Activation of naïve B lymphocytes via CD81, a pathogenetic mechanism for hepatitis C virus-associated B lymphocyte disorders

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Communicated by Rino Rappuoli, Chiron Corporation, Siena, Italy, October 28, 2005 (received for review September 30, 2005)

Infection with hepatitis C virus (HCV), a leading cause of chronic liver diseases, can associate with B lymphocyte proliferative disorders, such as mixed cryoglobulinemia and non-Hodgkin lymphoma. The major envelope protein of HCV (HCV-E2) binds, with high affinity CD81, a tetraspanin expressed on several cell types. Here, we show that engagement of CD81 on human B cells by a combination of HCV-E2 and an anti-CD81 mAb triggers the JNK pathway and leads to the preferential proliferation of the naïve (CD27–) B cell subset. In parallel, we have found that B lymphocytes from the great majority of chronic hepatitis C patients are activated and that naïve cells display a higher level of activation markers than memory (CD27+) B lymphocytes. Moreover, eradication of HCV infection by IFN therapy is associated with normalization of the activation-markers expression. We propose that CD81-mediated activation of B cells in vitro recapitulates the effects of HCV binding to B cell CD81 in vivo and that polyclonal proliferation of naïve B lymphocytes is a key initiating factor for the development of the HCV-associated B lymphocyte disorders.

**Materials and Methods**

**Patients.** We analyzed peripheral blood B lymphocytes from 64 patients (55% males and 45% females; median age of 62.5 years) with chronic HCV infection, as demonstrated by the presence of HCV-RNA and elevated serum levels (>40 units/liter) of alanine aminotransferase. Most of the patients (61%) were infected with HCV genotype 1; the remainder (39%) were infected by genotypes 2 and 3. A fraction of these patients (26 of 64) were diagnosed with cryoglobulinemia, as indicated by the presence of dosable levels of cryoglobulins (>1.0%) accompanied by weakness, arthralgias, and purpura (see Table 3, which is published as supporting information on the PNAS web site). Patients were therapy-naïve or had been off antiviral therapy for >12 months at the time of the sampling. None of them had clinical evidence of cirrhosis.

Twenty-two of 64 subjects were selected among therapy-naïve patients and underwent antiviral therapy. Patients received 1.5 μg of peginterferon α-2b (PEG-Intron, Shering–Plough) per kg of body weight s.c. once weekly and oral ribavirin (Rebetol, Shering–Plough) at 1,000–1,200 mg/day (those weighing >75 kg received the higher dose). Treatment lasted 6 months for patients with genotypes 2 and 3 (13 of 22) and 12 months for patients with genotype 1 (9 of 22). Analyses were performed before and at the end of therapy. In responders to therapy, as indicated by HCV-RNA negativity at PCR, an additional analysis after 12 months of follow-up was performed to identify patients who achieved sustained virological response. The main biochemical and virological characteristics of HCV-infected patients at the moment of enrollment are summarized in Table 3. Twenty-one healthy blood donors...
and 16 patients with chronic hepatitis B, matched for age and sex, were used as control groups. Study protocols were approved by the ethical committee of Trieste University School of Medicine. All subjects provided written informed consent.

**Clinical Tests.** Alanine aminotransferase was measured by using standard clinical tests. HCV RNA was determined by using RT-PCR by Amplicor HCV assay (Roche Diagnostics) and quantified with a REAL QUANT C kit (Genedia, Munich). HCV genotypes were determined by using the line-probe assay (Inno-Lipa HCV, Innogenetics, Ghent, Belgium). To evaluate the presence of cryoglobulins, blood samples were allowed to clot at 37°C; serum was centrifuged for 10 min at 350 × g at 4°C in graduated Wintrobe tubes, and the volume percent of the precipitate compared with the total volume of serum (cryocrit) was calculated. Rheumatoid factor titers were measured by using the PRP latex agglutination test (BRL and 10% FCS). A total volume of serum (cryocrit) was calculated. Rheumatoid factor (PRP latex agglutination test) (BRL and 10% FCS).

