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Antigen receptor stereotypy across B-cell lymphoproliferations: the case of IGHV4-59/IGKV3-20 receptors with rheumatoid factor activity

Rheumatoid factors (RFs) are the most frequent autoantibodies in autoimmune disorders, especially rheumatoid arthritis and primary Sjögren's syndrome (SS). RFs may also develop as a monoclonal component in B-cell lymphoproliferations, including type-II mixed cryoglobulinemia (MC-II).¹ MC-II develops almost exclusively in individuals infected with the hepatitis C virus (HCV) and is

characterized by the presence of immune complexes composed of monoclonal IgM binding to polyclonal IgG that exhibit anti-HCV reactivity. The monoclonal IgM almost always carries kappa light chains and represents autoantibodies with RF activity.¹

Chronic antigenic stimulation by HCV has been considered as a key mechanism in sustaining the proliferation of the RF-secreting B-cell clones in MC-II, as evidenced by the highly restricted immunoglobulin (IG) gene repertoire of the monoclonal IgM RF. Further, the regression of MC-II as well as HCV-associated splenic marginal-zone lymphoma (SMZL)² following antiviral therapy might be considered as evidence that the growth and survival

of monoclonal B cells in HCV-associated lymphoproliferations are dependent on continued exposure to the virus. However, it is unclear whether a direct stimulation by HCV itself is causative for any of these conditions or, alternatively, a virus-induced inflammatory condition may trigger a dysregulated (auto)immune response, eventually leading to the emergence of a monoclonal B-cell expansion.²⁻⁴

In recent years, chronic lymphocytic leukemia (CLL) has emerged as a paradigm for antigen-driven lymphomagenesis.⁵ However, despite recent progress, exactly which antigens are selecting and perhaps even continuously stimulating the leukemic clones have not been conclusively resolved yet. Here, we provide for the first time evidence linking a distinctive subset of CLL and isolated cases with other types of B-cell lymphoproliferations, including SMZL, B-cell expansions in SS and HCV-associated MC-II, all expressing 'stereotyped' IGHV4-59/IGKV3-20 BcR, with RFs.

Through a bioinformatics approach, we have previously identified three stereotyped IGHV-IGHD-IGHJ rearrangements from patients with CLL that were clustered in a subset referred to as subset no. 13.⁶ These rearrangements were highly similar to a RF from a healthy donor immunized with mismatched red

blood cells (GenBank, EMBL-Bank, DNA Data Bank of Japan and IMGT/LIGM-DB (GEDI) accession number: U85234) that also utilized the IGHV4-59 gene.⁷ They were also highly similar to an IGHV-IGHD-IGHJ sequence of a patient with CLL/small lymphocytic lymphoma (SLL) that developed in a setting of HCV-associated MC-II (GEDI: AF301516).⁸

In the present series, among 5798 productive IGHV-IGHD-IGHJ rearrangements from patients with CLL from our collaborating institutions, obtained as detailed in Supplementary Methods, 157 cases (2.71%) utilized the IGHV4-59 gene, of which 12 (0.21% of the entire cohort) carried antigen-binding sites characteristic of subset no. 13 (Table 1, Supplementary Table 1). All subset no. 13 cases expressed mutated heavy chains of the IgM/IgD isotypes and all the 10 tested cases were associated with IGKV3-20/IGKJ1 kappa light chains (Table 1, Supplementary Table 2). Other clinical, demographic and biological information available for subset no. 13 CLL cases is provided in Supplementary Table 3.

