Identification of a novel stereotypic *IGHV4-59/IGHJ5*-encoded B-cell receptor subset expressed by various B-cell lymphomas with high affinity rheumatoid factor activity

Subsets of mucosa-associated lymphoid tissue (MALT) lymphoma, splenic marginal zone B-cell lymphoma (MZBCL) and chronic lymphocytic leukemia (CLL) have been identified that express near-identical B-cell receptors (BCRs), strongly suggesting selection by restricted antigenic epitopes. We here report a new IGV mutated stereotypic IGHV4-59/IGHJ5-encoded BCR subset expressed in hepatitis C virus (HCV)-related B-cell lymphoma, MALT lymphoma and diffuse large B-cell lymphoma (DLBCL). We demonstrate that these mutated stereotypic IGHV4-59/IGHJ5 BCRs are high affinity monoreactive rheumatoid factors (RFs), underscoring the significance of IgG as a major auto-antigen in the pathogenesis of several types of B-cell lymphoma.

Stereotypic BCRs are encoded by highly homologous immunoglobulin variable region (IGV) heavy (H) and light chain rearrangements, having highly similar VH-CDR3. The VH-CDR3 is the most hypervariable region within IGHV, contributing most to the antigenic specificity of an IG. The antigenic binding capacity of an immunoglobulin is also determined by somatic mutations in the IGV regions. The majority of unmutated IG derived of pre-germinal center B cells are of low affinity, whereas somatically hypermutated antibodies of germinal center experienced memory B cells are most often monospecific and of high affinity. It has been shown that antibodies derived from CLL of different unmutated stereotypic BCR subsets display BCR subset-specific polyreactive binding patterns to various auto-antigens.[1] In contrast, antibodies from mutated stereotypic CLL subsets have, in general, more restricted binding characteristics. We recently obtained formal proof that antibodies of different members of a mutated stereotypic BCR subset of IGHV3-7-encoded CLL all displayed high affinity binding to a sugar epitope present in fungi.[2]

MALT lymphomas that express mutated IGV stereotypic RF BCRs were shown to have strong monoreactive RF activity, i.e. autoreactivity with IgG-Fc.[3] Stereotypic RFs are IgM antibodies encoded by typical combinations of restricted IG heavy variable and IG light variable genes with distinct VH-CDR3s. A total of four groups of stereo-

A

	IGHV	IGHV-CDR3 region	Homology	Genbank
Case 105	V1-69/JH4	C ARVFGYE-SNSYFYY WGQG	86%	AHI97205
WOL-RF		C AREYGFDTSDYYYYY WGQG		0707281C
Case 126	V1-69/JH4	C AREADYDSSDYYFFY WGQG	87%	AHI97231
WOL-RF		C AREYGFDTSDYYYYY WGQG		0707281C
Case 121	V1-69/JH4	C AREGQRAATNPFDY WGQG	79%	AHI97221
V1-69-RF, BOR-RF		C AREGRRMAINPFDY WGQG		1313976A
Case 103	V4-59/JH2	C ARDSYCSGGSCFDWYFDL WGRG	100%	AHI97201
V4-59-RF, MR20-RF		C ARDSYCSGGSCFDWYFDL WGRG		AAB58436
Case 110	V4-59/JH2	C ARD-YWCSGGSCFDWYFDL WGRG	94%	AHI97216
V4-59-RF, MR20-RF		C ARDSY-CSGGSCFDWYFDL WGRG		AAB58436

B

	IGHV	IGHV-CDR3 region	Homology	Genbank
Case 106	V4-59/JH5	C ASAGGGIGVTAPGGWFDP WGQG	72%	AHI97207
Case 108	V4-59/JH5	C ASGGGGIAVAGTGGWFDP WGQG	100%	AHI97211
Case 122	V4-59/JH5	C ASGGGGIAVAGTGGWFDP WGQG	100%	AHI97224
DLBCL (223)	V4-59/JH5	C ASGGGGLAVAGTGGWFDP WGQG	94%	CAA73051
OA MALT lymphoma (14)	V4-59/JH5	C ASGGGGISAPGTGGWLDP WGQG	83%	AFC97616

