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IL-25 Impact on Malignant B Cells Survival and T Cells Activation in Chronic Lymphocytic Leukemia

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ABSTRACT

T cell dysregulation and shift to T helper 2 responses, boosting tumor microenvironment support, contributes to the survival of leukemic B cells in Chronic Lymphocytic Leukemia. Interleukin (IL)-25 is involved in the initiation of T helper 2 cell responses. Signal transduction of IL-25 begins with the heterodimer receptor (IL-17RA/IL-17RB). The presence of IL-25 in the tumor microenvironment may affect the supportive effects of T cells in the surrounding tumor cell environment. The purpose of this study was to evaluate the role of IL-25 in the biology of CLL.

IL-17RB expression in $CD3^+$ and $CD19^+$ cells was assessed in isolated peripheral blood mononuclear cells (PBMCs) of nine CLL patients and nine healthy subjects by real-time polymerase chain reaction and flow cytometry. B cells were positively enriched from PBMCs using magnetic-activated cell sorting (MACS). PBMCs and purified leukemic B cells were cultured with recombinant human IL-25 (20ng/ml) for 72 hours, then the viability and apoptosis of cultured cells were measured by MTT assay and AnnexinV/7AAD. Furthermore, the levels of CD69 expression on T lymphocytes and IL-17RB in T and B cells were determined by flow cytometry.

The basal level of IL-17RB expression in CLL patients was significantly higher than that in control individuals. In addition, the percentage of $IL\text{-}17RB^+/CD3^+$, $IL\text{-}17RB^+/CD19^+$ cells and $CD69^+/CD3^+$ cells increased after 72 hours of culture with IL-25 in CLL patients compared to healthy subjects. IL-25 also reduces the apoptosis rate of tumor cells.

We found that IL-25 could stimulate T cells in CLL patients and lower B cell death. This suggests that IL-25 might have a role in enhancing the survival of tumor cell by expressing receptors for inflammation, such as IL-17RB, and might be involved in the development of CLL.

Keywords: Chronic lymphocytic leukemia; Interleukin-25; Tumor microenvironment; T helper 2 cells

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is an incurable hematological malignancy characterized by the accumulation of CD5/CD19/CD23 positive B lymphocytes in blood and lymphoid organs.[1] The etiology of CLL remains unknown, but several factors, such as environmental and hereditary factors, play key roles in disease development and progression.[2] Earlier studies have shown that malignant B cells are invaded by programmed cell death (apoptosis) by an arrest in the G0/G1 phase of the cell cycle.[3] B-CLL cells spontaneously died when cultured in vitro. Tumor microenvironment (TME) comprises malignant and non-malignant cells and pro-inflammatory mediators that contribute to tumor cell development and progression in CLL.[4]

Interleukin (IL)-25 (also known as IL-17E) is a pro-inflammatory member of IL-17 family cytokines that is produced by a variety of cells and is involved in the initiation of T helper 2 (Th2) cells mediated immune pathogenesis and causes allergic airway inflammation. IL-25 induces the development of eosinophils and B lymphocytes, increases antibody class switching to IgG1 and IgE, and stimulates the production of IL-9, IL-13 and IL-4.[5] Signal transduction of IL-25 begins through a heterodimeric receptor complex composed of IL-17RA/IL-17RB, which is known as IL-17RB and is expressed on monocytes, NKT, Th2, Th9, eosinophils, type 2 innate lymphoid cells (ILC2s) and keratinocytes.[6] IL-17RB is an inflammatory receptor and upregulation of IL-17RB expression in leukemic cells plays a pro-oncogenic role in leukemia.[7] In adult T-cell leukemia (ATL), IL-17RB overexpression has been reported in HTLV-1 transformed T cells; IL-17RB and IL-25 are required for T cell immortalization and survival via the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway.[8] Previous studies have shown that high IL-17RB expression is associated with poor patient prognosis in different cancer types.[9] In contrast, IL-25 in certain tumor cells, such as breast cancer cells, uses another signaling pathway that activates TNFRSF1A associated via death domain (TRADD) and Fas associated via death domain (FADD), which causes caspase activation and subsequently induces tumor cell apoptosis.[10]

Cluster of differentiation (CD) 69 is an early activation marker expressed on lymphocytes after activation and may affect T cell differentiation in vitro. In addition, CD69 is expressed on the surface of T cells at sites of chronic inflammation.[11] Increasing evidence of T cell dysfunction, imbalanced T cell expansion, and differentiation shift to Th2 in tumor microenvironments, which may contribute to disease progression, has been reported in patients with CLL.[12, 13] The activated phenotype ($CD69^+$) of T-cells in patients with CLL is associated with CLL progression.[14] IL-25 increases the activation and differentiation of Th2 cells.[15] Consequently, the presence of several pro-inflammatory cytokines, such as IL-25, in the tumor microenvironment could affect the activity and differentiation of T cells in the surrounding tumor cells.