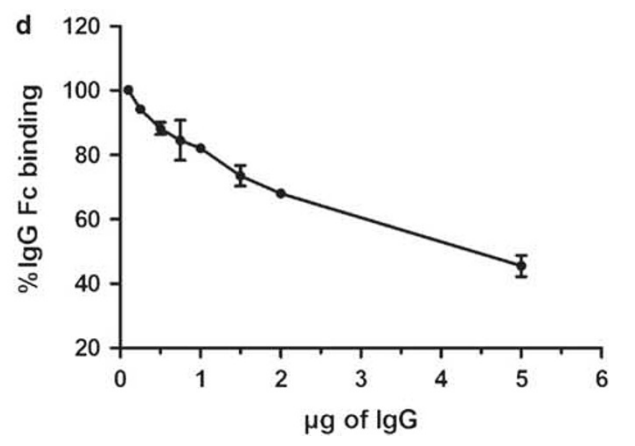
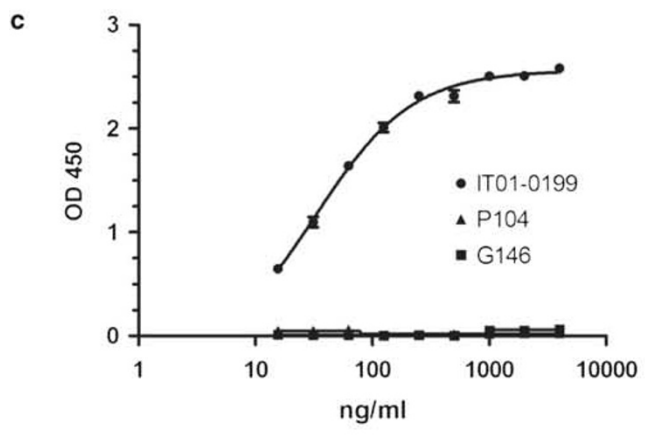
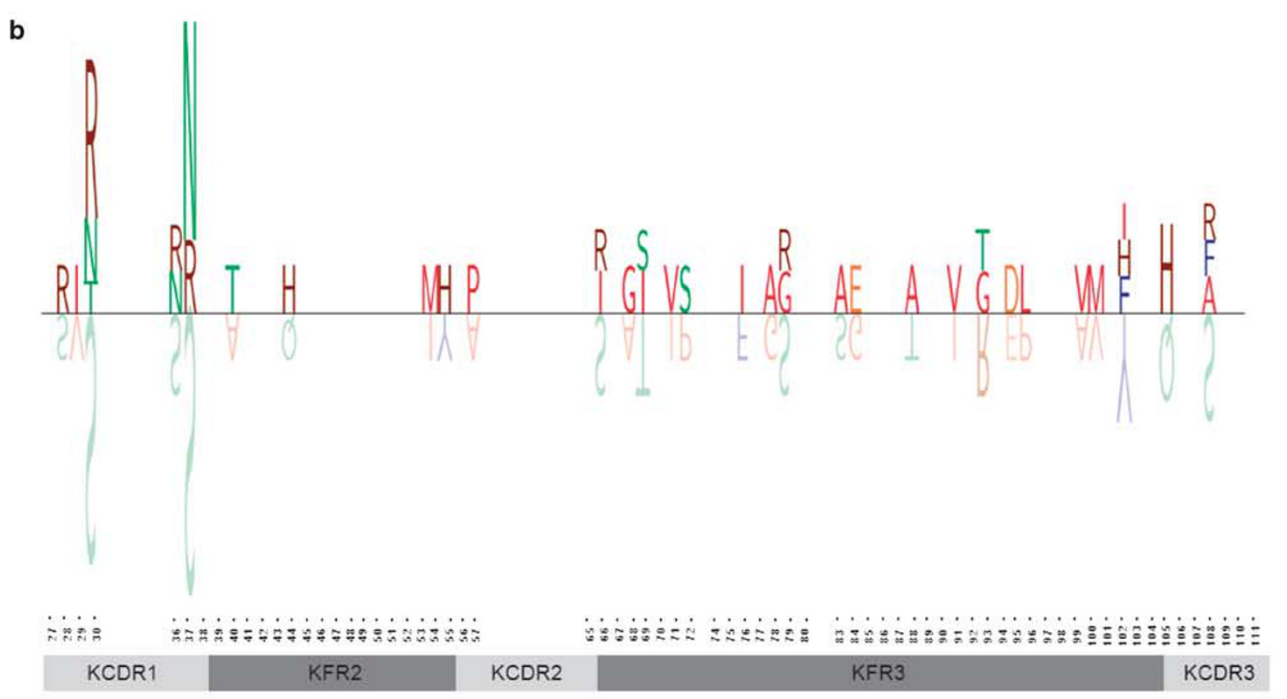
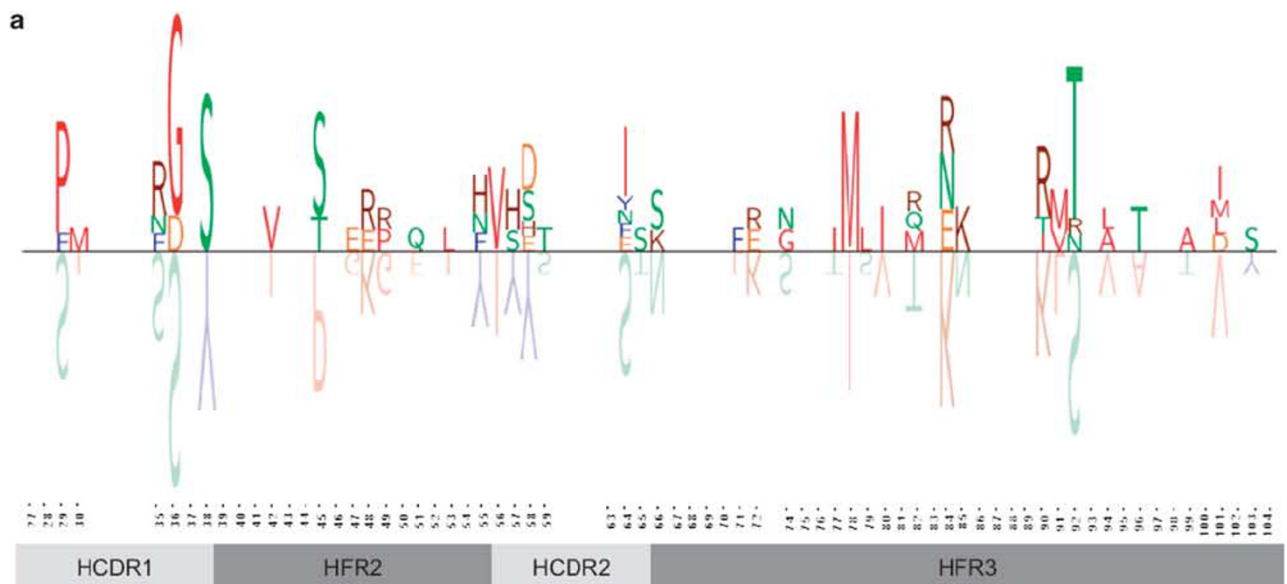
Comparisons of subset no. 13 IGHV4-59 CLL sequences with a comprehensive panel of IGHV4-59 sequences of various entities from our institutions and the IMGT/LIGM-DB database (retrieved as described in Supplementary Methods) identified highly similar