Figure 1. VH-CDR3 amino acid sequence homology of HCV-related B-cell lymphomas with stereotypic rheumatoid factors and with the newly identified IGHV4-59/IGHJ5-encoded stereotypic BCRs. (A) Sequence homology at amino acid level of the HCV lymphomas 105, 126, 121, 103 and 110 with stereotypic WOL-RF, V1-69-RF and V4-59-RF. (B) The HCV lymphomas 106, 108 and 122 as well as a DLBCL 223 and MALT lymphoma 14 from Genbank express highly homologous VH-CDR3 and belong to a newly identified stereotypic IGHV4-59/IGHJ5 BCR subset. Identical amino acids are highlighted in red and homologous amino acids are depicted in blue. A shared serine residue at position 106 is highlighted in green. VH-CDR3 regions are flanked by a Cysteine (C) and a Tryptophan (W). A detailed overview of VH-CDR3 homologies of the stereotypic RF HCV-related lymphomas is provided in the *Online Supplementary Figure S1*.

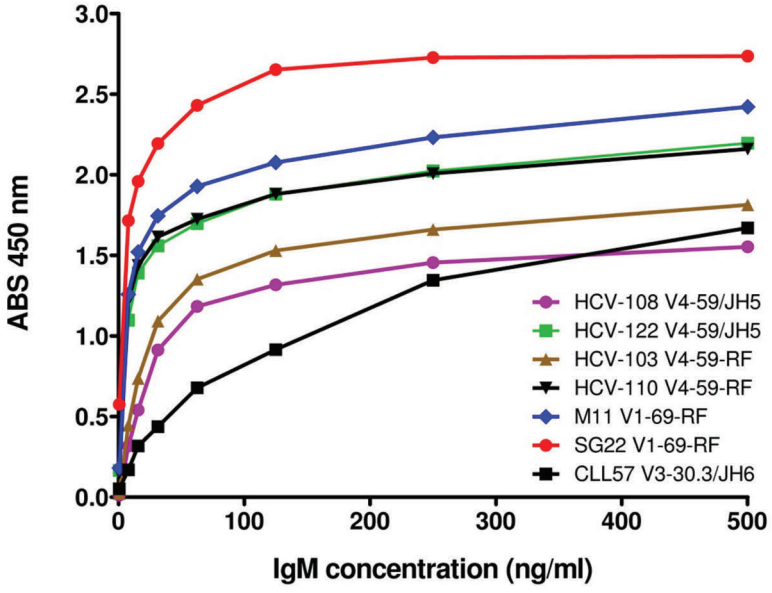
typic IGV-mutated RFs have been identified, encoded by two different IGHV1-69/IGHJ4 rearrangements, designated V1-69-RF and WOL-RF (also known as RFs of the Wa idiotype), and by two different IGHV3-7/IGHJ3 and IGHV4-59/IGHJ2 rearrangements, named V3-7-RF and V4-59-RF.[3,4] Of note, stereotypic V4-59-RFs are identical to CLL subset #13.[5] Stereotypic RF BCRs are expressed by 10%-40% of gastric- and Sjogren's syndrome-associated salivary gland-MALT lymphoma, HCV-related B-cell lymphoma, as well as more rarely by ocular adnexal MALT lymphoma, splenic MZBCL, DLBCL and CLL.[3,5-9]

Polyclonal stereotypic RFs are frequently found in donors immunized with mismatched red blood cells and in HCV-infected patients with type II mixed cryoglobulinemia.[10-14] In addition, none of the four groups of RF-BCRs have been identified to the same extent among the spectrum of B-cell lymphomas. For example, stereotypic V3-7-RFs and V4-59-RFs have both been identified in approximately 0.2% of CLL,[5,8] whereas V1-69-RFs and WOL-RFs have not been described in CLL. Moreover, in splenic MZBCL, 2 cases expressing V4-59-RF[5] and one case expressing WOL-RF[3] have been described, whereas V3-7-RFs and V1-69-RFs have as yet not been identified.

It has been shown in a mouse model that RF-expressing B cells are activated by IgG-chromatin complexes through the synergistic engagement of the RF-BCR and toll-like receptor 9 (TLR9).[15] We previously hypothesized that also human RF-B cells can be co-stimulated by nucleic acid-associated IgG-immune complexes, such as IgG-HCV and IgG-RNA/DNA-containing autoantigens, stimulating both RF-BCRs and TLR7/9.[3]