However, the effect of IL-25 and its receptor activity have not been described in some hematological malignancies, such as CLL. In the present study, the apoptotic or anti-apoptotic effects of IL-25 on B cells and the induction activity of T cells via IL-25 were evaluated in patients with CLL and compared to healthy subjects.

MATERIALS AND METHODS

Patients

After ethical committee approval (Semnan University of Medical Sciences, ethical code: 93/551669) and informed consent was obtained from all participants, peripheral blood samples (6 mL) were collected in heparinized tubes from nine indolent CLL patients who were referred to the Cancer Clinic at Semnan University of Medical Sciences with different disease stages. Patients were diagnosed according to the international guidelines for CLL diagnostic criteria.[16] Nine healthy volunteers who were age- and sex-matched to the patients were included in this study. The demographic and hematological data of patients with CLL are summarized in Table 1. Peripheral blood was obtained from all patients and healthy subjects after obtaining written informed consent according to the Ethical Committee of Semnan University of Medical Sciences.

PBMC Isolation and Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using a Ficoll Hypaque (Lymphodex, Inno-train, Germany). PBMCs were washed twice with sterile phosphate buffered saline and resuspended in RPMI-1640 cell culture medium supplemented with 10% human AB^+ serum (HABs), penicillin (100 IU), and streptomycin (100 μg/mL) (Gibco, Grand Island, NY, USA). Cell viability

was measured using the Trypan Blue test (>95%). In a pilot experiment, 3×10^5 PBMCs and B cells from patients with CLL were cultured in the presence or absence of different concentrations (10, 20, 40, and 80 ng/ml) of recombinant human IL-17E (BioSite, Täby, Sweden) for 48, 72, or 96 hours to determine the optimal concentration and incubation time of cytokine. In addition, different concentrations of hrIL-25 were used to assess IL-17RB expression in $CD3^+$ and $CD19^+$ cells.

Table 1. Demographic and hematological information of CLL patients

	Age	Sex	WBC*10^3	Lym (%)	Hb (g/dl)	PLT*10^3	Rai stage	Organomegaly
1	54	F	15.6	72	12.4	109	0	-
2	81	M	12.6	66	13.4	252	0	-
3	74	F	23.7	73	10.4	202	III	-
4	75	M	18.9	72	10	130	III	-
5	40	M	22.9	63	15	101	I	LAP
6	61	F	11.3	70	11.5	180	0	-
7	72	M	28.5	83	12.5	176	II	SM
8	63	M	54.1	85	13.5	201	0	-
9	60	M	29.6	80.7	15.7	124	0	-

Quantitative Real-time RT-PCR

Total cellular RNA was extracted using the Roche High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's protocol. The integrity of the RNA was checked using 1.5% agarose gel electrophoresis. cDNA was synthesized using a cDNA synthesis kit (Fermentas, EU), according to the manufacturer's guidelines. Quantitative real-time RT-PCR was performed using a Sybr-Green master mix (Ampliqon, Odense, Denmark) on an ABI 7900HT Real-Time PCR system. Primer sequences for the detection of target genes were acquired online from PrimerBank (https://pga.mgh.harvard.edu/primerbank/). Sequences of the primer pairs used were as follows: human glyceraldehyde 3-phosphate dehydrogenase (5′-GGAGCGAGATCCCTCCAAAAT-3′, 5′-GGCTGTTGTCATACTTCTCATGG-3′), and human IL-17RB (5′-ATGTCGCTCGTGCTGCTAAG-3′, 5′-AGCCACATTGAACGGTCGG-3′).

B Cells Isolation Using Magnetic-activated Cell Sorting

$CD19^+$ B cells were isolated from fresh PBMCs using the MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). Initially, PBMCs were incubated with CD19 MicroBeads for 15 min in a refrigerator, and $CD19^+$ B cells were positively selected using MS columns, according to the manufacturer's instructions. The viability and purity of the purified B cells were assessed using trypan blue staining and flow cytometry, respectively. The purity of the isolated $CD19^+$ cells was greater than 95%.

Flow Cytometry

To evaluate IL-17RB expression in $CD3^+$ and $CD19^+$ cells and the effect of IL-25 on $CD3^+$ cell activation, the following fluorochrome-conjugated monoclonal anti-human antibodies were used: CD3 FITC (BioLegend, San Diego, USA), CD19 PE (Becton Dickinson, New Jersey, USA), CD69 PE (BioLegend, San Diego, USA), and IL-17RB PerCP (R&D System, Abington, UK). Values for positive staining were set using the relevant isotype antibody as a negative control.