Table 1. Stereotyped IGHV4-59 rearrangements from CLL and other entities with sequence features of subset no. 13

Case	Entity	VH CDR3	V-KAPPA CDR3
<i>Internal sequences</i>			
CZ-01-0207	CLL	ARDRYCSGGSCFDWYFDL	QQYGSSPRT
CZ-01-0533	CLL	-----T-----	-----
CLL-188	CLL	---Q---T-----	-----
DK-01-0278	CLL	---Q--K-----F---	-----
FRA-P0127	CLL	---A-----T-----V	---F---
FRA-P0886	CLL	-----V-----	-H-----
FRA-P0898	CLL	---S-----F---	-----
GR-02-0214	CLL	-----Y-----	---A---
GR-02-0360	CLL	-----T-----	-H-----S
IT-01-0199	CLL	-----T-----	-----
IT-02-0445	CLL	-----	---R---
UK-01-0014	CLL	---G---T-----	-----
GR-02-0006	Atypical CLL	-----F-----	-----
UK-02-0067	Atypical CLL	-----	-----
89VB	CLL-like MBL	---A-----	-----
DK-01-0114	SMZL	---T-----	-----
505-12609	SMZL	---F-----	-----
<i>Sequences from public databases</i>			
U85234	RF	---S-----	-----
AF301516 ^a	CLL/SLL-MC-II	---F-----	-----
AF303917	SS-MESA	-----	-----
AF303914	SS-MESA	-----	-----

Abbreviations: CLL, chronic lymphocytic leukemia; CLL/SLL-MC-II, CLL/small lymphocytic lymphoma with a prior history of type II mixed cryoglobulinemia; CLL-like MBL, CLL-like monoclonal B lymphocytosis; RF, rheumatoid factor;⁷ SMZL, splenic marginal-zone lymphoma; SS-MESA, Sjögren's syndrome-associated myoepithelial sialadenitis. The table lists VH CDR3 and (when available) V-KAPPA CDR3 sequences. Highlighted in grey are junctional residues in both VH and V-KAPPA CDR3 that are shared by all available subset no. 13 sequences. ^aGEDI AF303897 for the associated light chain of AF301516.^{8,10}

