Clinical and Experimental Health Sciences

Intracellular Levels of IL-10 and STAT3 in Patients with Chronic Lymphocytic Leukemia

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ABSTRACT

Objective: Chronic Lymphocytic Leukemia (CLL) is characterized by the accumulation of $CD5^+CD19^+$ B cells in the bone marrow and peripheral blood. Recent studies indicated that expression of *IL-10*, AID and *mir-155* which are regulated by STAT3 are increased in CLL patients. $CD5^+CD19^+$ regulator B (B_{reg}) cells secrete IL-10 and suppress the immune system. While the CLL cells show similar immunophenotypic properties to Breg cells, they are also thought to be functionally similar. In this study, STAT3 and IL-10 levels of CLL patients were investigated.

Methods: Peripheral blood samples obtained from patients (n:24) and healthy controls (n:14). Peripheral blood mononuclear cells were cultured for 48 hours in the presence and absence of CpG for IL-10 expression and cultured with and without PMA for STAT3 expression. IL-10 and STAT3 expression were analyzed with anti-CD5, anti-CD19, anti-CD38, anti-STAT3 and anti-IL-10 monoclonal antibodies by using flow cytometry.

Results: Compared to healthy subjects, increased $IL\text{-}10^+$, $IL\text{-}10^+CD19^+$, $STAT3^+CD19^+$ were obtained in lymphocyte population of patients. Increased IL-10 was showed $CD19^+$ B cells of CLL patients. Our results showed that IL-10 levels had no significant difference between $CD5^+CD19^+$ cells, whereas STAT3 levels were found lower in patient compared to healthy controls.

Conclusion: These results made us thought that the levels of IL-10 and STAT3 expression in CLL B cells is clearly different from normal B lymphocytes might have a role in the biology of CLL. It is believed that the presented data will contribute to the studies that scrutinize the similarity of CLL cells to Breg cells.

Keywords: CLL, IL-10, STAT3, Breg, flow cytometry

1. INTRODUCTION

Chronic lymphocytic leukemia **(**CLL) is the most common leukemia in the western countries, and is characterized by the accumulation of $CD5^+CD19^+$ B cells in the bone marrow, peripheral blood and lymphoid organs (1). The clinical course of patients with CLL is highly variable and therefore difficult to predict (2)

Regulator B (B_{reg}) cells secrete IL-10 and suppress the immune system (3). There is no unique surface marker to identify B_{reg}, $CD19^+CD24^{hi}CD38^{hi}$ and $CD19^+CD5^+$ have been used B_{reg} marker in different studies (4). IL-10 is immunosuppressant and anti-inflammatory cytokine (5). Majority of IL-10 are produced by monocytes, remaining are done by lymphocytes (6). IL-10 inhibits proinflammatory responses of both innate and adaptive immune cells, and proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8 (3). IL-10 have a role to maintain the integrity and homeostasis of the epithelial layers of tissues (6).

The signal transducer and transcription activator 3 (STAT3) is a signal transduction molecule that is activated as a result of the binding of many cytokines, growth factors and hormones to their receptors in the cell membrane (7). Cytokine signal transduction is usually mediated by the JAK/STAT signaling pathway (8). After STAT3s are phosphorylated by JAKs, it becomes dimerized into active form (9). The activated STAT3s regulated the expression of their target genes which play a role in regulation of cell proliferation, angiogenesis and apoptosis (10, 11).

Recent studies indicated that expression of *IL-10* mRNA, *AID* mRNA and *mir-155* expressions which are regulated by *STAT3* are increased in CLL patients. IL-10 is an anti-inflammatory cytokine. In recent studies, it has been shown that IL-10 is also secreted in B cells. B_{reg} cells expressed CD5 and CD19, and suppress the immune system. B-CLL cells expressed CD5 surface molecule like B_{reg} cells. While the CLL cells show similar immunophenotypic properties to B_{reg} cells, they might

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also be thought to be functionally similar (10). In this study, it was aimed to determine the intracellular IL-10 and STAT3 levels of CLL cells by flow cytometry.

In this study, STAT3 and IL-10 expressions of CLL patients were investigated.

