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QUANTITATIVE ANALYSIS OF ANTI-HEPATITIS C VIRUS ANTIBODY-SECRETING B CELLS IN PATIENTS WITH CHRONIC HEPATITIS C

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Abbreviations: HCV, hepatitis C virus; ELISPOT, enzyme-linked immunospot; PBMC, peripheral blood mononuclear cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PBS, phosphate-buffered saline; SFC, spot forming cells; ROC, receiver operating characteristics; AUC, area under the curve; IQR, interquartile range.

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Abstract

To investigate quantitative characteristics of humoral immunity in patients with hepatitis C, we established an enzyme-linked immunospot (ELISPOT) assay for detection of anti-HCV-secreting B cells. ROC curve analysis demonstrated 100% specificity and 58% to 92% sensitivity for detecting B-cell responses to NS5b, NS3, E2 and core antigens. The median sum of anti-HCV secreting B cells to all HCV antigens tested was significantly higher in 39 patients with chronic hepatitis C (47.3 spot forming cells (SFC) /10^6 PBMC) than in 9 recovered subjects (15.3 SFC /10^6 PBMC; P=0.05) or 11 uninfected controls (5.3 SFC/10^6 PBMC; P<0.001); the significant difference (P=0.018) in chronic versus recovered patients was in reactivity to nonstructural antigens NS3 and NS5b. Anti-HCV IgM-secreting B cells were also readily detected and persisted decades into HCV infection; there was no difference in IgM positive cells between chronic and recovered patients. ELISPOT reactivity to genotype-1 derived antigens was equivalent in patients of genotypes 1, 2 or 3. There was significant correlation between the numbers of anti-HCV IgG-secreting B cells and serum transaminase and to the level of circulating antibody. In conclusion, ELISPOT assays can be adapted to study B cell as well as T cell responses to HCV. Measurement at the single-cell level suggests that humoral immunity plays a minor role in recovery from HCV infection and that B-cell immunity is strongest in those with persistent infection. The ELISPOT assay provides a new tool to study the mechanisms of viral persistence in the face of strong B-cell immune responses.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. More than half of patients with acute HCV infections develop chronic hepatitis that leads to liver cirrhosis and/or hepatocellular carcinoma in at least 20%. [1-3] Chronic HCV infection results in the induction of a strong humoral immune response, and measurement of anti-HCV antibodies in serum is widely used to screen for HCV infection. Although several studies have examined the features of the humoral immune response to HCV, [4-7] the quantitative characteristics of HCV-specific antibody production during infection remain undefined. In patients with acute hepatitis C, an early HCV-specific T cell response is associated with viral clearance, [8-11] but the role of humoral immune responses in HCV clearance is unclear and appears to be subsidiary since strong antibody responses are detected in all immunocompetent chronic HCV carriers. It is also unknown whether anti-HCV antibody serves to control the level of viremia during chronic infection and whether it ameliorates horizontal or vertical transmission.

An enzyme-linked immunospot (ELISPOT) assay, for detecting individual B cells secreting specific antibodies has enabled investigators to study B-cell immunity at a cellular level in a variety of clinical applications. [12, 13] The advantages of the ELISPOT assay are that the assay detects even a single cell out of 10^6 peripheral blood mononuclear cells (PBMCs), whose secretion level may not be sufficient for detection of circulating antibody, and distinguishes and quantifies only active immunoglobulin-secreting cells. The ELISPOT assay thus provides a useful tool for better understanding immunity to infectious diseases and improved analysis of the immune response to vaccination. [14] Although studies of antigen-specific antibody-secreting cells in various viral infections have been conducted, [15-19] there are no published data on detection and quantification of anti-HCV antibody-secreting B cells.

The objective of this study was to adapt the ELISPOT assay for the detection of anti-HCV antibody-secreting B cells, to clarify the HCV-specific humoral immune responses in patients with chronic hepatitis C, to examine the correlation between the numbers of anti-HCV antibody secreting B cells and clinical outcomes, and specifically, to examine humoral immune responses in patients with chronic hepatitis C compared to those who spontaneously clear HCV.

Patients and Methods

Subjects

Individuals who were identified by the Greater Chesapeake and Potomac Region of the American Red Cross as being positive for anti-HCV by enzyme immunoassay at the time of blood donation were referred to the Department of Transfusion Medicine at the National Institutes of Health for participation in a long-term study of the natural history of HCV infection; [20, 21] 750 participants were enrolled through September 2003. Of these, 48 subjects were selected randomly to assess humoral immune responses at the B-cell level. The “chronic hepatitis C” group included 39 subjects who were positive for anti-HCV antibodies (EIA-2 and RIBA-3) and positive for HCV RNA. The “recovered” group included 9 anti-HCV positive subjects who were HCV RNA-negative by qualitative polymerase chain reaction on at least two consecutive visits. The patients’ characteristics are summarized in Table 1. Eleven volunteer blood donors without a history of HCV infection served as controls. All subjects were negative for hepatitis B surface antigen and antibodies to the human immunodeficiency virus. The study protocols were reviewed and approved by the appropriate institutional review boards, and all subjects gave their written informed consent.

Laboratory Testing

Antibodies to HCV were measured in serum samples by a second-generation enzyme immunoassay (EIA-2, Abbott Laboratories, North Chicago, IL). EIA-2 reactive samples were subsequently tested by a third-generation recombinant immunoblot assay (RIBA-3^{TM}; Chiron Corporation, Emeryville, CA). Reactivity to at least two of four HCV antigens (5-1-1/C100-3, C33, C22, and NS5) was considered a positive RIBA-3^{TM} result, no reactivity, a negative result, and reactivity to only one antigen, an indeterminate result. The serum levels of HCV RNA were determined using the qualitative and quantitative COBAS AMPLICOR assays (Roche Diagnostic Systems, Branchburg, NJ), which amplify HCV RNA by reverse-transcription-polymerase chain reaction. HCV genotypes were determined by INNO-LiPA HCV II (Innogenetics, Gent, Belgium). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests were performed using standard methods.

Peripheral Blood Mononuclear Cells

PBMCs were isolated from whole blood using cellular preparation tubes (Becton Dickinson, Franklin Lakes, NJ), washed one time in phosphate-buffered saline (PBS) and three times in medium (RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2 mercaptoethanol, 50 U/mL penicillin, and 50 µg/mL streptomycin, and 10% fetal bovine serum), and were either studied immediately or cryopreserved in media containing 50% fetal bovine serum, 10% dimethyl sulfoxide (Sigma-Aldrich Corp. St. Louis, MO), and 10% RPMI 1640.

HCV proteins

The recombinant full-length HCV core protein (amino acid residues 1-191), E2 protein (amino acid residues 384-746), NS3 protein (amino acid residues 1027-1657), and NS5B protein (amino acid residues 2421-3011) were expressed and purified from *Escherichia coli* using the expression vector as described previously. [22, 23] Control proteins were expressed as carboxy-terminal fusion proteins with human superoxide dismutase in *Escherichia coli*.

ELISPOT Assay

Ninety six-well plates containing high-protein binding membranes (MAIP S4510; Millipore Co., Bedford, MA) were coated with a 10 µg/mL purified recombinant HCV core, E2, NS3, NS5b, or control antigens in carbonate coating buffer (0.1 mol/L Na_2CO_3, 0.1 mol/L $NaHCO_3$, pH 9.6). After incubation at 4°C overnight, the plates were washed twice with PBS and blocked with 3% bovine serum albumin for more than 30 minutes at 37°C. Cryopreserved PBMCs were thawed and incubated for 44 hours at 37°C in a humidified atmosphere of 5% CO_2 at 1.25×10^5 or 2.5×10^5 cells/well in AIM V Media (Invitrogen Carlsbad, CA). All determinations were run in triplicate. After incubation, the cells were removed by washing 6 times with PBS containing 0.05% NP-40, and the plates incubated with horseradish peroxidase-linked anti-human IgG or IgM antibodies (1:1000; KPL, Gaithesburg, MD) at 37°C for 2 hours. After the plates were washed twice with PBS and 6 times with PBS containing 0.05% NP-40, an optimal 4CN peroxidase substrate

(Bio-Rad Corp., Hercules, CA) was added and incubated for 20 to 30 minutes at room temperature to develop the spots. The reaction was stopped by washing with distilled water. The plates were dried overnight, and the spots were counted automatically by an ELISPOT reader (Carl Zeiss Vision, Hallbergmoos, Germany). The frequencies of anti-HCV antibodies-secreting B cells were calculated by subtracting the mean number of spots in the control wells from the HCV antigen-coated wells, and expressed as the mean of triplicates of spot forming cells (SFC) per 10^6 PBMCs. Assays with a high background (> 5 spots/well in the negative control) were excluded.

