

Downregulation of IL-17-producing T cells is associated with regulatory T cell expansion and disease progression in chronic lymphocytic leukemia

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Abstract Little is known about the immunobiology of interleukin-17 (IL-17)-producing T cells and regulatory T cells (Treg) in chronic lymphocytic leukemia (CLL). In this study, the frequencies of Th17, Tc17, and CD39⁺ Treg cells were enumerated in peripheral T cells isolated from 40 CLL patients and 15 normal subjects by flow cytometry. Our results showed a lower frequency of Th17 and Tc17 cells in progressive (0.99±0.12 % of total CD3⁺CD4⁺ cells; 0.44±0.09 % of total CD8⁺ cells) compared to indolent patients (1.57±0.24 %, $p=0.042$; 0.82±0.2 %, $p=0.09$) and normal subjects (1.78±0.2 %, $p=0.003$; 0.71±0.09 %, $p=0.04$). Decrease in IL-17-producing T cells was associated with CD39⁺ Treg cells expansion. Variation of IL-17-producing cells and Treg cells in indolent and progressive patients was neither associated to the expression levels of Th1- and

Th2-specific transcription factors T-bet and GATA-3 nor to the frequencies of IFN- γ and IL-4-producing CD4⁺ T cells in a selected number of samples. Additionally, suppressive potential of CD4⁺ Treg was similar in CLL patients and normal subjects. Our data indicate that progression of CLL is associated with downregulation of IL-17-producing T cells and expansion of Treg cells, implying contribution of these subsets of T cells in the progression of CLL.

Keywords Th17 · Tc17 · Regulatory T cell · Chronic lymphocytic leukemia · Interleukin 17

Introduction

Chronic lymphocytic leukemia (CLL) is a monoclonal B cell malignancy representing the most frequent leukemia in the Western world, but its prevalence in Asia is low [1–3]. Immune status in CLL is very complex with a spectrum of mild to severe systemic immune dysfunction [4]. Several prognostic markers have recently been reported for CLL including mutational status of immunoglobulin heavy chain variable (IGHV) region genes and expression of CD38 and ZAP-70 molecules [5]. Expression of unmutated IGHV genes and expression of CD38 and ZAP-70 were found to be associated with a poor prognosis [6–8].

Regulatory T cells (Treg) and IL-17-producing CD4⁺ (Th17) cells are two important T cell subsets, which have important roles in peripheral immune responses. An imbalance in these cells may lead to the development of

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tissue inflammation, autoimmune diseases, or cancer [9]. Although these cells are more studied in autoimmune diseases, information regarding their immunobiology in cancer is little. The current data regarding the role of Th17 cells in pathogenesis of cancer are scarce and controversial [10]. Th17 cells have been studied in a variety of solid tumors and a limited number of hematological malignancies including multiple myeloma [11, 12], monoclonal gammopathy of undetermined significance [12], acute myeloid leukemia [13], and non-Hodgkin lymphoma [14]. A subset of IL-17-producing CD8⁺ T (Tc17) cells has recently been identified predominantly displaying the CD27^{-/+}CD28⁺CD45RA⁻ memory phenotype. They were also found to express CCR6 and a high level of CCR5 with attenuated cytotoxic activity [15]. Tc17 cells express all hallmark molecules essential for Th17 differentiation, including retinoic acid receptor-related orphan receptor (ROR) γ t, ROR α , IL-21, and IL-23R [16]. There are only three reports regarding the function of Tc17 cells in cancer all performed in solid tumors, suggesting these cells as potent tools in tumor immunotherapy [17–19]. We and others have recently found that Treg cells are expanded in CLL patients, and this increase is associated to disease progression [20–24]. However, there is no comprehensive report regarding the frequency or function of IL-17-producing Th17 or Tc17 cells in CLL or other hematopoietic malignancies. Recent findings in autoimmune diseases indicate that imbalance between Treg cells and Th17 may contribute to immunopathogenesis of such diseases [25]. A subset of CD39⁺ Tregs was found to inhibit Th17 cells [26]. There are two reports on contribution of this subset of Treg cells in cancer, both of which confirmed the inhibitory effect of CD39⁺ Treg cells on Th17 generation and differentiation in tumor microenvironment [27, 28].

In the present study, we showed that the frequencies of Th17 and Tc17 cells are significantly lower in progressive CLL compared with indolent patients and normal subjects. Furthermore, we showed that this decrease is associated to expansion of Treg cells expressing CD39. This imbalance in IL-17⁺ T cells and CD39⁺ Treg cells seems to be independent of expansion of other T cell subsets, because frequencies of IL-4 and IFN- γ secreting cells and expression of the Th1- and Th2-specific transcription factors T-bet and GATA-3 were similarly represented in both progressive and indolent patients. The suppressive function of isolated CD4⁺ Treg cells from CLL patients on proliferation of polyclonally activated effector T cells was similar to those of the normal subjects. These novel findings suggest that imbalance in Th17/Tc17 and Treg cells might contribute to immunopathogenesis and prognosis of CLL.

Materials and methods

Patients and blood samples

A total number of 40 untreated CLL patients (26 males, 14 females; mean age 61 years, range 40–82 years) attending the Hematology and Oncology Clinics of Vali-Asr and Fir-ozgar Hospitals, affiliated to Tehran University of Medical Sciences, and 15 age-matched normal subjects, including ten males and five females with age range of 41–63 years and a mean of 58 years were included in this study. Fifty milliliters of heparinized peripheral blood was taken from all patients and normal subjects after written informed consent was obtained according to the Ethical Committee of Tehran University of Medical Sciences. The CLL diagnosis was based on immunophenotypic analysis, blood cell count, cell morphology, and clinical symptoms according to the criteria outlined by the World Health Organization [29]. Patients were classified in progressive ($n=20$) and indolent ($n=20$) or IGHV-mutated ($n=24$) and -unmutated ($n=16$) groups. Disease progression was identified according to the National Cancer Institute Working Group Criteria [30, 31]. Of the 40 CLL patients, 24, 5, 7, 1, and 3 were classified in stages 0, I, II, III, and IV, respectively, based on Rai staging system [32]. Major characteristics of our CLL patients are shown in Supplementary Table S1.

Peripheral blood mononuclear cell isolation

The fresh peripheral whole blood from both patients and healthy volunteers (HVs) were isolated using Histopaque (Sigma, St Louis, MO, USA) density-gradient centrifugation, as described previously [33]. Isolated peripheral blood mononuclear cell (PBMC) were washed twice with RPMI-1640 culture medium and resuspended in the same medium supplemented with 10 % fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 IU), and streptomycin (100 μ g/mL) (Biosera, Ringmer, East Sussex, UK) for immunophenotyping. The viability of isolated cells was more than 95 % as assessed by Trypan blue viability test.