**Cell Preparation and Purification.** Peripherical blood mononuclear cells (PBMCs) were obtained from the blood of patients and healthy volunteers by Ficoll-Hypaque (Amersham Pharmacia Biotech) gradient centrifugation. B lymphocytes were negatively purified from PBMCs of healthy donors. Briefly, the cells were first stained with purified mAbs specific for CD2 (clone RPA-2.10), CD14 (clone M5E2), CD16 (clone 3G8), and CD56 (clone B159) (BD Biosciences) at 4°C for 30 min; after washing, the cells were incubated with magnetic beads coated with goat anti-mouse IgG antibodies (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of selected B lymphocytes was >98%, as confirmed by FACS analysis using peridinin-chlorophyll–protein complex (PerCP)-anti-CD19 mAb (clone 4G7, BD Biosciences). Purified B lymphocytes were stained with phycoerythrin (PE)-anti-CD27 mAb (clone L-128, BD Biosciences), and naïve (CD27−) and memory (CD27+) subsets were sorted by FACSVantage SE (Becton Dickinson) with >96% purity. Human tonsils were obtained from patients undergoing routine tonsillectomy, and B lymphocytes were negatively isolated by using magnetic beads coated with an anti-CD2 mAb (Dynabeads, Oxoid, Basingstoke, U.K.). More than 97% of the isolated cells expressed CD19.

**Generation of mAbs Specific for Human CD81-LEL.** Monoclonal antibodies (MG81 and N81, IgG1 isotype) were obtained in our laboratory from BALB/c mice immunized with a recombinant human CD81-LEL fused with thioredoxin (10) by using standard technique. Hybridoma supernatants were screened for the ability to bind human CD81 and to neutralize the binding of HCV-E2 protein to CD81 on CD81-transfected NIH 3T3 cell line (American Type Culture Collection). The F(ab′)2 fragments were prepared by papain digestion by using the ImmunoPure F(ab′)2 kit (Pierce) according to the manufacturer's instructions. The retention of the ability of the F(ab′)2 fragment of N81 and MG81 mAbs to bind CD81 on the cell surface was confirmed by flow cytometry.

**Cell Cultures and Reagents.** All cultures were performed in RPMI medium 1640 (GIBCO/BRL) supplemented with vitamin, streptomycin, and glutamine, all from GIBCO/BRL and 10% FCS (HyClone) and incubated at 37°C in a humidified atmosphere containing 5% CO2. For the immunoglobulin-secretion experiment, complete medium containing 5% of ultralow IgG FCS (GIBCO/BRL) was used.

Cells were stimulated in vitro with the following reagents: anti-CD81 mAbs, N81 and MG81; F(ab′)2 fragments of N81 and MG81; recombinant purified HCV-E2 (56% pure) (18); anti-CD19 mAb (clone HIB19, BD Biosciences); anti-CD21 (clone B14y, BD Biosciences); recombinant human CD81-LEL (purity >90%) (10); formalinized Staphylococcus aureus Cowan I (SAC) (Pansorbin, Calbiochem); goat anti-human IgM F(ab′)2 fragments specific for μ-chains (Cappel ICN Biomedical).

**B Cell Proliferation Assay.** Triplicates of purified B lymphocytes were seeded at 2 × 105 cells per well in a 96-well flat-bottom plate (Costar) and stimulated with the following reagents: anti-CD81 mAbs (MG81 and N81), HCV-E2 protein, anti-CD19, anti-CD21, SAC, and anti-IgM. For inhibition experiments, MG81, HCV-E2, SAC, and anti-IgM were preincubated with recombinant CD81-LEL for 10 min at 37°C and added to the cells. After 4 days of culture, 1 μCi (1 Ci = 37 GBq) per well of [3H]thymidine was added, and cells were incubated for an additional 16 h and harvested onto filter plates (Packard). [3H]thymidine incorporation was measured by using a TopCount NXT β-counter (Packard).