Assay of anti-HCV/ NS3 antibodies

Anti-HCV/ NS3 IgG was assayed by ELISA as described previously.[23] Briefly, MaxiSorp Nunc-Immuno plates were coated with recombinant HCV NS3 protein at 6 μg/mL in coating buffer (20 mM sodium bicarbonate buffer, pH9.6, 0.15 M NaCl) and overcoated with 0.1% bovine serum albumin in PBS buffer, pH7.4.. The sera were tested by two-fold serial dilution in 0.3% IGEPAL CA-630 (Sigma), 5% milk diluent (Kirkegaard & Perry Laboratories), PBS, pH 7.4, with initial dilution at 1:250. Biotinylated anti-human IgGγ (Kirkegaard & Perry Laboratories) and strepavidin–horseradish peroxidase (Kirkegaard & Perry Laboratories) were added sequentially. One hundred μL per well ABTS micro-well peroxidase substrate was used to develop the color and 100 μL per well peroxidase stop solution (Kirkegaard & Perry Laboratories) was added to stop the reaction. Absorbance was read at 405 nm. The IgG titer was determined by end-point dilution.

Statistical Analysis

The Mann-Whitney *U* test or student's *t*-test was used to analyze continuous variables as appropriate. Spearman's rank order correlations were used to evaluate the frequencies of anti-HCV antibody-secreting B cells to each antigen and to the clinical features. A P value of ≤ 0.05 was considered significant. Although SFC/10^6 PBMCs was expressed in this study, the statistics were significant whether this was used or the raw counts were used. Statistical analyses were performed using SigmaStat (version 2.03, SPSS Inc., Chicago, IL). Receiver-operating characteristic (ROC) curve analysis was

performed with the MedCalc 7.0 program (http://www.medcalc.be). The best cutoff values of the ELISPOT assays were chosen automatically by the MedCalc 7.0 program as the spot forming cells with the highest diagnostic accuracy, i.e., the sum of the false-negative and false-positive rates was minimized. The respective overall diagnostic values were expressed using the area under the curve (AUC).

Results

Optimal Cutoff Values for ELISPOT Assay

To determine the optimal cutoff values for the B cell ELISPOT assay in differentiating patients with HCV infection from HCV seronegative blood donors, ROC curve analysis was performed. The ROC curves for the ELISPOT assay detecting anti-HCV IgG specific-B cells were obtained by calculations made using the values obtained from 48 patients with HCV infection and the 11 HCV-negative volunteer blood donors. The selection of the optimal cutoff point value was based on the level at which the accuracy was maximum (see Patients and Methods). The optimal cutoff values, sensitivity, specificity, positive predictive values, negative predictive values, and calculated AUCs to all HCV antigens are listed in Table 2A. In our ELISPOT assay, the values of sensitivity were from 58% to 92%, and the values of specificity were 100%. The results of AUC were constantly high in the ELISPOT assays to all antigens, and the values of AUC were between 0.71 (NS5B antigen) and 0.94 (core and E2 antigens).

After we defined the optimal cutoff value for each antigen, we determined the frequencies of anti-HCV IgG-secreting B cells in 48 patients with HCV infection. The prevalence of anti-HCV IgG-secreting B cells during HCV infection specific for the various antigens were core (92%), E2 (92%), NS3 (77%), and NS5B (58%) (Table 2A).

We further assessed the optimal cutoff values for the ELISPOT assay detecting anti-HCV IgM-secreting B cells using ROC curve analysis in 43 patients with HCV infection and in 6 HCV-negative blood donors (Table 2B). The values of AUC were from 0.73 (NS5B antigen) to 0.94 (core antigen). The prevalence of anti-HCV IgM-secreting B cells was from 54% (NS5B antigen) to 84% (core antigen) (Table 2B).

Detection and Quantitation of Anti-HCV Antibody-Secreting B Cells

Forty-eight PBMC samples obtained from patients with HCV infection and 11 samples from healthy volunteer blood donors were examined for detection of the anti-HCV IgG-secreting B cells. The median numbers of the sum of anti-HCV IgG-secreting B cells to all HCV antigens were significantly higher in patients with HCV infection (38.3 SFC/10^6 PBMCs; interquartile range (IQR), 10.7-149.3) compared with control anti-HCV negative donors (5.3 SFC/10^6 PBMCs; IQR, 2.7-8.0; P<0.001). Figure 1a shows box plots for the numbers of anti-HCV IgG-secreting B cells to all 4 HCV antigens in patients with HCV infection and in the controls. Among 48 patients with HCV infection, the range of median numbers of anti-HCV IgG-secreting B cells were from 10.7 SFC/10^6 PBMCs (NS5B antigen) to 119.0 SFC/10^6 PBMCs (E2 antigen). The median numbers of anti-HCV IgG-secreting B cells in patients with HCV infection were significantly higher than those in controls for each HCV antigen (Fig. 1A).

Subsequently, we developed an ELISPOT assay for detecting anti-HCV IgM-secreting B cells. Detection of the anti-HCV IgM-secreting B cells was performed in 43 patients with HCV infection and in 6 anti-HCV negative blood donors (Figure 1b). The median numbers of the sum of anti-HCV IgM-secreting B cells to all HCV antigens were significantly higher in patients with HCV infection (21.3 SFC/10^6 PBMCs; IQR, 9.2-48.0) compared with the controls (8.0 SFC/10^6 PBMCs; IQR, 0.0-10.7; P<0.001). The median numbers of anti-HCV IgM-secreting B SFC to the core (31.1 vs. 4.0 SFC/10^6 PBMCs; P<0.001) and E2 (32.0 vs. 8.0 SFC/10^6 PBMCs; P=0.005) antigens in patients with HCV infection were significantly higher than those in controls. (Fig. 1B).

Relationship between Anti-HCV Antibody Specific-B Cells and HCV Genotypes

Since the antigens utilized were derived from HCV genotype 1a, the numbers of anti-HCV IgG-secreting B cells were compared between 25 patients with HCV genotype 1 infection (ten with 1a, eleven with 1b, and four not subtyped) and 9 infected with another single genotype (one with 2a, four with 2b, two with 2 unsubtyped, and two with 3a). The median value of the anti-HCV IgG-secreting B cells to each antigen was not statistically different between the genotype 1 group and the other genotype groups (Fig. 2). In addition, there were no statistically significant differences in detecting anti-HCV

IgM-secreting B cells to all HCV antigens in those with genotype 1 versus non-1 infections. (data not shown).

Correlation between the anti-HCV IgG-secreting B cells and clinical features in patients with HCV infection

Several demographic (age and sex) and clinical findings (viral load, genotype, ALT, AST, alkaline phosphatase, total bilirubin, albumin, γ-glutamyltransferase, intensity of RIBA assay, and anti-HCV antibodies) were examined for their correlation with anti-HCV IgG-secreting B cell frequency in patients with HCV infection. The circulating anti-HCV IgG-secreting B cell frequency to the core antigen (Fig. 3A) was significantly correlated with the value of ALT (P= 0.048, r = 0.29) and inversely correlated with serum albumin (P=0.048, r = -0.33). Similarly, the number of anti-HCV IgG-secreting B cells to the E2 antigen was significantly correlated with the value of ALT (P=0.037, r = 0.30) (Fig. 3B), AST (P=0.033, r = 0.31) (Fig. 3C), and inversely correlated with serum albumin (P=0.029, r = -0.36). Furthermore, the number of SFCs to the NS3 antigen was significantly correlated with the circulating antibody level to the NS3 in 38 patients with available serum samples (P=0.008, r = 0.43) (Figure 3D). There was no significant correlation between the numbers of anti-HCV IgG-secreting B cells to NS3 or NS5b antigens and any of the biochemical, demographic or clinical parameters specified above.

Comparison of the number of anti-HCV Antibody-secreting B cells between patients with chronic hepatitis C and patients who recovered

As shown in Table 1, patients with chronic hepatitis C had significantly higher mean serum levels of ALT (58 vs. 25 IU/L, P=0.001), AST (48 vs. 24 IU/L, P=0.001), and γ-glutamyltransferase (48 vs. 27 IU/L, P=0.025) compared with the recovered patients. The mean HCV RNA level in the chronic group was 14.1×10^5 IU/mL. There were significant differences in the mean intensity of the RIBA assay against the C33 and C100 proteins in chronic vs. recovered subjects (C33; 3.2 vs. 2.1, P=0.042, C100; 3.7 vs. 2.6, P=0.011). We found no significant difference between patients with chronic hepatitis C

and patients who had recovered when their age, sex, race, source of infection, HCV genotypes, total bilirubin, or albumin were compared.