T cell enrichment by sheep red blood cell rosetting

Due to low frequency of T cells and extremely high numbers of leukemic B cells in PBMC of CLL patients, T cells were first enriched by rosette formation using 2-aminoethyl isothiuronium bromide (AET) (Sigma)-treated sheep red blood cells (SRBC), as previously described [34]. Briefly, 4 % AET solution were added to packed washed SRBC at 4:1 ratio and incubated for 20 min in 37 °C and washed by cold PBS two times. RPMI-1640 was added to packed washed AET-treated SRBC to prepare 4 % SRBC suspension. The suspension of PBMC (10^6 cell/ml) and AET-

treated SRBC was mixed at 1:1 ratio, centrifuged and incubated at 4 °C for 2 h, and subsequently overlaid on Histopaque. For deletion of non-specific bindings, fetal bovine serum was added to suspension of PBMC-treated SRBC in 1:10 ratio. Buffy coat layer on the top of Histopaque included non-T cells. The final pellet containing rosette-formed T cells was treated with RBC lysis buffer (0.8 % NH₄Cl, 0.1 mM EDTA, pH 7.2–7.6) to recover T cells. Using this simple approach, T cells were usually enriched more than 15- to 20-fold as assessed by flow cytometry (15–70 % of enriched population). Purity of B cells in buffy coat was usually more than 97 %, and viability of isolated cells was greater than 95 %.

Treg isolation by magnetic-activated cell sorting

CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ effector T cells were isolated from enriched T cells of patients and PBMC of normal subjects using CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, non-CD4⁺ cells were negatively selected by labeling with a cocktail of biotin-conjugated monoclonal antibodies (mAbs) against non-CD4⁺ cells and subsequently with antibiotin mAbs conjugated to microbeads. Subsequently, CD25⁺ cells were positively selected through labeling of purified CD4⁺ cells with anti-CD25-coupled microbeads. Purity was checked by fluorescence-activated cell sorting (FACS) and was usually greater than 90 %.

Flow cytometry

Pre-enriched T cells were analyzed by FACS using optimized amount of the following fluorochrome-conjugated monoclonal antibodies (mAbs): CD19-FITC (HIB19), CD5-PE (UCHT2), ZAP-70-FITC (1E7.2), CD38-PE (HIT2), CD3-PECY5 (UCHT1), CD4-FITC (RPA-T4), CD4-PECY5 (RPA-T4), CD8-FITC (HIT8a), CD39-FITC (eBioA1), FoxP3-PE (236A/E7), IL-17-PE (eBio64DEC17), interferon-gamma (IFN- γ)-PE (4S.B3), IL-4-PE (8D4-8), FITC-mouse IgG1 κ isotype control (P3.6.2.8.1), PE-mouse IgG1 κ isotype control (P3.6.2.8.1), and PECY5-mouse IgG1 κ isotype control (P3.6.2.8.1). All mAbs, with the exception of CD38-PE (HIT2) (BD Biosciences, San Jose, CA, USA), were purchased from eBioscience (San Diego, CA, USA). Briefly, after washing twice with washing buffer (PBS 0.15 M, 0.5 % BSA, 0.1 % Na₃) 1×10^6 cells were resuspended in 100- μ l washing buffer and incubated with appropriate specific fluorochrome-conjugated mAbs for 45 min at 4 °C in the dark. For intracellular FoxP3 staining, after surface staining with appropriate mAbs, cells were washed twice with Perm/Wash buffer (supplemented in BD Cytotfix/Cytoperm

Fixation/Permeabilization kit) and subsequently fixed and permeabilized with BD Cytotfix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) for 20 min at 4 °C in the dark. After washing twice with Perm/Wash buffer, fixed and permeabilized cells were incubated with an optimized amount of FoxP3-PE mAb for 45 min at 4 °C in the dark. Cells were then washed twice with Perm/Wash buffer before scanning by flow cytometer.

For detection of IL-17, IL-4, and IFN- γ -producing T cells, pre-enriched T cells were initially stimulated for 5 h with PMA (25 ng/mL) (Sigma) plus ionomycin (500 ng/mL) (Sigma) in the presence of Brefeldin A (supplemented in BD Cytotfix/Cytoperm Fixation/Permeabilization kit) (BD Biosciences). After stimulation, cells were washed twice with washing buffer resuspended in 100- μ l washing buffer and incubated with appropriate surface mAbs for 45 min at 4 °C in the dark. For intracellular IL-17, IFN- γ , and IL-4 staining, the same procedure was adapted as that employed for FoxP3 staining. Stained cells were assessed in Partec PAS flow cytometer (Partec, Nuremberg, Germany), and the data were analyzed by Flomax flow cytometry analysis software (Partec).

Quantitative real-time polymerase chain reaction

Expression of the lineage-specific transcription factors Tbet, GATA-3, ROR γ t, and FoxP3 was determined by real-time PCR. Total RNA was extracted from PBMC of 12 CLL patients (six progressive and six indolent patients), and cDNA was synthesized as described [35]. Quantitative real-time PCR was performed on an ABI StepOnePlus system (Applied Biosystems) using the following specific primers (5'-3' forward, reverse): Tbet: CCAACAATGTGAC CCAGATGATT, TATGCGTGTTGGAAGCGTTG; GATA-3: CCCTACTACGGAAACTCGGTCA, GTAG GGATCCATGAAGCAGAGG; Foxp3: ACAGCACATT CCCAGAGTTCCT, GATGAGCGTGGCGTAGGTG; ROR γ t: ACAGCACCGAGCCTCACG, CAGACGACT TGTCCCCACAGA; and β -actin: CAGCAGATGTGGAT CAGCAAG, GCATTGCGGTGGACGAT. All PCRs were performed using SYBR Green Master Mix (PrimerDesign, Southampton, UK) with a total volume of 20 μ l. All samples were first denatured for 5 min at 95 °C. Samples were then amplified for 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Gene-specific amplification was tested by melting curve analysis. Relative quantification method was used for data analysis based on the relative expression of target gene mRNA to β -actin mRNA level as a housekeeping. Relative expression of each target gene in T cells was calculated, taking into consideration the percent of CD3⁺ T cells in PBMC of each patient as determined by flow cytometry.