2. MATERIAL AND METHODS

2.1. Study Population

CLL patients who were newly diagnosed or being followed up in the outpatient clinic of Hematology Division were included in the study. Patients receiving treatment were excluded from the study. Twenty-four patients (17 males, 7 females) and 14 healthy individuals (8 males, 6 females) were included in the study. The mean age of the 24 patients in the study was 65 (53-83 years), while the mean age of the healthy control group was 57 (52-68 years). The clinical features of the patients are shown in the Table-1. The clinic data of the patients was collected retrospectively.

All patients met the National Cancer Institute (NCI) diagnostic criteria for CLL, and all samples showed the characteristic immunophenotype: cells expressed CD5 and CD19. Written informed consent was obtained according to the Declaration of Helsinki and the study was approved by the local ethics committee (Ethical Number:1135/09.06.2015).

Table 1. *The clinical features of the patients*

Patient No	Age	Gender	Cytogenetic Status	BINET	RAI	WBC (10^9/L)	Lymphocyte (10^3/µL)	CD38
1	63	M	no mutation	A	0	56	52.00	N
2	68	M	no mutation	C	1	34.6	30.01	N
3	48	M	no mutation	A	0	15.9	9.00	N
4	54	M	no data	A	1	25.4	21.59	N
5	56	M	no mutation	B	2	44.2	37.80	N
6	77	M	tri12	C	3	55	46.60	N
7	68	M	no mutation	A	2	47.83	43.77	N
8	65	F	del11q	B	2	54.5	46.90	P
9	73	M	tri12	A	0	28.2	19.60	N
10	60	F	no mutation	C	2	15.6	12.30	N
11	64	M	del13q	A	1	9.7	4.60	N
12	63	F	no data	A	1	63	52.20	N
13	68	F	no mutation	C	1	226.3	186.70	N
14	83	M	del13q	A	1	44.9	36.50	N
15	62	F	tri12	A	0	36.3	30.00	N
16	65	F	no mutation	C	1	13.7	8.50	N
17	86	F	tri12	C	4	149.2	137.10	N
18	70	M	no mutation	C	4	89.9	86.30	N
19	70	M	no data	A	1	37.2	30.20	N
20	53	M	del13q+del11q	C	4	20.8	17.68	P
21	62	M	del13q14	B	3	225.5	207.20	N
22	56	M	no mutation	B	2	111.8	87.10	N
23	66	M	del13q+del11q	B	2	196.2	178.70	N
24	55	M	no mutation	B	1	100.5	87.10	N

%30 ≥ CD38 positive (P); %30 < CD38 negative (N); M: Male; F: Female

2.2. Cell Cultures and Stimulation

2.2.1. IL-10 Expression

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by density gradient centrifugation using Ficoll-Paque (Histopaque-1077; Biochrom, Cambridge, UK) at 2100×rpm for 30 min. The acquired PBMCs were washed twice with PBS and suspended in a complete cell culture medium RPMI containing 10% Fetal Bovine Serum (Sigma Chem. Co., Hamburg, Germany), 1% L-glutamine (Sigma Chem. Co., Hamburg, Germany), 1% anti-micotic and antibiotic solutions (Sigma Chem. Co., Hamburg, Germany). Viability was determined by trypan blue dye exclusion. PBMCs ($1x10^6$) were stimulated with CpG (1 ug/ml) at 48 hours and PMA (50 ng/ml-final concentrations, Sigma Chem. Co, Hamburg, Germany) and Ionomycine (1 µg/

ml-final concentration, Sigma Chem. Co, Hamburg, Germany) and Brefeldin A (BFA; Sigma Chem. Co., Hamburg, Germany) (final concentration of 10 µg/mL) was added for 4 hours to promote intracytoplasmic cytokine accumulation.