**Flow Cytometry.** PBMCs or purified B cells were stained in an one-step procedure incubating cells for 20 min at 4°C with the following FITC-, PE- and PerCP-conjugated mAbs: anti-CD19 (clone 4G7), anti-CD27, anti-CD69 (clone L78), anti-CD71 (clone L01.1), anti-CD86 (clone 2331), and anti-CXCR3 (clone 49801). Mouse isotype-matched FITC, PE, or PerCP were used as negative controls. All mAbs were purchased from Becton Dickinson or Pharmingen, except for the CXCR3 mAb, which was purchased from R & D Systems. After washing, samples were acquired on a FACS Calibur flow cytometer (Becton Dickinson), and data were processed by using the program CELLQUEST (Becton Dickinson). Analyses of B cells on total PBMCs were performed, gating on CD19-positive cells.

**Carboxyfluorescein Succinimidyl Ester (CFSE) Labeling.** Purified naive and memory B lymphocytes were resuspended at 2 × 105 cells per ml in PBS, and CFSE (Molecular Probes) was added at a final concentration of 0.5 μM (19). The CFSE-labeled cells were incubated with complete medium, anti-CD81 mAbs (MG81 and N81), or SAC for 96 h, stained with PE-anti-CD27, and analyzed by flow cytometry by using FACS Calibur (Becton Dickinson). Cell viability was measured by propidium iodide incorporation.

**In Vitro Ig Production.** For Ig measurements, 1 × 105 purified naive and memory B lymphocytes were cultured with CD81 mAbs (MG81 and N81) or SAC. At day 10, IgM and IgG concentration in culture supernatants were measured by standard ELISA techniques, as described in ref. 20.

**Western Blot Analysis.** B lymphocytes purified from human tonsils (30 × 106 cells per ml) were stimulated with of MG81 (5 μg/ml) and N81 (5 μg/ml) antibodies or goat anti-human IgM F(ab′)2 fragments (IgM) (10 μg/ml) at 37°C. At the indicated time, cells were washed with cold PBS and lysed for 1 h on ice in 300 mM NaCl, 50 mM Tris-HCl (pH7.6), 1% Triton X-100, 1 mM NaF, 1 mM Na3VO4, 10 mM sodium pyrophosphate, 1 mM PMSF, and 1× EDTA-free complete protease-inhibitor mixture (Roche Diagnostics). Equal amounts of protein extracts were resolved on 10% SDS/PAGE gel and transferred onto nitrocellulose membrane. Blotting was performed by using horseradish-peroxidase-conjugated 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), phospho-e-Jun (KM1) and total e-Jun (H-79) antibodies (Santa Cruz Biotechnology), total CD19 and phospho-CD19 (Tyr-531) antibodies (Cell Signaling Technology, Beverly, MA), phospho JNK1 and -2 (pTpY185/185), and total JNK1 (BioSource International, Camarillo, CA). The JNK inhibition experiments were conducted by preincubating tonsil-derived B cells for 10 min at 37°C with increasing concentrations of SP600125 (BioSource International, Camarillo, CA).

**Statistical Analysis.** Comparison of the percentages of activation markers and CXCR3 on B cells from HCV patients before therapy, at the end of therapy, and at the end of follow-up was performed by using two-tailed P values, Wilcoxon matched-pairs test (INSTAT, GraphPad).
CD81 engagement induces human B cell proliferation. B lymphocytes purified from healthy donor PBMCs were cultured for 5 days in the presence of increasing concentrations of MG81 mAb (0.15 to 10 μg/ml) and 5 μg/ml HCV-E2 (Fig. 1A). N81 mAb (anti-CD19), or anti-CD21 mAbs (A); MG81 F(ab')2 (0.15–10 μg/ml) and 5 μg/ml N81 F(ab')2 (B); CD81-LEL (0.03–5 μg/ml) combined with MG81 plus HCV-E2 (C) (5 μg/ml each), goat anti-human IgM F(ab')2 fragments (C) (10 μg/ml), or SAC (C) (1:30,000, vol/vol) (C). Cell proliferation was assessed by [3H]thymidine incorporation. In A and B, data are shown as the mean of cpm × 10^3 ± SD of triplicate wells. In C, data are expressed as the percentage of inhibition of the B cell proliferation rate calculated in the absence of CD81-LEL. Data are representative of at least five different experiments performed with independent donors.

