Molecular analysis of immunoglobulin variable genes in human immunodeficiency virus-related non-Hodgkin's lymphoma reveals implications for disease pathogenesis and histogenesis

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ABSTRACT

Background
Human immunodeficiency virus (HIV)-related non-Hodgkin's lymphomas (HIV-NHL) are heterogeneous and associated with distinct molecular pathways. Analysis of immunoglobulin variable genes (*IGV*) may provide insights into the pathogenesis and histogenesis of HIV-NHL.

Design and Methods
IGV rearrangements were amplified from genomic DNA by polymerase chain reaction and directly sequenced in 87 cases of HIV-NHL (17 Burkitt/Burkitt-like lymphomas, 38 diffuse large B-cell lymphomas, and 32 primary central nervous system lymphomas).

Results
A skewed *IGHV* repertoire in specific HIV-NHL clinico-pathological categories was observed. Systemic HIV-diffuse large B-cell lymphomas displayed underrepresentation of the *IGHV3* family (11/38, 28.9%; p=0.0047) and, in particular, of the *IGHV3-23* gene (0/38; p<0.001). These same cases were also characterized by significant overrepresentation of the *IGHV4* family (18/38; 47.4%; p=0.0044) and, in particular, of the *IGHV4-34* gene (10/38; 26.3%; p=0.003). HIV-primary central nervous system lymphomas displayed a preferential usage of *IGLV6-57*, with stereotyped B-cell receptor in two cases. Somatic hypermutation of *IGHV* genes was detected in 81/87 (93.1%) HIV-NHL. Unmutated cases were restricted to six HIV-primary central nervous system lymphomas with immunoblastic plasmacytoid morphology. A mutational profile suggesting a tendency to maintain antigen binding and antigen selection was observed in more than 50% of the cases of *IGV* mutated HIV-NHL.

Conclusions
Our data show evidence of a skewed *IGHV* repertoire in specific HIV-NHL categories and suggest B-cell receptor restriction in some HIV-primary central nervous system lymphomas. The heterogeneous representation of *IGHV* genes in HIV-NHL may be related to specific pathways of antigen stimulation, or to differences in host's immune dysregulation and lymphoma histogenesis.

Key words: HIV, lymphoma, immunoglobulin genes, pathogenesis, histogenesis.

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The online version of this article contains a supplemental appendix.

Introduction

Non-Hodgkin's lymphomas (NHL) are a major complication of human immunodeficiency virus (HIV) infection, and since 1985 have been recognized as an AIDS-defining illness.[1-3] Although the incidence of HIV-related NHL (HIV-NHL) has diminished since the introduction of highly active antiretroviral therapy, NHL constitute an increasing proportion of AIDS-defining events diagnosed in recent years.[4,5]

The vast majority of HIV-NHL are clinically aggressive monoclonal B-cell lymphomas displaying distinctive clinical features, including widespread disease at presentation, poor prognosis and frequent involvement of extranodal sites.[1-3] The pathological spectrum of HIV-NHL includes systemic HIV-NHL, primary central nervous system lymphoma, primary effusion lymphoma, and plasmablastic lymphoma of the oral cavity.[6] Systemic HIV-NHL are histologically classified into HIV-related Burkitt/Burkitt-like lymphoma and HIV-related diffuse large B-cell lymphoma.[6]

The clinico-biological heterogeneity of HIV-NHL might reflect the presence of multiple pathogenetic pathways that have been only partially elucidated so far.[7,8] Analysis of immunoglobulin variable genes (*IGV*) rearranged by HIV-NHL may provide insights into the mechanisms involved in the neoplastic transformation of B cells. A biased usage of immunoglobulin heavy chain variable genes (*IGHV*) and/or immunoglobulin light chain variable gene families or gene segments suggests restricted antigen/superantigen binding. Moreover, analysis of *IGV* mutational profile may provide information on the pressure imposed by the stimulating antigen on the expanding clone.[9-11]

That antigen stimulation plays a critical role in the pathogenesis of HIV-NHL has been suggested by several observations. First, serum immunoglobulins exhibiting specificities for HIV-associated proteins and autoantigens have been isolated in HIV-positive patients with follicular hyperplasia and oligoclonal hypergammaglobulinemia.[12-14] Second, HIV gp120 is a natural ligand for a subset of *IGHV3* genes and may act as a superantigen for *IGHV3* expressing B cells.[15,16] Finally, production of immunoglobulins with specificities for HIV-associated proteins and autoantigens has been observed in a number of HIV-NHL.[17-19] Evidence for a biased usage of *IGHV* genes in HIV-NHL is currently controversial, whereas the usage of *IGV* light chain genes in these lymphomas has not been explored.[20-23] We, therefore, performed a comprehensive analysis exploring usage and mutational profile of *IGV* genes in a large panel of HIV-NHL representative of different clinico-pathological types.