Treg functional assay

Suppressive potential of Treg cells on the proliferation of effector T cells was investigated in six CLL patients (three progressive and three indolent) and six normal subjects by using [³H] thymidine incorporation assay. For demonstration of Treg cell suppressive function on proliferation of pre-stimulated T cells, 9×10^4 CD4⁺CD25⁻ effector T cells purified by magnetic beads were cultured and stimulated with Phytohaemagglutinin (PHA) (Sigma) at 10 µg/ml in sterile 96-well flat-bottomed plates (NUNC, Roskilde, Denmark) in triplicate at 37 °C in a humidified 5 % CO₂ incubator for 36 h. Concomitantly, 10^4 purified CD4⁺CD25⁺ Tregs were also cultured separately. After 36 h, effector T cells were cocultured with CD4⁺CD25⁺ Treg cells for an additional 36 h. As a control, 9×10^4 polyclonally activated CD4⁺CD25⁻ effector T cells were cultured alone. Finally, the suppressive capacity of Tregs on effector T cell proliferation was determined by addition of 1 µCi per well [³H] thymidine (PerkinElmer, Boston, USA). Cells were subsequently harvested after 18 h of incubation and transferred to scintillation fluid for measurement of [³H] thymidine incorporation by a beta counter (Wallac 1410 Liquid Scintillation Counter, Pharmacia, Sweden).

Assessment of IGHV mutation status

We analyzed the IGHV expressed by the CLL cells of all patients as previously described [2]. Sequences with less than 98 % homology with the corresponding germline IGHV genes were considered mutated.

Statistics

Statistical analysis was performed using the SPSS statistical package (SPSS, Chicago, IL, USA). The results were evaluated by independent samples *t* test, Mann–Whitney *U* test, and Pearson and Spearman correlation tests as appropriate. Probability values of less than 0.05 were considered significant. The results presented in text and tables represent mean ± standard error of mean (SEM).

Results

Enumeration of Th17 cells in CLL patients and normal subjects

Representative flow cytometry dot plots illustrating the analysis method for detection and enumeration of CD3⁺CD4⁺IL-17⁺ Th17 cells are shown in Fig. 1. Our results showed that the frequency of CD3⁺CD4⁺IL-17⁺ (Th17) cells was significantly lower in progressive (0.99 ±

0.12 % of total CD3⁺CD4⁺ cells) compared to indolent (1.57 ± 0.24 %, *p* = 0.04) patients and normal subjects (1.78 ± 0.2 %, *p* = 0.003) (Fig. 2a), though the differences were marginal. Additionally, Th17 cells were decreased in patients at Rai stages II–IV (*n* = 11) (1.05 ± 0.17 %) compared to those at early stages 0–I (*n* = 29) (1.37 ± 0.18 %). The difference, however, was not statistically significant. When the data were analyzed based on the mutational status of IGHV, we observed a significant decrease of Th17 cells in unmutated IGHV compared to mutated samples (0.88 ± 0.13 and 1.56 ± 0.2 %, respectively, *p* = 0.007). No significant difference in the frequency of Th17 cells was observed between ZAP-70-positive and ZAP-70-negative (1.33 ± 0.28 vs. 1.26 ± 0.16 %) as well as CD38-positive and CD38-negative (1.21 ± 0.2 vs. 1.32 ± 0.18 %) groups. The mean fluorescence intensity (MFI) of IL-17 in CD3⁺CD4⁺ cells was also lower in progressive (9.93 ± 1.67) as compared with indolent patients (14.83 ± 1.77, *p* = 0.052) and normal subjects (16.79 ± 1.77, *p* = 0.007) (Fig. 2b). The frequency of Th17 cells highly correlated with the frequency of CD8⁺IL-17⁺ (*r* = 0.63, *p* < 0.001).

The frequency of CD8⁺IL-17⁺ (Tc17) cells in CLL patients and normal subjects

Investigation of the frequency of CD8⁺IL-17⁺ (Tc17) cells in isolated T cells from CLL patients and PBMC of normal subjects showed that Tc17 cell are decreased in progressive (0.44 ± 0.09 % of total CD8⁺ T cells) and IGHV-unmutated (0.44 ± 0.1 %) patients compared to indolent (0.82 ± 0.2 %, *p* = 0.09) and IGHV-mutated (0.76 ± 0.17 %, *p* = 0.12) patients, though these differences were not significant. This subset of IL-17-producing T cells was also significantly decreased in progressive patients compared to normal subjects (0.71 ± 0.09 %, *p* = 0.04) (Fig. 2c). Moreover, the frequency of Tc17 cells was significantly decreased in patients at advanced Rai stages II–IV compared to those at early stages 0–I (0.35 ± 0.1 vs. 0.73 ± 0.14 %, *p* = 0.042). The frequency of Tc17 cells was similarly represented in ZAP-70-positive and -negative (0.67 ± 0.2 and 0.61 ± 0.13 %) as well as CD38-positive and -negative (0.65 ± 0.13 and 0.62 ± 0.15 %) groups, respectively (Table 1).

Comparison of IL-17 MFI in CD8⁺ (Tc17) cells showed a lower intensity of IL-17 in progressive (16.41 ± 5.6) compared with indolent patients (35.57 ± 8.6, *p* = 0.072), though the difference was not significant (Fig. 2d).

The frequency of CD4⁺FoxP3⁺CD39⁺ regulatory T cells in CLL patients and normal subjects

Our results showed that a significantly higher proportion of CD4⁺FoxP3⁺ Treg cells from progressive patients express CD39 (80.4 ± 4.2 % of total CD4⁺FoxP3⁺ cells) compared to