Results

Multimeric Engagement of CD81 Induces B Cell Activation and Proliferation. It is well documented that CD81 cross-linking by HCV-E2 protein or by anti-CD81 mAbs inhibits NK cells (17) and costimulates T lymphocytes (16). Therefore, we assessed the effect of CD81 cross-linking on human B lymphocytes. Engagement of CD81 on freshly purified B cells from healthy individuals through HCV-E2 coated on plastic or anti-CD81 mAb cross-linked by anti-mouse antibodies did not affect expression of activation markers, maturation, or proliferation (data not shown). However, when B lymphocytes were cultured for 5 days with a combination of two anti-CD81 mAbs, MG81 and N81, in soluble form, we detected a robust B cell proliferation (Fig. 1A). Both MG81 and N81 are directed against the recombinant CD81-LEL protein; however, only N81 is capable of neutralizing HCV-E2 binding to CD81. No stimulation of proliferation was detected when B cells were treated with MG81 or N81 alone (data not shown) or combined with mAbs directed against the other components of the B cell coreceptor, CD19 and CD21 (Fig. 1A). The stimulation of B cell proliferation was preserved, although to a lesser extent, when N81 was replaced by a recombinant form of the HCV-E2 glycoprotein, whereas HCV-E2 alone did not have any effect (Fig. 1A). We found that B cells stimulated with F(ab')2; MG81 and N81 mAbs proliferate as efficiently as cells stimulated with intact immunoglobulins, excluding the involvement of Fc-mediated activities (Fig. 1B). To further validate that B cell proliferation was mediated by CD81, recombinant CD81-LEL protein was combined with HCV-E2 plus MG81, SAC, or anti-IgM. The proliferation induced by CD81 engagement was strongly inhibited (50% inhibition at 0.25 μg/ml of CD81-LEL), whereas CD81-LEL did not affect SAC or anti-IgM stimulation (Fig. 1C). The same results were obtained when HCV-E2 was replaced by N81 or when CD81-LEL was substituted with an anti-HCV-E2 mAb, which is known to interfere with the binding of HCV-E2 to CD81 (data not shown).

CD81 engagement resulted in an increased percentage of B cells expressing the early activation marker CD69, the transferrin receptor CD71, and the costimulatory molecule CD86. Similar results were obtained when well characterized polyclonal B cell stimuli (anti-IgM or SAC) were used (Table 1). Table 1 also shows that CXCR3, which is expressed in a small fraction of resting B cells, is up-regulated by anti-CD81 mAbs but not by anti-IgM and SAC. Taken together, these results demonstrate that multimeric engagement of CD81, in the absence of exogenous cytokines and BCR engagement, induces activation, proliferation, and increased expression of CXCR3 molecules on B lymphocytes and that the epitopes involved in HCV binding to target cells are required for this process.

CD81 Engagement Preferentially Activates Naive B Cell Proliferation. B lymphocytes can be schematically divided into naive and memory subsets on the basis of CD27 expression (21). To better assess the effect of CD81-mediated activation on these B cell subsets, peripheral blood B lymphocytes were separated as CD27+ and CD27– cells, labeled with CFSE, and activated by CD81, IgM, or SAC.

We first assessed the proliferative response through CFSE labeling. Fig. 2 shows that naive and memory B lymphocytes displayed a similar proliferation rate when activated by SAC. In contrast, CD81 engagement resulted in the preferential division of naive B cells (45% vs. 1% control), with a minor proliferative effect in the memory B cell subset (15% vs. 9% control) (Fig. 2 Top). In a control experiment, we found no differences in the cell death rate of memory and naive B cells activated through CD81 engagement (data not shown).

CD81 multimeric engagement and SAC stimulation induced the differentiation of the majority of naive cells into memory B lymphocytes. In addition, both CD81 and SAC treatments resulted in the up-regulation of activation molecule CD71 in the naive and memory B cell subsets (Fig. 2 Middle).

