High frequency of chronic lymphocytic leukemia-like low-count monoclonal B-cell lymphocytosis in Japanese descendants living in Brazil

Mariane de Faria-Moss,[1] Mihoko Yamamoto,[1*] Celso Arrais-Rodrigues,[1] Ignacio Criado,[2] Caio Perez Gomes,[1,3] Maria de Lourdes Chauffaille,[1] Matheus Vescovi Gonçalves,[1] Eliza Kimura,[1] Efstathios Koulieris,[2] Fabio Borges,[1] Guilhermo Dighiero,[4] Joao Bosco Pesquero,[3] Julia Almeida[2#] and Alberto Orfao[2#]*

[*]MdFM and MY contributed equally as co-first authors.

[#]JA and AO are co-senior authors.

[1]Division of Hematology, Universidade Federal de São Paulo (EPM-UNIFESP), Sao Paulo, Brazil; [2]Cancer Research Center, Cytometry Service (NUCLEUS) and Department of Medicine, University of Salamanca, Instituto de Investigación Biomédica de Salamanca (IBSAL) and CIBERONC CB16/12/00400 (Centro de Investigación Biomédica en Red de CÁNCER), Salamanca, Spain; [3]Department of Biophysics (EPM-UNIFESP), Sao Paulo, Brazil and [4]Institut Pasteur of Montevideo, Montevideo, Uruguay

Correspondence: ALBERTO ORFAO - orfao@usal.es
MIHOKO YAMAMOTO - yamamoto@unifesp.br

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Subjects and samples: all volunteer participants filled a clinical and epidemiological questionnaire that also included data on their current and past personal health, together with the medical history of three (ancestor) family generations (Supplemental Table S1) and gave their informed consent to participate prior to entering the study, in accordance with the Declaration of Helsinki. Individuals with hematological or other neoplastic diseases, autoimmune / infectious diseases, previous chemotherapy and radiotherapy or immunosuppressant drugs use, were excluded from the study.

Peripheral blood (PB) samples (~15mL/subject) were collected in EDTA-containing tubes and processed immediately after obtained, for both a complete blood cell count (CBC) (CELL-DIN Rubi, Abbott Laboratories, Libertyville, IL) and multiparameter flow cytometry (MFC) screening for MBL cells.

Complete blood cell counts (CBC). Eleven of 258 subjects (4.3%) showed an altered CBC, including leukocytosis (14 x 10^9/L) in one individual (suffering from diabetes mellitus and associated morbidities treated with statins), isolated thrombocytopenia in 3 cases (106, 108 and 117 x 10^9 platelets/L), leukopenia (2.8 x 10^9 leukocytes/L) and lymphopenia (0.66 x 10^9 lymphocytes/L) in one case each, and low B-cell counts in three cases (0.003 x 10^9 B-cells/L in a 74y male, 0.004 x 10^9 B-cells/L in another 75y male, and 0.005 x 10^9 B-cells/L in a 78y female). The other 2 cases showed lymphocytosis (4.0 and 4.6 x 10^9 lymphocytes/L) at the expense of T-cells (Table 1). Median (range) hemoglobin, white blood cell (WBC), lymphocyte and platelet counts were of: 146 g/L (112–180 g/L), 5.7 x 10^9 leukocytes/L (2.8–14 x 10^9/L), 1.8 x 10^9 lymphocytes/L (0.66–4.6 x 10^9/L) and 216 x 10^9 platelets/L (106–492 x 10^9/L), respectively.

