B-cell receptor usage correlates with the sensitivity to CD40 stimulation and the occurrence of CD4+ T-cell clonality in chronic lymphocytic leukemia

Nadja Zaborsky,[1,2] Claudia Holler,[1,2] Roland Geisberger,[1,2] Daniela Asslaber,[1,2] Franz Josef Gassner,[1,2] Viktoria Egger,[1,2] Josephina Piñón-Hofbauer,[1,2] Thomas Kocher,[1,2] Tanja Nicole Hartmann,[1,2] Richard Greil,[1,2] and Alexander Egle[1,2]

[1]Laboratory for Immunological and Molecular Cancer Research, 3rd Medical Department with Hematology, Medical Oncology, Hemostaseology, Infectious Diseases and Rheumatology, Oncologic Center, Paracelsus Medical University Salzburg, and [2]Salzburg Cancer Research Institute, Austria

Correspondence: n.zaborsky@salk.at
doi:10.3324/haematol.2015.124719

Supplemental Materials and Methods

CLL samples.

Peripheral blood samples from CLL patients at our medical department were collected upon informed consent in accordance with the Declaration of Helsinki and upon approval by the ethics committee of Salzburg, Austria (Ref. No. 415-E/1287/4–2011 and 415-E/1287/8–2011). The patients' characteristics are shown in Supplemental Table 1 and 2. Peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation using Biocoll (Biochrom AG). The determination of prognostic markers were performed as previously described [1].

Analysis of BCR IgVH gene usage and mutational status were performed as described previously [2;3]. PCR amplification of VH gene rearrangements was performed on cDNA from CLL cells using seven VH-family specific 5'-leader-primers (VH1/VH7 5'-atg gac tgg acc tgg agg-3', VH2 5'-cac (AG)ct cct gct gct gac ca-3', VH3a 5'-gct ggg ttt tcc ttg ttg c-3', VH3b 5'-atg gag tt(gt) gg(ag) ctg agc tg-3', VH4 5'-gct ccc aga tgg ggt cct g-3', VH5 5'-ctc ctc ctg gct gtt ctc c-3', VH6 5'-ctg tct cct tcc tca tct tc-3') in combination with an IgM specific 3' primer 5'-cag gag aaa gtg atg gag tcg-3'. The PCR amplicons were gel purified (Qiagen) and sequenced (Eurofins Operon, Germany). The sequences were aligned to germline immunoglobulin sequences from the IMGT database (www.imgt.org) [4-6] to determine IgVH mutation status, stereotypy and VDJ usage. IgVH gene sequences with less than 98% homology to the corresponding germline sequence were defined as mutated. BCR stereotypy was defined according to Stamatopoulos et al [7], where BCRs were classified as stereotyped in case their CDR3 amino acid sequences share ≥60% similarity and do not differ in length in more than three amino acids with sequences from published datasets [8-10].

TCR Vβ spectratyping.

$CD4^+$ T cells were isolated from PBMCs using anti-CD4 magnetic beads according to the manufacturer's instructions (Miltenyi Biotech, Germany). Total RNA was isolated (Qiagen) and first strand cDNA was generated (iScript, Biorad). Rearranged TCR Vβ genes were PCR amplified from cDNA using a panel of 20 TCR Vβ specific 5' primers and a TCR constant region-specific 3' WellRED-D4PA labeled primer (5'-TTC TGA TGG CTC AAA CAC-3'; PROLIGO Primers & Probes). Primer sequences are described in [11]. Spectratyping was carried out on a CEQ 8000 (Beckmann Coulter).

Flow cytometry.

For the characterization of T cell subsets, fresh blood samples were stained and subsequently red blood cells were lysed using FACS lysing solution (Becton Dickinson). The following antibodies were used: CD8 PE-Cy7, CD4 ECD, CD45RA FITC, CD3 PE, CD62L PE-Cy5 (all from Beckman Coulter), CD8 Pacific Orange (Invitrogen), CD4 PC7, CD3 Alexa Fluor 700 (eBioscience) PD1 Brilliant Violet 421 and isotype control (Biolegend). For determination of TCR Vβ usage, PBMCs were stained with the IOTest® Beta Mark Kit (Beckman Coulter) according to manufacturer's instructions in combination with antibodies for CD3 and CD4, where, eight sets of three TCR Vβ - specific antibodies (each set comprises a FITC, PE and FITC/PE conjugated antibody) are combined in a single test. Analysis of CLL cell proliferation upon stimulation with CD40L expressing fibroblasts (NIH3T3) or mock transfected NIH3T3 cells (MOCK) was performed by intracellular Ki67 staining as previously described [12]. Samples were measured on a Gallios flow cytometer (Beckman Coulter) and analysed using FlowJo software 7.6.5 (TreeStar, USA) and Kaluza 1.2 (Beckman Coulter).