We then measured the concentration of immunoglobulins in

Table 1. In vitro analysis of B lymphocytes from healthy donors stimulated by multimeric CD81 engagement

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control*</th>
<th>CD81†</th>
<th>SAC†</th>
<th>IgM§</th>
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<tr>
<td>CD69</td>
<td>6 ± 1</td>
<td>81 ± 5</td>
<td>57 ± 2</td>
<td>76 ± 10</td>
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<td>CD71</td>
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<td>56 ± 3</td>
<td>79 ± 6</td>
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<tr>
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<td>CXCR3</td>
<td>8 ± 4</td>
<td>27 ± 4</td>
<td>4 ± 2</td>
<td>9 ± 6</td>
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</table>

Data are shown as percentage of CD19 cells expressing the markers indicated on the left (mean ± SD). B lymphocytes from healthy donors (n = 10) stimulated in vitro for 24 h by using: *, complete medium; †, MG81 mAb (5 μg/ml) + N81 mAb (5 μg/ml); ‡, SAC (1:30,000 vol/vol); §, Goat anti-human IgM F(ab')2 (10 μg/ml).
culture supernatants from naïve and memory B cells stimulated through CD81 or SAC. As expected, activated naïve B cells did not produce IgG but a low (in the case of SAC) or very low (in the case of CD81) amount of IgM, whereas memory B cells produced a high amount of IgM and IgG in response to SAC activation and a low amount of IgM and IgG in response to the modest CD81 activation (Fig. 2 Bottom).

In summary, these results demonstrate that, although CD81 engagement up-regulates activation markers in both the naïve and the memory B cell subsets, it promotes a preferential polyclonal expansion of naïve B lymphocytes.

**CD81-Mediated Activation of B Cells Triggers JNK Activity.** We next assessed the intracellular signaling events induced by multimeric CD81 engagement on B cells. We found that, in contrast to BCR stimulation by anti-IgM, multimeric CD81 engagement did not result in a significant increase in total tyrosine phosphorylation levels in purified tonsil-derived B cells (Fig. 3A). Despite the extensive difference observed in the tyrosine phosphorylation pattern, both CD81 and BCR engagement activated the JNK pathway, as shown by the increased phosphorylation of JNK1 and -2 and by the accumulation of the activated form of c-Jun (pJun) 2–4 h after treatment (Fig. 3A). When B cells from the same experiment were monitored for activation markers and proliferation, we obtained the same results shown in Table 1 and Fig. 1 for PBMC-derived B cells (data not shown). In addition, JNK inhibitor SP600125 (22) abolished both c-Jun activation and proliferation of tonsil B cells induced by CD81 engagement (see Fig. 6, which is published as supporting information on the PNAS web site). Because CD81 is part of the coreceptor complex with CD19, we assessed whether multimeric CD81 engagement could signal through the activation of CD19, which occurs through phosphorylation of its cytoplasmic domain. BCR engagement by anti-IgM stimulated CD19 phosphorylation, whereas CD81 engagement resulted in a transient inhibition of CD19 phosphorylation (Fig. 3B). The absence of a significant change in tyrosine phosphorylation levels, together with the inhibition of CD19 activity, suggests that CD81-mediated activation of B cells is independent from the B cell receptor and coreceptor complexes. The activation of the JNK pathway, monitored by phospho c-Jun accumulation, was specific for multimeric CD81 engagement (MG81 plus N81) and did not occur when MG81 and N81 mAbs were provided alone (Fig. 3C). In contrast, the transient dephosphorylation of CD19 was observed in response to both multimeric engagement and N81 alone (Fig. 3C).

**Chronic HCV Infection Associates with B Lymphocyte Activation.** We reasoned that polyclonal B cell activation obtained by multimeric CD81 engagement in vitro could mimic what occurs in HCV-infected patients when whole HCV particles bind CD81 on the B cell surface in vivo. To test this hypothesis, we compared the phenotype of peripheral blood B cells from 64 chronic hepatitis C patients with (n = 26) or without (n = 38) cryoglobulinemia with 21 healthy controls and 16 patients with chronic hepatitis B. We found that B cells from the great majority of HCV patients (54 of 64) expressed elevated levels of the activation markers CD69, CD71, and CD86 and of the chemokine receptor CXCR3, whereas B cells from all 16 patients with chronic hepatitis B had the same levels of activation markers and CXCR3 as healthy controls (Table 2). The up-regulation of the activation markers and CXCR3 did not associate with a particular HCV genotype, RNA levels (Table 3), or the presence of cryoglobulinemia (Table 2). In contrast with previous observations showing that the frequency of B cells is higher in HCV patients (23), we found the same percentage of B lymphocytes (CD19+ cells) in healthy controls and HCV- and hepatitis B virus (HBV)-infected patients (data not shown). We then analyzed the relative expression of activation markers and CXCR3 on naïve (CD27−) and memory (CD27+) subsets from peripheral B cells of healthy controls and HCV patients. We found more activated naïve and memory B cells in HCV-infected patients than in healthy controls; however, the difference in the frequency of B cells expressing activation markers and CXCR3 between healthy controls and HCV patients was more pronounced in the naïve B cell subset (Fig. 4). Because CD27-negative cells up-regulate CD27 a few days after activation, the presence of a sizeable fraction of CD27-negative cells that expressed activation markers ex vivo suggests that a continuous activation of naïve B cells occurs.
during chronic HCV infection. Accordingly, we also found that HCV patients display a higher percentage of memory (CD27+) B cells compared with healthy controls and HBV patients (Table 2).