Recently, Ng *et al.*[16] reported that the recombinantly produced BCRs, such as IgG1 antibodies, of 19 B-cell lymphomas of HCV-infected patients did not react with HCV proteins nor displayed RF reactivity. Prompted by this observation, we analyzed their published IGHV-rearrangements for VH-CDR3 stereotypy, in particular for RF stereotypy. VH-CDR3s were defined to be stereotypic when they shared at least 60% amino acid sequence homology with a maximal gap of 3 amino acids between the amino acid sequences of the VH-CDR3s. The IGHV1-69/IGKV3-20 combination, used by stereotypic V1-69-RFs and WOL-RFs, was expressed by 6 of the 19 lymphomas and 3 of these (numbered 105, 126 and 121) all show, with a maximal gap of 1 amino acid, more than 75% VH-CDR3 amino acid sequence homology with stereotypic RFs, i.e. 2 with WOL-RFs and one with V1-69-RFs (Figure 1A). In addition, the IGHV1-69-

A



B

Patient	IG rearrangement	Sterotypic RF	Actin	Insulin	LPS	dsDNA	ssDNA	human IgG
HCV108	V4-59/JH5							
HCV122	V4-59/JH5							
HCV103	V4-59/JH2	V4-59-RF						
HCV110	V4-59/JH2	V4-59-RF						
M11	V1-69/JH4	V1-69-RF						
SG22	V1-69/JH4	V1-69-RF						
M8	V3-30/JH5							
CLL57	V3-30.3/JH6							

Figure 2. Recombinant antibodies derived from HCV-related lymphomas have strong monoreactive rheumatoid factor activity. Recombinant IgM antibodies derived from HCV lymphomas 108, 122, 103 and 110, control IgM V1-69-RF antibodies M11 and SG22 and a polyreactive IgM CLL57 antibody derived of an IGV-unmutated CLL were tested for binding in ELISA on coated human IgG (A) and on Actin, Insulin, LPS, dsDNA, ssDNA and human IgG (B). M8 is an IgM derived from a Sjögren's syndrome-associated MALT lymphoma with unknown specificity. Red colored boxes indicate positive ELISA results at all IgM concentrations tested, i.e. 125, 250, 500 and 1000 ng/mL (with ABS 450 nm signal/backround levels >3).

expressing lymphoma 101 harbored 69% VH-CDR3 amino acid sequence homology to the IGHV1-69 expressed by an ABC-type DLBCL (*Online Supplementary Figure S1*). Two of five IGHV4-59-expressing lymphomas 103 and 110 showed even more than 90% IGHV-CDR3 amino acid sequence homology to stereotypic V4-59-RFs. Of note, the VH-CDR3 of case 103 is identical to a stereotypic V4-59-RF named MR20-RF (Figure 1A).[11]

More interestingly, we noticed that the HCV-related lymphomas 106, 108 and 122 express stereotypic VH-CDR3 regions, encoded by IGHV4-59/IGHD6-19/IGHJ5 rearrangements. Intriguingly, the VH-CDR3s of lymphoma 108 and 122 are even identical (Figure 1B). Moreover, the IGHV regions of lymphomas 108 and 122 share 5 somatic mutations on a total of 21 and 20 somatic mutations, respectively. All these lymphomas co-express a IGKV3-15/IGKJ1-encoded light chain, further indicating that they, indeed, represent a genuine newly identified stereotypic BCR group. Using the NCBI Protein-BLAST algorithm and literature IGHV searching, in addition, we also identified an ocular adnexal MALT lymphoma[17] and a DLBCL,[18] expressing highly similar VH-CDR3s, encoded by IGHV4-59/IGHJ5 rearrangements (Figure 1B). The frequency of this new stereotypic BCR group is high in the HCV-related lymphoma series of Ng *et al.*[16] (3/19 = 15.8%) but low in DLBCL (1/483 = 0.2%) and in ocular adnexal MALT lymphoma (1/199 = 0.5%), whereas it has as yet not been identified in other B-cell lymphoma entities. An overview of all IGHV sequences used to search for the new stereotypic VH-CDR3 is provided in the *Online Supplementary Table S1*. These five stereotypic IGHV4-59/IGHJ5-encoded lymphomas have in common possession of a serine instead of an arginine residue at position 106 of VH-CDR3, and all have one shared somatic mutation at position 57 in CDR2, resulting in a tyrosine to histidine replacement. Of note, these five lymphomas displayed more than 78% VH-CDR3 amino acid sequence identity (Figure 1B).