Flow cytometry immunophenotypic studies:

Detection and characterization of clonal B cells. Fresh PB samples were stained according to the EuroFlow Bulk Lysis standard operating procedure, as described elsewhere.[1,2] Briefly, cells were stained with 8-color panels of monoclonal antibodies combined in a "screening tube" for analysis of the distribution of the different lymphocyte subsets, and a "characterization tube", for the identification and characterization of the B-cell clone(s). Details about the combination of monoclonal antibodies used are provided in Supplemental Table S2*,* panels A and B. Data acquisition was performed in a FACSCanto II flow cytometer (Becton/Dickinson Biosciences –BD-San Jose, CA) using the FACSDiva software (BD). For data analysis, the INFINICYT^{TM} software (Cytognos SL, Salamanca, Spain) was used. Every stained sample was analyzed in parallel by two independent experts at UNIFESP and at the University of Salamanca (USAL, Salamanca, Spain), using a sequential "boolean gating strategy", as reported elsewhere.[3] MBL was defined based on the presence of a cluster of ≥30 clonal B-cells, with i) a typical CLL-like MBL phenotype –defined by co-expression of CD5^{+} CD20^{lo} CD23^{+} CD43^{+} and CD79b$^{-/lo}$ on CD19^{+} cells–, ii) an atypical CLL-like MBL phenotype–MBL clones with a CD23^{-} and/or CD20^{bright} CLL-like phenotype– and, iii) a non-CLL-like CD5^{-} or CD5^{+} MBL phenotype.[4] Clonality was defined by MFC whenever a smIgκ:smIgλ ratio of either >3:1 or <1:3, and/or a phenotypically aberrant B-cell population was detected. Biphenotypic MBL was defined when two distinct B-cell clones [with a surface membrane immunoglobulin (smIg) κ+ (phenotypically aberrant) MBL clone coexisting with a smIgλ+ (phenotypically aberrant) clone]. Absolute MBL cell counts were calculated using a dual-platform assay.

Analysis of normal residual PB T, B and natural killer (NK) cells and their subsets. PB distribution of total T and B lymphocytes, plasma cells and NK cells, and their major subsets, was systematically analyzed (n=258 cases) using the screening tube (Supplemental Table S2, panel A). In a subset of 143 samples, an additional stain with 8-color combinations of the monoclonal antibodies (Supplemental Table S2, panel C) was performed, in order to further subset normal

residual PB B-cells into: immature ($CD19^+$ $CD20^+$ $CD10^{lo}$ $CD27^-$ $CD38^+$ $smIgM^+$), naïve ($CD19^+$ $CD20^+$ $CD10^-$ $CD27^-$ $CD38^{-/lo}$ $smIgM^+$), unswitched- ($CD19^+$ $CD20^+$ $CD10^-$ $CD27^+$ $CD38^{-/+}$ $smIgM^+$) and switched-memory B cells ($CD19^+$ $CD20^+$ $CD10^-$ $CD27^+$ $CD38^{-/+}$ $smIgM^-$), as well as plasma cells ($CD19^+$ $CD20^{-/lo}$ $CD10^-$ $CD27^{bright}$ $CD38^{bright}$), as previously described.[5]

***IGHV* gene sequencing, somatic hypermutation (SHM) profiling and interphase fluorescence in situ hybridization (iFISH) studies:** PB MBL cells were purified using a FACSAria II flow cytometer (BD) and the FACSDiva (BD) software (final purity of 98% ± 0.8%) and used for *IGHV* gene sequencing and iFISH studies.

IGHV sequencing. RNA was extracted from FACS-purified MBL cells in TRIzol Reagent (Thermo-Fisher Scientific) using the *PureLink RNA Micro Kit* (Thermo-Fisher Scientific, Waltham, MA), and reverse-transcribed into cDNA with the SuperScript III First-Strand (Thermo-Fisher Scientific). Afterward, the resulting cDNA was amplified by PCR using a mixture of VH1-VH6 (sense) and JH (antisense)[6] primers and the GoTaq® Green Master MIX (Promega, Madison, WI). Amplicons were analyzed by electrophoresis and purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). For the sequencing reaction, the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) was used, strictly following the manufacturer instructions. The resulting DNA fragments were sequenced in an automatic DNA sequencer (Genetic Analyzer 3500 XL, Applied Biosystems) using the Geneious R6 software (Biomatters, Auckland, NZ). The resulting *IGHV* gene sequences were compared to a reference germline sequence, using the Ig basic local alignment search tool (IgBLAST) directory (http://www.ncbi.nlm.nih.gov/igblast) and classified as either unmutated (sequence homology of ≥98%) or mutated *IGHV* sequences (germline sequence homology of <98%).[7]

iFISH studies. The LSI p53/LSI ATM & LSI D13S319/LSI 13q34/CEP 12 multicolor probe set (Vysis Inc, Des Plaines, IL) was used for simultaneously targeting the 13q14.3 (*D13S319*), 13q34 (*Proz*, *CUL4A*, *LAMP1*), 17p13.1 (*TP53*) and 11q22 (*ATM*) chromosomal regions, together with the chromosome 12 centromere, following the procedures recommended by the manufacturer.