PBMCs were washed, then were labeled with anti-human CD19-APC (HIB19 clone), anti-human CD5-PE.Cy7 (UCHT2), anti-human CD38-FITC (HIT2 clone) and incubated for 20 min at room temperature. PBMCs were washed with PBS; fixed and permeabilized using Cytofix&Cytoperm Kit, (Nordic, MUbio, Netherlands), and then stained with anti-human IL-10 PE (JES3-9D7 clone) (all from Biolegend, San Diego,USA) mAbs for 20 min at room temperature and finally washed and data collected by FACS Calibur. Analyses were carried out using CellQUEST Software (Becton Dickinson, San Jose, USA) on FACS Calibur cytometer Becton Dickinson San Jose, USA.

Lymphocytes were gated on SSC (side scatter)/FSC (forward scatter) dot-plot per tube, then $CD19^+$ cells and $CD5^+CD19^+$ were gated in SSC/CD19 dot-plot (Figure 1). IL-10 expression were analyzed in lymphocytes, $CD19^+$ and $CD5^+CD19^+$ cells.

Table 2. *IL-10+CD19+, IL-10-CD19+, STAT3+CD19+ and STAT3-CD19+ levels*

	$IL\text{-}10^+CD19^+$ % in Lymphocyte Median (min-max)	$IL\text{-}10^+$ % in $CD19^+$ cells Median (min-max)	$IL\text{-}10^+$ % in $CD5^+CD19^+$ cells Median (min-max)	$STAT3^+CD19^+$ % in Lymphocyte Median (min-max)	STAT3% in $CD19^+$ cells Median (min-max)	STAT3% in $CD5^+CD19^+$ cells Median (min-max)
Heathy Control	0.39 (0.07-1.60)	2.76 (0.54-3.36)	6.97 (0.00-18.47)	0.16 (0.01-1.15)	5.14 (0.71-9.57)	6.77 (1.82-25.00)
CLL	6.73 (1.06-33.68)	7.67 (1.75, 38.09)	8.82 (1.08-38.09)	1.86 (0.37-12.64)	2.28 (0.48-21.59)	2.25 (0.41-29.38)
CD38 Negative	9.02 (1.06 – 33.68)	9.91 (1.75-38.09)	9.42 (1.08-38.09)	2.89 (0.37-12.64)	4.98 (0.48-21.59)	4.96 (0.41-29.38)
CD38 Positive	21.28 (14.43 – 27.85)	22.90 (13.66-32.13)	23.32 (14.49-32.02)	5.24 (0.48-11.58)	6.54 (0.93-16.00)	7.58 (1.78-14.41)
Deletion Negative	8.96 (1.23-19.63)	12.16 (1.70-24.70)	10.84 (1.08-25.26)	3.08 (0.37-12.64)	3.93 (0.48-13.32)	3.61 (0.41-13.88)
Deletion Positive	9.46 (1.06 – 27.85)	10.87 (2.01-32.13)	11.01 (2.01-32.02)	2.44 (0.48-11.58)	3.52 (0.89-16.00)	3.22 (0.81-14.41)
Rai 0	10.87 (1.03-29.80)	14.35 (2.01-32.36)	14.14 (2.01 – 35.33)	4.51 (0.84-11.58)	6.73 (2.00-16.00)	6.19 (1.91-14.41)
Rai I	6.88 (1.23-33.68)	5.62 (1.75-38.09)	4.97 (1-08-38.09)	2.50 (0.48-5.78)	4.10 (0.98-11.22)	3.99 (0.97-11.09)
Rai II	15.69 (9.54-21.5)	18.22 (10.33 – 24.70)	17.56 (10.66-25.26)	4.81 (0.37-12.64)	5.66 (0.48-13.32)	5.30 (0.41-13.88)
Rai III+ Rai IV	10.77 (2.67-27.85)	12.71 (2.82 – 32.13)	12.30 (2.86-32.02)	1.25 (0.89-1.89)	1.43 (0.89-2.32)	1.32 (0.81-2.38)
Binet A	10.78 (1.06-33.68)	10.38 (2.01-38.09)	9.58 (2.01-38.09)	4.74 (0.48-12.64)	6.60 (1.85-16.00)	6.13 (1.78-14.41)
Binet B	13.29 (6.73-21.57)	15.33 (4.54-24.70)	15. 59 (3.48-25.26)	1.39 (0.37-3.55)	2.06 (0.48-6.73)	2.02 (0.41-6.81)
Binet C	8.23 (1.23-27.85)	10.23 (1.75-32.13)	9.84 (1.08-32.02)	3.49 (0.95-11.40)	4.00 (0.98-11.61)	3.81 (0.94-11.74)