Design and Methods

Patients and pathological specimens

Eighty-seven HIV-NHL specimens, collected from Caucasian HIV-infected patients, formed the basis of this study. Genomic DNA was isolated as previously reported.[24] Approval was obtained from the local Institutional Review Board and informed consent was provided according to the Declaration of Helsinki.

Amplification of IGV gene rearrangements

Rearrangements of *IGHV* and *IGV* light chain genes were amplified with family-specific primers that hybridize to sequences in the IGHV, IGV κ (IGKV) and λ (IGLV) leader, framework region (FR1) 1 or FR2 in conjunction with the corresponding IGHJ, IGKJ or IGLJ primers.[24] Polymerase chain reaction (PCR) was performed for 35 cycles (45 for paraffin-embedded biopsy specimens) with an annealing temperature of 60°C. Inactivation of the *IGKV* locus by rearrangements involving the kappa-deleting element was analyzed as previously reported.[25]

Sequencing and analysis of PCR products

PCR products were directly sequenced with the ABI PRISM BigDye Terminator v1.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Milan, Italy) using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Sequences were aligned to ImMunoGeneTics sequence directory (*http://imgt.cines.fr/*). The *IGHD* germline segment was assigned to the DNA stretch with the highest nucleotide homology, with a minimum of seven successive matches or eight matches interrupted by one mismatch.[26] *IGHV* complementarity determining region 3 (CDR3) length was determined as previously reported.[27] The length of *IGKV* and *IGLV* CDR3 was determined by counting the number of amino acids between position 88 at the end of FR3 and position 97 at the beginning of FR4 (a conserved phenylalanine in all J segments). *IGV* sequences have been submitted to the EMBL database, and accession numbers are listed in *Online Supplementary Table S1*.

Intraclonal analysis of IGV genes

Clonal *IGHV* rearrangements were amplified using the high-fidelity PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) and cloned into the pCR4-TOPO plasmid vector (Invitrogen, Milan, Italy). For each sample, at least 20 clones were sequenced and analyzed using Multiple Sequence Alignment Software.[28] For evaluation of ongoing somatic hypermutation of *IGHV* genes, only clones with identical or near identical CDR3 were considered. In our laboratory, the PfuTurbo DNA polymerase error rate was 0.01%, which amounts to about 0.04 mutations/*IGHV* clone.[24] The frequency of mutation in the tumor clones was compared to Taq error and statistical significance was assessed using the Student's *t* test. The following definitions were also used: unconfirmed mutations – mutations observed in only one clone; confirmed mutations – mutations observed in more than one clone.

Statistical analysis

The Statistical Product and Service Solutions (SPSS) software v.15.0 (Chicago, IL, USA) was used for statistical analyses. The normal B-cell repertoire was compared using previously published data on 206 *IGHV*,

321 *IGKV* and 172 *IGLV* productive rearrangements.[29-31] Fisher's exact test with two-tailed p and χ^2 test, with Bonferroni's adjustment for multiple comparisons, were used to calculate the significance of differences in *IGHV*, *IGHD*, and *IGHJ* use. The parametric t test and the non-parametric Mann-Whitney test were used to calculate the significance of differences in CDR3 length and mutation frequency. Binomial and multinomial distribution models were used to evaluate the distribution of mutations among CDR and FR gene segments.[32,33]

Analysis of viral infection, immunohistochemistry and in situ hybridization

Infection by EBV was investigated by EBER *in situ* hybridization and PCR analysis as previously reported.[24]

Results

Characteristics of the HIV-NHL panel

The HIV-NHL samples (n=87) included 55 cases of systemic HIV-NHL and, for comparative purposes, 32 HIV-primary central nervous system lymphomas. Based on the World Health Organization classification of hematopoietic tumors,[6] systemic HIV-NHL were histologically classified into Burkitt/Burkitt-like lymphomas (n=17) and HIV-diffuse large B-cell lymphomas (n= 38). Depending on the presence of immunoblastic features, HIV-diffuse large B-cell lymphomas were further distinguished into large non-cleaved cell/centroblastic lymphomas (n=22) and large cell immunoblastic plasmacytoid lymphomas (n=16). HIV-primary central nervous system lymphomas were represented by large non-cleaved cell/centroblastic lymphomas (n=7) and large cell immunoblastic plasmacytoid lymphomas (n=25). Representative histological pictures of the different lymphoma subtypes are shown in Figure 1.