Hybridized slides were analyzed using an epifluorescence microscope (Olympus BMax 60, Tokyo, Japan) with appropriate filters, and the MacProbe 44 Power Gene System software (Applied Imaging Corporation, Santa Clara, CA). Cutoff levels for normal diploid values were established by the β inverse analysis test.

Measurement of soluble plasma levels of anti-viral-specific antibodies: soluble levels of IgG-specific antibodies against cytomegalovirus (CMV; Abbott Ireland Diagnostic Division, Sligo, Ireland), Epstein-Barr virus (EBV) viral capsid antigen and nuclear antigen (EBNA-EBV) (Abbott, Wiesbaden, Germany) were measured using commercially available kits for chemiluminescent immune assays, strictly following the recommendations of the manufacturers.

Statistical Methods: The Pearson chi-square test (for categorical variables) and either the non-parametric Mann-Whitney *U* and Kruskal Wallis tests or the parametric Student t and ANOVA tests (for continuous variables) were used to evaluate the statistical significance of differences observed among groups of subjects. Direct comparison between virus-specific immunoglobulin plasma levels and age were performed via the Spearman's correlation test, and the corresponding Rho correlation coefficients were calculated. Multiple linear regression test was used to study the impact of age and the MBL^{lo} vs. non-MBL status on PB NK-cell counts (considering the former as independent variables and the NK-cell count as dependent variable).

References

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SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE S1.- Epidemiological characteristics of MBL^{lo} vs. non-MBL subjects included in the study.

	MBL^{lo} (n=27)	Non-MBL (n=231)	*P*-value
Country of birth			
Brazil	24 / 27 (89%)	201 / 229 (87%)	NS
Japan	3 / 27 (11%)	28 / 229 (13%)	
Generation of ancestors living in Brazil			
First generation	3 / 27 (11%)	29 / 226 (13%)	
Second generation	23 / 27 (85%)	172 / 226 (76%)	NS
Third generation	1 / 27 (4%)	25 / 226 (11%)	
Body mass index (BMI)			
BMI median (range)	23 (18 – 37)	24 (16 – 33)	NS
Habits			
Tobacco			
Non smoker	19 / 27 (70%)	170 / 226 (75%)	
Former smoker	6 / 27 (22%)	43 / 226 (19%)	NS
Current smoker	2 / 27 (7.4%)	13 / 226 (5.7%)	
Alcohol consumption			
None	12 / 25 (48%)	153 / 217 (70%)	
Weekend	8 / 25 (32%)	36 / 217 (17%)	NS
2-4 times per week	4 / 25 (16%)	23 / 217 (11%)	
Every day	1 / 25 (4.0%)	5 / 217 (2.3%)	
Diet			
Meat			
0-2 days a week	11 / 27 (41%)	70 / 229 (31%)	NS
3-7 days a week	16 / 27 (59%)	159 / 229 (69%)	
Fish			
0-2 days a week	15 / 27 (56%)	107 / 229 (47%)	NS
3-7 days a week	12 / 27 (44%)	122 / 229 (53%)	
Eggs and poultry			
0-2 days a week	12 / 27 (44%)	77 / 228 (34%)	NS
3-7 days a week	15 / 27 (56%)	151 / 228 (66%)	
Dairy products			
0-2 days a week	5 / 27 (19%)	42 / 226 (19%)	NS
3-7 days a week	22 / 27 (81%)	184 / 226 (81%)	
Olive oil, dried fruit and nuts			
0-2 days a week	4 / 27 (15%)	36 / 227 (16%)	NS
3-7 days a week	23 / 27 (85%)	191 / 227 (84%)	
Fresh fruit			
0-2 days a week	2 / 27 (7.4%)	14 / 227 (6.1%)	NS
3-7 days a week	25 / 27 (93%)	213 / 227 (94%)	
Vegetables			
0-2 days a week	0 / 27 (0%)	5 / 229 (2.1%)	NS
3-7 days a week	27 / 27 (100%)	224 / 229 (98%)	
Legumes			
0-2 days a week	3 / 27 (11%)	33 / 228 (14%)	NS
3-7 days a week	24 / 27 (89%)	195 / 228 (86%)	
Bread, grain products and tubers			
0-2 days a week	1 / 27 (3.7%)	12 / 229 (5.2%)	NS
3-7 days a week	26 / 27 (96%)	217 / 229 (95%)	
Cold meat			
0-2 days a week	22 / 27 (81%)	173 / 224 (77%)	NS