Figure 1. Sequence logos for alignments of the VH (a) and V-KAPPA (b) regions of 12 CLL cases in subset no. 13. These logos represent all studied sequences and at each position a stack of upright amino-acid symbols is displayed, showing the amino acids actually used in that particular position. The height of each one-letter amino-acid symbol is directly proportional to the relative frequency of that amino acid at a position in the alignment among VH and V-KAPPA sequences, respectively. The germline amino acids are shown upside down: the height of the inverted germline amino-acid symbol is the sum of the heights of the upright amino acids. Positions with a distribution identical to germline are left blank. Positions are according to the IMGT unique numbering for V domains. (c) Subset no. 13 IG exhibits RF activity. The graph depicts IgM binding against IgG-Fc fragment using ELISA. Serial dilutions, from 4 µg/ml to 15.6 ng/ml, of IgM monoclonal antibodies produced by CLL heterohybridomas established from subset no. 13 CLL case IT01-0199 as well as two other non-subset no. 13 CLL cases (P104 and G146) were used as primary antibodies. The subset no. 13 IG displayed strong RF activity, in contrast to the other unrelated, CLL-derived IGs. The s.d. refers to replicates of the same experiments. (d) IgG inhibition of IT-01-0199-IgM binding to human IgG Fc. Increasing amounts of human IgG (0.1–5 µg) were used as inhibitors. A dose-dependent decrease in the absorbance value was observed confirming the specificity of IgM binding to IgG. The results were plotted as the percent bound versus the concentration of the competitor. The percent bound was calculated by using the optical density (OD), taking the OD without a competitor as 100% bound.



rearrangements (Supplementary Tables 3–5) from: (1) two cases (GR-02-0006 and UK-02-0067) classified as atypical CLL (both CD23– and *CCND1/IGH*– by fluorescence in situ hybridization); HCV serology data were available for one case that was found positive for anti-HCV antibodies; (2) two cases (505-12609 and DK-01-0114) with SMZL, both positive for anti-HCV antibodies; (3) a case of CLL-like monoclonal B-cell lymphocytosis (MBL) from our published series⁹ (case 89Vb), also HCV seropositive; (4) two cases with SS-associated monoclonal myoepithelial sialadenitis (SS-MESA) from the public database (cases 7 and 11 in De Re *et al.*¹⁰; GEDI: AF303917 and GEDI: AF303914).

The stereotyped VH domains in both CLL and non-CLL cases, carried a distinctive CDR3 created by the association of the IGHV4-59, IGHD2-15 and IGHJ2 genes. The ‘intra-subset’ amino acid identity exceeded 80% and it was underscored by ‘stereotyped’ N region sequences (Table 1).

We analyzed the expressed light chain in 12 cases (including both CLL and non-CLL) and all of them carried stereotyped IGKV3-20 rearrangements with a similarly high degree of CDR3 amino acid identity (Table 1, Supplementary Table 2). Alignment with public database sequences revealed six related IGKV3-20 rearrangements, including: (1) GEDI: AF303897, the partner IGKV3-20 light chain of GEDI: AF301516 from a HCV + CLL/SLL case⁸ (see above); (2) GEDI: AF301528, the clonotypic kappa light chain rearrangement from a HCV + SMZL case;⁸ (3) GEDI: AJ236551, the kappa light chain from an antibody against HCV glycoprotein E2;¹¹ (4) GEDI: EF624093, EF624200 and EF624214, the rearrangements of clonally expanded B cells in HCV-associated MC-II.¹²

All IGHV-D-J and IGKV-J stereotyped sequences from our series, including both CLL and non-CLL cases, were mutated with median identity values of 93.8% for IGHV- and 96.1% for IGKV-rearranged genes. Replacement to silent mutation ratios was significantly higher in VH CDR vs FR, a pattern typical of antigen-selected cells, and slightly higher for V-KAPPA CDR vs FR (Supplementary Table 6). Both VH and V-KAPPA of subset no. 13 cases were distinctive for shared, ‘stereotyped’ somatic hypermutations (Figures 1a and b).