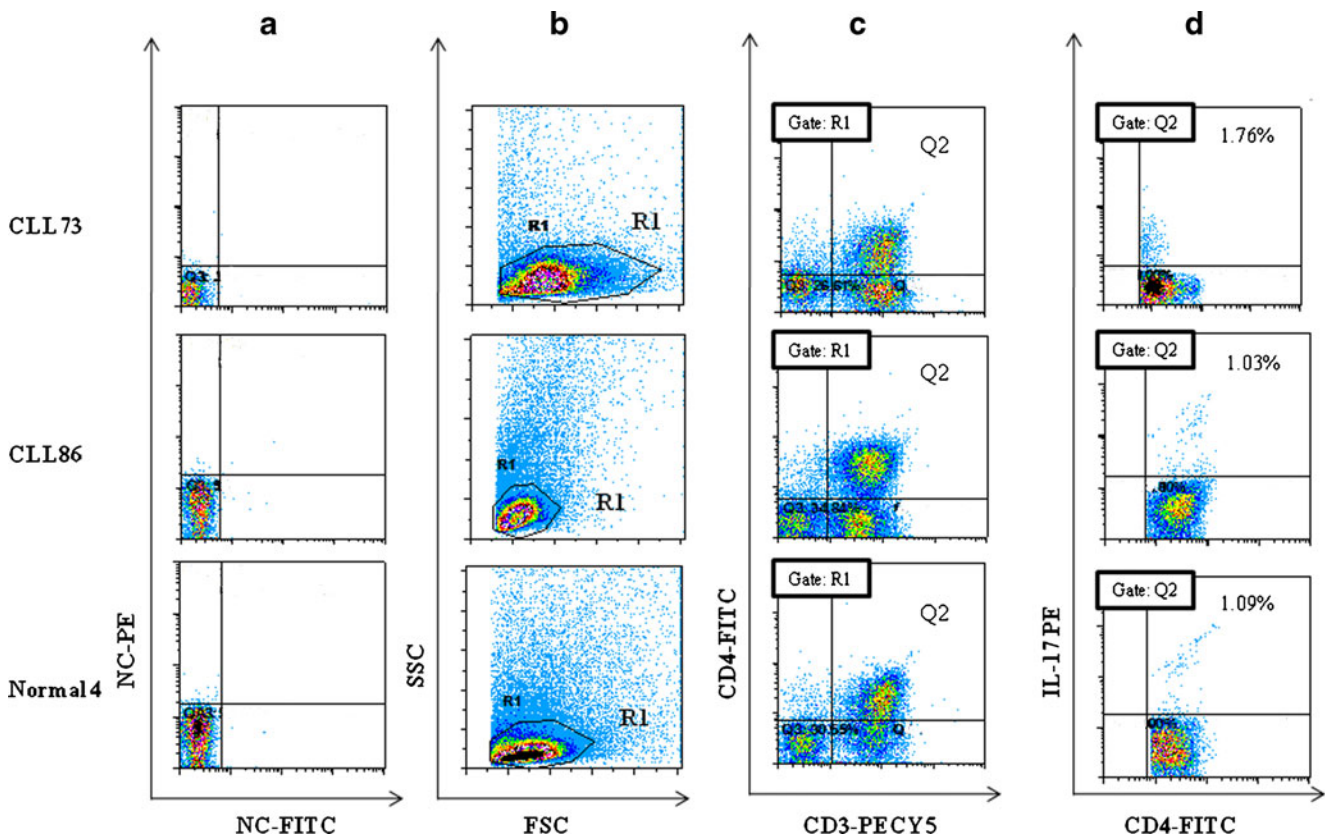


Fig. 1 Representative dot plots demonstrating the analysis method for identification of Th17 cells among the CD3⁺CD4⁺ T cell population. Dot plots obtained for two CLL patients and one normal subject are shown. **a** The isotype-matched negative controls (*NC-FITC* and *NC-PE*) are shown. **b** The lymphocyte population was gated (*R1*) based on

forward vs. side scatter (*FSC/SSC*) characteristics. **c** The dot plots show CD3⁺ vs. CD4⁺ T cell distribution and the *Q2* region was used to select CD3⁺CD4⁺ T cells. **d** The *Q2* gated events were then selected for enumeration of CD4⁺IL-17⁺ cells

those from indolent patients ($67.4 \pm 4\%$, $p=0.032$) or normal individuals ($60.09 \pm 3.77\%$, $p=0.001$) (Table 1). The frequency of CD39⁺CD4⁺FoxP3⁺ Treg subset was found to be significantly higher in progressive patients ($9.62 \pm 0.78\%$ of total CD4⁺ T cells) compared to indolent patients ($5.25 \pm 0.72\%$, $p<0.001$) and normal subjects (2.31 ± 0.25 , $p<0.001$) (Fig. 2e). Representative flow cytometry dot plots illustrating the analysis method for detection and enumeration of CD4⁺FoxP3⁺CD39⁺ regulatory T cells are shown in Fig. 3. The frequency of this subset of Treg cells was similarly represented in IGHV-mutated and -unmutated, ZAP-70-positive and -negative, as well as CD38⁺-positive and -negative CLL samples. No correlation was found between the CD39 expressing CD4⁺ Treg cells with IL-17-producing T cells, age, serum Hb concentration, platelet and white blood cell count, Rai stage, or lymphocytosis.

The frequencies of IFN- γ and IL-4 secreting CD4⁺ T cells in CLL patients

We evaluated the frequencies of IFN- γ ⁺ and IL-4⁺ cells in CD4⁺ T cells of 12 CLL patients (six progressive and six indolent

patients). Representative dot plots illustrating the analysis method for CD4⁺IFN- γ ⁺ Th1 cells and CD4⁺IL4⁺ Th2 cells have been shown in Fig. 4. There was no significant difference in the frequencies of these two subsets of cells between progressive (10.6 ± 1.1 and $10.6 \pm 2.7\%$ of total CD4⁺ T cells, respectively) and indolent (13.8 ± 1.4 and $10.3 \pm 1.1\%$, respectively) patients, while there was a significant difference in the frequency of CD39⁺ Treg cells (11 ± 1.5 vs. $5 \pm 0.9\%$, $p=0.015$) and to a lesser extent in the frequency of Th17 cells (1 ± 0.3 vs. $2.2 \pm 0.4\%$, $p=0.065$) between the same selected groups of patients (Fig. 5a).

Expression levels of lineage-specific transcription factors in PBMC of CLL patients

We analyzed the mRNA levels of some lineage-specific transcription factors in the same groups of patients described above (six progressive and six indolent patients) by real-time PCR. As shown in Fig. 5b, the expression levels of T-bet (Th1 lineage-specific transcription factor) and GATA-3 (Th2 lineage-specific transcription factor) were similarly represented in progressive and indolent patients. However, the mRNA levels of ROR γ t (Th17 lineage-specific