3-7 days a week	5 / 27 (19%)	51 / 224 (23%)	
Sweets			
0-2 days a week	15 / 27 (56%)	106 / 228 (46%)	NS
3-7 days a week	12 / 27 (44%)	122 / 228 (54%)	
Coffee			
0-3 cups per day	8 / 24 (33%)	70 / 227 (31%)	NS
>3 cups per day	16 / 24 (67%)	157 / 227 (69%)	
Tea			
0-3 cups per day	11 / 25 (44%)	104 / 225 (46%)	NS
>3 cups per day	14 / 25 (56%)	121 / 225 (54%)	
Fizzy drinks			
0-3 cups per day	22 / 24 (92%)	189 / 219 (86%)	NS
>3 cups per day	2 / 24 (8.0%)	30 / 219 (14%)	
Physical exercise			
Type of physical activity during work			
Sitting	16 / 26 (61%)	113 / 220 (51%)	NS
Standing	8 / 26 (31%)	55 / 220 (25%)	
In motion	1 / 26 (3.9%)	49 / 220 (22%)	
Hard physical effort	1 / 26 (3.9%)	3 / 220 (1.4%)	
Exercise on free time			
None	4 / 25 (16%)	67 / 228 (30%)	**.009**
Light (e.g. walking)	16 / 25 (64%)	69 / 228 (30%)	
Moderate (e.g. tennis, jogging)	4 / 25 (16%)	72 / 228 (31%)	
Exhausting	1 / 25 (4.0%)	20 / 228 (8.8%)	
Work and professional environment			
Current work status			
Active	13 / 27 (48%)	105 / 227 (46%)	NS
Unemployed	0 / 27 (0%)	2 / 227 (0.88%)	
Retired	14 / 27 (52%)	120 / 227 (53%)	
Exposure to toxics or (potentially) hazard products			
No	22 / 27 (81%)	196 / 224 (88%)	NS
Yes	5 / 27 (19%)	28 / 224 (12%)	
Stressful work			
Not stressful	4 / 25 (16%)	35 / 223 (16%)	NS
Slightly stressful	8 / 25 (32%)	62 / 223 (28%)	
Regular	6 / 25 (24%)	59 / 223 (26%)	
Quite stressful	7 / 25 (28%)	53 / 223 (24%)	
Very stressful	0 / 25 (0%)	14 / 223 (6.3%)	
General health status and family history of severe diseases			
Relevant diseases (e.g. cancer, heart diseases, diabetes, etc.)			
No	15 / 27 (56%)	151 / 229 (66%)	NS
Yes	12 / 27 (44%)	78 / 229 (34%)	
Severe infections throughout life			
No	24 / 27 (89%)	214 / 229 (93%)	NS
Yes	3 / 27 (11%)	15 / 229 (6.6%)	
First degree relatives suffering from relevant diseases			
No	4 / 26 (15%)	38 / 190 (20%)	NS
Yes	22 / 26 (85%)	152 / 190 (80%)	

Results expressed as number of cases / total cases (percentage).

Abbreviations (alphabetical order): **BMI:** body mass index; **NS:** Not statistically significantly different ($P > .05$); **MBL^{lo}:** low-count monoclonal B-cell lymphocytosis.

