Detection of monoclonal T populations in patients with KIR-restricted chronic lymphoproliferative disorder of NK cells

Cristina Gattazzo,[1,2] Antonella Teramo,[2] Francesca Passeri,[1,2] Elena De March,[1] Samuela Carraro,[1] Valentina Trimarco,[1,2] Federica Frezzato,[1,2] Tamara Berno,[1] Gregorio Barilà,[1] Veronica Martini,[1,2] Francesco Piazza,[1,2] Livio Trentin,[1,2] Monica Facco,[1,2] Gianpietro Semenzato,[1,2] and Renato Zambello[1,2]

[1]Department of Medicine, Hematology and Clinical Immunology Branch, Padua University School of Medicine; and [2]Venetian Institute of Molecular Medicine (VIMM), Padua, Italy

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Correspondence: g.semenzato@unipd.it

MATERIALS AND METHODS

Flow cytometry analysis

The collection of peripheral blood samples from patients was performed during medical examination.

Peripheral blood mononuclear cells (PBMCs) were isolated through Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) and washed twice in phosphate-buffered saline. PBMCs were studied for the expression of cell surface antigens with direct four color analysis using a panel of commercially available fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy5 and allophycocyanin-conjugated (APC) mAbs with the specific isotype-matched control reagents (BD Pharmingen, San Diego, CA, USA; R&D Systems, Toronto, Canada; Caltag Laboratories; Immunotech, Marseille, France), that included mouse anti-human -CD16, -CD3, -CD4, -CD8, -CD57, and -CD56 markers. The following mAbs were kindly provided by Drs A. Moretta and M. Vitale (Genova, Italy): EB6 (IgG1, anti-CD158a), GL183 (IgG1, anti-CD158b), FES172 (IgG2a, anti-CD158i), Z27 (IgG1, anti-CD158e), Q66 (IgM, anti-CD158k), XA185 (IgG1, anti-CD94), Z199 (IgG2b, anti-NKG2A). The expression of the above quoted antigens on LGLs was assessed by flow cytometry analysis using direct or indirect immunofluorescence assays. The analyses were performed on freshly recovered LGLs. Briefly, cells were stained with the appropriate mAbs either unlabeled or labeled; staining with unlabeled mAb was followed by PE- or FITC-conjugated isotype-specific goat anti mouse second reagent (Southern Biotechnology, Birmingham, AL, or Caltag, Burlingame, CA). A PE-Cy5 CD16/APC CD3 gate was set and the status of the antigens of interest was analyzed only on CD16+/CD3-, CD16+/CD3+ or CD57+/CD3+ gated cells. Stained cells were scored using a FACSCcanto analyzer (Becton Dickinson). 15,000 events were analyzed and data were processed using CELLQuest PRO software (Becton Dickinson).

PCR analysis for TCRγ clonality and STAT3 mutations

TCRγ gene rearrangement analysis was performed in all the patients using PCR analysis as already reported.[24] Briefly, PCR was carried out on DNA purified by PBMCs or CD3+/CD57+ cells of

patients. Primers specific for the variable region of the rearranged TCRγ chain gene were used as two mixes: mix I (Set I primers), which contained TCRγV 2, 3, 4, 8, and 9; and mix II (Set II primers) which contained TCRγV 5, 10, 11, and 12. For the J segments, three primers were included: JGT12 for J1.3 and J2.3, JGT3 for J1.1 and J1.2, and JGT4 for J1.2. PCR products were considered monoclonal only if at least one discrete band within the expected size range (clonal bands ranged from 170 to 230 bp) was amplified and observed on agarose gel electrophoresis. Twenty-nine patients were available for at least two (from 2 up to 5) measurements over 3 to 14 years.

T Cell Receptor Beta Variable (Vβ) region repertoire expression was performed, as already reported,[25-28] in 6 patients over 3-14 years follow up. Briefly, RNA was purified from PBMCs of patients and transcribed into cDNA. Detection of TCRγ repertoire restriction was performed by PCR using Vβ family (from 1 to 24) -specific primers. The amplified products were separated on 2% agarose gel electrophoresis (Vβ bands ranged from 170 to 220 bp). Agarose gels were acquired with the CHEMI DOC XRS supply (Bio-Rad Laboratories; Milan, Italy).

Analysis of D661V, D661Y, D661H, Y640F, N647I and K658N STAT3 mutations were performed according to previously reported methods[21] on DNA from purified NK or PBMCs. As healthy control, DNA from both CD16+/CD3- cells and PBMCs of buffy coats was used. DNA was sequenced as previously reported.[20] The presence of D661Y and Y640F mutations non-detectable by direct sequencing, due to the low sensitivity of the method (reaching 25% of positive cells) was also analyzed by a DNA tetra-primer amplification refractory mutation system assay (ARMS-PCR), as reported. ARMS-PCR allows to identify STAT3 mutations in the pathologic clone with a sensitivity of less than 10% of mutated cells.[20,22]

Investigation on sensitivity of PCR analysis for TCRγ clonality

To investigate the sensibility of PCR analysis for TCRγ clonality, we performed PCR using DNA purified by a monoclonal CD57+ population with rearranged TCRγ. DNA was serially diluted with DNA purified by CD3+ population with polyclonal TCRγ. We used 200ng of total DNA in each

amplification, where DNA from monoclonal rearranged CD57+ cells represented respectively 100%, 75%, 50%, 25%, 12.5%, 6.25%, 2.5%, 1.25% and 0% of total DNA (Figure 1 Supplemental). 6.25% was the limit for the detection of TCR rearrangements.