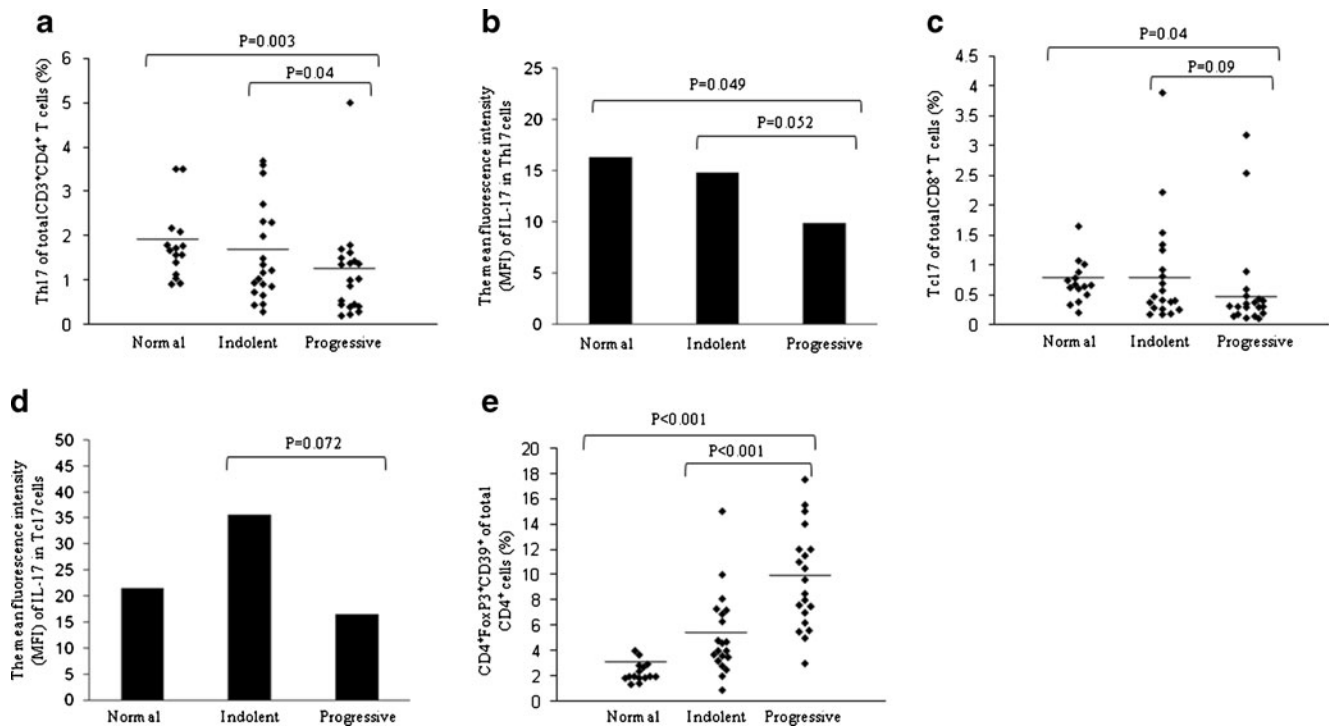


Fig. 2 Frequency and mean fluorescence intensity of IL-17⁺ T cells and CD39⁺ Treg cells from CLL patients and normal subjects. **a** Frequency of CD3⁺CD4⁺IL-17⁺ cells among CD3⁺CD4⁺ T cells. **b** The mean fluorescence intensity (MFI) of IL-17 in CD3⁺CD4⁺ T cells.

c Frequency of CD8⁺ IL-17⁺ Tc17 cells among the CD8⁺ T cells. **d** The MFI of IL-17 in CD8⁺ T cells. **e** Frequency of CD4⁺FoxP3⁺CD39⁺ Treg among CD4⁺FoxP3⁺ T cells. Horizontal lines represent mean levels

transcription factor) was expressed at several folds higher in indolent compared to progressive patients and FoxP3 (Treg lineage-specific transcription factor) was slightly lower in indolent compared to progressive patients. However, it should be noted that due to low sample size (six progressive and six indolent patients) applied in these experiments, differences did not reach statistical significance as analyzed by Mann–Whitney *U* test.

Treg cells inhibit proliferation of effector T cells

Our results showed that purified CD4⁺CD25⁺ Treg cells from six CLL patients (three progressive and three indolent) and six

normal subjects similarly inhibited PHA-mediated proliferation of effector T cells in patients and normal controls. Despite the small sample size, when the inhibition levels obtained for all six CLL patients (31.2±6.5 %) were compared with those of the normal subjects (25.4±8.1 %), no statistically significant differences were achieved (Fig. 6).

Discussion

It is generally accepted that progression of autoimmune diseases is associated with Treg cell decrease and Th17 expansion [36, 37]. On the other hand, cancer development

Table 1 Mean frequency of different subsets of IL-17-producing cells and CD39⁺ regulatory T cells in CLL patients and normal subjects

Phenotype	Total CLL patients	IGHV mutated	IGHV unmutated	ZAP-70 ⁺	ZAP-70 ⁻	CD38 ⁺	CD38 ⁻
CD3 ⁺ CD4 ⁺ IL-17 ⁺	1.28±0.14	1.56±0.2	0.88±0.13	1.33±0.28	1.26±0.16	1.21±0.2	1.32±0.18
CD8 ⁺ IL-17 ⁺	0.63±0.11	0.76±0.17	0.44±0.1	0.67±0.2	0.61±0.13	0.65±0.13	0.62±0.15
CD4 ⁺ FoxP3 ⁺	10.1±0.68	10.29±0.95	9.83±0.95	10.85±1.33	9.79±0.79	10.1±1.25	10.11±0.82
CD39 ⁺ (from total CD4 ⁺ FoxP3 ⁺)	73.88±3.1	75.3±3.9	71.75±4.95	75.1±5.9	73.3±3.6	71.9±7	74.8±3.1
CD39 ⁺ FoxP3 ⁺ (from total CD4 ⁺ cells)	7.4±0.6	7.73±0.92	7±0.92	8.2±1.4	7.1±0.73	7.4±1.29	7.45±0.77

Results represent mean ± SEM

CLL chronic lymphocytic leukemia, IGHV immunoglobulin heavy chain variable region

