receptor available for binding of ligand, nor decreased expression of CD81 reflecting down-regulation due to internalisation or as a consequence of repeated binding, correlate with viral infection of the cell. Our data provides additional evidence that this molecule does not function as a route of infection and supports the notion that CD81 is more likely to act as a signalling molecule effecting the behaviour of lymphocyte populations involved in host anti-viral immunity [40].

Recent studies have demonstrated that CD81:HCV interactions result in activation or inhibition of NK/T lymphocyte subsets and may thus be important in determining the outcome of HCV infection [40,41]. CD81 expression on the surface of B cells is coupled with the B cell receptor complex and when appropriately engaged with ligand results in reduction of the activation threshold leading to the production of autoantibodies and immune complexes characteristic of HCV disease. Proliferation of B cells in response to B cell receptor crosslinking is impaired in CD81-deficient mice [53]. Thus, direct viral stimulation *via* CD81 may initiate B cell clonal proliferation. Translocation of the *bcl-2* gene from chromosome 18 to a position close to the immunoglobulin heavy chain locus on chromosome 14 during proliferation prevents apoptosis and leads to lymphoproliferative disease [54,55]. Thus, the interaction between HCV and CD81 may result in stimulation of B cells with resultant B cell proliferation.

Increased expression of the CD81 molecule on populations of innate B cells that are expanded in the livers and peripheral blood of chronic HCV infected patients suggests that it may have a role in viral specific activation and clonal proliferation of this population in chronic HCV infection. Two recent studies [39,56] have described increased CD81 on B cells in HCV infection. B cell CD81 expression correlated with serum viral load and rheumatoid factor [56] and was downregulated by interferon- α both *in vitro* and *in vivo* [39]. In our study, we have demonstrated increased expression of CD81 on all CD5^{pos} B cells even in normal controls. Zuckerman [56] and Kronenberger [39] only looked at total B cell populations, therefore, the observed increase of CD81 on B cells and downregulation by treatment could be accounted for by increased or depleted CD5^{pos} B cell subset within the whole B cell population.

Signalling through CD81 may represent an important HCV survival strategy as suggested by the potential to inhibit natural killer activity [40,41]. Expansion of the subpopulation of CD5^{pos} B cells, which expresses high levels of CD81, in HCV infection has exciting implications for our understanding of how innate immune mechanisms might contribute to HCV-associated disease pathogenesis.