The median numbers of the sum of anti-HCV IgG-secreting B cells to all HCV antigens were significantly higher in patients with chronic hepatitis C (47.3 SFC/10^6 PBMCs; IQR, 13.3-149.7) than in the recovered patients (15.3 SFC/10^6 PBMCs; IQR, 3.3-142.7; P=0.05) and normal controls (5.3 SFC/10^6 PBMCs; IQR, 2.7-8.0; P<0.001). The median numbers of the sum of anti-IgG-secreting B cells to structural antigens were not significantly higher in patients with chronic hepatitis C (108.3 SFC/10^6 PBMCs) than in those who recovered (97.4 SFC/10^6 PBMCs) (Fig. 4A). In contrast, the median numbers of the sum of anti-HCV IgG-secreting B cells to nonstructural antigens were significantly higher in patients with chronic hepatitis C (19.0 SFC/10^6 PBMCs) than in patients who were recovered (4.9 SFC/10^6 PBMCs; P=0.018), particularly for NS3 antigen (26.7 vs. 5.3 SFC/10^6 PBMCs; P=0.032) (Fig. 4B). Furthermore, patients with chronic hepatitis C had a significantly higher frequency of anti-HCV IgG-secreting B cells to NS3 antigen than those who were recovered (85% vs. 44%, P=0.02, Figure 4C).

The median numbers of the sum of anti-HCV IgM-secreting B cells to all HCV antigens were similar in the patients with chronic hepatitis C (22.0 SFC/10^6 PBMCs; IQR, 8.2-49.3) and recovered subjects (20.7SFC/10^6 PBMCs; IQR, 12.2-36.7) and significantly higher than in the controls (8.0 SFC/10^6 PBMCs; IQR, 0.0-10.7; P<0.001) (Fig. 4A). When the responses were analyzed for structural and nonstructural antigens, the median numbers of the sum of anti-HCV IgM-secreting B cells were not significantly different in patients with chronic hepatitis C and recovered subjects for either structural antigens (30.7 vs. 31.6 SFC /10^6 PBMC) or nonstructural antigens (20.7 vs. 12.7 SFC/10^6 PBMCs) (Fig. 4A).

Discussion

In the present study, we developed an ELISPOT assay for sensitive quantitative assessment of anti-HCV antibody-secreting B cells in PBMCs from patients with HCV infection and used this technique to analyze the induction of humoral immune responses at the single-cell level. IgG and IgM anti-HCV antibody secreting B cells to core, E2, NS3, and NS5 were detected and quantified in patients with chronic HCV infection and

compared to patients who recovered and uninfected controls. The key findings were that: (1) anti-HCV secreting B cell responses were greater in chronically-infected than recovered subjects suggesting that antibody does not play a major role in recovery from acute HCV infection, as also indicated by recently developed pseudotype assays for HCV neutralizing antibodies [24, 25]; (2) the primary difference between chronically-infected and recovered subjects was in the greater reactivity of the former to non-structural antigens; (3) in chronic infection, HCV antibodies were cross-reactive against genotypes, again consistent with recent findings by neutralizing antibody assays [26, 27] ; (4) the ELISPOT assay can measure IgM as well as IgG responses at the single cell level providing a new means to measure the more elusive IgM response; (5) IgM responses were surprisingly well maintained during chronic infection; (6) IgG responses correlated positively with serum transaminase levels.

In this study, the B-cell ELISPOT assay showed high specificity (91% to 100%) and sensitivity (58% to 92%) to all HCV antigens by analysis of the ROC curves and thus achieved high diagnostic accuracy. Although there was a general problem that raw numbers of SFC were low, statistical analysis and prior publications [28, 29] suggest that these small differences are consistent and relevant. Of note, individuals infected with non-genotype1 variants were strongly positive in this assay which utilized only genotype1-derived antigens. This suggests that genotype 1 contains conserved epitopes that will allow the ELISPOT assay to assess humoral immune responses to HCV irrespective of genotype (Fig. 2), with the caveat that we did not assess genotypes 4, 5 and 6 that are rare in the US.

ELISPOT assays have been used as sensitive and specific tools to measure B cell responses in autoimmune diseases [28, 29] and viral infections including cytomegalovirus [15], rotavirus [16], measles virus [17], and hepatitis B virus, [18, 19] as well as to evaluate responses to bacterial [30] and viral vaccines. [18, 19] Other reports demonstrate that ELISPOT is able to detect and numerate antigen-specific memory B cells in PBMCs after *in vitro* stimulation in both autoimmune diseases and viral infection. [31, 32] Thus, the B-cell ELISPOT assay might be a useful tool to detect anti-HCV-specific memory B cells, and to monitor the efficacy of future HCV vaccines.

Interestingly, this study showed a strong correlation between the numbers of anti-HCV IgG-secreting B cells to the core and E2 antigens and the values of serum transaminases. The clinical significance of these observations is unknown, but raises the possibility that antibody can contribute to liver cell injury. In addition, Ni et al. [33] recently reported that 10 of 36 hepatitis C patient samples showed increased B cell frequencies that correlated with the degree of hepatic fibrosis. There are insufficient histologic data in our study to assess whether the numbers of anti-HCV antibody-secreting B cells correlate with the degree of fibrosis as well as biochemical evidence of inflammation.

The median numbers of the sum of anti-HCV IgG-secreting B cells to nonstructural antigens were significantly higher in patients with chronic hepatitis C than in the recovered patients. Similarly, an HCV-specific B cell response was more frequently detected in patients with chronic hepatitis C than in recovered subjects (92% vs. 56%, P=0.017) and was directed against a broader range of HCV antigens, particularly to NS3. In contrast, CD4 T-cell responses to NS3 epitopes are greatest in patients who recover from HCV infection. [34, 35]

We have also developed and evaluated the ELISPOT assay for detecting anti-HCV IgM-secreting B cells. It has been reported that IgM anti-HCV in serum might be predictive of viral clearance in acute hepatitis C or response to interferon therapy. [36-40] However, these results have been controversial and other studies have shown a significant correlation between IgM anti-HCV levels in serum and the recurrence of hepatitis C post-liver transplantation. [41, 42] In this study, we found that IgM secreting B cells persisted during chronic infection so that the usefulness of IgM detection for assessing acute versus chronic HCV infection would have to depend on quantitative differences in IgM level rather than the simple presence or absence of IgM antibody. The fact that there are no standardized assays for measuring IgM anti-HCV in serum and the ready detection of IgM secreting B cells in this study suggests that the ELISPOT assay could be used to better define the clinical relevance of IgM antibody in acute and chronic HCV infection.

Overall, this study, as do studies of HCV-specific neutralizing antibodies, [26, 27] suggest that the humoral arm of the HCV immune response is not a critical element of spontaneous viral clearance. However, because of the difficulty in obtaining serial

acute-phase PBMC collections from recovering subjects, our study does not exclude a role for antibody mediated viral clearance early in HCV infection. Sequential acute phase ELISPOT IgM testing of PBMC is planned in forthcoming chimpanzee infectivity studies. Nonetheless, studies of neutralizing and anti-envelope antibodies [26, 27], that measured serial acute phase serum samples from recovering subjects, did not show that such antibodies correlated with viral clearance. Rather, it appears in those studies and the current study that antibodies to HCV increase in strength and broadness of reactivity during the course of chronic infection, presumably because of persistent antigenic stimulation. This is in contrast to cell-mediated immunity that is markedly diminished in chronically infected as compared to recovered subjects. This dichotomy between the humoral and cellular immune response to HCV is intriguing and suggests T-cell tolerance in the absence of B-cell tolerance.

It is interesting to speculate on the role that antibody might play in HCV infection. First, it seems reasonable that such antibodies complexed to virus would reduce the level of free virus and diminish transmission to others. This reduction in free virus in addition to lowered viral load might explain the relative rarity of sexual and perinatal transmission during chronic HCV infection. More intriguing is whether such antibodies establish the set point for viral load during chronic infection. It is known that viral loads are highest early in HCV infection prior to the appearance of antibody [43] and that chronically infected patients establish a lower and relatively constant level of viremia. [44] It appears that production and elimination of virus achieve a steady state. This steady state is probably multifactorial in origin, but antibody may play a key role. When patients in a steady state are immunosuppressed at the time of transplantation [45] or when coinfected with human immunodeficiency virus [46], the viral load increases, supporting an immunologic role for viral containment even in the absence of clearance. A deleterious function of anti-HCV is that it serves to drive quasispecies evolution making it increasingly hard for the immune system to achieve viral clearance. Farci et al. have shown in both humans and chimpanzees [47] , that the appearance of antibody coincides with increasing viral diversity and complexity and predicts progression to chronic infection.

There is much to explore regarding the function and relevance of IgG and IgM antibodies in HCV infection and we believe the ELISPOT assay, by measuring antibody production at the single cell level, provides a new and useful tool for understanding the complex interplay between HCV and the host immune response.