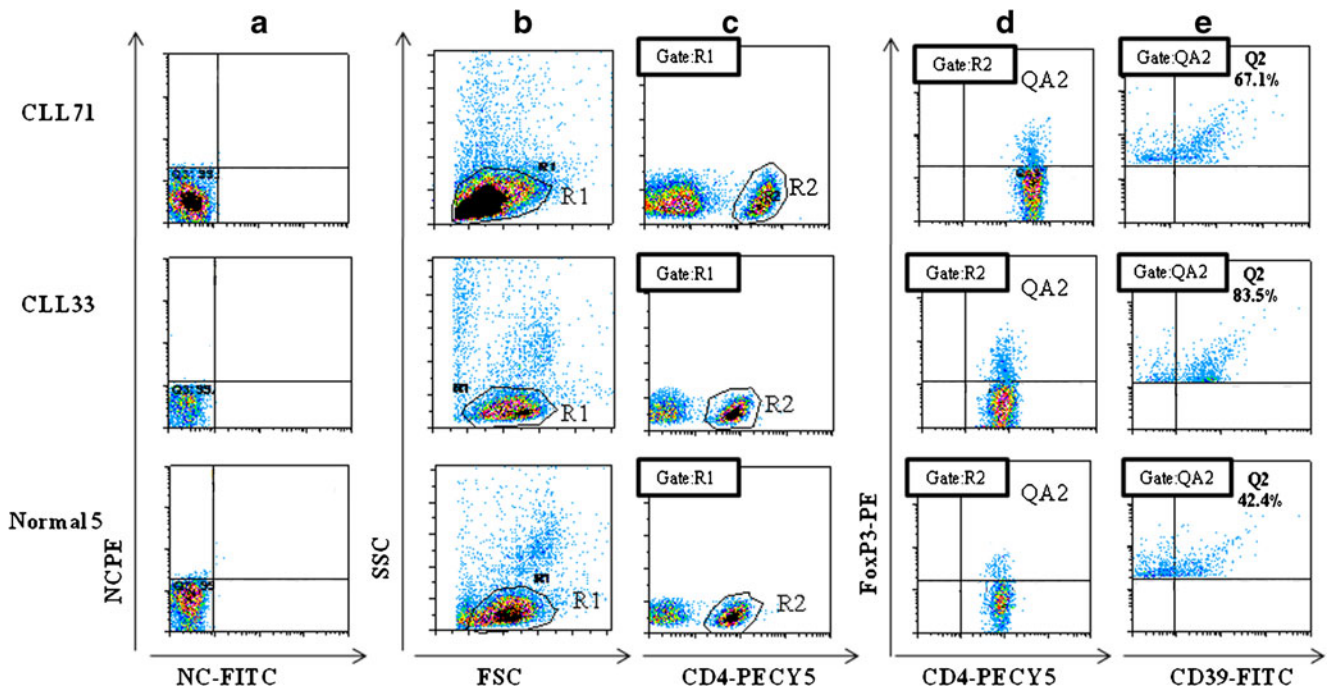


Fig. 3 Representative dot plots demonstrating the analysis method for identification of CD39⁺ Treg cells. Dot plots obtained for two CLL patients and one normal subject are shown. **a** The isotype-matched negative controls (*NC-FITC* and *NC-PE*) are shown. **b** The lymphocyte population was gated (*R1*) based on forward vs. side scatter (*FSC/SSC*)

characteristics. **c** The dot plots show CD4⁺ vs. *SSC* distribution and the *R2* region was used to select CD4⁺ T cells. **d** The *R2* gated events were then selected for discrimination of CD4⁺FoxP3⁺ Treg cells in *QA2* region. **e** Events included in *QA2* region were gated for discrimination of CD39⁺ Treg cells among the CD4⁺FoxP3⁺ Treg cells

is thought to be linked to Treg burden and Th17 regression [38, 39]. However, there are no sufficient data regarding the role of Th17 cells in cancer progression [40].

In the present study, we showed that the frequencies of both Th17 and to a lesser extent Tc17 cells are inversely associated to disease progression, implying their possible protective role in the pathogenesis of CLL. Contribution of these subsets of T cells in CLL pathogenesis should be taken with caution, considering their low frequency and the massive expansion of leukemic B cells as well as the lack of data on their biological function in CLL. The mechanism by which Th17 cells exert their protective role is not clear and

needs further investigation. The current data regarding the role of Th17 cells in immunopathogenesis of cancer diseases are limited. Antitumorigenic function of Th17 cells has been demonstrated in melanoma [41], B16 melanoma mouse model [42], prostate [43], pancreas [44], ovarian [45], and breast [46] cancers. Contrary to solid tumors, there are few studies regarding the role of Th17 cells in hematologic malignancies. It has been reported that serum levels of IL-17 is increased in multiple myeloma patients and correlated to disease prognosis which suggest IL-17 as tumor-promoting factor in multiple myeloma [11, 47]. The Th17 cells were found to be accumulated in the bone marrow of

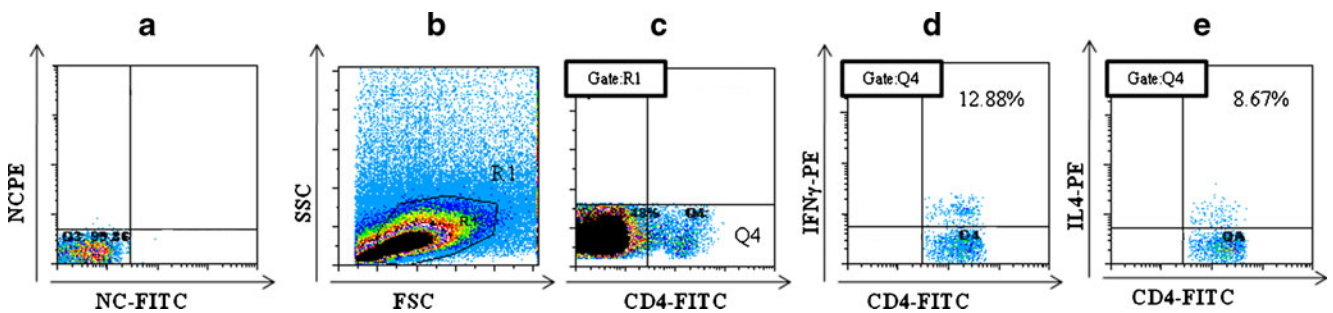


Fig. 4 Representative dot plots illustrating the analysis method for identification of CD4⁺IFN- γ ⁺ Th1 cells and CD4⁺IL4⁺ Th2 cells. **a** The isotype-matched negative controls (*NC-FITC* and *NC-PE*) are shown. **b** The lymphocyte population was gated (*R1*) based on forward

vs. side scatter (*FSC/SSC*) characteristics. **c** The dot plots show CD4⁺ vs. *SSC* distribution, and the *Q4* region was used to select CD4⁺ T cells. The *Q4* gated events were then selected for discrimination of **d** CD4⁺IFN- γ ⁺ Th1 and **e** CD4⁺IL-4⁺ Th2 cells

