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Oligoclonal TCRV β gene usage among CD8⁺ T cells in monoclonal B lymphocytosis and CLL

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Whereas most malignancies are associated with decreased numbers of circulating T cells, in chronic lymphocytic leukemia (CLL) T cell numbers are elevated 2 to 4 times normal. Though T cells are increased in number, the T cell repertoire is significantly contracted in CLL, with oligoclonal and monoclonal subsets (Goolsby, *et al* 2000, Rezvany, *et al* 1999). Rather than promoting an anti-tumor response, these qualitative and quantitative T cell abnormalities may contribute to a tumor microenvironment that promotes progression of the malignant clone (Ghia and Caligaris-Cappio 2000).

CLL patients with mutated immunoglobulin heavy chain variable region (IgV_H) genes follow a more indolent disease course than those with unmutated IgV_H. It has been proposed that IgV_H unmutated CLL arise from B cells that mature through a T cell independent pathway, because in the germinal center reaction CD4⁺ T cell help is required for somatic mutation and isotype switching. Therefore, IgV_H mutated and unmutated CLL may have differing capacities for T cell interaction and immune modulation. A recent report by Palmer *et al* showed that increased numbers of T and “natural killer” (NK) cells relative to the number of CLL lymphocytes in patients with newly diagnosed CLL was associated with both a mutated IgV_H and improved prognosis, suggesting that T and NK cell responses precede clinical CLL (Palmer, *et al* 2008).

Using sensitive flow cytometry, we and other groups have found that small populations of cells with a typical CLL immunophenotype can be found in older adults (Ghia, *et al* 2004, Rawstron, *et al* 2002). This condition is termed monoclonal B-lymphocytosis (MBL). Because MBL may progress to CLL (Rawstron, *et al* 2008), we sought to clarify if either the number or T cell receptor (TCR) repertoire of T cells differed between IgV_H mutated CLL, and IgV_H unmutated CLL, and MBL.

Twenty untreated CLL patients were identified from a longitudinal CLL patient cohort at Duke University or the Durham VA Medical Centers between October 2006 and August 2007. The clinical and molecular characteristics of this longitudinal cohort of patients have

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been previously described (Weinberg, *et al* 2007). *A priori*, an equal number of IgV_H mutated and unmutated patients were selected for this study. No other clinical or molecular features were used to identify or stratify patients. A diagnosis of CLL was based on standard criteria (Weinberg, *et al* 2007). MBL subjects were identified by flow cytometric screening of unaffected relatives of patients with familial CLL at Duke University or the Durham VA Medical Center. Eight subjects from 5 different families with MBL provided blood for T cell analysis. This study was approved by the Institutional Review Boards at Duke University and the Durham VA Medical Centers, and all subjects provided written informed consent.

CLL and MBL subject characteristics are summarized in Table 1. Patients with IgV_H unmutated and mutated CLL were similar in regards to WBC count, age, lymphocyte doubling time, and duration since diagnosis. Similar to prior reports, unmutated IgV_H correlated with ZAP70 expression (Fishers exact test, $p < 0.01$) and surface expression of CD38 (Fishers exact test, $p < 0.05$). Patients with IgV_H unmutated CLL were significantly more likely to be intermediate or high Rai Stage ($\chi^2_{df=2}$, $p < 0.01$). The mean age of CLL patients was 62, and the mean MBL age was 71. This difference was not statistically significant (2-sided t -test, $p = 0.1$).

Lymphocyte subset enumeration was performed using multi-channel flow cytometry (Table 2 and Supporting Information). The absolute B cell count was not significantly different in IgV_H unmutated compared to mutated CLL patients. Among MBL subjects, the mean absolute B cell count was 0.6×10^9 cells/L and the MBL clone size was small ($< 150 \times 10^6$ cells/L) in 7 of the 8 subjects. The CD4⁺ count was similar in IgV_H unmutated CLL, IgV_H mutated CLL and MBL subjects. The CD8⁺ count was higher in IgV_H unmutated compared to IgV_H mutated CLL patients (891 vs. 593×10^6 cells/L, *n.s.*), and was significantly higher in IgV_H unmutated than MBL subjects (891 vs. 273×10^6 cells/L, $p < 0.05$). Similarly, the ratio of CD4⁺:CD8⁺ T cells was significantly lower in IgV_H unmutated CLL compared to IgV_H mutated CLL or MBL (1.6 vs. 2.6, $p < 0.05$, and 1.6 vs. 4.5, $p < 0.01$, respectively). NK T cell numbers were not significantly different among IgV_H unmutated CLL compared to IgV_H mutated CLL or MBL.

To estimate the CD4⁺ and CD8⁺ T cell repertoire diversity, we coupled PCR amplification of the CDR3 length polymorphism of 23 TCRV β families ("spectratyping") with a novel, quantitative method for analysis of TCR repertoires by using SpA (Kepler, *et al* 2005) (Supporting information). CD4⁺ and CD8⁺ T cells were positively selected using anti-CD4 and anti-CD8 monoclonal antibodies conjugated to magnetic beads according to the manufacturer's directions (Dynabeads®, Invitrogen, Carlsbad, CA). The calculated mean divergence coefficients (a calculated unitless value to quantify the degree of "skewness" from a pseudo-Gaussian distribution) for the T cell spectratypes are provided in Table 2. Combined analysis of all CLL patients showed the mean CD8⁺ TCR spectratype divergence was 0.32, significantly higher than the mean CD4⁺ divergence (0.07, 2 sided t -test, $p < 10^{-6}$). Oligoclonal and monoclonal V β families were frequently observed among CD8⁺ cells, but rarely observed among CD4⁺ cells. We did not observe restriction of any specific V β families across patients. Given our hypothesis that IgV_H unmutated CLL and mutated CLL may have different capacities for T cell interaction, we compared the TCR repertoire between these patient groups. The mean CD8⁺ TCR spectratype divergence was greater in IgV_H unmutated CLL than in mutated CLL among CD8⁺ cells, but this difference was not statistically significant (0.36 vs. 0.29, 2-sided t -test, $p = 0.08$, *n.s.*).