The median CD4 count at the time of diagnosis of lymphoma was significantly lower for HIV-primary central nervous lymphomas (36 cells/µL; range, 12-52 cells/µL) than for HIV-Burkitt/Burkitt-like lymphomas (137 cells/µL; range, 5-663 cells/µL; p<0.0001) and for HIV-diffuse large B-cell lymphomas (90 cells/µL; range, 4-446 cells/µL; p=0.0004). The median time from the diagnosis of HIV infection to the diagnosis of lymphoma was 36 months (range, 0-171 months) for HIV-diffuse large B-cell lymphomas, 42 months (range, 0-100 months) for HIV-Burkitt/Burkitt-like lymphomas, and 60 months (range, 36-76 months) for HIV-primary central nervous system lymphomas.

Clonal EBV infection was detected in 62/87 (71.3%) cases, including 9/17 (52.9%) HIV- HIV-Burkitt/Burkitt-like lymphomas, 21/37 (56.8%) systemic diffuse large B-cell lymphomas (7/22, 31.8% diffuse large B-cell lymphomas centroblastic and 14/16; 87.5% diffuse large B-cell lymphomas immunoblastic), and 32/32 (100%) HIV-primary central nervous system lymphomas.

Biased usage of IGHV genes in HIV-NHL

A clonal *IGHV* rearrangement could be identified in all 87 cases of HIV-NHL. A functional *IGHV* rearrangement was identified in 84/87 (96.6%) HIV-NHL. In three cases, represented by EBV-positive systemic diffuse large B-cell lymphoma with immunoblastic plasmacytoid features, a stop-codon was found within the originally productive IGHV rearrangement, leading to a crippled *IGHV* sequence.

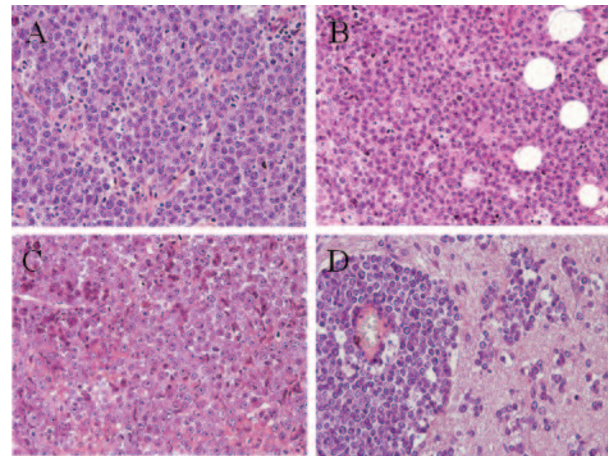

Figure 1. (A) Classic Burkitt's lymphoma (case 10). The tumor displays diffuse proliferation of neoplastic cells containing scant cytoplasm, and round uniform nuclei with two to five small but distinct nucleoli. A starry-sky pattern due to the presence of many stainable macrophages containing abundant clear cytoplasm is also seen (hematoxylin-eosin stain, magnification x400). (B) Atypical Burkitt/Burkitt-like lymphoma (case 15). This case of atypical Burkitt/Burkitt-like lymphoma has nuclear features similar to those of classic Burkitt lymphoma, but it has cells that are more pleiomorphic in size and shape. Also, the nuclei may contain more prominent nucleoli (hematoxylin-eosin stain, magnification x400). (C) Diffuse large B-cell lymphoma with immunoblastic features (case 64). Tumor cells contain abundant, deeply basophilic cytoplasm, with plasmacytoid differentiation; round, oval, or ovoid nuclei show a solitary, prominent, central nucleolus (hematoxylin-eosin stain, magnification x400). (D) Primary central nervous system lymphoma (case 19). Large tumor cells are located close to small vessels (hematoxylin-eosin stain, magnification x400).

The analysis of *IGHV* rearrangements showed evidence for positive and negative selection of specific IGHV families and *IGHV* genes in different clinico-pathological groups of HIV-NHL (Table 1 and Figure 2). In particular a significant overrepresentation of the IGHV4 family (18/38; 47.4%; p=0.0044) and significant underrepresentation of the IGHV3 family (11/38, 28.9%; p=0.0047) were observed in systemic HIV-diffuse large B-cell lymphomas, when compared to the normal B-cell repertoire (25% and 54%, respectively).[29] Conversely, this bias was not observed in HIV-Burkitt/Burkitt-like lymphomas and HIV-primary central nervous system lymphomas (Table 1).