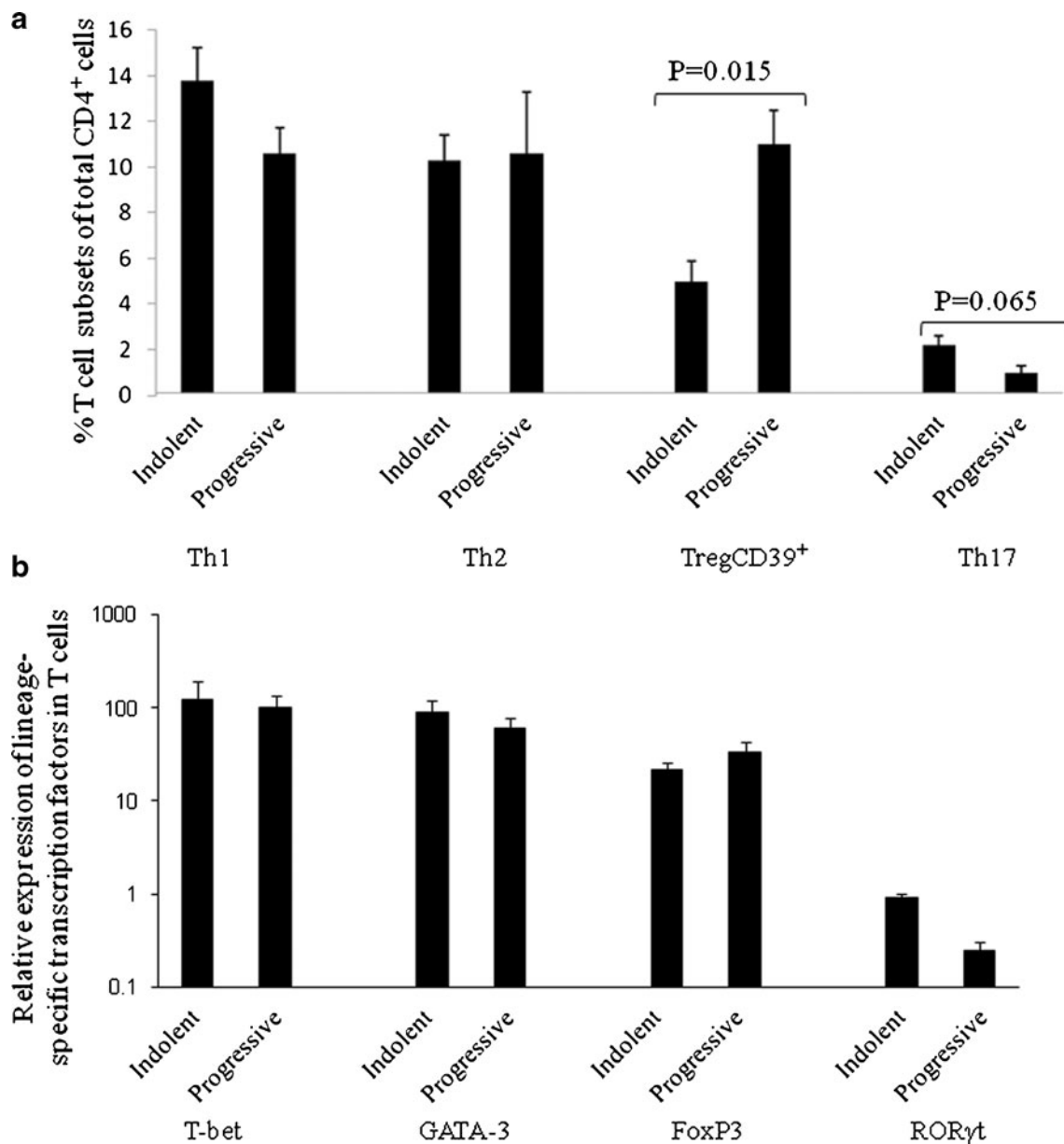


Fig. 5 Expression of IFN- γ , IL-4, and lineage-specific transcription factors in a selected group of CLL patients. **a** Frequencies of IFN- γ ⁺ and IL-4⁺ T cells were investigated in a selected number of CLL patients (six progressive and six indolent patients) by flow cytometry. The results obtained for CD39⁺ Treg cells and Th17 cells in the same

group of patients have also been shown for comparison. **b** Relative expression of the lineage-specific transcription factors T-bet (for Th1), GATA-3 (for Th2), FoxP3 (for Treg), and ROR γ t (for IL-17⁺ cells) was determined in mRNA obtained from the same selected CLL patients by real-time PCR

myeloma patients [12]. In AML patients, the frequency of Th17 cells was significantly higher compared to healthy controls and correlated with serum IL-17 concentration [13]. In contrast to AML, Yang et al. reported a lower frequency of Th17 cells in patients with non-Hodgkin lymphoma (0.44 % of total CD4⁺ T cells) compared to normal controls (1.25 %) [14].

Enumeration of IL-17-producing T cells in CLL has recently been reported in a limited number of patients showing highly variable frequencies. Thus, while the frequency

of IL-17-producing T cells constituted 3 % of the total CD4⁺ T cells in a limited number of CLL/small lymphocytic lymphoma patients [14], highly variable number of CD4⁺ (0.5–46 %) and CD8⁺ (0.2–31 %) [48, 49] T cells were found to secrete IL-17 in only three and six CLL patients, respectively. These controversial results were obtained in a limited number of CLL patients, and lack of information on the association of these cells with disease progression has promoted us to perform this study. Thus, we enumerated Th17 cells in the peripheral blood of different subtypes of

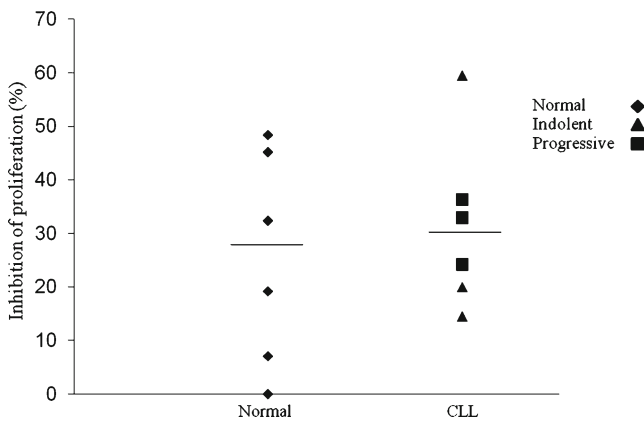


Fig. 6 CD4⁺ Treg cells inhibit the proliferation of polyclonally activated effector T cells in CLL patients and normal subjects. The proliferation assay was performed by 3H-thymidine incorporation assay. All tests were performed in triplicate. Horizontal lines represent mean levels

CLL patients and showed that the frequency of Th17 cells was significantly decreased in progressive patients compared to indolent patients, suggesting their protective role in CLL pathogenesis. Attention to this point is essential that this decrease in IL-17-producing T cells might be, in part, due to decreased MFI of IL-17 in T cells in progressive compared to indolent patients and normal controls. Association between CLL progression and mutational status of IGHV as well as expression of CD38 and ZAP-70 molecules in leukemic B cells has been reported by many investigators [2, 8, 50, 51]. We, however, failed to find any association between the number of IL-17-producing T cells and CD38 or ZAP-70 expression in our patients (Table 1). Although we showed that the frequency of Treg cells was increased in progressive patients compared to indolent patients, we did not find this difference when data were analyzed based on IGHV mutational status. This may be, in part, due to the fact that we also could not find any correlation between disease progression and IGHV mutational status in our patients. Lack of correlation could be due to the low sample size employed in this analysis.