Next, we evaluated whether MBL have a restricted TCR repertoire. The mean CD8⁺ and CD4⁺ divergence among MBL subjects was similar to that observed in CLL patients (mean CD8⁺ divergence: 0.35 for MBL vs. 0.32 for all CLL; mean CD4⁺ divergence: 0.06 for MBL vs. 0.07 for all CLL, Table 2). We performed a multivariate correlation to identify

variables associated with restriction of the CD8⁺ repertoire in MBL. Patient characteristics, the absolute number of different lymphocyte subsets, and “MBL characteristics” (ZAP70 status, CD38 status, and the “MBL percentage,” defined as the number of MBL cells divided by the total number of CD19⁺ B cells) were considered. A correlation between the absolute number of CD4⁺ cells and the diversity of the CD8⁺ repertoire was observed: higher numbers of CD4⁺ cells correlated with more diverse CD8⁺ repertoire ($\rho = -0.82$, $p < 0.05$). Loss of specific CD4⁺ subsets may cause contraction of the CD8⁺ repertoire; alternatively some CLL and MBL may have the capacity to modulate both the CD4⁺ and CD8⁺ subsets as independent effects. Of the remaining variables, a correlation was observed between the MBL percentage and mean CD8⁺ divergence ($\rho = 0.52$, *n.s.*). There was no correlation of mean CD8⁺ divergence with MBL subject age ($\rho = 0.20$, *n.s.*).

Because abnormalities in the T cell compartment are detectable at time of CLL diagnosis (Palmer, *et al* 2008), we speculate that MBL lymphocytes may have a capacity for T cell immune modulation similar to CLL lymphocytes. The absolute number of MBL lymphocytes was quite small in this cohort of MBL subjects, suggesting that acquisition of an immunomodulatory phenotype is an early event in CLL leukemogenesis. Further investigation is required to test this hypothesis. Furthermore, MBL is relatively common among the elderly (Ghia, *et al* 2004), and the great majority of MBL likely do not progress to CLL (Rawstron, *et al* 2008). We identified qualitative abnormalities in the T cell repertoire in this otherwise healthy group of elderly individuals, suggesting that MBL may contribute to accelerated immune senescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Subject Characteristics

Characteristic ^a	IgV _H Mutated ^b CLL (N=10)	IgV _H Unmut. CLL (N=10)	MBL (N=8)
Age	64 (57 – 75)	61 (48 – 76)	71 (58 – 94)
Male Gender	8	7	2
Rai Stage (Low, intermediate, high)	9; 0; 1	4; 4; 2	-
Time since diagnosis (yrs)	5.7 (0.4 – 14.4)	4.4 (0.3 – 16.7)	-
Doubling Time < 2 yrs	3	5	-
Zap-70+ (>20%)	2	9	3
CD38+ (>30%)	0	4	5
Hemoglobin (g/dL)	13.9 (11.4 – 17.0)	14.3 (11.6 – 18.4)	13.6 (10.1 – 17.2)
Platelets (× 10 ⁹ /L)	170 (97 – 251)	169 (66 – 278)	210 (150 – 287)
WBC (× 10 ⁹ /L)	34.0 (8.0 – 87.7)	53.5 (16.4 – 161.3)	7.3 (5.5 – 10.3)

WBC: white blood cells

^aFor continuous variables, mean and range are presented^b≥98% homology with germline sequence

Table 2

Lymphocyte Enumeration and T Cell Repertoire Divergence Coefficients

	ABLC ($\times 10^9/L$)	CD4 ⁺ ($\times 10^6/L$)	CD8 ⁺ ($\times 10^6/L$)	CD4 ⁺ :CD8 ⁺ Ratio	NK ($\times 10^6/L$)	D _{KL} CD4 ⁺ ^a	D _{KL} CD8 ⁺ ^b
All CLL (N=20)	33.9 $\pm$ 34.1	1081 $\pm$ 637	742 $\pm$ 688	2.1 $\pm$ 1.1	310 $\pm$ 329	0.07 $\pm$ 0.03	0.32 $\pm$ 0.10
IgV _H unmutated CLL (N=10)	42.0 $\pm$ 41.7	1198 $\pm$ 841	891 $\pm$ 637	1.6 $\pm$ 0.8	372 $\pm$ 440	0.08 $\pm$ 0.04	0.36 $\pm$ 0.11
IgV _H mutated CLL (N=10)	25.8 $\pm$ 23.9	965 $\pm$ 333	593 $\pm$ 737	2.6 $\pm$ 1.2	246 $\pm$ 159	0.06 $\pm$ 0.02	0.29 $\pm$ 0.07
MBL (N=8)	0.6 $\pm$ 1.2	875 $\pm$ 481	237 $\pm$ 192	4.5 $\pm$ 2.3	275 $\pm$ 165	0.06 $\pm$ 0.03	0.35 $\pm$ 0.14

Mean and standard deviation are presented for each variable; ABLC: Absolute B-lymphocyte count, NK: CD56⁺ NK T cells;

^a Divergence coefficient (unitless) among CD4⁺ T cells

^b Divergence coefficient among CD8⁺ T cell