Overall, 13 genes represented 65% of the *IGHV* repertoire of HIV-NHL (Figure 2). The *IGHV* genes most frequently rearranged in HIV-NHL were *IGHV4-34* (14/87; 16.1%) and *IGHV4-39* (6/87; 6.90%). When compared to the normal B-cell repertoire,[29] systemic HIV-diffuse large B-cell lymphomas used *IGHV4-34* at a frequency significantly higher than expected (10/38; 26.3%; versus 5%; p=0.003). Biased usage of *IGHV4-34* was not observed in HIV-Burkitt/Burkitt-like lymphomas (2/17; 11.8%; p=ns) or in HIV-primary central nervous lymphomas (2/32; 6.25%; p=ns). Regarding the IGHV3 family, the *IGHV3-23* gene, which repre-

sents more than 10% of all rearrangements found in the normal B-cell repertoire,[29] was totally absent in HIV-diffuse large B-cell lymphomas (0/38; p<0.001).

Biased usage of immunoglobulin light chain variable genes in HIV-NHL

Clonal *IGLV* and *IGKV* rearrangements were investigated in 54 HIV-NHL, including 14 Burkitt/Burkitt-like lymphomas, 21 systemic HIV-diffuse large B-cell lymphomas, and 19 HIV-primary central nervous system lymphomas. A functional rearrangement was identified in 49/54 (90.7%) cases. Twenty-seven out of 49 (55.1%) cases rearranged a functional *IGLV* gene and 22/49 (44.9%) cases rearranged a functional *IGKV* gene. A crippled IGKV sequence was identified in one HIV-diffuse large B-cell lymphoma, which also carried a crippled *IGHV* rearrangement. Four cases displayed two in-frame, unmutated, *IGKV* rearrangements. In these cases, analysis of the kappa-deleting element showed inactivation of both *IGKV* loci, suggesting the existence of an *IGLV* rearrangement, as confirmed by immunohistochemical analysis of immunoglobulin expression (*data not shown*).

The distribution of the *IGLV* and *IGKV* families rearranged in HIV-NHL differed from that in the normal B-cell repertoire.[30,31] In particular, the *IGLV6* family, represented by the single gene *IGLV6-57*, was positively selected in HIV-NHL (7/27; 25.9%) compared to the non-neoplastic B-cell repertoire (3.5%; p<0.001).[31] Five out of seven *IGLV6-57* rearrangements of HIV-NHL clustered with HIV-primary central nervous system lymphomas. HIV-NHL also displayed a trend toward a biased usage of the *IGLV3* family, occurring in 8/27 (29.6%) cases but in only 16% of normal B cells (p=0.07).[31] Usage of the *IGLV1* family, though recurring in five cases of HIV-NHL, did not differ statistically from that in the normal B-cell repertoire.[31]

The most frequently rearranged *IGKV* family was *IGKV1* (11/22; 50.0%), followed by *IGKV3* and *IGKV4* (5/22; 22.7% each family). The frequency of *IGKV4* family usage in HIV-NHL was higher than expected compared to the usage in the normal B-cell repertoire (5%; p=0.0012),[30] whereas the *IGKV2* family was underrepresented in HIV-NHL (0/22) compared to in normal B cells (19%; p=0.024).[30] When the distribution of individual *IGKV* genes was examined, *IGKV4-1* was the most frequently rearranged *IGKV* gene in HIV-NHL (5/22, 22.7%), followed by *IGKV1-5* and *IGKV1-39/1D-39* (4/22; 18.2%, each gene). *IGKV4-1* gene usage was significantly higher in HIV-NHL than in normal B cells (5%; p=0.0018).[30]

Analysis of CDR3 in HIV-NHL

According to the criteria adopted, a precise *IGHD* family could be assigned to 68/87 (78.2%) *IGHD* segments. The most frequently rearranged *IGHD* family was *IGHD3* (29/68; 42.6%), followed by *IGHD2* (16/68; 23.5%) and *IGHD6* (4/41; 14.6%). The most frequently rearranged *IGHD* segments were *IGHD2-2* and *IGHD3-10* (9/63, 14.3% each segment), followed by *IGHD3-22* (8/63; 12.7%).

Use of *IGHJ* genes in HIV-NHL was consistent with that observed in normal B cells.[29,34,35] In particular, the most frequently rearranged *IGHJ* family was *IGHJ4* (43/87; 49.4%), followed by *IGHJ6* (19/87; 21.8%) and *IGHJ2* (8/87; 9.20%).