Data regarding the role of Tc17 cells in cancer are little; however, there is evidence which suggests their protective role in tumor microenvironment [17, 52]. Antitumor activity of Tc17 cells was reported in an animal model by Hinrichs and coworkers [18]. They showed that after adoptive transfer, these cells are converted to IFN- γ -producing effector cells and mediate regression of established tumors. This improved antitumor immunity was associated with increased expression of IL-7R α , decreased expression of killer cell lectin-like receptor G1, and enhanced persistence of the transferred cells [18]. In other study, *adoptive* transfer of Tc17 cells was found to control tumor growth in early- and late-stage melanoma [17]. Moreover, it was shown that tumor-activated monocytes secrete a set of key cytokines

(IL-1 β , IL-6, and IL-23) to stimulate the proliferation of Tc17 cells [19].

Here, we showed that the frequency of Tc17 cells was decreased in progressive as compared with indolent CLL patients, but the difference did not reach statistical significance. Thus, our results could not prove a protective role for these cells in CLL. Although some data regarding the protective mechanisms exerted by Tc17 cells have already been reported [53], little is known regarding the protective mechanisms of IL-17-producing cells for the control of tumor cells. However, considering pro-inflammatory function of these cells, it seems that they provide an inflammatory microenvironment in which tumoral cells could be killed. Th17 cells have been shown to secrete both IFN- γ and IL-17 cytokines which are effective factors in mediating antitumor responses [54]. It doesn't seem that Th17 cells use contact-dependent mechanisms in their antitumor responses. The main effective antitumor CD4⁺ T cell subset involved in the control of B cell malignancies are thought to be Th1 cells [55]. However, contrasting results regarding the frequency and balance status between Th1/Th2 cells have been reported in CLL patients. While a shift from Th1 towards Th2 cells has been shown to be associated with CLL progression [56], decreased Th2 [57] or no change in Th1 [58] cell number have also been reported in CLL patients.

Our results obtained from a limited number of selected progressive and indolent patients did not display a significant difference between these two subsets of T cells both at the level of cytokine secretion profile (IFN- γ and IL-4) and expression of the lineage-specific transcription factors T-bet and GATA-3 (Fig. 5). Our data suggest that imbalance between IL-17-producing T cells and Treg cells in CLL patients is not due to relative shift in T cell populations such as Th1 or Th2 cells which has already been reported in these patients following some viral infections, such as cytomegalovirus infection [59, 60].

In order to investigate the balance between IL-17-producing T cells and Treg, we enumerated different subsets of Treg cells. We found that Treg cells are increased with CLL progression, which is consistent with previous reports in other malignancies [20–23, 61, 62]. Our results suggest that this increase in Treg frequency is associated with a decrease in Th17 and Tc17 frequencies. Two most recent published reports showed that CD39⁺ Treg cells could suppress generation and differentiation of Th17 cells in human [28] and animal cancer models [27]. Moreover, recent report by Pulte et al. [63] showed that the percentage of cells expressing CD39 was higher in both CD4⁺ cells and CD8⁺ cells in CLL patients compared to normal controls. Interestingly, they found that higher percentage of T cells expressing CD39 was associated with advanced disease stage which is consistent with our data [63]. In the present study, we also enumerated the CD39⁺ Treg subset for the first time in CLL

patients and detected significantly higher number of these cells in progressive as compared with indolent patients and normal subjects. Although increased frequency of CD4⁺ Treg cells has already been reported in CLL patients, this is the first paper which reports upregulation of CD39⁺ Treg cells in CLL. Importance of this subset of Treg cells is due to their inhibitory potential on Th17 cells. These cells have recently been shown to suppress IL-17-producing T cells through adenosine production from extracellular ATP [26]. However, the relevance of increased frequency of Treg cells to the expansion of leukemic B cells and also disease progression is not fully substantiated. Treg cells have also been shown to suppress other effector T cell subsets [19, 61] and thereby could negatively influence the control of leukemic B cell expansion.

We could not find significant correlation between the frequencies of CD39⁺ Treg and IL-17-producing T cells in our CLL patients. Thus, it seems that these two subsets of T cells are regulated differently in CLL patients. Moreover, our real-time PCR results regarding the mRNA levels of lineage-specific transcription factors including FoxP3 and ROR γ t confirmed the imbalance between CD39⁺ Treg cells and IL-17-producing T cells. However, it should be noted that due to low sample size (six progressive and six indolent patients) employed in this set of experiments, differences did not reach statistical significance. A recent report showed that malignant B cells could suppress IL-17-producing T cells and induce Treg cells in non-Hodgkin lymphoma patients, and this effect was mediated in part through CD27-CD70 or CD28-B7.1,2 interactions [14]. The leukemic B cells of CLL produce large amount of TGF- β [64] and, to some extent, IL-2 [61] which are, in combination, potent inducers of Treg cells and inhibitors of IL-17⁺ T cells [37]. Moreover, it has been shown that IL-10 produced by B cells can suppress Th17 cells [65], and since B CLL cells are the main source of IL-10 and Th17 cells express IL-10 receptor [66], thus Th17 cells might be downregulated by IL-10 produced by the leukemic B cells independent of Treg cells. There are other Th17 attenuators which are not studied in CLL patients including cytokines such as IL-25 and IL-27 [9]. Investigation of these mediators may shed more light on the reciprocal relationship between Treg and IL-17⁺ T cells observed in our CLL patients. We tried to measure some cytokines such as IL-17 and IL-10 in serum of our CLL patients, but, due to the very low serum levels of these cytokines and the sensitivity of our ELISA kits, serum samples from most patients were negative.

It should be noted that Treg and particularly IL-17⁺ T cells constitute a very small fraction of the total human T cell pool, and T cells form a small fraction of the PBMC in many CLL patients due to massive expansion of the peripheral leukemic B cells which may constitute up to 95 % of the total PBMC. Thus, we decided to enrich patients' T cells to

be able to enumerate peripheral IL-17⁺ T cells and Treg cells more precisely. We adapted this approach to determine the frequency of Treg and IL-17⁺ T cells in total T cells, but this approach unfortunately does not allow us to determine the absolute count of these cells.

Our results on Treg suppressive function showed that the inhibitory effects of Treg cells on the proliferation of effector T cells in indolent and progressive CLL patients were similar to those of normal subjects. Although it is difficult to discuss about the difference in suppressive function of Treg cells between indolent and progressive patients as well as normal controls due to the small sample size, it seems that the increased frequency of the Treg cells in CLL patients and particularly patients with progressive disease could induce higher suppressive function on the immune cells, leading to expansion of leukemic cells and disease progression.

In conclusion, our novel findings of decreased frequency of CD4⁺ and CD8⁺ IL-17-producing cells in progressive CLL patients, concomitant with elevated frequency of CD4⁺CD39⁺ Treg cells in the CLL patients, imply their important role in the progression of CLL and their potential implication in immunotherapy of this malignancy.

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Conflicts of interest None.

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