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Table 1. Demographic and Clinical Characteristics of Patients with HCV infection

Characteristics	All (n=48)	Chronic (n=39)	Recovered (n=9)	*P* Value
Mean Age, *yr* (range)	51 (33-83)	52 (37-83)	49 (33-78)	0.46
Male, n (%)	23 (48)	17 (44)	6 (67)	0.28
Race, n (%)				
White	43 (90)	35 (90)	8 (89)	1.00
Black	5 (10)	4 (10)	1 (11)	
Source of infection, n (%)				
Transfusion	16 (33)	14 (36)	2 (22)	0.30
Injection drug use	19 (40)	15 (38)	4 (44)	
Nasal cocaine use	4 (8)	2 (5)	2 (22)	
Occupational	6 (13)	6 (15)	0 (0)	
Unknown	3 (6)	2 (5)	1 (11)	
Genotype, n (%)				
1	25 (52)	24 (62)	1 (11)	0.074
2	7 (15)	6 (15)	1 (11)	
3	2 (4)	1 (3)	1 (11)	
Unknown	14 (29)	8 (21)	6 (67)	
Mean values (range)				
ALT (IU/L)	52 (15-251)	58 (28-251)	25 (15-52)	0.001
AST (IU/L)	43 (12-145)	48 (12-145)	24 (13-37)	0.001
ALP (IU/L)	69 (32-171)	71 (35-171)	59 (32-74)	0.20

Total bilirubin (mg/dL)	0.7 (0.3-1.5)	0.7 (0.3-1.5)	0.7 (0.4-1.4)	0.72
Albumin (g/dL)	3.9 (3.3-4.5)	3.9 (3.3-4.5)	4.0 (3.6-4.3)	0.53
GGTP (g/dL)	44 (8-286)	48 (8-286)	27 (8-102)	0.025
HCV RNA level (10^5 IU/mL)	11.2 (<0.5 -73)	14.1 (<0.5-73)	<0.5 (<0.5)	<0.001
RIBA assay				
C100	3.0 (0-4)	3.2 (0-4)	2.1 (0-4)	0.042
C33	3.5 (1-4)	3.7 (1-4)	2.6 (1-4)	0.011
C22	3.8 (0-4)	3.9 (1-4)	3.1 (0-4)	0.068
NS5	2.1 (0-4)	2.3 (0-4)	1.3 (0-4)	0.18

ALP, alkaline phosphatase; GGTP, γ-glutamyltransferase.

Table 2A. Optimal Cutoff Values, Sensitivity, Specificity, AUC, and Predictive Values of Anti-HCV IgG-Secreting B Cells in ELISPOT Assay in 48 Patients with Chronic Hepatitis C and 11 Volunteer Blood Donors

Antigen	Cutoff value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
Core	13.4	92 (80-98)	100 (71-100)	0.94 (0.84-0.98)	100	73
E2	10.7	92 (80-98)	100 (71-100)	0.94 (0.85-0.99)	100	73
NS3	5.4	77 (63-88)	100 (71-100)	0.83 (0.71-0.92)	100	50
NS5B	5.4	58 (43-72)	100 (71-100)	0.71 (0.58-0.82)	100	36

NOTE: All the AUC were significantly higher than a 0.50 nonpredictive value (P<0.001 for all comparisons).

Abbreviations: AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

Cutoff values were determined by making ROC curves and are expressed as spot forming cells/10^6 PBMCs.

Table 2B. Optimal Cutoff Values, Sensitivity, Specificity, AUC, and Predictive Values of Anti-HCV IgM-Secreting B Cells in ELISPOT Assay in 43 Patients with Chronic Hepatitis C and 6 Volunteer Blood Donors

Antigen	Cutoff value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
Core	12.1	84 (69-93)	100 (54-100)	0.94 (0.84-0.99)	100	46
E2	17.4	72 (56-85)	100 (54-100)	0.86 (0.73-0.94)	100	33
NS3	10.7	70 (54-83)	100 (54-100)	0.74 (0.60-0.86)	100	32
NS5B	8.1	54 (38-69)	100 (54-100)	0.73 (0.58-0.85)	100	23

NOTE: All the AUC were significantly higher than a 0.500 nonpredictive value (P<0.001 for all comparisons).

Abbreviations: AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

Cutoff values were determined by making ROC curves and are expressed as spot forming cells/10^6 PBMCs.

Figure Legends

Figure 1. Detection of anti-HCV antibody-secreting B cells in patients with HCV infection and volunteer blood donors.

The box represents the interquartile range of the data. The line across the box indicates the median value. The whiskers above and below the box indicate the 90th and 10th percentiles for each group. (A) The frequencies of anti-HCV IgG-secreting B cells to 4 HCV antigens were detected in 48 patients with HCV infection and in 11 volunteer blood donors. (B) The frequencies of anti-HCV IgM-secreting B cells were detected in 43 patients with HCV infection and in 6 volunteer blood donors.

Blood donors, BDs.

Figure 2. Detection of anti-HCV IgG-secreting B cells in patients infected with HCV of genotype 1 and non-genotype 1.

The box represents the interquartile range of the data. The line across the box indicates the median value. The whiskers above and below the box indicate the 90th and 10th percentiles for each group. The frequencies of anti-HCV secreting B cells were detected in patients infected with genotype 1 (n=25) and in those with other genotypes (n=9).

Non-genotype 1, Non-1.

Figure 3. Correlation of the number of anti-HCV IgG-secreting B cells and clinical characteristics in 48 patients with HCV infection.

Frequency of circulating anti-HCV IgG-secreting B cells to core antigen was significantly correlated with the value of ALT (A; r=0.29, P=0.048). Frequency of circulating anti-HCV IgG-secreting to E2 antigen was correlated with the value of ALT (B; r=0.30, P=0.037) and AST (C; r=0.31, P=0.033), respectively. Frequency of circulating anti-IgG-secreting B cells to NS3 antigen was correlated with the value of anti-HCV antibodies to NS3 antigen (D; r=0.43, P=0.0085).

Figure 4. Detection of anti-HCV antibody-secreting B cells in patients with chronic hepatitis C and in patients who had recovered from HCV.

(A) Circulating anti-HCV IgG-secreting B cells were detected in 39 patients with chronic hepatitis C, 9 patients who had recovered from HCV infection, and 11 volunteer blood donors. Circulating anti-HCV IgM-secreting B cells were detected in 34 patients with chronic hepatitis C, 9 patinets who had recovered from HCV infection, and 6 volunteer blood donors. (B) Frequency of circulating anti-HCV IgG-secreting B cells to 4 HCV antigens were detected in 39 patients with chronic hepatitis C and in 9 recovered patients. (C) The prevalence of anti-HCV IgG-secreting B cells in 39 patients with chronic hepatitis C and 9 recovered patients.

CH-C, chronic hepatitis C; Rec, recovered; BDs, blood donors.