Among HIV-NHL with a productive *IGKV* or *IGLV* rearrangement, the distribution of *IGKJ* segments was consistent with that found in normal B cells.[30] Conversely, evidence for positive and negative selection was noted for specific *IGLJ* segments.[31] In particular, the *IGLJ2/3* families were positively selected in HIV-NHL (21/27; 77.8%) compared to in non-neoplastic B cells (39%; p<0.001), whereas the *IGLJ7* family was negatively selected in HIV-NHL (6/27; 22.2%) compared to in non-neoplastic B-cells (54%; p<0.001).

The mean length of the *IGHV* CDR3 was 12.8±5.86 codons for *IGHV*, 10.4±0.87 for *IGLV*, and 9.22±0.67 for *IGKV* rearrangements. The mean lengths of *IGHV*, *IGKV* and *IGLV* CDR3 did not differ significantly among the clinico-pathological types of HIV-NHL.

Table 1. Distribution of *IGHV* families in HIV-NHL.

Histology	*IGHV family*				
	IGHV1	*IGHV2*	*IGHV3*	*IGHV4*	*IGHV5-7*
HIV-NHL	9/87 (10.3%)	3/87 (3.45%)	39/87 (44.8%)	31/87 (35.6%)	5/87 (5.75%)
HIV-Burkitt's/Burkitt like lymphoma	1/17 (5.88%)	1/17 (5.88%)	8/17 (47.0%)	5/17 (29.4%)	2/17 (11.7%)
HIV-diffuse large B-cell lymphoma	6/38 (15.8%)	2/38 (5.26%)	11/38 (28.9%)[a]	18/38 (47.4%)[a]	1/38 (2.63%)
HIV-primary central nervous system lymphoma	2/32 (6.25%)	0/32	20/32 (62.5%)	8/32 (25.0%)	2/32 (6.25%)

[a]*Significant difference between HIV-diffuse large B-cell lymphoma and normal B-cell repertoire.*

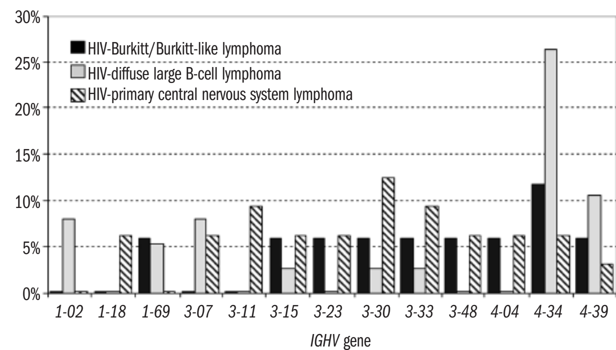

Figure 2. Distribution of *IGHV* genes rearranged in HIV-NHL. The figure shows only genes that, overall, represent >60% of the total *IGHV* rearrangements detected in HIV-NHL. Systemic HIV-diffuse large B-cell lymphomas used *IGHV4-34* at a frequency significantly higher than expected (10/38, 26.3%; p=0.003). Biased usage of *IGHV4-34* was not observed in HIV-Burkitt/Burkitt-like lymphomas (2/17, 11.8%; p=ns) or HIV-primary central nervous system lymphomas (2/32, 6.25%; p=ns). With regards to the *IGHV3* family, the *IGHV3-23* gene, which represents more than 10% of all rearrangements found in the normal B-cell repertoire,[30-32] was absent in HIV-diffuse large B-cell lymphomas (0/38; p<0.001).

Table 2. Characteristics of *IGHV* and *IGV* light chain gene rearrangements in *IGLV6-57* positive HIV-NHL.

Case	Histology	IGH					IGL			
		IGHV	IGHD[a]	IGHJ	Mutation (%)	CDR3[b]	IGLV	IGLJ	Mutation (%)	CDR3[b]
26	HIV-primary central nervous system lymphoma	1-18	3-16	4	8.15	*R***D**L*R***DD**VWG**DD**Y*R*LF**D**YW	6-57	2/3	2.37	CQSY**D**SSN*R*AVF
81	HIV-primary central nervous system lymphoma	1-18	na	4	5.71	*R***D**L*R***DD**IFG**D**Y*R*LF**D**YW	6-57	2/3	1.71	CQSY**D**SSH*K*AVF
25	HIV-primary central nervous system lymphoma	3-15	6-6	4	7.10	GG*R*VGA*R*PLP**D**YW	6-57	3	3.50	CQSY**D**SSNPVF
72	HIV-primary central nervous system lymphoma	3-30	na	4	7.12	*R*SYA*R*GIPW	6-57	2/3	6.96	CQSS**D**NSNNVIF
74	HIV-primary central nervous system lymphoma	3-7	6-19	6	13.70	*R*HGVGGGWFYFYYAM**D**VW	6-57	7	1.88	CQSY**D**SSNNVVF
57	HIV-diffuse large B-cell lymphoma	1-69	3-10	4	22.20	*K***E**GL*R*TMSNPN**D**YW	6-57	7	4.24	CQSY**D**SSNLWVF
66	HIV-diffuse large B-cell lymphoma	4-34	3-16	5	13.20	*R*FGS*R*GGNHYISWF**D**PW	6-57	7	7.45	CQSY**D**SSNPYAVF

[a]na, not assignable; [b]positively charged residues are in italics and underlined; negatively charged residues are represented in bold type.