In a previous study performed on cryopreserved peripheral blood lymphocytes from patients with chronic HCV infection, activated B cells were not found (23). We therefore assessed B cell phenotype in peripheral blood from HCV patients on either freshly separated cells or the same samples that had been cryopreserved. Freshly isolated B cells displayed an increased expression of CD69, CD71, CD86, and CXCR3, as described above. In contrast, cryopreserved B cells from the same samples, although viable and responding to polyclonal stimuli, did not display the activated phenotype (see Fig. 7, which is published as supporting information on the PNAS web site). This finding indicates that assessment of activation markers on human B cells should be performed in freshly isolated rather than cryopreserved peripheral blood lymphocytes.

**Table 2. Ex vivo analysis of B lymphocytes from HCV-infected patients**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control*</th>
<th>HCV cryo†</th>
<th>HCV‡</th>
<th>HBV§</th>
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<tr>
<td>CD27</td>
<td>20 ± 4</td>
<td>43 ± 20</td>
<td>42 ± 20</td>
<td>23 ± 5</td>
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<td>CD69</td>
<td>5 ± 1</td>
<td>33 ± 11</td>
<td>35 ± 12</td>
<td>6 ± 2</td>
</tr>
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<td>19 ± 6</td>
<td>52 ± 16</td>
<td>38 ± 13</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>CD86</td>
<td>12 ± 3</td>
<td>34 ± 14</td>
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<td>8 ± 2</td>
</tr>
<tr>
<td>CXCR3</td>
<td>9 ± 2</td>
<td>44 ± 16</td>
<td>38 ± 12</td>
<td>9 ± 3</td>
</tr>
</tbody>
</table>

Data are shown as percentage of CD19 cells expressing the markers indicated on the left (mean ± SD). * Healthy donors (n = 21); †, HCV-infected patients with cryoglobulinemia (n = 26); ‡, HCV-infected patients (n = 38); §, HBV-infected patients (n = 16); ††, ‡, characteristics of patients are summarized in Table 3. HBV, hepatitis B virus.

**Fig. 5.** Eradication of HCV infection associates with a decreased expression of activation markers and CXCR3. Ex vivo expression of activation molecules (CD69, CD71, and CD86) and the chemokine receptor CXCR3 was measured on peripheral B cells derived from 22 HCV-infected patients treated with IFN plus ribavirin therapy (for details, see Materials and Methods) and 21 healthy subjects (Control). Fifteen patients responded to therapy (Responders), whereas seven did not eradicate HCV infection (Non Responders), as demonstrated by HCV-RNA PCR. B lymphocytes from HCV-infected patients were monitored before and soon after the end of therapy in responders, an additional analysis was performed 12 months after the end of therapy (follow-up). All responders achieved sustained virological response. Data is shown as the percentage of CD19-positive cells expressing the indicated markers (mean ± SD). (*, P < 0.005 as compared with patients before therapy)

**Fig. 4.** HCV patients display high percentage of activated naïve B cells. B lymphocytes from HCV patients (HCV) and healthy subjects (Control) were analyzed ex vivo for the surface expression of activation markers (CD69, CD71, and CD86) and CXCR3 by flow cytometry. For each sample, 2 × 10⁶ events were acquired. Data is shown as the percentage of naïve B cells (CD19⁺/CD27⁻) or memory B cells (CD19⁺/CD27⁺) expressing the indicated molecules (mean ± SD). Numbers on bars represent the ratio between the level of expression of each marker in HCV patients over control.