2.2.2. STAT3 Expression

PBMCs ($1x10^6$ cell/ml) were cultured for STAT3 expression with and without PMA (20 ng/ml) (Sigma Chem. Co, Hamburg, Germany) at 15 min. After washing step, cells were stained with anti-CD5-PE-Cy7 (UCHT2 clone), anti-CD19-APC (HIB19 clone) and anti-CD38-FITC (HIT2 clone) (Biolegend, San Diego,USA) for 15 min at room temperature. Cells were fixed with fix-perm kit (Nordic MUbio, Susteren, Netherlands) for 20 min after washing, the cells were permeabilized with the Perm Buffer solution (Nordic MUbio, Susteren, Nederland) and anti-STAT3 PE (Biolegend, San Diego, USA) for 15 min and finally washed and data collected by FACS Calibur. Analyses were carried out using CellQUEST Software (Becton Dickinson, San Jose, USA) on FACS Calibur cytometer Becton Dickinson San Jose USA.

2.3. Statistics

The Mann–Whitney U-test was used to analyze for the measurement variables when the numbers within the groups were low and not normally distributed. P-value less than 0.05 was considered statistically significant. All calculations were performed using GraphPad InStat version 5.03 (GraphPad Software Inc., San Diego, CA).

3. RESULTS

3.1. Clinical Features of Patients

Twenty-four patients with CLL were evaluated and 70% of them were male. The patients' clinical features are summarized in Table 1. All patients were currently not under treatment and not taking any medicine at the time of blood sampling. None of the patients had any finding of acute or chronic infection. According to Binet stage; 41% of patients had Binet A (n: 10), 25% of patients had Binet B (n: 6), and 34% of patients had Binet C (n: 8) disease. Four (16%), 9 (38%), 6 (25%), 2 (8%) and 3 (13%) patients had Rai stage 0, I, II, III and IV disease, respectively.

3.2. Increased IL-10 levels in lymphocyte and CD19+ cells but not in CD5+CD19+ cells were detected in CLL patients

Increased $IL\text{-}10^+CD19^+$ levels were detected in lymphocyte of CLL patients compared to healthy controls ($p<0.0001$). There is a significant difference in $IL\text{-}10^+CD19^+$ of CD38 negative and positive ($p=0.009$, $p=0.0001$, respectively), chromosomal deletion negative and positive ($p=0.004$ and $p=0.005$, respectively) patient groups when compared to healthy

controls. CD38 positive patient groups have high IL-10^{+}CD19^{+} levels than CD38 negative patient group (p=0.04). High IL-10^{+}CD19^{+} levels were found in Rai 0, I, II, III+IV stage of CLL patients than healthy controls (p=0.006, p<0.0001, p=0.0006, p=0.009, respectively). Similarly, high IL-10^{+}CD19^{+} levels were found in Binet A, B, C stage of CLL patients (p<0.0001, p=0.0006, p=0.0002, respectively). On the other hand, there were no differences in IL-10^{+}CD19^{+} cells for deletion status, Rai or Binet staging system within the CLL cohort (Figure 1).