Figure 1A

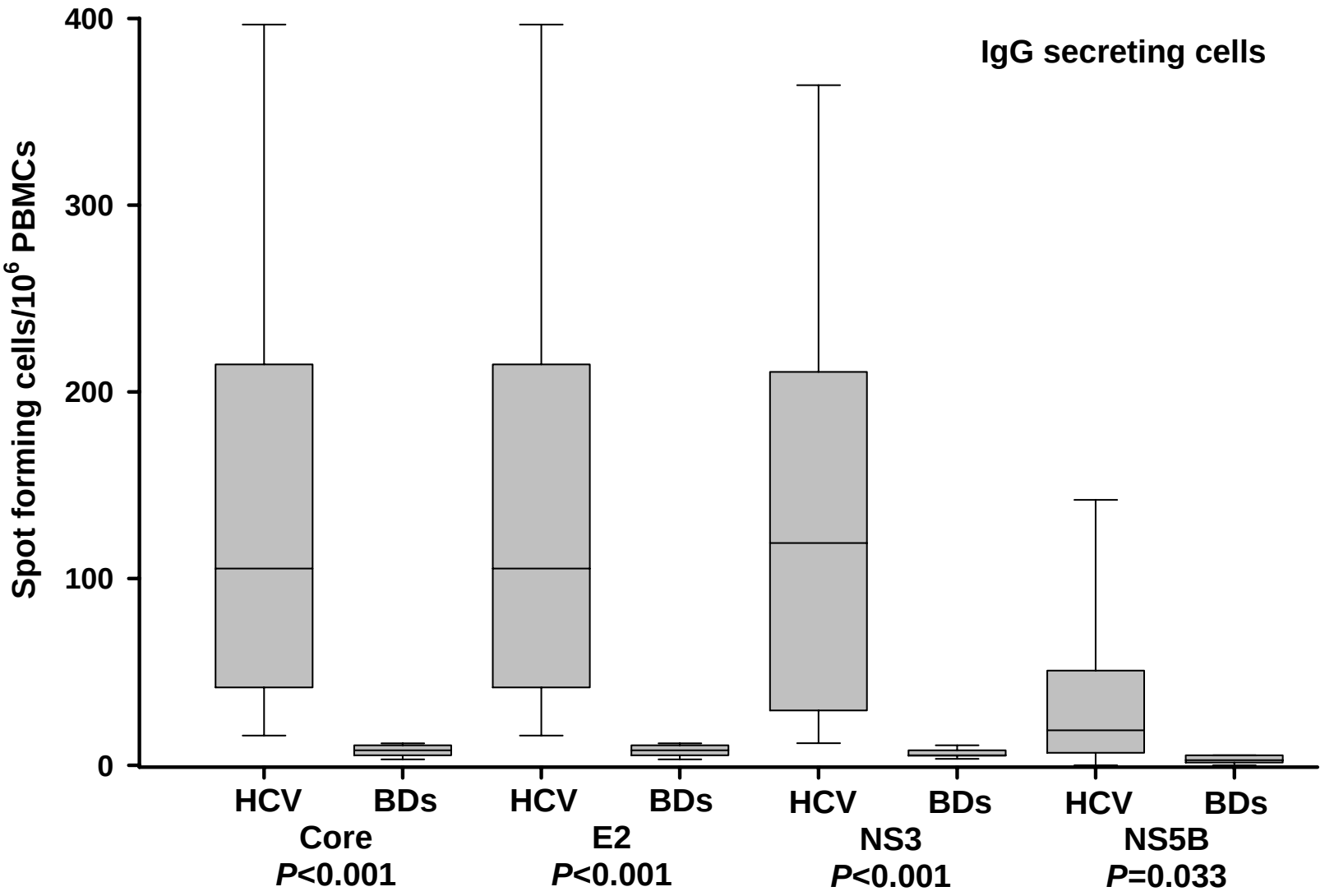
IgG secreting cells
Spot forming cells/10^6 PBMCs
400
300
200
100
0
HCV
BDs
HCV
BDs
HCV
BDs
HCV
BDs
Core
P<0.001
E2
P<0.001
NS3
P<0.001
NS5B
P=0.033

Figure 1B

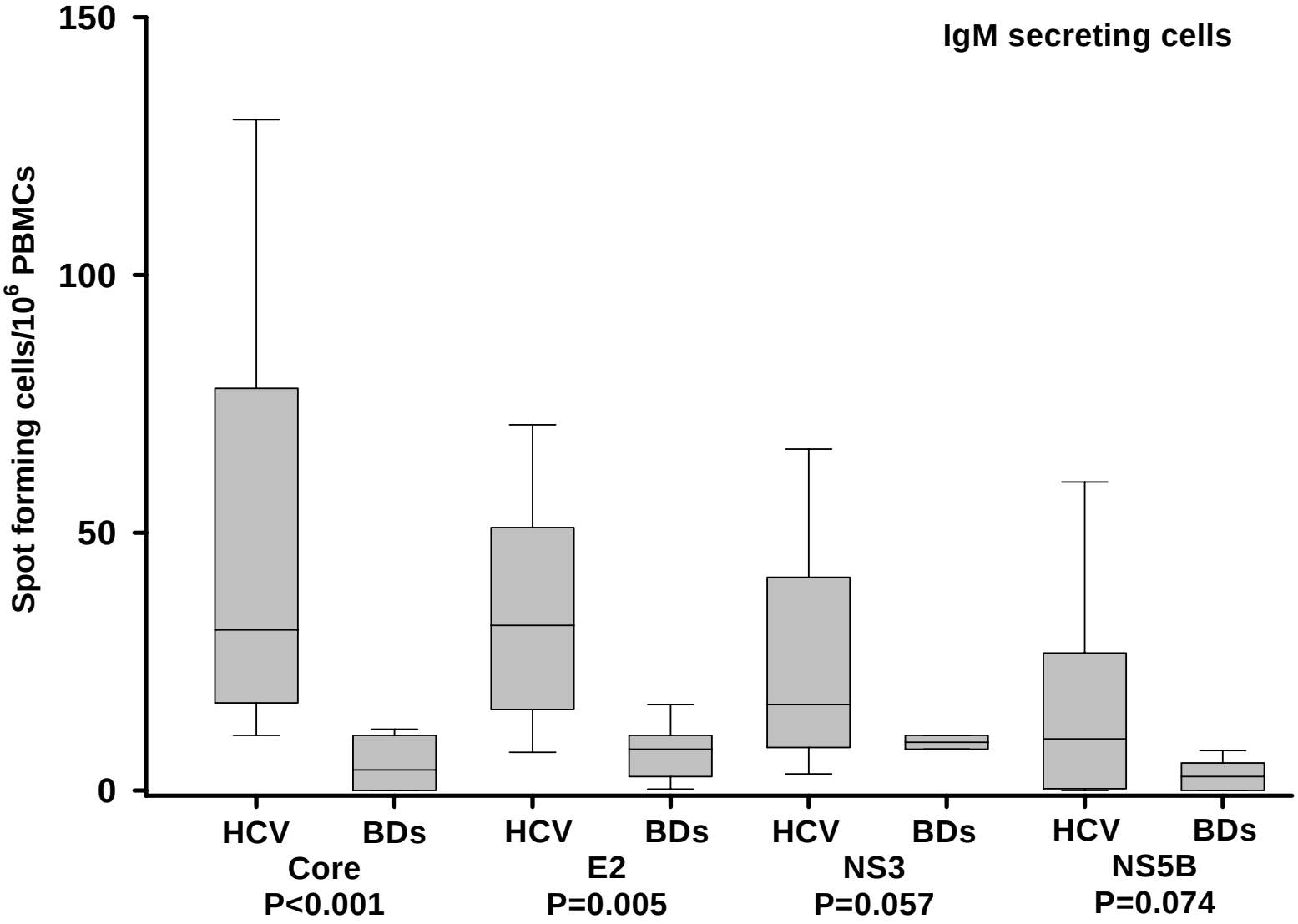
IgM secreting cells
Spot forming cells/10^6 PBMCs
150
100
50
0
HCV
BDs
HCV
BDs
HCV
BDs
HCV
BDs
Core
P<0.001
E2
P=0.005
NS3
P=0.057
NS5B
P=0.074

Figure 2

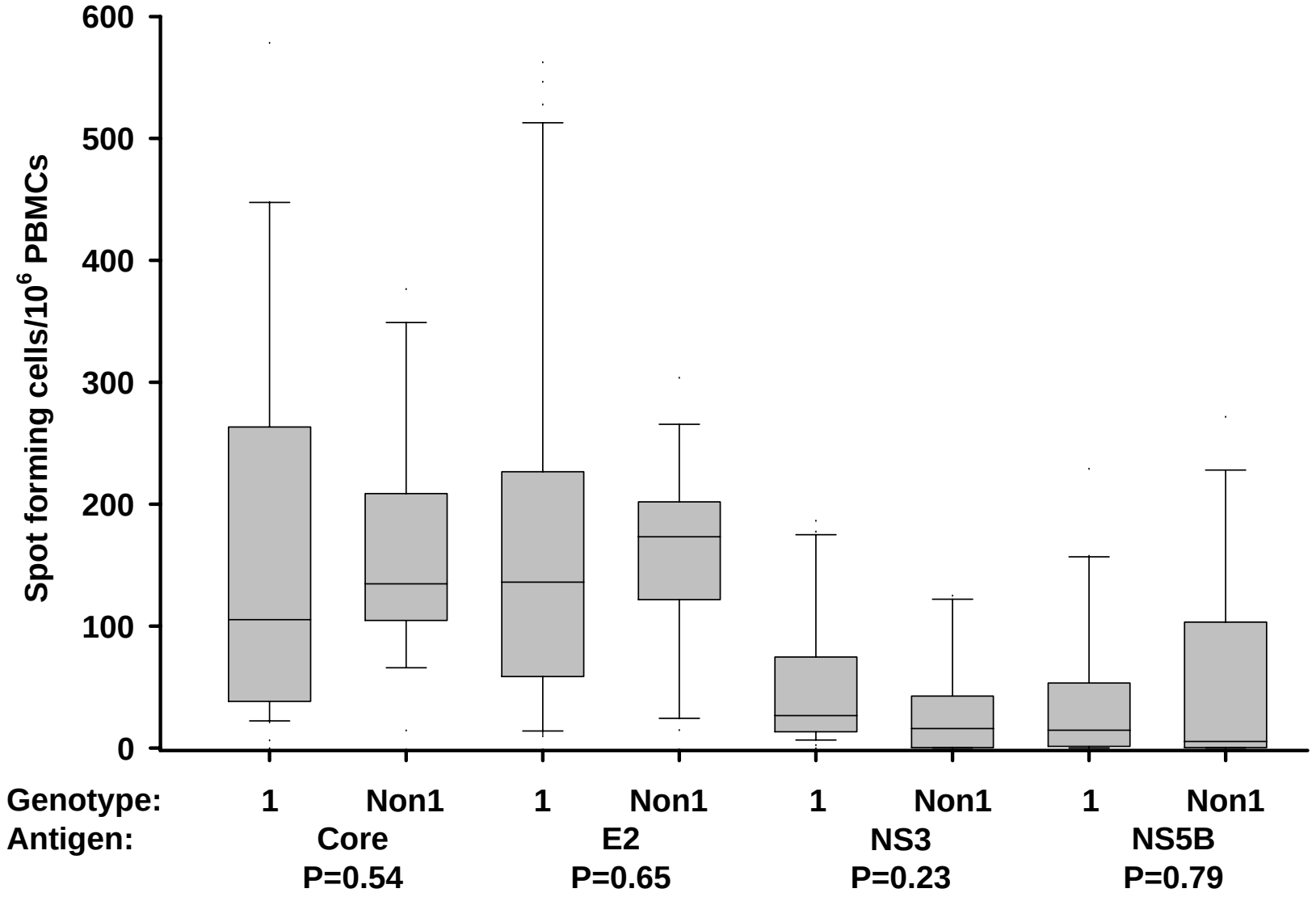
600
500
400
300
200
100
0
Spot forming cells/10^6 PBMCs
Genotype: 1 Non1 1 Non1 1 Non1 1 Non1
Antigen: Core E2 NS3 NS5B
P=0.54 P=0.65 P=0.23 P=0.79