**Discussion**

We found that engagement of the HCV receptor CD81 activates human B cells in the absence of BCR coligation. CD81-mediated activation differs from other polyclonal B cell stimuli in that it induces preferential proliferation of naïve B cells, whereas anti-IgM and SAC activate naïve and memory B cell proliferation equally well, and CpGs selectively activate memory B cells (24).

CD81-mediated B cell activation occurred through a combination of two soluble CD81 ligands, the HCV envelope protein E2 and one anti-CD81 mAb. Because a single antibody should be able to induce capping, our finding indicates that membrane reorganization is not the only key to the CD81-mediated B cell activation. The multimeric CD81 engagement required for activation of B cells is in apparent contrast with the requirement of a single CD81 ligand and a cross-linking agent in CD81-mediated inhibition of NK cells (17) and costimulation of T lymphocytes (16). However, because CD81 associates with specific complexes on different cell lineages (12), it is likely that CD81 promotes cell-type-restricted signaling events that might or might not require multiple ligands or receptor cross-linking.
CD81 has very short cytoplasmic regions without any recognizable signaling motif and is believed to signal through interaction with associated partners. CD19 directly binds to CD81, representing an obvious candidate to exert such a function (25). Our data, however, show that CD19 is not activated during CD81 engagement, arguing against this hypothesis. Other proteins interacting with CD81 on the B cell surface, namely integrin α4β1 and MHC-II, have been identified (26, 27), but more work is required to assess whether these proteins play any role in CD81-mediated B cell activation. We have found that CD81 engagement activates the JNK pathway through a mechanism that, at variance with the BCR-dependent JNK activation, is not associated with general tyrosine phosphorylation, as detected by Western blot. The finding that the CD81 signaling pathway diverges from the BCR pathway upstream of JNK activation might reveal new targets for the treatment of lymphoproliferative diseases resulting from uncontrolled B cells stimulation.

We have found that chronic HCV infection associates in 85% of cases with a high percentage of activated B cells and that therapeutic eradication of HCV coincides with the reduction of this activated B cell phenotype. Our data demonstrate that polyclonal B cell activation is a general feature of chronic HCV infections, because cells of target organs are observed in the presence of mixed cryoglobulinemia. It is tempting to speculate that the polyclonal B cell activation obtained by multimeric CD81 engagement in vitro mimics what occurs in HCV-infected patients when the virus binds CD81 on the B cell surface. Notably, our model does not require the assumption that B cells are infected by HCV. Indeed, the surface contact between HCV and CD81 could activate B cells in the absence of infection. Our finding that CD81 engagement by HCV envelope protein preferentially activates naïve B lymphocyte proliferation may be relevant to explain the high rate of autoantibodies found in HCV infections (28) and the association of HCV with cryoglobulinemia (5). It is possible that the polyclonal-antigen-independent activation of naïve B cells induced by HCV engagement of CD81 is the first step toward the polyclonal expansion of autoreactive clones of naïve B cells that, by becoming memory cells, can be more readily activated in a bystander mode to produce autoantibodies (24). In a few cases, this B cell autoreactivity, most likely in combination with still unidentified genetic/environmental factors, could evolve into frank lymphoproliferative disorders, such as type II cryoglobulinemia (5) or non-Hodgkin lymphoma (6, 7).

The envelope of HCV preferentially activates naïve B lymphocytes in the absence of infection. Our finding that CD81 engagement by the HCV envelope protein inhibits NK cells (17), costimulates T lymphocytes (16), and activates B cells (this article). Interestingly, it has been recently reported that the engagement of B cells by purified E2 induced double-strand DNA breaks specifically in the variable region of Ig (VH) gene loci, leading to hypermutation in the VH genes of B cells (34). Altogether, one can depict a scenario where interaction between HCV and CD81 facilitates the establishment of chronic infection, creating an escape mechanism for the virus through the inhibition of the innate immune response and the dilution of the adaptive immune response resulting from polyclonal expansion.