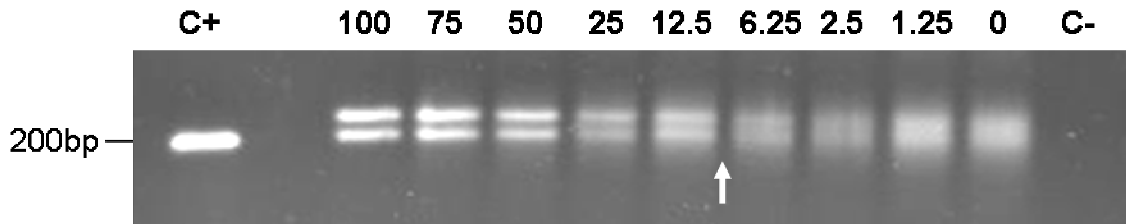

Table 1 Supplemental. Comparison of immunophenotype in patients with or without clonal TCRγ rearrangement, ranked according to the pattern of KIR restriction.

Patient #	TCR rearrangement	% CD16+	% CD3+/16+	% CD3+	% CD3+/57+	% CD8+	CD4/8 ratio	KIR RESTRICTION
4	no clonal	78	0	18	2	26	1.12	CD158a
25	no clonal	76	3	22	2	11	0.87	CD158a
36	no clonal	62	1	18	3	42	0.49	CD158b
37	no clonal	42	0	41	3	28	1.07	CD158b
15	no clonal	80	0	14	3	22	0.32	CD158a-b
30	no clonal	50	0	38	14	42	0.64	CD158a-b
1	no clonal	48	0	40	4	21	2.19	CD158e
20	no clonal	49	0	44	4	28	1.14	CD158e
33	no clonal	49	0	32	25	49	0.43	CD158e
40	no clonal	89	1	8	5	34	0.56	CD158e
9	no clonal	62	0	16	5	25	0.98	CD158i
17	no clonal	47	0	44	9	34	1.03	CD158i
31	no clonal	89	0	9	2	13	0.62	CD158i
48	no clonal	47	2	48	4	36	0.86	CD158i
5	no clonal	42	0	40	14	36	0.79	KIR NEG
6	no clonal	89	1	8	1	30	0.89	KIR NEG
10	no clonal	91	2	4	2	17	1.47	KIR NEG
18	no clonal	59	0	35	4	27	1.56	KIR NEG
19	no clonal	78	0	19	3	29	0.79	KIR NEG
22	no clonal	49	3	38	7	49	0.47	KIR NEG
29	no clonal	48	1	46	13	43	0.56	KIR NEG
35	no clonal	88	5	10	1	15	1.33	KIR NEG
38	no clonal	53	2	41	6	26	0.69	KIR NEG
45	no clonal	74	0	12	6	64	0.13	KIR NEG
46	no clonal	45	0	34	6	27	1.00	KIR NEG
16	clonal	49	1	27	15	39	0.48	CD158a
47	clonal	49	0	39	24	33	0.82	CD158a
8	clonal	80	0	18	9	27	0.52	CD158b
11	clonal	48	0	39	28	40	0.95	CD158b
12	clonal	49	1	41	14	40	1.43	CD158b
13	clonal	59	1	33	20	37	1.24	CD158b
21	clonal	48	0	51	22	24	1.42	CD158b
27	clonal	76	2	20	18	23	0.87	CD158b
32	clonal	77	1	21	13	49	0.37	CD158b
43	clonal	68	0	20	13	24	2.04	CD158b
26	clonal	56	1	31	22	11	2.19	CD158a-b
7	clonal	76	2	38	18	55	0.42	CD158e
24	clonal	68	0	24	16	24	0.32	CD158i
2	clonal	50	0	42	20	32	1.31	KIR NEG
3	clonal	49	4	47	26	53	0.43	KIR NEG
14	clonal	69	1	28	18	29	0.48	KIR NEG
23	clonal	76	0	22	20	17	0.88	KIR NEG
28	clonal	50	0	42	18	36	0.58	KIR NEG
34	clonal	78	0	18	16	85	0.13	KIR NEG
39	clonal	69	0	27	16	58	0.19	KIR NEG
41	clonal	47	2	41	27	49	0.63	KIR NEG
42	clonal	77	0	9	20	76	0.07	KIR NEG
44	clonal	76	1	18	14	55	0.45	KIR NEG