Figure 3A

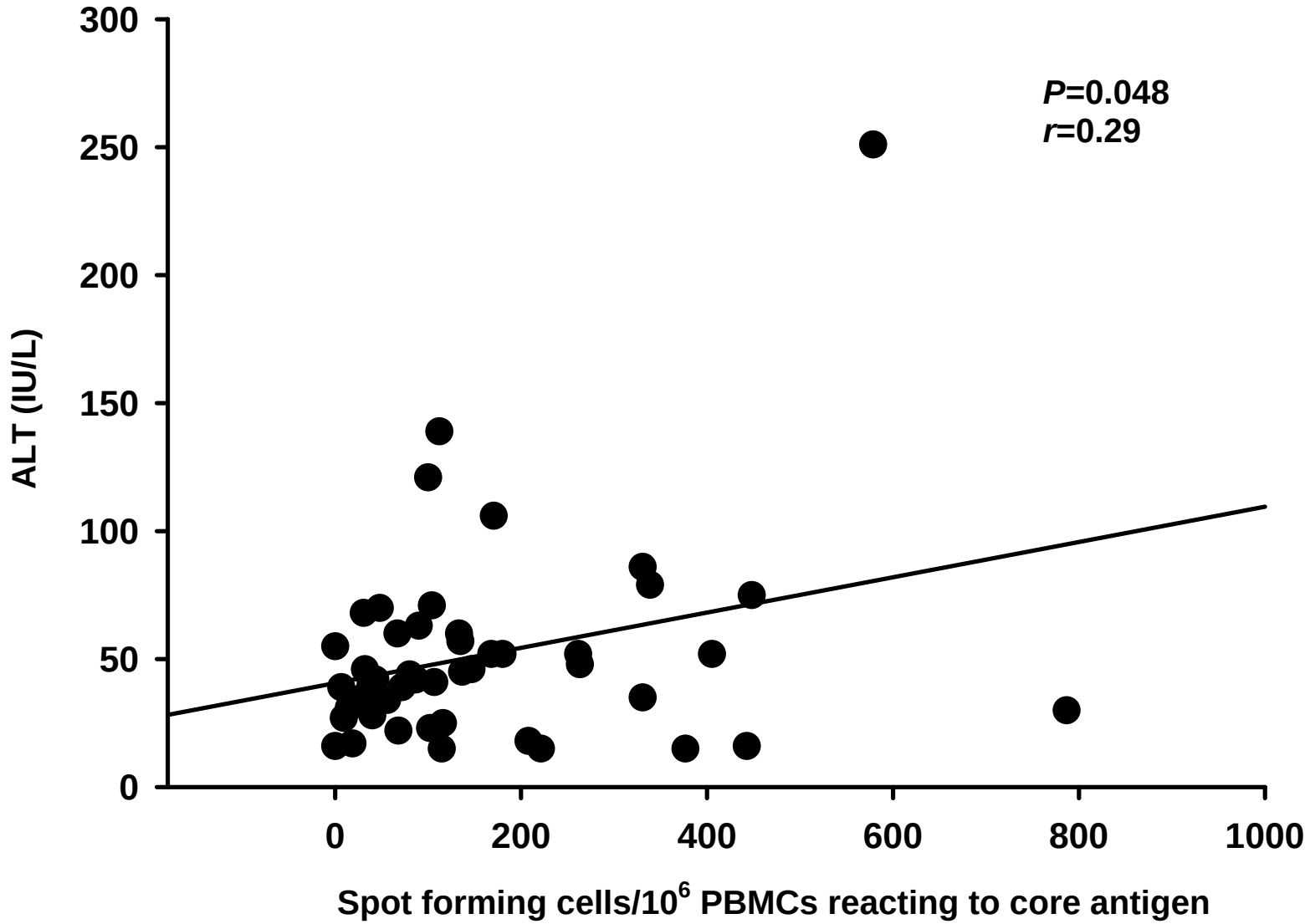
P=0.048
r=0.29
ALT (IU/L)
300
250
200
150
100
50
0
0
200
400
600
800
1000
Spot forming cells/10^6 PBMCs reacting to core antigen

Figure 3B

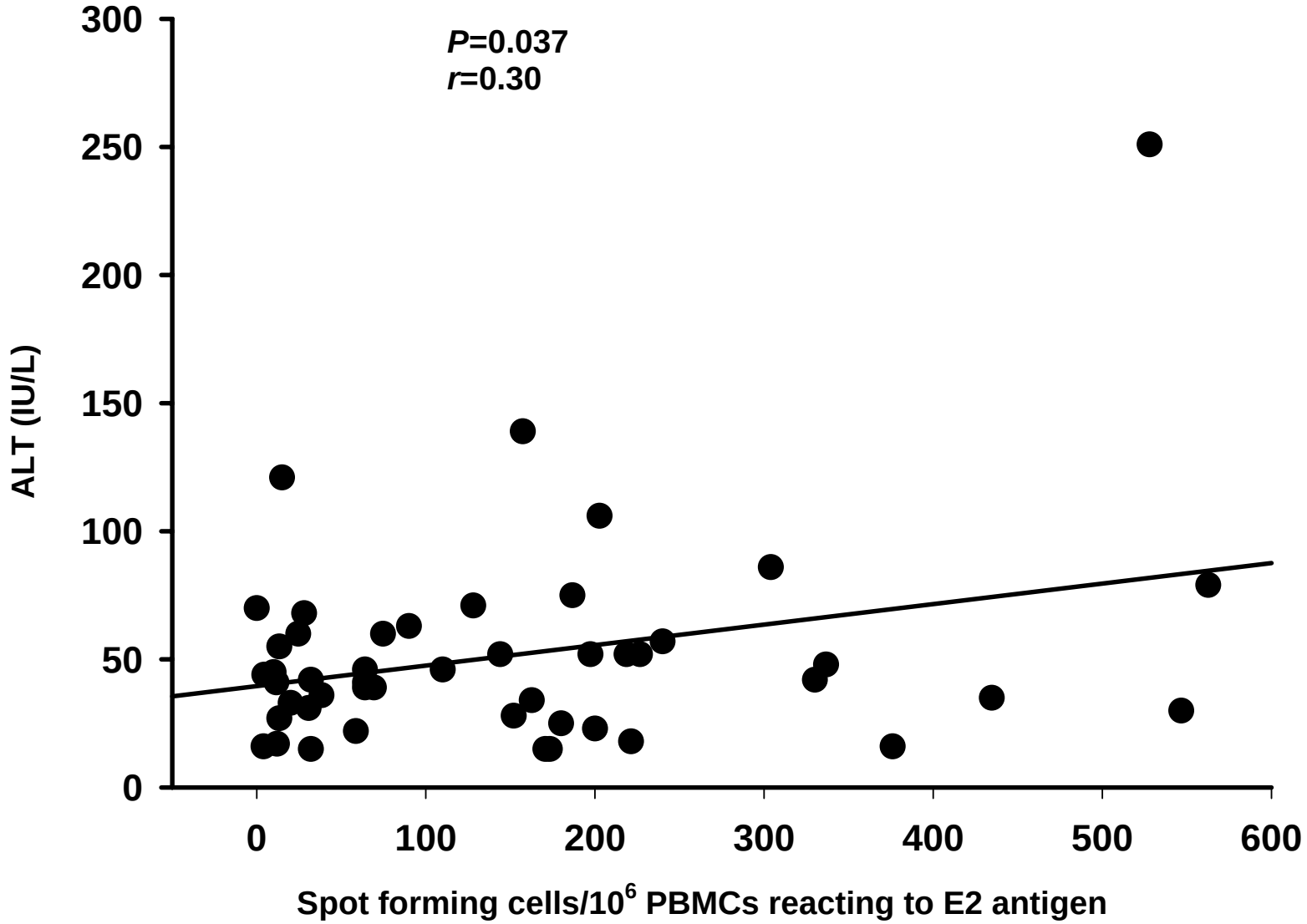
P=0.037
r=0.30
ALT (IU/L)
300
250
200
150
100
50
0
0
100
200
300
400
500
600
Spot forming cells/10^6 PBMCs reacting to E2 antigen