As stated above, the *IGLV6-57* gene was positively selected in HIV-NHL. Detailed analysis revealed that two *IGLV6-57* cases (cases 26 and 81 in Table 2) rearranged the *IGHV1-18* gene and had highly similar *IGHV* CDR3 amino acid sequences, differing only by conservative substitutions. The *IGLV6-57* CDR3 sequence of both these *IGHV1-18* HIV-primary central nervous system lymphomas was characterized by the presence of one basic amino acid (an arginine in case 26 and a lysine in case 81), which was not observed in the other *IGLV6-57* cases.

Analysis of mutational profile of IGV genes in HIV-NHL

Somatic hypermutation of *IGHV* genes was detected in 81/87 (93.1%) cases of HIV-NHL. Unmutated cases were restricted to six HIV-primary central nervous system lymphomas with large cell immunoblastic plasmacytoid morphology. Among mutated cases, the average mutation frequency was 8.96±5.43% (median 7.19; range, 2.00–23.6%) for *IGHV*; 5.49±2.98% (median 5.00; range, 1.71-11.7%) for *IGLV*; and 5.01±2.69% (median 3.59; range, 2.10-11.6%) for *IGKV* rearrangements. The mutation frequency was comparable among the different clinico-pathological categories of HIV-NHL and no differences were observed between EBV-positive and EBV-negative cases (*data not shown*).

The distribution of mutations was analyzed using binomial and multinomial distribution models on all HIV-NHL carrying functionally rearranged and somatically mutated *IGHV* genes. A lower than expected number of replacement mutations in the FR was observed in 48/78 (61.5%) HIV-NHL, namely 8/17 (47%) HIV-Burkitt/Burkitt-like lymphomas, 25/35 (71.4%) systemic HIV-diffuse large B-cell lymphomas, and 15/26 (57.7%) HIV-primary central nervous system lymphomas. A higher than expected number of replacement mutations in the CDR was observed in 36/78 (46.1%) HIV-NHL, namely 5/17 (29.4%) HIV-Burkitt/Burkitt-like lymphomas, 19/35 (54.3%) systemic HIV-diffuse large B-cell lymphomas, and 12/26 (46.1%) HIV-primary central nervous system lymphomas.

Analysis of ongoing somatic hypermutation of IGHV genes

Intraclonal variation of *IGHV* genes was assessed by extensive molecular cloning of 15 *IGHV* gene isolates derived from 15 different HIV-NHL specimens, including four HIV-Burkitt/Burkitt-like lymphomas, seven systemic HIV-diffuse large B-cell lymphomas, and four HIV-primary central nervous system lymphomas. In all cases, the clonal *IGHV* sequence had been previously established by direct DNA sequencing of the PCR product. In 13/15 (86.7%) HIV-NHL, the clonal *IGHV* isolates did not show intraclonal heterogeneity, indicating absence of ongoing *IGHV* mutations (Table 3). Conversely, evidence of ongoing somatic hypermutation was detected in 1/4 HIV-Burkitt/Burkitt-like lymphomas and in 1/7 HIV-diffuse large B-cell lymphomas with centroblastic morphology (Table 3).

Discussion

By analyzing the mutational profile and usage of *IGHV, IGKV*, and *IGLV* genes in a series of 87 HIV-NHL, we show an abnormal distribution of *IGV* gene usage in these lymphomas, suggesting the presence of selective forces acting on the *IGV* repertoire expressed by HIV-NHL. Analysis of *IGV* gene usage in HIV-NHL showed evidence of a skewed *IGHV* repertoire in specific clinico-pathological categories of the disease. First, systemic HIV-diffuse large B-cell lymphomas display underrepresentation of the *IGHV3* family and, in particular, of the *IGHV3-23* gene. Second, systemic HIV-diffuse large B-cell lymphomas are characterized by significant overrepresentation of the *IGHV4* family and, in particular, of the *IGHV4-34* gene. Third, HIV-primary central nervous system lymphomas show a preferential usage of *IGLV6-57*. Conversely, HIV-Burkitt/Burkitt-like lymphomas show an *IGV* repertoire similar to that observed in normal, mature B cells. The heterogeneous representation of *IGHV* genes in HIV-NHL may be related to differences in the host's immune dysregulation and/or in lymphoma histogenesis.

