B Cell Receptors Expressed by Lymphomas of Hepatitis C virus (HCV)-Infected Patients Rarely React with the Viral Proteins

Patrick P. Ng¹, Chiung-Chi Kuo¹, Stanley Wang¹, Shirit Einav¹, Luca Arcaini², Marco Paulli², Carol S. Portlock³, Joe Marcotrigiano⁴, Alexander Tarr⁵, Jonathan Ball⁵, Ronald Levy¹ and Shoshana Levy¹

¹Stanford University Medical Center, Stanford, CA; ²University of Pavia, Pavia, Italy; ³Memorial Sloan Kettering Cancer Center, NY, NY; ⁴Rutgers University, Piscataway, NJ; ⁵University of Nottingham, Nottingham, UK

Corresponding Author:
Shoshana Levy
slevy@stanford.edu
Phone: 650-725-6425
Fax: 650-736-1454

Short Title: HCV-induced Lymphomagenesis
Key points:
- We tested the hypothesis that B-cell lymphomas arising in HCV-infected patients express BCRs specific to the virus.
- We analyzed the reactivity of these BCRs with HCV proteins using several experimental approaches, none of which supported the hypothesis.

Abstract

Chronic HCV infection has been implicated in the induction and maintenance of B-cell lymphomas. The strongest evidence for this comes from clinical observations of tumor regressions upon anti-viral treatments. Here we used multiple methods to test the hypothesis that the expansion of HCV-specific B cells gives rise to lymphomas. We obtained lymphoma tissues from HCV-infected lymphoma patients, including some that later regressed upon anti-viral treatments. We expressed the lymphoma B-cell receptors (BCRs) as soluble IgGs and membrane IgMs, and analyzed their reactivity with HCV proteins and with HCV virions. We confirmed previous reports that HCV-associated lymphomas use a restricted immunoglobulin variable region (V) gene repertoire. However, we found no evidence for their binding to the HCV antigens. We conclude that most lymphomas of HCV-infected patients do not arise from B cells aimed at eliminating the virus.
Introduction

Survival of B cells requires the expression of BCR, as demonstrated in knockout mice\(^1,2\) and in some patients with non-X-linked agammaglobulinemia. Lymphoma B cells undergo somatic hypermutation in their V genes, which would be expected to generate protein loss variants. However, in the various lymphoma types, the BCR is retained\(^3\), suggesting importance for lymphoma cell survival. Yet, lymphomas’ cognate antigens are not known. B-cell proliferative diseases such as mixed cryoglobulinemia (MC) and Non-Hodgkin lymphoma (B-NHL) that arise in HCV-infected patients represent a special opportunity to study antigenic drive in lymphomagenesis. First, both MC and B-NHL use a restricted V gene repertoire shared by anti-HCV envelope antibodies\(^4,5\). Second, elimination of HCV by anti-viral therapy in patients with these B-cell diseases has been associated with their regression\(^6\). Moreover, we previously identified an HCV-associated lymphoma whose BCR bound the HCV envelope protein E2\(^7\). Normal B cells aimed at eliminating HCV would be expected to bind the virus via two receptors, the cognate BCR and the viral entry receptor, CD81, which is a member of a costimulatory complex with CD19/CD21. Such B cells would receive dual stimulatory signals and might undergo unchecked proliferation during chronic HCV infection.

Here we tested this hypothesis by expressing BCRs from lymphomas of HCV-infected patients as soluble IgG and as membrane IgM. We included patients that had tumor regressions after anti-viral therapy\(^8\) expecting that they would be more likely to express anti-HCV BCRs. We used several methods to test the reactivity of the rescued lymphoma BCRs with viral proteins and particles. However, we found no reactivity and therefore no evidence to support the hypothesis that viral antigens drive B-cell lymphomas.

Methods

Patients
Biopsies of patients with B-NHL and chronic HCV infection were collected at Stanford University Medical Center, Sloan Kettering Memorial Cancer Center and at the University of Pavia Medical School. Patients’ medical record numbers were de-identified and reassigned numbers. Institutional review boards at each center approved this study,
and written informed consents were obtained from all patients in accordance with the Declaration of Helsinki.

V gene rescue
mRNA was isolated using RNeasy (Qiagen, Valencia, CA) and cDNA amplified using SMARTer RACE (Clontech, Mountain View, CA), V region amplification used 5′ RACE and the following constant regions primers:

IgM 5'-ggggcctgaggagacggtgacc-3'
IgG 5'- ggagsagggygccagggggaagac-3'
κ 5'-tggtcagcgcgctcaggccct-3'
λ 5'-gcggtcagcagcataagctgctgga-3'.