We established heterohybridomas (Supplementary Methods) from the leukemic cells of a subset no. 13 CLL case (IT01-0199) that secreted large amounts of human IG mu and kappa chains. The identity of the produced soluble antibody to the IG expressed by the CLL clone was confirmed by nucleotide sequencing analysis of IGHV-IGHD-IGHJ and IGKV-IGKJ rearrangements. The secreted monoclonal antibody (mAb) was collected and tested against a panel of various autoantigens and HCV-associated antigens (see Supplementary Methods). The mAb from patient IT01-0199 proved to have RF reactivity and its specificity was confirmed by inhibition assays (Figures 1c and d). In contrast, it did not react against other common autoantigens (nuclear antigens, cardiolipin and beta2-glycoprotein I), previously demonstrated to be targets of other CLL mAbs or a panel of commercially available HCV-associated antigens (HCr43, c200, c100-3 and NSS). Finally, soluble mAbs produced by several CLL-specific heterohybridomas established from CLL cases with IGs unrelated to subset no. 13 were also tested using ELISA and none displayed RF activity, to further prove the specificity of our findings with the mAb from patient IT01-0199.^{10,12}

The subset of CLL cases reported here (subset no. 13) is defined by the expression of mutated stereotyped IGHV4-59/IGKV3-20 mIg, suggesting strong selection of B cells with unique molecular features within their antigen-binding sites. Interestingly, closely related sequences were identified by public database searches originating from patients with different types of B-cell lymphoproliferations, including MC-II, MBL, CLL/SLL, SMZL and SS-MESA. All such cases employed the IGKV3-20 gene, which is also preferentially utilized in HCV-associated immunocytomas¹³ and IgM⁺CD27⁺ B cells with RF activity in HCV-associated MC-II.¹

MC-II is seen as an *in vivo* model of B-cell lymphoproliferation driven either indirectly through immune complexes or directly by HCV-associated antigens.¹² Notably, strong similarities have been

reported between the RF in HCV-associated MC-II and the IG expressed by salivary gland B-cell oligo/monoclonal expansions in primary SS, which can also progress into overt lymphomas, though not generally associated with HCV, suggesting that immune cross-reactivity or molecular mimicry may be involved in the development of these as well as other entities.¹⁰

Taken together, these findings indicate potential parallels between subset no. 13 IG and RFs developing in various disease states, some of which are associated with HCV infection. Intriguingly, some of the entities with stereotyped IGHV4-59/IGKV3-20 BcR described in this study were also positive for serum HCV antibodies. It is perhaps relevant to recall that previous studies have shown that the frequency of HCV seropositivity could be as high as 33% among patients with CLL, well above the controls.^{14,15}

Despite a potential association with HCV infection, the mAb secreted from heterohybridomas of a typical CLL case in subset no. 13 was not directly binding to HCV, whereas it was exhibiting a readily detectable RF activity. Overall, this supports the idea, in this as in other disorders, that HCV may not be directly stimulating the B-cell clone, as one would surmise by the fact that most subset no. 13 cases are HCV negative. However, it does not exclude the possibility that an inflammatory reaction triggered and fuelled by infectious agents (in some cases perhaps HCV) may drive B-cell progenitors or even the malignant B cells themselves by continuously triggering this distinctive BcR. In this scenario, the stereotyped IGHV4-59/IGKV3-20 mIg with RF activity would not be binding directly to the pathogen but receiving indirect stimulation, as RF may bind to immune complexes involving polyclonal IgG directed against indeterminate microbial antigens, similar to what has been long proposed for MC-II and salivary gland B-cell lymphoproliferations in SS.

Subset no. 13 is relatively minor in CLL (12/5798 cases; 0.2%), compared with other CLL subsets with stereotyped mIg.⁶ Yet, the findings reported here indicate that it may be considered as an *in vivo* model to study the role of antigenic and/or infective stimulation in the pathogenesis of CLL. In addition, the existence of the stereotyped IGHV4-59 mIg in a wide range of B-cell lymphoproliferative disorders besides CLL points to the operation of shared, pathogen-triggered, immune-mediated mechanisms, which nevertheless result in clearly distinct pathologic conditions. It remains a matter of speculation how such differences in terms of phenotype and clinical presentation may occur. Perhaps they may be related to the targeting of a distinct B-cell subset and/or the action of a specific microenvironment where the stimulation takes place, yet by similar if not identical antigenic elements.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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