Published data on *IGV* usage in HIV-positive patients

Table 3. Analysis of ongoing somatic hypermutation of *IGHV* genes in HIV-NHL.

Case	*Histology*	*EBV status*	*IGHV gene*	*% mutations*	*N. of DNA clones analyzed*	*N. of confirmed mutations*[a]	*N. of unconfirmed mutations*[b]	*Ongoing somatic hypermutation*[c]
6	HIV-diffuse large B-cell lymphoma, centroblastic	–	3-7	6.12	20	0	0	No
8	HIV-diffuse large B-cell lymphoma, centroblastic	POS	2-70	2.68	25	0	0	No
43	HIV-diffuse large B-cell lymphoma, centroblastic	–	4-34	4.12	21	0	1	No
49	HIV-diffuse large B-cell lymphoma, centroblastic	POS	4-34	3.75	20	0	1	No
51	HIV-diffuse large B-cell lymphoma, centroblastic	–	4-34	11.60	25	11	24	Yes
14	HIV-diffuse large B-cell lymphoma, immunoblastic	–	4-59	21.60	20	0	3	No
64	HIV-diffuse large B-cell lymphoma, immunoblastic	–	4-34	5.49	22	0	2	No
27	HIV-primary central nervous system lymphoma, centroblastic	POS	3-23	6.18	23	0	0	No
29	HIV-primary central nervous system lymphoma, immunoblastic	POS	4-4	8.33	20	0	0	No
32	HIV-primary central nervous system lymphoma, immunoblastic	POS	4-34	21.60	24	4	2	No
28	HIV-primary central nervous system lymphoma, immunoblastic	POS	6-1	7.92	22	0	2	No
10	HIV-Burkitt/Burkitt-like lymphoma	–	4-39	11.20	25	0	0	No
15	HIV-Burkitt/Burkitt-like lymphoma	–	4-4	18.50	22	13	15	Yes
17	HIV-Burkitt/Burkitt-like lymphoma	–	4-61	8.08	20	1	1	No
62	HIV-Burkitt/Burkitt-like lymphoma	POS	3-33	5.78	24	2	2	No

[a]*Mutations observed in more than one clone.* [b]*Mutations observed in only one clone.* [c]*Frequency of mutation differing significantly from the observed PfuTurbo DNA polymerase error rate (p<0.05 based on Student's t test).*

show that the naïve B-cell repertoire is similar in these patients to that in HIV-negative controls. Conversely, the pool of non-neoplastic, activated/memory B cells of HIV-positive patients displays reduced usage of the IGHV3 family.[36-41] In particular, the depletion of *IGHV3* B cells is directly correlated to the reduction of the CD4 count and/or to viral load.[16,39,42] The reduction of *IGHV3* B cells mainly involves the *IGHV3-23* and *IGHV3-30/3-30.5* genes that directly bind HIV gp120, and may be accompanied by an increase in the relative representation of *IGHV4* B-cells.[41,42] Our results, compared to published data, may explain, at least in part, the underrepresentation of the most commonly used *IGHV3* genes in HIV-diffuse large B-cell lymphoma, and suggest that a subset of HIV-diffuse large B-cell lymphomas, but not of HIV-Burkitt/Burkitt-like lymphomas or HIV-primary central nervous system lymphomas, may derive from activated, post-germinal center, *IGHV3*-depleted B cells. A biased usage of the *IGHV4-34* gene occurs in systemic HIV-diffuse large B-cell lymphomas, but not in HIV-Burkitt/Burkitt-like lymphomas or HIV-primary central nervous system lymphomas. The high prevalence of *IGHV4-34* that we detected in systemic HIV-diffuse large B-cell lymphomas may suggest a specific role for an autoreactive antigen/superantigen that drives the growth of these lymphomas. This hypothesis is corroborated by the observation that *IGHV4-34* encoded antibodies are intrinsically autoreactive.[43-45] In healthy individuals, *IGHV4-34* cells are predominantly expressed and expanded in the naïve B-cell repertoire, but are underrepresented in the germinal center and memory compartments, probably because these cells are prevented from differentiating into antibody–producing plama cells.[44] However, in the context of immune dysregulation and HIV infection, censoring of *IGHV4-34* B cells may be bypassed and lymphoproliferative disorders expressing the *IGHV4-34* genes might be favored.[46]