Reference List

1. Tinhofer I, Rubenzer G, Holler C et al. Expression levels of CD38 in T cells predict course of disease in male patients with B-chronic lymphocytic leukemia. Blood 2006;108:2950-2956.

2. Campbell MJ, Zelenetz AD, Levy S, Levy R. Use of Family Specific Leader Region Primers for Pcr Amplification of the Human Heavy-Chain Variable Region Gene Repertoire. Molecular Immunology 1992;29:193-203.

3. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94:1848-1854.

4. Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Research 2008;36:W503-W508.

5. Giudicelli V, Wu Y, Brochet X et al. Databases and tools IMGT (R): use in hematological cancers and in the humanization of antibodies. Bulletin du Cancer 2009;96:S31.

6. Lefranc MP, Giudicelli V, Ginestoux C et al. IMGT (R), the international ImMunoGeneTics information system (R). Nucleic Acids Research 2009;37:D1006-D1012.

7. Stamatopoulos K, Belessi C, Moreno C et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: pathogenetic implications and clinical correlations. Blood 2007;109:259-270.

8. Stamatopoulos K, Belessi C, Moreno C et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: pathogenetic implications and clinical correlations. Blood 2007;109:259-270.

9. Murray F, Darzentas N, Hadzidimitriou A et al. Stereotyped patterns of somatic hypermutation in subsets of patients with chronic lymphocytic leukemia: implications for the role of antigen selection in leukemogenesis. Blood 2008;111:1524-1533.

10. Rossi D, Spina V, Cerri M et al. Stereotyped B-Cell Receptor Is an Independent Risk Factor of Chronic Lymphocytic Leukemia Transformation to Richter Syndrome. Clinical Cancer Research 2009;15:4415-4422.

11. Rezvany MR, Jeddi-Tehrani M, Osterborg A et al. Oligoclonal TCRBV gene usage in B-cell chronic lymphocytic leukemia: Major perturbations are preferentially seen within the CD4 T-Cell subset. Blood 1999;94:1063-1069.

12. Asslaber D, Groessinger EM, Girbl T et al. Mimicking the microenvironment in chronic lymphocytic leukaemia where does the journey go? British Journal of Haematology 2013;160:711-714.

Supplemental Table 1. Clinical parameters of patients

Clinical parameters	Patients [n (%)]
Age (years) [mean, (range)]	70 (49 – 83)
Sex	
male	28 (51%)
female	27 (49%)
IgVH mutational status/stereotypy	
mutated (stereo/non-stereo)	15/28 (35%/65%)
unmutated (stereo/non-stereo)	7/5 (58%/42%)
RAI stage	
0	22 (40%)
1	18 (33%)
2	13 (24%)
3	2 (4%)
Genomic aberrations	
Favourable (absent, del13q)	48 (87%)
Intermediated (tris12)	4 (7%)
Unfavourable (del11q, del17p)	3 (6%)

Supplemental Table 2. Amino acid sequences of CDR3 regions of IgHV genes from CLL samples.

BCR stereotypy was defined according to Stamatopoulos et al (Stamatopoulos *et al*, 2007), where BCRs where classified as stereotyped in case their CDR3 amino acid sequences share ≥60% similarity and do not differ in length in more than three amino acids with sequences from published datasets (Stamatopoulos *et al*, 2007;Murray *et al*, 2008;Rossi *et al*, 2009).

mutated – non stereotyped n=28

>2575
CARHGSNFLGMDVW
>2499
CAGQVWSLFDPW
>3265
CAREERFFSWMLYGGRSNNYGMDVW
>3421
CARDQQPSGSYSISFDYW
>2826
CARPQFFSGWNAFQHW
>3106
CTTDCSSPNCSPYMDVW
>3323
CARAGHVDISAFDIW
>2625
CARGGGIGFGDYGDSW
>2772
CARELLGWTWWFDPW
>2508
CVAGPSQWFDPW
>2593
CARHSRAVDGHEGKIDYW
>2453
CANSHQWELFNYW
>3261
CARDLVVASIPLDNW
>2568
CARVAVAGRWEGFGDLEEIW
>2609
CARREELWTYAVGDGLDIW
>2739
CTIGDHGHHDAFDMW
>3595
CTRGGVGDGTNPFDPW
>2463
CVRDWGVTIFGVAYPDYW
>2727
CAHRQYGDYTLGYW
>2719