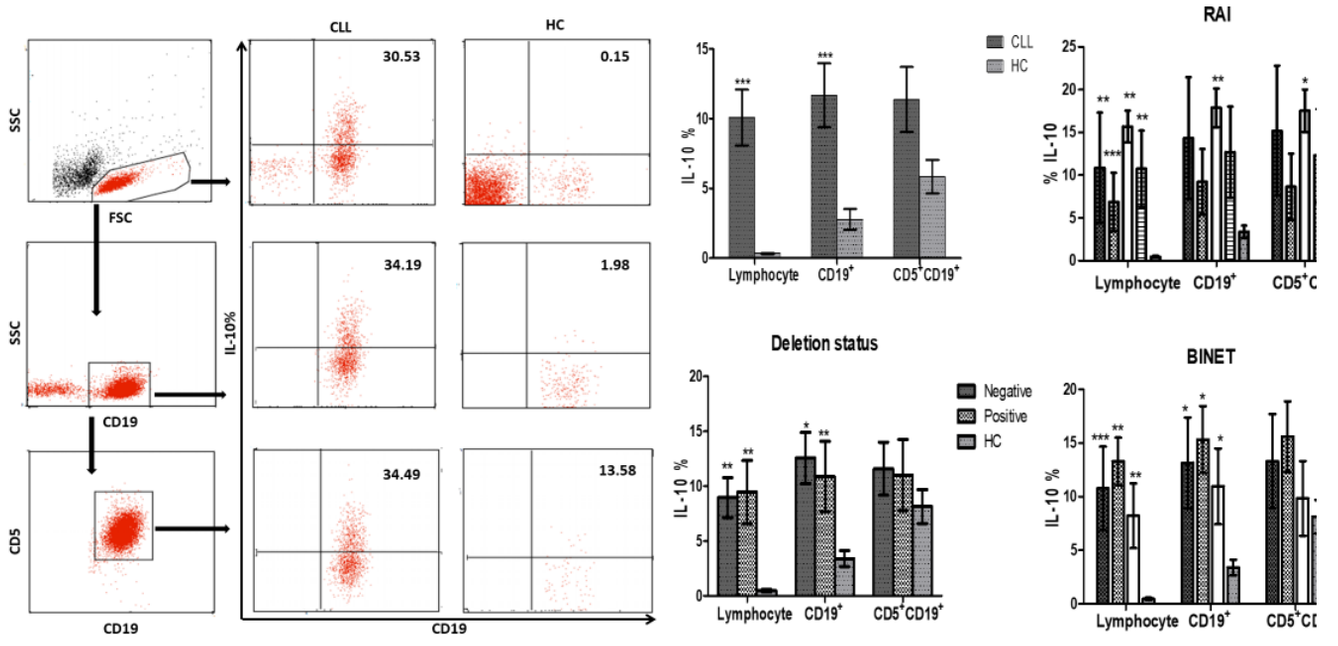

Figure 1. *IL-10+CD19+ levels in CLL patients and healthy controls obtained from lymphocytes (A), CD19+ B (B), CD5+CD19+ cells (C). Stim: Stimule-CpG (48-h), PMA (4 hours min.)*