SUPPLEMENTAL TABLE S2.- Eight-color monoclonal antibodies used for the identification and characterization of MBL cells and other normal residual T-, B- and NK-cell subsets

Panel A: Antibody combination used for the screening test

Fluorochrome	FITC	PE	PerCPCy5.5	PECy7	APC	APC-H7	PacB	PacO
Marker	CD8 / anti-λ	CD56 / anti-κ	CD5	CD19	CD3	CD38	CD20 / CD4	CD45
Clone	UCH-T4 / polyclonal	C5.9 / polyclonal	L17F12	J3119	SK7	HB7	2H7 / RPA-TA	HI30
Manufacturer	Cytognos	Cytognos / Cytognos	BD	Beckman Counter	BD	BD	BioLegend / BD	Invitrogen

Panel B: Antibody combination used for the specific characterization of clonal B cells

Fluorochrome	**FITC**	**PE**	**PerCPCy5.5**	**PECy7**	**APC**	**APC-H7**	**PacB**	**PacO**
Marker	CD23	CD10	CD79b	CD19	CD5	CD43	CD20	CD45
Clone	MHM6	ALB1	SN8	J3119	UCHT2	1G10	2H7	HI30
Manufacturer	Dako	Immunotech	BD	Beckman Counter	BD	BD	BioLegend	Invitrogen

Panel C: Antibody combination used for the characterization of normal B-cells and their subsets

Fluorochrome	**FITC**	**PE**	**PerCPCy5.5**	**PECy7**	**APC**	**APC-H7**	**PacB**	**PacO**
Marker	smIgM	CD10	CD5	CD19	CD27	CD38	CD20	CD45
Clone	polyclonal	ALB1	L17F12	J3119	L128	HB7	2H7	HI30
Manufacturer	Dako	Immunotech	BD	Beckman Counter	BD	BD	BioLegend	Invitrogen

Abbreviations (alphabetical order): **APC,** Allophycocyanin; **APCH7**, Allophycocyanin- Hilite®7; **BD,** Becton/Dickinson Biosciences®; **FITC,** fluorescein isothiocyanate; **PacB,** Pacific Blue; **PacO**, pacific orange; **PE,** phycoerythrin; **PECy7,** phycoerythrin-cyanin 7; **PerCPCy5.5,** peridinin chlorophyll cyanin 5.5.

SUPPLEMENTAL TABLE S3.- Gender, age and peripheral blood cell counts in the MBL^{lo} Japanese individuals living in Brazil identified in this study.