Figure 3C

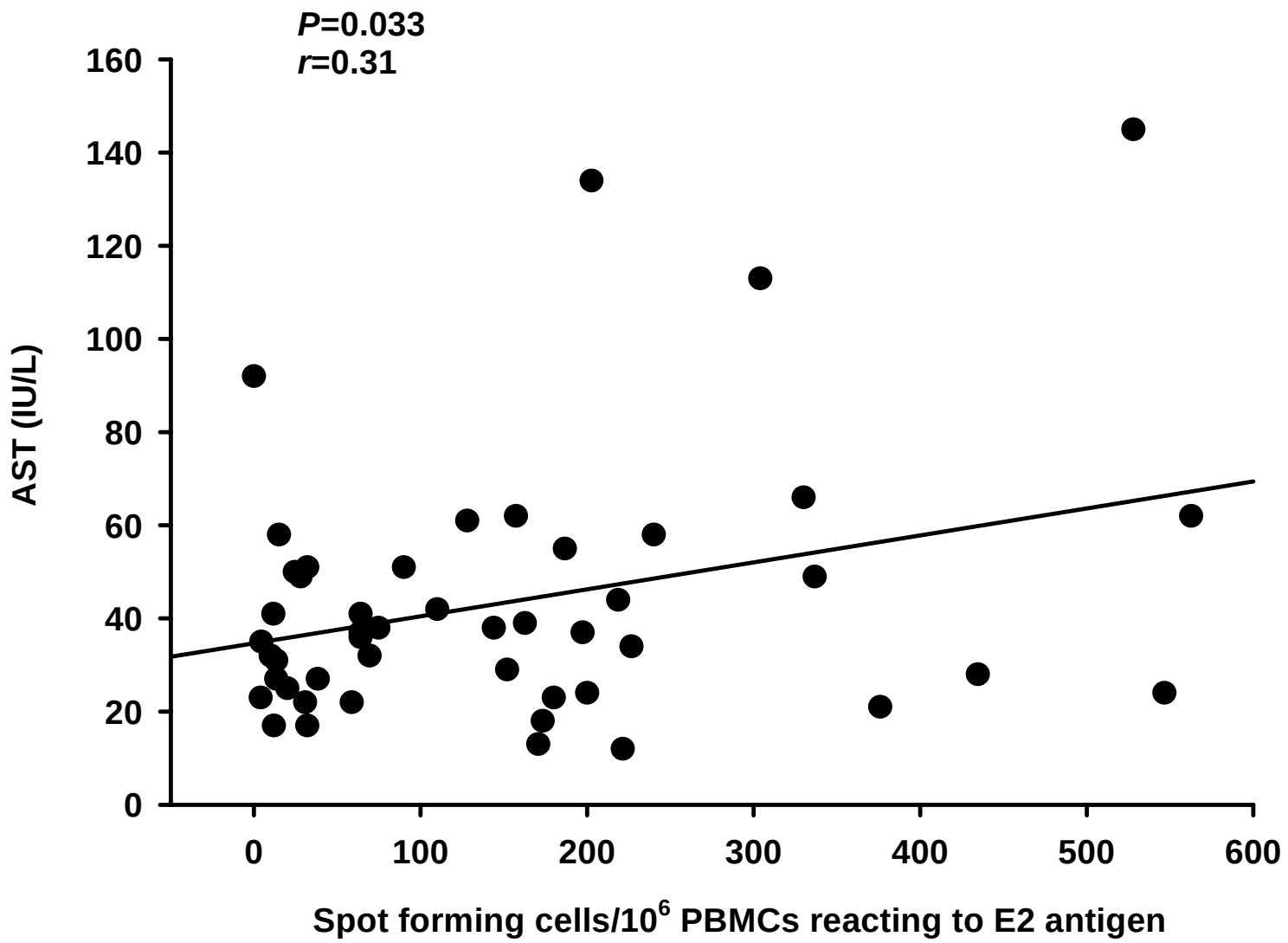
P=0.033
r=0.31
AST (IU/L)
160
140
120
100
80
60
40
20
0
0
100
200
300
400
500
600
Spot forming cells/10^6 PBMCs reacting to E2 antigen

Figure 3D

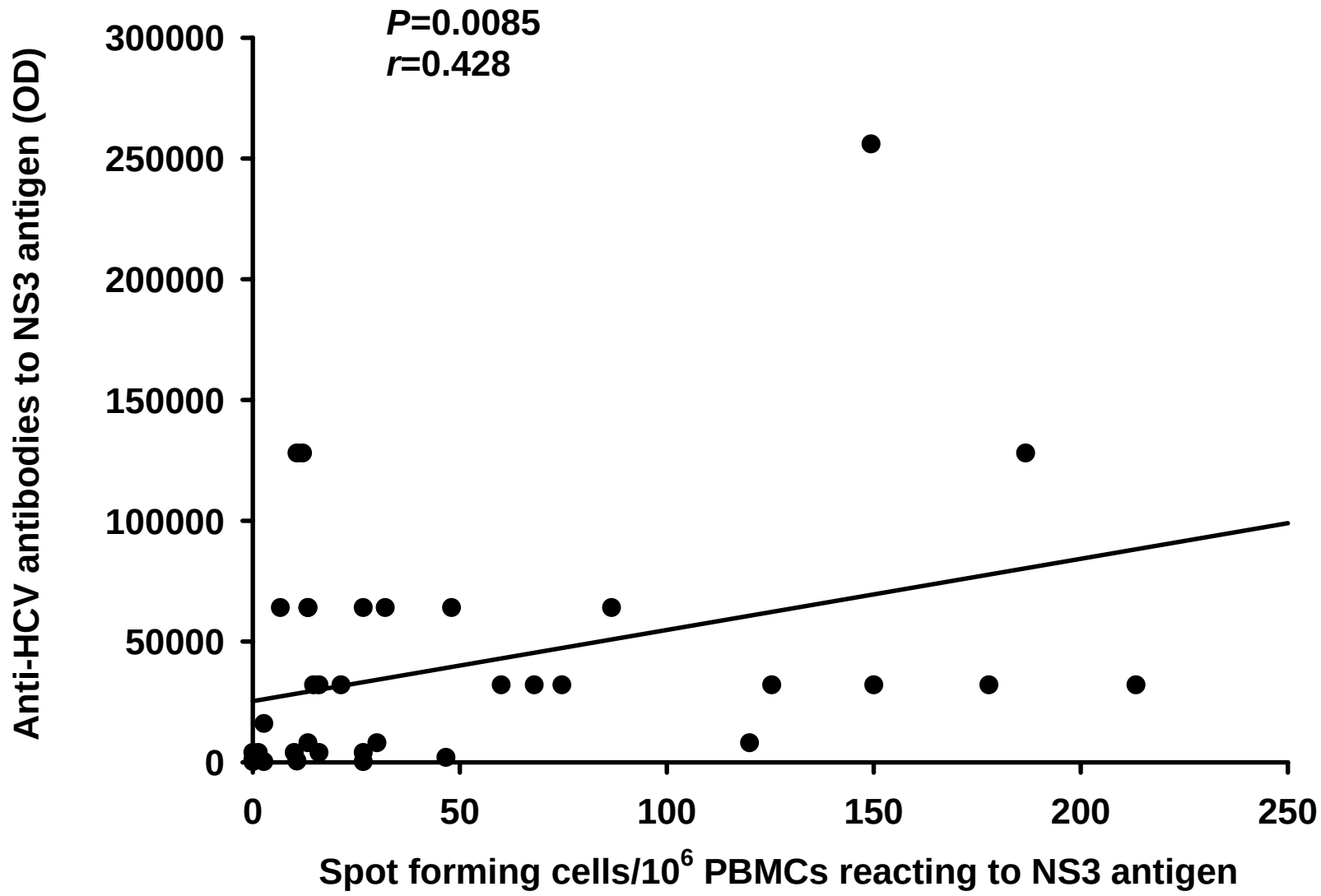
P=0.0085
r=0.428
Anti-HCV antibodies to NS3 antigen (OD)
300000
250000
200000
150000
100000
50000
0
0
50
100
150
200
250
Spot forming cells/10^6 PBMCs reacting to NS3 antigen