CAGAPLTRYW
>2578
CASQGHWNFDYW
>2669
CARNGGSLRSQPWDFDY
>3636
CAGGRTCFDLW
>2671
CAKDSLGFGGYFDYW
>2790
CAHSSKHMVFFFYGSGTHWDHFDYW
>3816
CARTTTVAGTGVGYFFEYW
>2598
CASEAGSGGPPPYPALTF
>2736
CAKDRGGSGWFFDYW

mutated – stereotyped n=15

>2574
CAKERSDWYYFDYW
>2663
CARGFGYSYGNEYFDSW
>2735
CSKSGVTYYDSSGYYGGYW
>2798
CAKLSSGSGNYGCMDVW
>3175
CAREYKFDNWFFDLW
>2731
CARYDRISYKYYMGVW
>2947
CVADRNVMDVW
>2783
CARDGWEPPPDAFDVW
>3270
CARGRDAYSSCPFDFW
>3407
CARTRYCSSTTCRGAFDLW
>2561
CAKHQQLSVNYYYYYYMDVW
>3817
CAKVTRMGAIEEFYYYGVDVW
>2557
CAKDIGSGFYHPFDYW
>2653
CAGGPGTPGDFDYW
>2837
CARGPNQSGWNEFDYW

unmutated – non stereotyped n=19

>2793
CARGPRIKQWLGMGAFDIW
>2579
CARDGVDTMVRGVITGLDAFDIW
>2523
CARDNWGVRFLEWLSSYYFDYW
>3091
CARGVEMATIRGLGYYYYYGMDVW
>3546
CAGLNWGGDCYFCGAFDIW
>4552
CARDTSRGACSGGSCYSGAFDIW
>6893
CAHSDFWSGYLNFDYW
>5535
CARGPRIKQWLGMGAFDIW
>4296
CARGSANYDFWKNYYYYMDVW
>8228
CARGPYCSSTSCYFYGMDVW
>8096
CTTDPKVDIVVVPPGRVVYADTLDYW
>7812
CAKDIRRNDDYDSSIWGGMDVW
>5913
CARDRPYVWGSYRYYYYYYMDVW
>7331
CAREFIGDSSGYYYYYYYGMDVW
>7767
CARDGDRLPKYSSGWYSHRYWYFDLW
>6328
CARGRGYCSSTSCYVDTTMVTELDYW
>6146
CAKDYSQEGSTFDYW
>7493
CAKDEGYGYYDFWSGLPFDYW
>6354
CARQQIAVAGNWYFDLW

unmutated – stereotyped n=10

>2632
CVADRNVMDVW
>2715
CARDHSHRDDFWSGYYHYFDYW
>2774
CARHRLGYCSSTSCYYYYYGMDVW
>2835
CAREGQWLDTYYYYYGMDVW
>3172
CARALPQYYDFWSGFPSVAFDIW
>2902
CARGGNYDYIWGSYRSNDAFDIW