Expression of lymphoma idiotypes (Ids)
Amplified products were inserted into an IgG1 expression vector9, then expressed, as previously4. IgGs in the supernatant of transiently transfected COS-7 cells were quantitated by ELISA. Expression of the rescued V regions in A20 cells as membrane IgM was as previously10.

HCV proteins
Expression of E2661 and J6E2 were as previously7,11. HCV-E1E2 of various genotypes were encoded by pCR 3.1-UKN1B12.16, -UKN1B25.23, -UKN2A1.2, and -UKN2A2.412. The E1E2 sequences from these plasmids, and the E1E2 of the H77c strain (genotype 1a) were ligated into pCDM8 expression plasmids and transiently transfected into 293T cells. An anti-HCV ELISA kit (DIAsource, Louvain-la-Neuve, Belgium) analyzed interaction of patient IgGs with core, NS3, NS5A and NS5B proteins.

Binding of rescued IgG and IgM
ELISA detecting binding to HCV-E2 was as previously7. Flow cytometry was used to detect rescue IgG binding to intracellular E1E2 in permeabilized 293T cells, and the binding of A20 cell surface IgM to soluble E2.
Results and Discussion

The incidence of B-cell proliferative diseases, including MC and NHL is higher in HCV-infected patients than in non-infected individuals, especially in certain geographical areas, such as Italy. Moreover, the regression of B-cell diseases in response to successful anti-viral therapies implies a causative link between HCV infection and B-cell proliferative diseases. Here, we aimed to validate the hypothesis that B-cell lymphomas arise from expansion of anti-viral B cells in HCV-infected patients by analyzing the reactivity of their lymphoma BCRs with HCV. Patients were diagnosed in the US and in Italy, the latter received anti-viral therapy and included oncological responders and non-responders. Analysis of V gene usage showed a restricted repertoire, specifically, usage of VH-169 and Vκ3-20 (Table 1).

We sequenced the V region genes and expressed them as secreted human IgG1/κ in transfected cells. We then analyzed the reactivity of all rescued IgG1 with the soluble HCV E2 ectodomain, E2661 (genotype 1a) or J6E2 (genotype 2a). However, except for the anti-E2 mAb controls, none of the tested IgG1 reacted with E2661 (Table 1) or with J6E2 (Fig 1A). Next, we tested all the rescued IgG1s with internal HCV antigens, which are included in a diagnostic kit; however, none reacted with core, NS3, NS5A and NS5B proteins (Fig 1B).

HCV is enveloped by two heterodimeric proteins, E1 and E2. We tested the possibility that the rescued IgG1s recognize the heterodimer in its native membrane-bound form by using 293T cells transfected with constructs encoding full E1E2 polypeptides. We specifically selected E1E2 of the HCV genotypes 1b and 2a matching the infected patients genotypes, as well as E1E2 derived from HCV isolate H77 of genotype 1a. The heterodimers were expressed intracellularly, as detected by flow cytometry using anti-E1 and anti-E2 mAb (Fig 1C, top panels). However, none of the patients’ rescued IgG1 showed reactivity (Fig 1C, bottom panels). Chronic HCV infection is thought to have a causative role in MC, characterized by the benign proliferation of B cell secreting IgM with rheumatoid factor (RF) activity. Evidence also exists that implicate MC as the precursor to frank NHL. However, we did not find RF activity in any of the rescued IgG1s (data not shown).
The VH-1\textsubscript{69} gene is also repetitively used in human mAbs that react with HIV\textsuperscript{15} and with the influenza hemagglutinin (HA) protein\textsuperscript{16}. Importantly, in a recent study germline VH-1\textsubscript{69} expressed as surface IgM reacted with HA, while soluble versions of the mAb were unreactive. It was proposed that clustering of surface BCR on a naïve B cell increases the avidity of the otherwise low-affinity germline BCR to HA to a level sufficient to trigger B-cell activation\textsuperscript{17}. We therefore expressed the lymphoma Ids as human IgMs on A20, a mouse B cell line (Fig 1D top panel) and tested their reactivity with soluble E2 proteins. However, only positive control cells expressing IgM with known anti-E2 reactivity showed binding to soluble E2\textsubscript{661} and J6E2 proteins (Fig 1D, middle and bottom panels, respectively). In addition, these cell-surface-expressed lymphoma Id IgMs did not bind HCV-core, or NS proteins (data not shown).