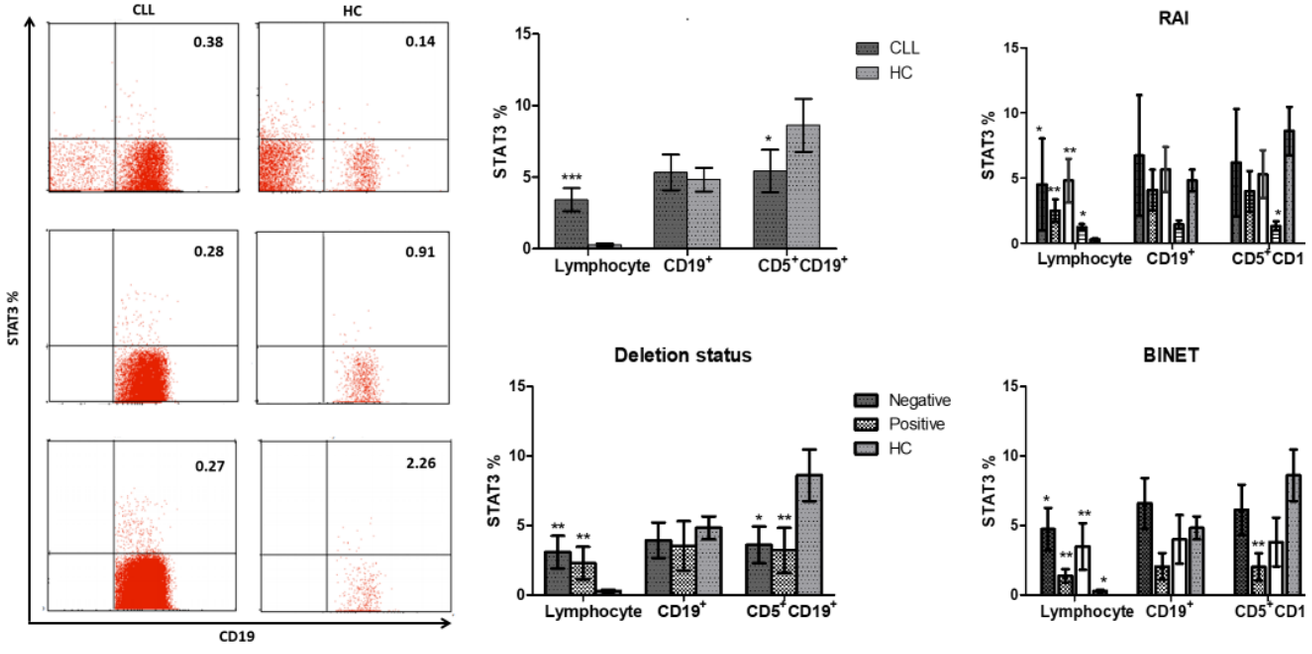

Figure 2. *STAT3+CD19+ levels in CLL patients and healthy controls obtained from lymphocytes (A), CD19+ B (B), CD5+CD19+ cells (C).*

According to IL-10$^+$ levels in CD19$^+$ gate, increased IL-10$^+$ levels were detected in CLL patients compared to healthy controls (p<0.0001). There is a significant difference in IL-10 level of CD38 negative and positive (p=0.009 and p=0.02, respectively), deletion negative and positive (p=0.02 and p=0.002, respectively), when compared to healthy controls. According to Rai staging system, there were no significant differences to healthy controls, except Rai II (p=0.0006). High IL-10 levels were determined in all Binet stage compared to healthy control, (Binet A; p=0.03, Binet B; p=0.002 and Binet C; p=0.02). There were no differences in IL-10 levels of CD19$^+$ cells for chromosomal deletions, CD38 expression status.

In the CD5$^+$CD19$^+$ cells, there were no difference in IL-10 levels between CLL patient and healthy controls. Similarly, there were no differences in IL-10 levels of CD5$^+$CD19$^+$ cells for chromosomal deletion or CD38 expression status. However, increased IL-10 level of CD5$^+$CD19$^+$ in CD38 positive patients compared to healthy control (p=0.01). According to Rai and Binet staging systems, there were no significant differences between CLL patients and healthy controls, except Rai II (p=0.02).

3.3. STAT3+CD19+ levels in lymphocyte of CLL patients

High STAT3$^+$CD19$^+$ cells in lymphocyte were found in CLL patients compared to healthy controls (p<0.0001). There is a significant difference in STAT3$^+$CD19$^+$ cells of CD38 negative and positive (p<0.0001 and p=0.004, respectively), chromosomal deletion negative and positive (p=0.0003 and p=0.0007, respectively) patient groups when compared to healthy controls. According to Rai and Binet staging systems, increased STAT3$^+$CD19$^+$ cells were detected in all stage of patients, except Rai III compared to healthy controls (Rai 0; p=0.01, Rai I; p=0.0008, Rai II; p=0.0006, Rai III+IV; p=0.01, Binet A; p=0.0003, Binet B; p=0.003, and Binet C; p=0.001, respectively). On the other hand, there were no differences in STAT3$^+$CD19$^+$ cells for deletion or CD38 expression status and staging system within the CLL cohort.

In the CD19$^+$ cells, there were no differences in STAT3 levels between CLL patients and healthy controls. Similarly, we did not found any differences in STAT3 level for deletions or CD38 expression status, Rai and Binet staging system within the CLL cohort.

In CD5$^+$CD19$^+$ cells, decreased STAT3 levels were found in CLL patients compared to health controls (p<0.04). There is a significant difference in STAT3$^+$ levels in CD5$^+$CD19$^+$ cells of CD38 negative (p=0.01), chromosomal deletion negative and positive (p=0.01 and p=0.008, respectively) when compared to healthy controls. Except decreased STAT3 level in Rai III+IV (p=0.01) and Binet B (p=0.007), there were no differences in STAT3 levels in CD5$^+$CD19$^+$ cells for Rai and Binet staging system. On the other hand, there were no differences in STAT3 levels for deletion or CD38 expression status and staging system within the CLL cohort (Figure 2).