Figure 4A

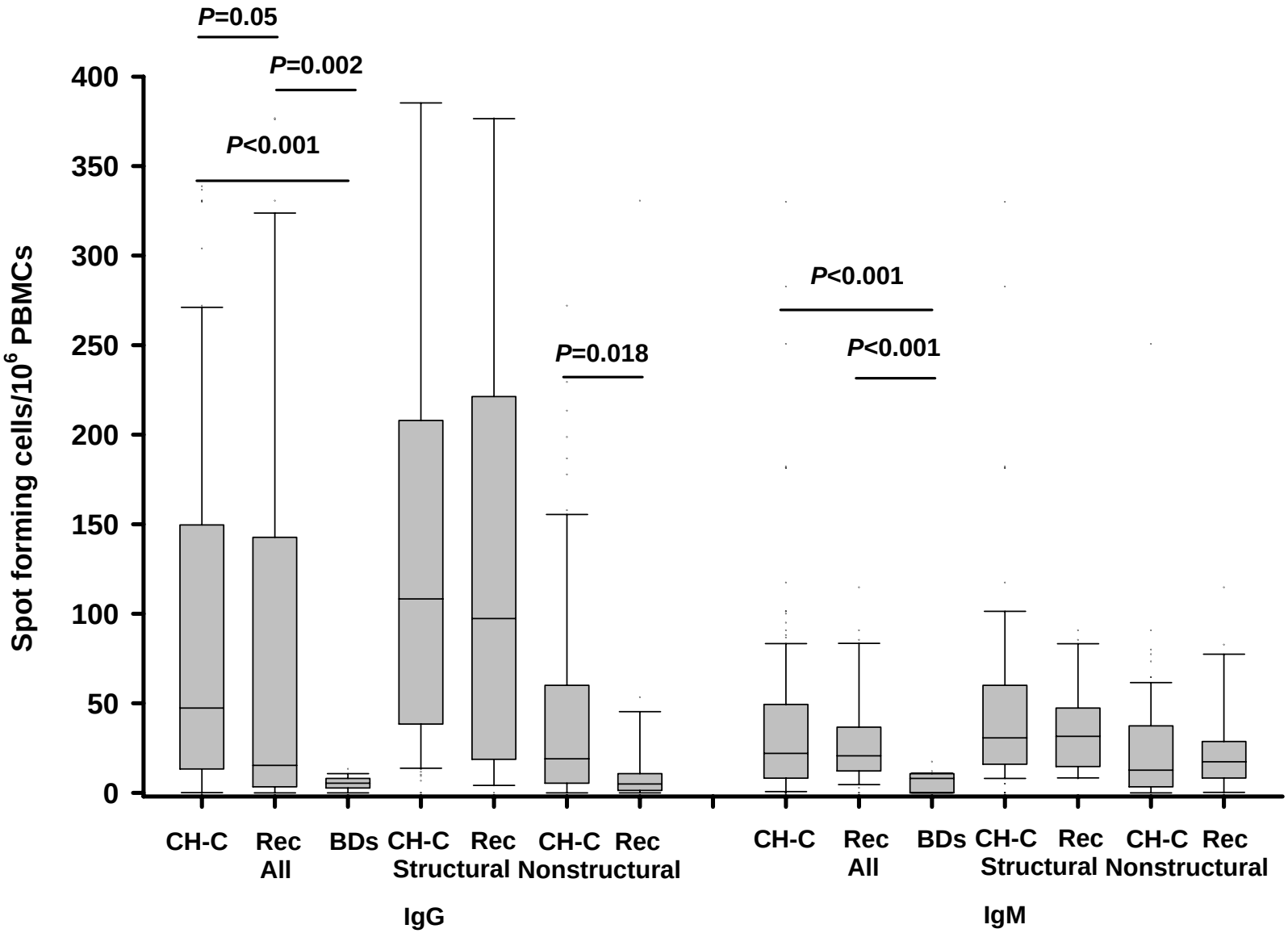
P=0.05
P=0.002
P<0.001
P=0.018
P<0.001
P<0.001
Spot forming cells/10^6 PBMCs
400
350
300
250
200
150
100
50
0
CH-C
Rec All
BDs
CH-C Structural
Rec
CH-C Nonstructural
Rec
IgG
CH-C
Rec All
BDs
CH-C Structural
Rec
CH-C Nonstructural
Rec
IgM

Figure 4B

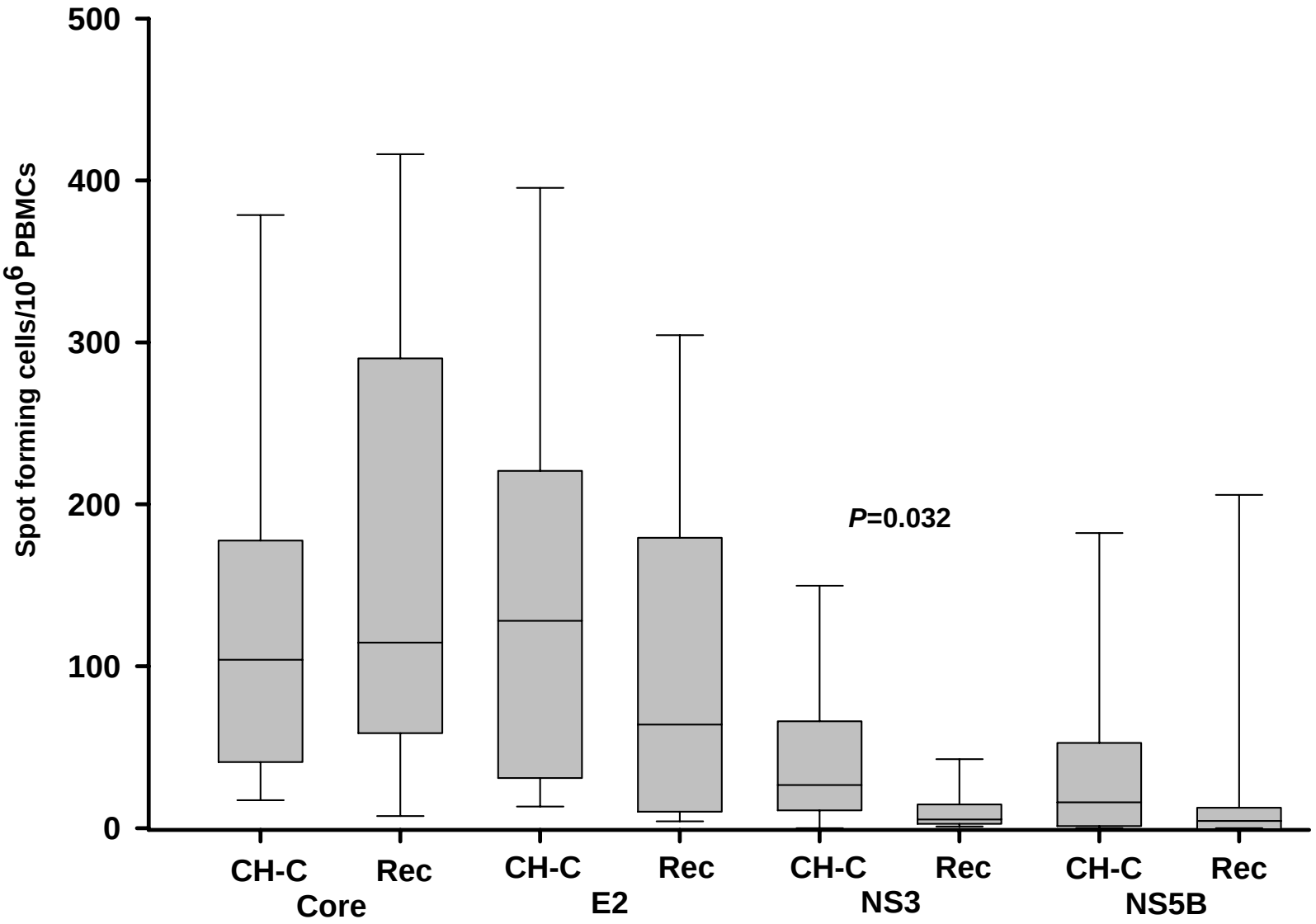
500
400
300
200
100
0
Spot forming cells/10^6 PBMCs
P=0.032
CH-C
Rec
CH-C
Rec
CH-C
Rec
CH-C
Rec
Core
E2
NS3
NS5B

Figure 4C