N	Sex	Age (years)	Leukocytes (x10^9/L)	B cells (x10^9/L)[a]	MBL cells/ total leukocyte events	% MBL from PB B-cells	N. of MBL PB cells (x10^3/L)	MBL type
1	F	54	5.5	0.31	33/5,535,631	0.01	31	CLL-like λ
2	F	85	6.0	0.16	30/5,096,220	0.02	33	CLL-like λ
3	M	72	6.0	0.22	42/2,549,700	0.02	44	CLL-like κ
4	M	63	4.6	0.10	60/5,000,000	0.05	49	CLL-like κ
5	F	60	6.4	0.50	24 κ; 26 λ /5,355,506	0.01	50	CLL-like κ and λ
6	F	60	5.9	0.18	19 κ; 40 λ /5,141,548	0.03	54	CLL-like κ and λ
7	M	75	4.7	0.16	42 κ;15 λ /5,000,000	0.04	62	CLL-like κ and λ
8	M	72	4.9	0.33	14 k; 42 λ /5,000,000	0.02	65	CLL-like κ and λ
9	F	58	6.1	0.38	31 κ; 32 λ /6,214,018	0.02	72	CLL-like κ and λ
10	M	96	6.0	0.08	80/5,000,000	0.10	76	CLL-like κ
11	F	83	4.8	0.11	23 κ; 41 λ /7,583,368	0.08	88	CLL-like κ and λ
12	F	70	4.7	0.18	44 κ; 26 λ /5,000,000	0.05	91	CLL-like κ and λ
13[a]	F	66	7.1	0.19	62/5,000,000	0.05	95	Atypical CLL-like κ
14	M	73	6.3	0.19	113/5,000,000	0.06	116	CLL-like κ
15	M	63	5.8	0.44	174 κ; 40 λ /5,000,000	0.04	178	CLL-like κ and λ
16	F	65	5.0	0.16	148 κ; 74 λ /5,000,000	0.12	192	CLL-like κ and λ
17	F	66	6.0	0.22	101/3,664,160	0.09	198	CLL-like κ
18	M	66	5.4	0.15	65 κ; 141 λ /5,000,000	0.13	202	CLL-like κ and λ
19	M	77	4.4	0.05	177 κ; 174 λ /5,263,546	0.42	222	CLL-like κ and λ
20	M	68	7.4	0.12	186 k; 164 λ /6,047,723	0.35	395	CLL-like κ and λ
21	F	60	4.7	0.20	223 κ; 377 λ /5,000,000	0.20	400	CLL-like κ and λ
22	M	76	5.7	0.20	431/5,686,465	0.25	495	CLL-like κ
23	M	77	6.1	0.29	474/5,000,000	0.17	498	CLL-like κ
24	M	42	6.3	0.36	463/5,000,000	0.14	508	CLL-like smIg$^-$
25	M	82	6.3	0.13	400/5,704,522	0.42	551	CLL-like κ
26	M	80	4.5	0.15	11662/5,000,000	6.0	9,000	CLL-like λ
27	M	88	5.2	0.10	16636/5,114,440	19.8	19,800	CLL-like κ

[a] Atypical CLL-like case (CD19$^+$/CD5$^+$/CD23$^-$), MBL-negative after 2 years and 8 months since recruitment).

Abbreviations: **MBL:** monoclonal B-cell lymphocytosis

SUPPLEMENTAL TABLE S4.- Identification of *IGHV* genes rearranged sequences in MBL^{lo} subjects

n		Rearranged sequence	Germline Homology	*Productive nature*
1	*IGHV3*	*IGHV3-7*	95.6%	no
2	*IGHV3*	*IGHV3-48*2*	90.7%	yes
3	*IGHV3*	*IGHV3-48*2*	91.6%	yes
4	*IGHV3*	*IGHV3-21.01*	91.2%	yes
5	*IGVH1*	*IGHV1-08*	100%	yes

Abbreviations: ***IGHV***: immunoglobulin heavy chain variable region; **MBL:** monoclonal B-cell lymphocytosis.

SUPPLEMENTAL TABLE S5.- Seroprevalence and virus-specific immunoglobulin (Ig) plasma levels in MBL^{lo} vs. non-MBL individuals included in the study.

A.- Frequency of seropositive cases for anti-CMV IgG and anti-EBV IgG in MBL^{lo} vs. non-MBL subjects.

Serologic tests	Total	MBL^{lo} (n=27)	Non-MBL (n=222)	*P*-value
Anti-CMV-IgG + **n=249**	234 (94%)	25 (93%)	209 (94%)	NS
Anti-VCA EBV-IgG + ***n=249***	246 (99%)	27 (100%)	219 (99%)	NS
Anti-EBNA EBV-IgG + ***n= 249***	247 (99%)	27 (100%)	220 (99%)	NS

B.- Virus-specific immunoglobulin (Ig) plasma levels in MBL^{lo} vs. non-MBL seropositive individuals for each pathogen studied.