Lastly, we took a step further to explore whether lymphoma Ids interact with HCV proteins on an assembled virion. HCV produced in cell culture, HCVcc are associated with lipids\textsuperscript{18,19} and may contain other antigens not studied in previous experiments. However, incubation of HCVcc with A20 cells expressing surface lymphoma Id IgMs did not reduce their infectivity, whereas neutralizing anti-E2 mAbs (CBH-2 and CBH-5) blocked infection (Fig 1E). Non-neutralizing anti-E2 mAbs (CBH-4B, CBH-4G) expressed as cell surface IgMs (Fig 1E), or as soluble CBH-4G mAb (data not shown), did not block infection.

This study tested the hypothesis that B cells aimed at eliminating the virus give rise to HCV-associated B-cell lymphomas. We included patients that responded to anti-viral therapy expecting them to be more likely to bind the virus. However, while confirming a restricted usage of VH-1\textsubscript{69}, we did not identify a single BCR that reacted with HCV (Table 1), hence, no evidence to support the hypothesis.
Acknowledgments

We thank Drs. William Robinson and Jeremy Sokolove for providing RF positive and negative plasma, and technical expertise in testing RF activity. The research was supported by a grant from Stanford's Institute for Immunity, Transplantation, and Infection. S. Einav was supported by K08 AI079406 from the National Institute of Allergy and Infectious Diseases.

Authorship

Contribution: P.P.N., C-C.K., and S.W. performed the experiments; P.P.N. and S.W. analyzed the results and made the figures; L.A., C.S.P, and R.L. provided biopsy specimens; J.M. provided the J6E2; A.T. and J.B. provided the plasmids encoding E1E2 glycoproteins; P.P.N., S.W., S.E., R.L. and S.L. designed the research and wrote the paper.

Conflict-of-interest: The authors declare no conflict of interest.
References

<table>
<thead>
<tr>
<th>Patient</th>
<th>Isotype</th>
<th>Diagnosis</th>
<th>Lymphoma response to anti-viral therapy</th>
<th>V_H</th>
<th>V_L</th>
<th>Accession #</th>
<th>HCV genotype</th>
<th>E2661</th>
<th>J6E2</th>
<th>E1E2</th>
<th>NS + Core</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>IgM/k</td>
<td>DLBCL</td>
<td>ND</td>
<td>V_H1-69</td>
<td>V_L3-20</td>
<td>KF895775/6</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>IgM/k</td>
<td>FL</td>
<td>ND</td>
<td>V_H1-39</td>
<td>V_L1-39</td>
<td>KF895777/8</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>lgM/k</td>
<td>MZL</td>
<td>ND</td>
<td>V_H4-59</td>
<td>V_L3-20</td>
<td>KF895779/80</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>104</td>
<td>lgM/k</td>
<td>DLBCL</td>
<td>ND</td>
<td>V_H1-69</td>
<td>V_L1-16</td>
<td>KF895781/2</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>lgM/k</td>
<td>DLBCL</td>
<td>ND</td>
<td>V_H1-69</td>
<td>V_L3-20</td>
<td>KF895783/4</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>lgM/k</td>
<td>NHL</td>
<td>ND</td>
<td>V_H4-59</td>
<td>V_L3-15</td>
<td>KF895785/6</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>107</td>
<td>lgM/k</td>
<td>NA</td>
<td>ND</td>
<td>V_H1-31</td>
<td>V_L1-39</td>
<td>KF895787/8</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>lgM/k</td>
<td>NA</td>
<td>ND</td>
<td>V_H4-59</td>
<td>V_L3-15</td>
<td>KF895789/90</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>109</td>
<td>lgM/k</td>
<td>NA</td>
<td>ND</td>
<td>V_H4-34</td>
<td>V_L3-20</td>
<td>KF895791/2</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>lgM/k</td>
<td>NA</td>
<td>ND</td>
<td>V_H4-59</td>
<td>V_L3-20</td>
<td>KF895793/4</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>111</td>
<td>lgM/k</td>
<td>NA</td>
<td>ND</td>
<td>V_H1-02</td>
<td>V_L2-30</td>
<td>KF895795/6</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>112</td>
<td>lgM/k</td>
<td>NA</td>
<td>ND</td>
<td>V_H4-34</td>
<td>V_L3-20</td>
<td>KF895797/8</td>
<td>ND Neg ND ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>lgM/k</td>
<td>MALT MZL</td>
<td>Yes</td>
<td>V_H1-69</td>
<td>V_L3-20</td>
<td>KF895799/800</td>
<td>2a/2c Neg ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>lgM/k</td>
<td>SMZL</td>
<td>No</td>
<td>V_H4-59</td>
<td>V_L3D-15</td>
<td>KF895801/2</td>
<td>1b Neg ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>123</td>
<td>lgM/k</td>
<td>SMZL</td>
<td>Yes</td>
<td>V_H1-69</td>
<td>V_L3-20</td>
<td>KF895803/4</td>
<td>2a/2c Neg ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>lgM/k</td>
<td>MALT MZL</td>
<td>Yes</td>
<td>V_H4-30</td>
<td>V_L3-15</td>
<td>KF895805/6</td>
<td>2a/2c Neg ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>lgM/k</td>
<td>SMZL</td>
<td>Yes</td>
<td>V_H3-30</td>
<td>V_L1-8</td>
<td>KF895807/8</td>
<td>2a/2c Neg ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>126</td>
<td>lgM/k</td>
<td>MALT MZL</td>
<td>Yes</td>
<td>V_H1-69</td>
<td>V_L3-20</td>
<td>KF895809/10</td>
<td>1b Neg ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>lgM/k</td>
<td>NHL</td>
<td>No</td>
<td>V_H1-69</td>
<td>V_L3-20</td>
<td>KF895811/2</td>
<td>1b Neg ND ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E2661, soluble envelope protein of HCV genotype 1a; J6E2, envelope protein of HCV genotype J6; E1E2, HCV envelope proteins expressed intracellularly. NS+Core, HCV non-structural + core proteins.

DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; MALT, mucosa-associated lymphoid tissue; MZL, marginal zone lymphoma; NHL, Non-Hodgkin lymphoma; SMZL, splenic marginal zone lymphoma; NA, not available. ND, not done; Neg, negative.

Table 1
Figure legend

Rescued HCV-associated lymphoma idiotypes do not react with viral proteins when expressed as soluble IgGs or as cell surface IgMs. (A) Purified J6E2 captured on lectin-coated 96-well plates was incubated with the indicated patients' Id IgG1s, with anti-E2 mAbs CBH-4G or CBH-5, or with a human IgG1/κ isotype control. Plate-bound IgGs were detected with an HRP-conjugated anti-human IgG. (B) 96-well plates coated with HCV core, NS3 and NS5 antigens were incubated with the indicated patients’ Id IgG1s, with anti-HCV plasma, or with negative control plasma, diluted as indicated. After wash, plate-bound Igs were detected with HRP-conjugated HCV core, NS3 and NS5 antigens. (A, B) Each bar represents the mean O.D. of wells incubated with each group of Id IgGs ± standard deviation. Representative results of two experiments for each assay are shown. (C) Single-cell suspensions of 293T cells transfected with empty pCDM8 vector (filled gray) or with pCDM8 vectors encoding E1E2 of the indicated genotypes were fixed and permeabilized. The cells were then stained with the anti-E2, anti-E1, a human IgG1/κ isotype control mAb, or with a mixture of the indicated patients’ IgG1 containing 0.5 μg of each Id. Cells were then washed, stained with PE-conjugated anti-human IgG, and analyzed by flow cytometry. (D) The patients’ Ids were expressed as human IgMs on the surface of the mouse B cell line, A20. Positive controls were A20 cells expressing CBH-4B or CBH-4G. Cells were stained with FITC conjugated anti-human IgM (top panel); Cells were incubated for 1 h on ice with cell culture supernatant containing soluble E2661 or mock supernatant. Cells were then washed, stained with AlexaFluor® 647-conjugated mouse anti-E2 mAb (H53)(middle panel); Cells were incubated for 1 h on ice with soluble J6E2 or BSA, washed and further incubated for 1 h on ice with a 1:1 mixture of the anti-E2 mAbs CBH-2 and CBH-5. After wash, cells were stained with AlexaFluor® 647-conjugated anti-human IgG (bottom panel). (C, D) Cells were washed and analyzed by flow cytometry. (E) A20 cells expressing the indicated surface lymphoma patients’ Id IgM or an IgM of irrelevant specificity (SIC5); neutralizing anti-E2 mAbs (CBH2 or CBH5) or a control human IgG1 mAb were incubated with luciferase reporter HCVcc (J6/JFH(p7-Rluc2A) HCV20, titer: 6.3×10^5 TCID_{50}/ml) for 1hr at 37°C. These samples were then used to inoculate naïve huh-7.5 cells. To measure infectivity, cells were lysed at 48hr and subjected to standard luciferase assays. Y-axis represents HCVcc infection relative to the A20 SIC5 control. Data represent means and s.d. (error bars).
Fig. 1 (Ng et al.)
B cell receptors expressed by lymphomas of hepatitis C virus (HCV)-infected patients rarely react with the viral proteins

Patrick P. Ng, Chiung-Chi Kuo, Stanley Wang, Shirit Einav, Luca Arcaini, Marco Paulli, Carol S. Portlock, Joe Marcotrigiano, Alexander Tarr, Jonathan Ball, Ronald Levy and Shoshana Levy