4. DISCUSSION

CLL is a chronic lymphoproliferative disease characterized by monoclonal B cell accumulation and it is the most common type of leukemia in adults in western countries (13). Most of the CLL cells have low proliferative capacity and most of them are in G_0 phase of cell cycle (14). Since the mechanism of apoptosis in CLL is disrupted, the tumor cells accumulate in the lymph nodes and bone marrow. Rai and Binet staging systems have been used in CLL patients for about 40 years to predict the clinical outcome (15).

STAT3 is a transcription factor and activate many genes like cytokines, growth factors etc. Receptor-ligand interaction was induced phosphorylation to activate the STAT3. It was shown that, *STAT3* expression were increased in many solid tumor cells or hematologic malignancies (16, 17). Anti-inflmatory cytokine IL-10 is regulated by STAT3 (5).

In 2014, Antosz et al. investigated mRNA transcript levels of *IL-6*, *IL-10*, *c-jun* and *STAT3* in CLL patients and, found that high *IL-10* mRNA expression in normal lymphocytes than in CLL cells in the non-stimulated condition, but after stimulation with LPS for 30 min increased *IL-10* mRNA expression were found in patients with CLL compared to healthy controls (18). Dilillo et al. showed that CD5$^+$CD19$^+$ B_{reg} cells suppress the immune system by secreting IL-10 and these cells are similar in both immunophenotipically and functionally as a B cell suppressant (12).

In our study, high IL-10$^+$ levels were detected in lymphocytes and CD19$^+$ cells of patients with CLL after 48 hours of CpG stimulation than healthy controls, but in the CD5$^+$CD19$^+$ cells, there is no difference were found in expressing IL-10 cells. These findings might suggest that CLL cells can be functionally similar, in addition to immunophenotypic properties similar to those of B_{reg} cells. Gary-Gouy et al. reported that an increase in IL-10 expression may cause an excessive increase in CD5 expression in CLL and that CD5 enhances IL-10 production as an immunosuppressive cytokine (19).

IL-10 signals through the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) signaling pathway (6). Antosz et al. found increased *STAT3* mRNA expression in CLL patients (18). In our study, STAT3$^+$CD19$^+$ levels in lymphocytes were higher in patients with CLL than healthy controls and our data were consistent with this study. However, no significant difference was observed in STAT3$^+$ levels in CD19$^+$ B cells, whereas in CD5$^+$CD19$^+$ cells, STAT3$^+$ levels of patients with CLL after stimulation were found to be lower than healthy individuals. In patients with CLL, absolute number of B cell are increased due to disease. However, B cell number in healthy subjects is low in normal physiological conditions. Increased number of B cell may cause enhanced ratio of STAT3 cells in CLL patients compared to healthy subjects. In our study analysis of STAT3 were carried out by flow cytometry, but that of Antosz et al. were made by real time PCR in the RNA isolation obtained in total cells (18). Therefore, Antosz et al. was found *STAT3* mRNA expression to be high due to the high B cells in the patients (18).

These results made us thought that the levels of IL-10 and STAT3 expression in CLL B cells is clearly different from healthy controls B lymphocytes and might have a role in the biology of CLL. It is though that the presented data will contribute to the studies that scrutinize the similarity of CLL cells to B_{reg} cells.

Conflicts Of Interest/Disclosures

The authors declare that they have no financial or other conflicts of interest in relation to this research and its publication.

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Author Contributions:

Interpretation of data for the study: OO, MYG, SC, GD, MA.
Drafting the manuscript: OO, MYG, SC, GD, MA.
Revising it critically for important intellectual content: OO, MYG, SC, GD, MA.
Final approval of the version to be published: OO, MYG, SC, GD, MA.
Research idea: OO, MYG, MA.
Design of the study: OO, MYG, GD, MA.
Acquisition of data for the study: OO, SC, MYG.
Analysis of data for the study: OO, SC, MYG.

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