To investigate the antigen specificity of the newly identified IGHV4-59/IGHJ5-encoded stereotypic group, we produced the BCRs of lymphomas 108 and 122 recombinantly as soluble IgM antibodies.[2,3,8] The stereotypic V4-59-RFs of HCV lymphomas 103 and 110 were also produced as IgM antibodies, and they did indeed display strong IgG-binding capacity, as previously also shown of a V4-59-RF derived from a CLL,[5] and which is comparable to that of two control lymphoma-derived V1-69-RF IgM antibodies M11 and SG22 originating from Sjögren's syndrome patients.[3,9] Interestingly, also the IgM antibodies 108 and 122 of the newly identified IGHV4-59/IGHJ5 stereotypic BCR group showed strong RF activity (Figure 2A). Of note, none of these four stereotypic RF antibodies were found to be reactive in ELISA with actin, insulin, dsDNA, ssDNA or LPS (Figure 2B).[19] This contrasts with a recombinant IgM antibody CLL57 originating from an IGV-unmutated CLL, which showed RF activity, as well as binding to all the coated antigens of the standard ELISA used to assess polyreactivity (Figure 2B).[2] As a negative control, an IGV-mutated IgM antibody M8 derived from a Sjögren's syndrome-associated MALT lymphoma with unknown specificity was used.[3] Surface plasmon affinity measurements demonstrated that the IGHV4-59/IGHJ5 IgM antibodies 108 and 122 as well as V4-59-RFs IgM antibodies 103 and 110 bound human IgG (RF activity) with KD values of 22.5 (±6.9), 7.2 (±1.6), 10.1 (±0.8) and 3.9 (±0.1) nM, respectively. The KD values for IgG binding of the control V1-69-RF IgM antibodies M11 and SG22 were 5.9 (±1.8) and 4.1 (±0.3) nM, respectively. The lack of *in vitro* RF activity, as observed by Ng *et al.*, is most likely explained by the fact that the HCV lymphoma BCRs were expressed as IgG1 instead of IgM. Charles *et al.*[20] have shown that *in vitro* RF activity of stereotypic RFs is not observed when they are expressed as IgG or as Fab fragments, most likely since IgG antibodies with specificity for IgG-Fc will cross-interact in solution and form IgG complexes precluding binding to the coated polyclonal IgGs in ELISA. Of note, the V1-69-RFs and WOL-RFs have the highest binding activity for IgG1-Fc.[3,20]

In conclusion, we have identified a novel IGHV4-59/IGHJ5-encoded stereotypic subset of somatically mutated BCRs, expressed by three HCV-related B-cell lymphomas, a MALT lymphoma and a DLBCL. Recombinant IgM of both the known V4-59-RF and this new IGHV4-59/IGHJ5 BCR subset were found to bind IgG-Fc with high affinity. Their binding characteristics are fully comparable with those of two stereotypic V1-69-RFs derived from Sjögren's syndrome-related lymphomas and contrast with the broad polyreactivity of an IgM derived from an IGV-unmutated CLL (Figure 2). Moreover, within the series of 19 HCV-related B-cell lymphomas described by Ng *et al.*,[16] as many as eight lymphomas express stereotypic RFs, five of which belong to known stereotypic RF subsets, i.e. two WOL-RFs, one V1-69-RF and two V4-59-RFs, and three representing the novel stereotypic IGHV4-59/IGHJ5-encoded subset. In this study and our earlier work, we further substantiate the high frequency of RF-producing malignant B cells, particularly in inflammation-related B-cell lymphomas, such as HCV-related lymphoma and MALT lymphoma, respectively. These findings further highlight the significance of IgG as a major auto-antigen in chronic inflammatory environments, able to stimulate and provide growth advantage to IgG-Fc-specific B cells and therewith being a key player in the pathogenesis of several types of B-cell lymphomas.[3,5-8]

Richard J. Bende,[1] Jerry Janssen,[1] Thera A.M. Wormhoudt,[1] Koen Wagner,[2] Jeroen E.J. Guikema,[1] and Carel J.M. van Noesel[1]

[1]Department of Pathology and Lymphoma and Myeloma Center Amsterdam (LYMMCARE), Academic Medical Center; and [2]AIMM Therapeutics, Amsterdam, The Netherlands

The online version of this letter has a SupplementaryAppendix.

Correspondence: r.j.bende@amc.uva.nl

doi:10.3324/haematol.2015.139626

Key words: B-cell lymphoma, B-cell receptor, rheumatoid factor, IGHV4-59/IGHJ5.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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