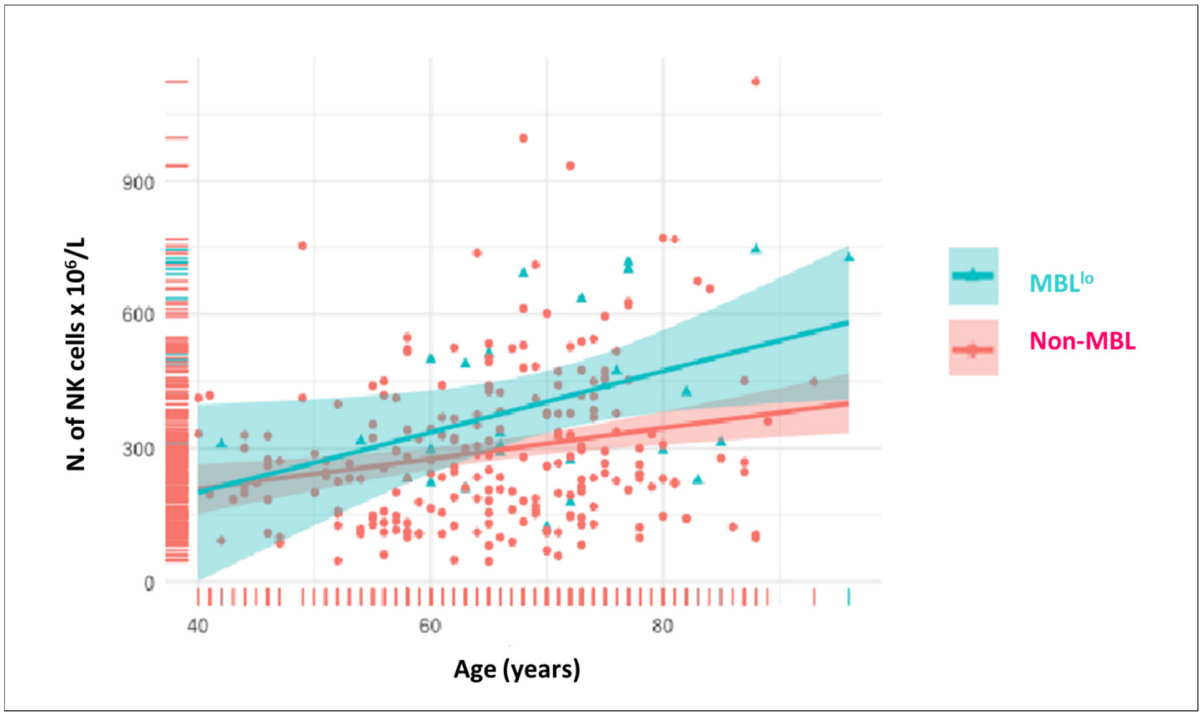
Serologic tests	Total	MBL^{lo}	Non-MBL	*P*-value
Anti-CMV-IgG (AU/mL)* **n=234**	190 (19 – 1606)	221 (64 – 1606) n=25	187 (19 – 1392) n=209	NS
Anti-VCA EBV-IgG (S/CO)* ***n=246***	60 (2.7 – 83)	58 (14 – 79) n=27	61 (2.7 – 83) n=219	NS
Anti-EBNA EBV-IgG (S/CO)* ***n= 247***	19 (1.9 – 26)	19 (1.9 – 25) n=27	19 (2.5 – 26) n=220	NS

Results expressed as number of cases (percentage) or * median (range). Only those individuals who were seropositive (according to the manufacturers' instructions) were studied.

Abbreviations: **AU:** arbitrary units; **CMV:** cytomegalovirus; **EBNA:** Epstein-Barr nuclear antigen; **EBV:** Epstein-Barr virus; **MBL^{lo}:** low-count monoclonal B-cell lymphocytosis; **S/CO:** signal to cut-off ratio; **VCA:** viral capsid antigen.