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References

- [1] Alter MJ. Epidemiology of hepatitis C. *Hepatology* 1997;26:62S–65S.
- [2] Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, et al. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N Engl J Med* 1999;341:556–562.
- [3] Fattovich G, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, et al. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. *Gastroenterology* 1997;112:463–472.
- [4] Hu KQ, Tong MJ. The long-term outcomes of patients with compensated hepatitis C virus-related cirrhosis and history of parenteral exposure in the United States. *Hepatology* 1999;29:1311–1316.
- [5] Belle SH, Beringer KC, Detre KM. Trends in liver transplantation in the United States. *Clin Transpl* 1993;19–35.
- [6] Sansonno D, Iacobelli AR, Cornacchiulo V, Distasi M, Dammacco F. Immunohistochemical detection of hepatitis C virus-related proteins in liver tissue. *Clin Exp Rheumatol* 1995;13(Suppl. 13):S29–S32.
- [7] Muratori L. In situ reverse transcriptase-polymerase chain reaction: an innovative tool for hepatitis C virus RNA detection and localisation, and for quantification of infected cells. *Eur J Histochem* 1998;42:133–136.
- [8] Kao JH, Chen PJ, Lai MY, Wang TH, Chen DS. Positive and negative strand of hepatitis C virus RNA sequences in peripheral blood mononuclear cells in patients with chronic hepatitis C: no correlation with viral genotypes 1b, 2a, and 2b. *J Med Virol* 1997;52:270–274.
- [9] Crovatto M, Pozzato G, Zorat F, Pussini E, Nascimben F, Baracetti S, et al. Peripheral blood neutrophils from hepatitis C virus-infected patients are replication sites of the virus. *Haematologica* 2000;85:356–361.
- [10] Radkowski M, Wang LF, Vargas HE, Rakela J, Laskus T. Detection of hepatitis C virus replication in peripheral blood mononuclear cells after orthotopic liver transplantation. *Transplantation* 1998;66:664–666.
- [11] Moldvay J, Deny P, Pol S, Brechot C, Lamas E. Detection of hepatitis C virus RNA in peripheral blood mononuclear cells of infected patients by in situ hybridization. *Blood* 1994;83:269–273.
- [12] Moldvay J, Deny P, Pol S, Brechot C, Lamas E. In situ hybridization for the detection of hepatitis C virus RNA in mononuclear cells of peripheral blood from infected patients. *Orv Hetil* 1995;136:2267–2271.
- [13] Rodriguez-Inigo E, Casqueiro M, Navas S, Bartolome J, Pardo M, Carreno V. Fluorescent in situ hybridization of hepatitis C virus RNA in peripheral blood mononuclear cells from patients with chronic hepatitis C. *J Med Virol* 2000;60:269–274.
- [14] Maggi F, Fornai C, Morrica A, Vatteroni ML, Giorgi M, Marchi S, et al. Divergent evolution of hepatitis C virus in liver and peripheral blood mononuclear cells of infected patients. *J Med Virol* 1999;57:57–63.
- [15] Manzin A, Candela M, Paolucci S, Caniglia ML, Gabrielli A, Clementi M. Presence of hepatitis C virus (HCV) genomic RNA and viral replicative intermediates in bone marrow and peripheral blood mononuclear cells from HCV-infected patients. *Clin Diagn Lab Immunol* 1994;1:160–163.
- [16] Sansonno D, Lotesoriere C, Cornacchiulo V, Fanelli M, Gatti P, Iodice G, et al. Hepatitis C virus infection involves CD34(+) hematopoietic progenitor cells in hepatitis C virus chronic carriers. *Blood* 1998;92:3328–3337.
- [17] Agnello V, Chung RT, Kaplan LM. A role for hepatitis C virus infection in type II cryoglobulinemia. *N Engl J Med* 1992;327:1490–1495.
- [18] Bichard P, Ounanian A, Girard M, Baccard C, Rolachon A, Renversez JC, et al. High prevalence of hepatitis C virus RNA in the supernatant and the cryoprecipitate of patients with essential and secondary type II mixed cryoglobulinemia. *J Hepatol* 1994;21:58–63.
- [19] Dammacco F, Gatti P, Sansonno D. Hepatitis C virus infection, mixed cryoglobulinemia, and non-Hodgkin's lymphoma: an emerging picture. *Leuk Lymphoma* 1998;31:463–476.
- [20] Sansonno D, Iacobelli AR, Cornacchiulo V, Lauletta G, Distasi MA, Gatti P, et al. Immunochemical and biomolecular studies of circulating immune complexes isolated from patients with acute and chronic hepatitis C virus infection. *Eur J Clin Invest* 1996;26:465–475.
- [21] Franzin F, Efremov DG, Pozzato G, Tulissi P, Batista F, Burrone OR. Clonal B-cell expansions in peripheral blood of HCV-infected patients. *Br J Haematol* 1995;90:548–552.
- [22] Mazzaaro C, Franzin F, Tulissi P, Pussini E, Crovatto M, Carniello GS, et al. Regression of monoclonal B-cell expansion in patients affected by mixed cryoglobulinemia responsive to alpha-interferon therapy. *Cancer* 1996;77:2604–2613.
- [23] Sansonno D, De Vita S, Iacobelli AR, Cornacchiulo V, Boiocchi M, Dammacco F. Clonal analysis of intrahepatic B cells from HCV-infected patients with and without mixed cryoglobulinemia. *J Immunol* 1998;160:3594–3601.
- [24] Magalini AR, Facchetti F, Salvi L, Fontana L, Puoti M, Scarpa A. Clonality of B-cells in portal lymphoid infiltrates of HCV-infected livers. *J Pathol* 1998;185:86–90.
- [25] Murakami J, Shimizu Y, Kashii Y, Kato T, Minemura M, Okada K, et al. An innovative B-cell response in intrahepatic lymphoid follicles in chronic hepatitis C. *Hepatology* 1999;30:143–150.
- [26] Muller HM, Kallinowski B, Solbach C, Theilmann L, Goeser T, Pfaff E. B-lymphocytes are predominantly involved in viral propagation of hepatitis C virus (HCV). *Arch Virol Suppl* 1994;9:307–316.
- [27] Zehender G, Meroni L, De Maddalena C, Varchetta S, Monti G, Galli M. Detection of hepatitis C virus RNA in CD19 peripheral blood mononuclear cells of chronically infected patients. *J Infect Dis* 1997;176:1209–1214.
- [28] Curry MP, Golden-Mason L, Nolan N, Parfrey NA, Hegarty JE, O'Farrelly C. Expansion of peripheral blood CD5⁺ B cells is associated with mild disease in chronic hepatitis C virus infection. *J Hepatol* 2000;32:121–125.
- [29] Pietrogrande M, Corona M, Milani S, Rosti A, Ramella M, Tordato G. Relationship between rheumatoid factor and the immune response against hepatitis C virus in essential mixed cryoglobulinemia. *Clin Exp Rheumatol* 1995;13(Suppl. 13):S109–S113.
- [30] Tatu C, Ye J, Arnold LW, Clarke SH. Selection at multiple checkpoints focuses V(H)12 B cell differentiation toward single B-1 cell specificity. *J Exp Med* 1999;190:903–914.