>3406
CAHARALRNDDSGYYFGFDVW
>8174
CARVTIFGVVSSNYYYYYYMDVW
>7827
CARQGAYYDFWSGYYLPGWFDPW
>7301
CARHLWFGEYHFDYW

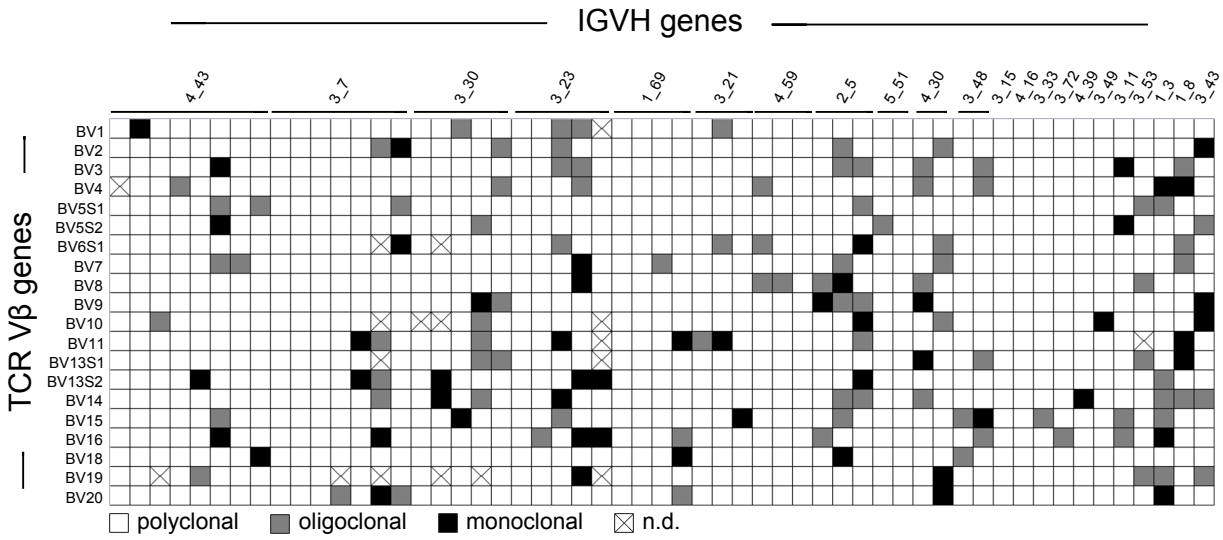
Supplemental Table 3. Clinical parameters of selected patients for Fig 2F and G (selected for the presence of overrepresented CD4$^+$ T cells and patients with unmutated IgHV)

Additional Pat for Figure 2F

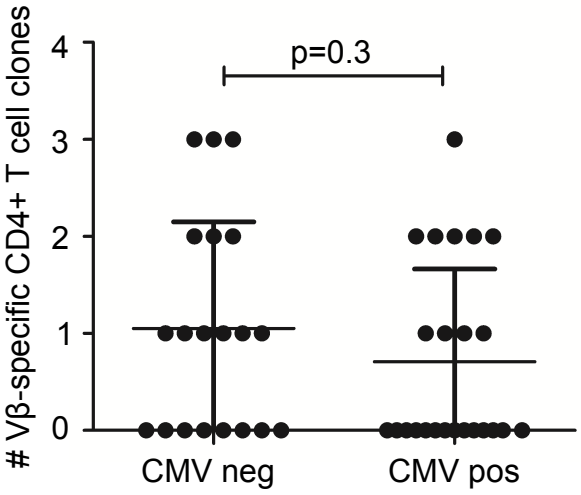
Pat ID	Mutation status	Stereotypy	Age	Sex	Rai	Genomic aberrations	CD38	Zap70	therapy
73	M	n.d.	78	f	1	del13q	low risk	low risk	therapy

Additional Pat for Figure 2G

Pat ID	Mutation status	Stereotypy	Age	Sex	Rai	Genomic aberrations	CD38	Zap70	therapy
326	UMut	NST	81	f	2	absent	low risk	low risk	chemonaiv
270	UMut	NST	87	f	2	absent	high risk	high risk	therapy
182	UMut	NST	51	m	3	absent	high risk	high risk	therapy
424	UMut	NST	66	f	1	del13q, del17p	low risk	low risk	chemonaiv
430	UMut	NST	51	m	0	absent	high risk	low risk	chemonaiv
293	UMut	NST	80	f	0-1	absent	high risk	low risk	chemonaiv
153	UMut	ST	87	m	1	absent	high risk	high risk	therapy
462	UMut	NST	67	f	0-1	tris12	low risk	low risk	chemonaiv
242	UMut	NST	61	f	1	del13q	low risk	high risk	chemonaiv
337	UMut	NST	69	m	0	del11q, del13q	high risk	low risk	therapy
379	UMut	ST	83	f	0	tris12	high risk	low risk	chemonaiv
353	UMut	NST	73	m	2	del13q	low risk	high risk	chemonaiv
81	UMut	NST	75	m	2	tris12	high risk	n.d.	therapy
449	UMut	NST	61	f	0	del13q	low risk	high risk	chemonaiv
381	UMut	ST	79	m	0	absent	high risk	high risk	chemonaiv
411	UMut	NST	77	f	0	del13q	low risk	low risk	chemonaiv
230	UMut	NST	74	f	0	absent	high risk	high risk	chemonaiv



Supplemental Figure 1. Heat map of TCR Vß clonality in patients classified according to IgHV gene usage.



Supplemental Figure 2. Frequency of clonal CD4+ T cells in CMV seropositive versus seronegative patients.