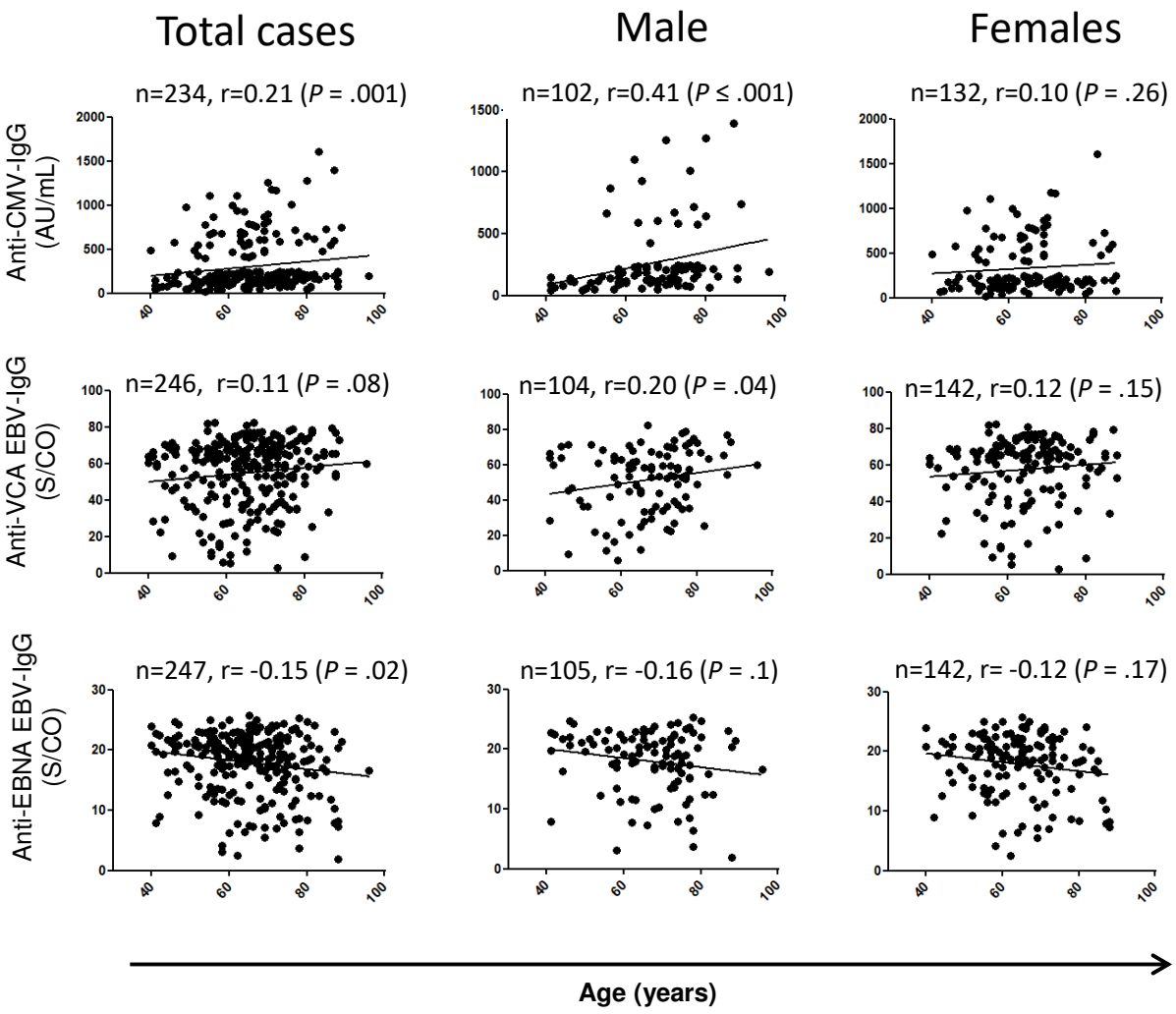
SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE S1.- Peripheral blood (PB) NK-cell count in 256 Japanese descendants (27 MBL^{lo} and 229 non-MB, coded as blue and red, respectively), distributed by age



PB NK-cell counts increase with age (*P*=.0001), particularly in the MBL^{lo} subjects vs. non-MBL (*P*=.01)

SUPPLEMENTAL FIGURE S2.- Correlation between virus-specific immunoglobulin plasma levels and both gender and age of seropositive individuals.



Abbreviations: **AU:** arbitrary units; **CMV:** cytomegalovirus; **EBNA:** Epstein-Barr nuclear antigen; **EBV:** Epstein-Barr virus; **Ig:** immunoglobulin; **MBL^{lo}:** low-count monoclonal B-cell lymphocytosis; **S/CO:** signal to cut-off ratio; **VCA:** viral capsid antigen.