- [31] Hayakawa K, Asano M, Shinton SA, Gui M, Altman D, Stewart CL, et al. Positive selection of natural autoreactive B cells. *Science* 1999;285:113–116.
- [32] Martin F, Kearney JF. Positive selection from newly formed to marginal B cells depends on the rate of clonal production, CD19 and btk. *Immunity* 2000;12:39–49.
- [33] Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. *Curr Opin Immunol* 2001;13:195–201.
- [34] Hayakawa K, Hardy RR. Development and function of B-1 cells. *Curr Opin Immunol* 2000;12:346–353.
- [35] Dammacco F, Sansonno D, Piccoli C, Racanelli V, D'Amore FP, Lauletta G. The lymphoid system in hepatitis C virus infection: autoimmunity, mixed cryoglobulinemia, and Overt B-cell malignancy. *Semin Liver Dis* 2000;20:143–157.
- [36] Sansonno D, Cornacchiulo V, Iacobelli AR, Gatti P, Distasi M, Dammacco F. Hepatitis C virus infection and clonal B-cell expansion. *Clin Exp Rheumatol* 1996;14(Suppl. 14):S45–S50.
- [37] Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. *Immunol Rev* 2000;174:5–20.
- [38] Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, et al. Binding of hepatitis C virus to CD81. *Science* 1998;282:938–941.
- [39] Kronenberger B, Ruster B, Elez R, Weber S, Piiper A, Lee J-H, et al. Interferon alfa down-regulates CD81 in patients with chronic hepatitis C. *Hepatology* 2001;33:1518–1526.
- [40] Crotta S, Stilla A, Wack A, D'Andrea A, Nuti S, D'Oro U, et al. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J Exp Med* 2002;195:35–41.
- [41] Tseng CT, Klimpel GR. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *J Exp Med* 2002;195:43–49.
- [42] Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. *Irish Hepatology Research Group. N Engl J Med* 1999;340:1228–1233.
- [43] Bedossa P. The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in biopsy interpretation in patients with chronic hepatitis C. *Hepatology* 1994;20:15–20.
- [44] Curry MP, Norris S, Golden-Mason L, Doherty DG, Deignan T, Collins C, et al. Isolation of lymphocytes from normal adult human liver suitable for phenotypic and functional characterisation. *J Immunol Methods* 2000;242:21–31.
- [45] Davidson F, Simmonds P, Ferguson JC, Jarvis LM, Dow BC, Follett EA, et al. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5' non-coding region. *J Gen Virol* 1995;76:1197–1204.
- [46] Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, et al. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J Gen Virol* 1993;74:2391–2399.
- [47] Chan SW, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, et al. Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. *J Gen Virol* 1992;73:1131–1141.
- [48] Kawarabayashi N, Seki S, Hatsuse K, Ohkawa K, Koike Y, Aihara T, et al. Decrease of CD56⁺ T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. *Hepatology* 2000;32:962–969.
- [49] Deignan T, Curry MP, Doherty DG, Golden-Mason L, Volkov Y, Norris S, et al. Decrease in hepatic CD56⁺ T cells and V α 24⁺ natural killer T cells in chronic hepatitis C viral infection. *J Hepatol* 2002;37:101–108.
- [50] Mazzaro C, Zagonel V, Monfardini S, Tulissi P, Pussini E, Fanni M, et al. Hepatitis C virus and non-Hodgkin's lymphomas. *Br J Haematol* 1996;94:544–550.
- [51] Mazzaro C, Tulissi P, Moretti M, Mazzoran L, Pussini E, Crovatto M, et al. Clinical and virological findings in mixed cryoglobulinaemia. *J Intern Med* 1995;238:153–160.
- [52] Hezode C, Duvoux C, Germanidis G, Roudot-Thoraval F, Vincens AL, Gaulard P, et al. Role of hepatitis C virus in lymphoproliferative disorders after liver transplantation. *Hepatology* 1999;30:775–778.
- [53] Miyazaki T, Muller M, Campbell KS. Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81. *EMBO J* 1997;16:4217–4225.
- [54] Zignego AL, Giannelli F, Marrochi ME, Mazzocca A, Ferri C, Giannini C, et al. T(14;18) translocation in chronic hepatitis C virus infection. *Hepatology* 2000;31:474–479.
- [55] Zignego AL, Giannelli F, Marrochi ME, Giannini C, Gentilini P, Innocenti F, et al. Frequency of bcl-2 rearrangement in patients with mixed cryoglobulinemia and HCV-positive liver diseases [letter]. *Clin Exp Rheumatol* 1997;15:711–712.
- [56] Zuckerman E, Slobodin G, Kessel A, Sabo E, Yeshurun D, Halas K, et al. Peripheral B-cell CD5 expansion and CD81 overexpression and their association with disease severity and autoimmune markers in chronic hepatitis C virus infection. *Clin Exp Immunol* 2002;128:353–358.