In HIV-primary central nervous system lymphomas, we failed to detect a prevalence of *IGHV4-34*, which, conversely, is frequently utilized by HIV-negative primary central nervous system lymphomas.[47] Our data are in agreement with those of a previous study that reported no biased usage of *IGHV* families in HIV-primary central nervous system lymphomas.[22] The difference between *IGHV4-34* usage in HIV-primary central nervous system lymphomas and non-HIV related cases supports the concept that HIV-related and HIV-unrelated primary central nervous system lymphomas represent separate disease entities with distinct pathogeneses. On the other hand, we observed a biased usage of the *IGLV6-57* gene among HIV-primary central nervous system lymphomas. Notably, two HIV-primary central nervous system lymphomas expressing the *IGLV6-57* gene also used the same *IGHV1-18* gene and their CDR3 displayed a high degree of identity. These data provide the first documentation of B-cell receptor restriction in this category of lymphoma. Evidence of B-cell receptor restriction in B-cell malignancies has been reported for B-cell chronic lymphocytic leukemia and hepatitis C virus-related lymphomas associated with autoimmune disorders, and is regarded as a proof of B-cell stimulation and selection by a common antigen in lymphomagenesis.[48,49] In the context of the pathogenesis of primary central nervous system lymphomas, antigens of the central nervous system microenvironment might favor the expansion of lymphomas bearing particular B-cell receptor features recognizing such molecules.

The role of antigen stimulation in the pathogenesis of most HIV-NHL is further supported by a mutational profile consistent with a tendency to maintain antigen binding and antigen selection in more than 50% of *IGV*-mutated HIV-NHL. These features are at variance

with those observed in other lymphomas associated with immunodeficiency, namely post-transplant lymphoproliferative disorders, whose molecular features suggest a minor role for antigen stimulation.[24,50,51] In particular, the different role exerted by B-cell receptor stimulation in the pathogenesis of HIV-NHL and post-transplant lymphoproliferative disorders is indicated by the presence of *IGV* inactivation by crippling mutations introduced by somatic hypermutation in nearly a quarter of post-transplant lymphoproliferative disorders,[24,50,51] whereas crippling mutations of *IGV* rearrangements are a very rare finding among HIV-NHL (3%; this study). Interestingly, each case of HIV-NHL carrying *IGV* inactivation by crippling mutations was positive for EBV infection of the tumor clone. This is reminiscent of the situation observed in classical Hodgkin's lymphomas, in which all cases with destructive mutations in *IGV* genes were found to be EBV-positive.[52] Also, EBV infection is found in many post-transplant lymphoproliferative disorders with crippled *IGV* genes.[24,50,51] Overall, these observations support a central role of EBV in the pathogenesis of lymphomas with impaired B-cell receptor.

Our results show that over 90% of HIV-NHL have highly mutated *IGV* genes. These data are in agreement with previous observations, and support the notion that HIV-NHL originate from B cells that have persistently experienced the germinal center reaction.[18,20-23,53] Because ongoing somatic hypermutation of *IGV* genes is a rare event in HIV-NHL (as shown by this study and Delecluse *et al.*[21] and Bellan *et al.*[53]), conceivably both HIV-Burkitt/Burkitt-like lymphomas and HIV-diffuse large B-cell lymphomas, regardless of EBV infection status, are histogenetically related to B cells that have terminated the germinal center reaction.

In this study, HIV-NHL devoid of somatic hypermutation were restricted to a fraction of HIV-primary central nervous system lymphomas with large cell immunoblastic plasmacytoid morphology. HIV-diffuse large B-cell lymphomas with unmutated *IGHV* genes have also been reported in a previous study, suggesting that the origin of these HIV-NHL subsets can be traced to naïve B cells that have not experienced the germinal center reaction and microenvironment.[21] Because both HIV-primary central nervous system lymphomas with large cell immunoblastic plasmacytoid morphology and plasmablastic lymphomas of the oral cavity express well-established markers of post-germinal center B cells,[8] these findings indicate that this fraction of HIV-NHL devoid of somatic hypermutation may represent the transformation of B cells experiencing a preterminal differentiation independent of the germinal center reaction.

Authorship and Disclosures

DC designed the study, performed molecular analyses, analyzed data and wrote the manuscript; MM and AG provided patients' samples and performed and interpreted immunophenotypic analyses; MC, SR, CD and DR performed molecular analyses; LML and AC provided patients' samples and revised the article for intellectual content; GG supervised the whole study and revised the last version of the manuscript. All authors approved the final version of the manuscript. The authors reported no potential conflicts of interest.

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