Apoptosis Assay

Apoptosis was detected using the FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, San Diego, USA), according to the manufacturer's instructions. Briefly, 3×10^5 cells were seeded in each well of a 96-well flat-bottom plate, and cultured in the presence or absence of 20 ng/ml IL-25 and incubate in 37º, 5 % CO2 for 72 hours. Fludarabine (1 μM) was used as the positive control. After 72 hours of incubation, the

cells were collected and washed twice in 1x binding buffer and subsequently resuspended in 100 µL binding buffer. The cells were incubated with 1 µL of Annexin V and 7AAD for 30 min at room temperature in the dark and then 500 µL of binding buffer was added to each sample and immediately analyzed by flow cytometry using Flow Max software (Partec, Germany).

Viability Assay

To evaluate cell viability, MTT assay (Carl Roth, Karlsruhe, Germany) was performed. In brief, 1.5×10^5 cells/well were seeded in 96-well flat-bottom plates and incubated with or without IL-25. IL-4 and PHA were used as positive controls for the CLL cells and healthy subjects, respectively. After incubation for 72 hours, MTT solution (1 mg/mL) was added to the culture medium in a dark room. After 5 h of incubation at 37˚C, a stop solution was added to solubilize the blue formazan crystals. The absorbance was read at 570 nm using a plate reader (SPECTRA III, Germany).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc.). Values are expressed as mean ± standard error of the mean (SEM) and median. The results were evaluated using the Kolmogorov-Smirnov test for normality, independent-samples t-test, and Mann–Whitney U test. Differences were considered statistically significant with a *p value* less than 0.05.

RESULTS

Expression of IL-17RB Is Significantly Up-regulated in CLL Patients

To determine the expression levels of IL-17RB in patients with CLL, we compared them with those in healthy volunteers. We observed increased expression of the IL-17RB mRNA level in the PBMCs of CLL patients compared to that in the PBMCs of age-matched healthy individuals (Figure 1A). To determine the expression level of IL-17RB protein in $CD3^+$ and $CD19^+$ cells in PBMCs of CLL patients and healthy subjects, we assessed IL-17RB expression in $CD3^+$ and $CD19^+$ cells using flow cytometry. No difference was observed in IL-17RB expression in $CD3^+$ cells between patients with CLL and healthy subjects (Figure 1B). Moreover, no difference was observed in IL-17RB expression in $CD19^+$ cells between patients with CLL and healthy subjects (Figure 1C). These data showed that the difference in the mRNA expression level of IL-17RB between patients with CLL and healthy controls highlighted the possible role of this receptor in the tumor microenvironment.

The IL-25 Induces High Expression Level of IL-17RB on Surface of $CD3^+$ and $CD19^+$ Cells in CLL Patients

IL-25 has a high affinity for IL-17RB and activates signaling pathways through IL-17RB. To elucidate the role of IL-25 in CLL cells survival, we determined the optimal concentration of IL-25 by viability testing and evaluation of IL-17RB expression on $CD3^+$ and $CD19^+$ cells after IL-25 treatment (from 10 to 80 ng/ml) in PBMCs of CLL patients. IL-25 enhanced the viability of CLL cells in a dose-dependent manner, especially at a concentration of 20 ng/mL for 72 hours (Figure 2A). In addition, the highest IL-17RB level in $CD3^+$ and $CD19^+$ cells after exposure to IL-25 (20 ng/mL) was observed after 72 hours than time-zero (*p value*= 0.0257 and *p value*= 0.0110, n=3; respectively) (Figure 2B). Thus, to investigate the effect of IL-25 on its receptor expression on the surfaces of $CD3^+$ and $CD19^+$ cells, we evaluated IL-25 (20ng/ml) effect on PBMCs after 72 hours of incubation. IL-17RB levels significantly increased in $CD3^+$ cells of CLL patients upon treatment with IL-25 (from 9.78±1.58% at time zero to 14.42±0.58% upon IL-25 treatment, mean± SEM, n=5) (Figure 3A), whereas, IL-25 induced a significant decrease in the expression of IL-17RB in $CD3^+$ cells of healthy subjects (from 16.44±1.14% at time zero to 7.46±0.49% upon IL-25 treatment, mean± SEM, n=5) (Figure 3B). On the other hand, treatment with IL-25 (20ng/mL) for 72 hours resulted in an increased expression level of IL-17RB on $CD19^+$ surface cells from CLL patients (from 4.57±1.02% at time zero to 13.11±1.24% upon IL-25 treatment, mean± SEM, n=5) (Figure 3C), while, IL-25 did not induce a significant change in the expression level of IL-17RB in $CD19^+$ cells of healthy subjects (from 5.66±0.27% at time zero to 4.51±0.61% upon IL-25 treatment, mean± SEM, n=5) (Figure 3D). These data showed that 20ng/ml IL-25 after 72 hours significantly induces overexpression of IL-17RB on $CD3^+$ and $CD19^+$ surface cells in the PBMCs of CLL patients. Induction of IL-17RB increase by IL-25 was not observed in the healthy subjects. It seems that the presence of IL-25 in the tumor microenvironment can help tumor progression by inducing an increase in IL-17RB expression.

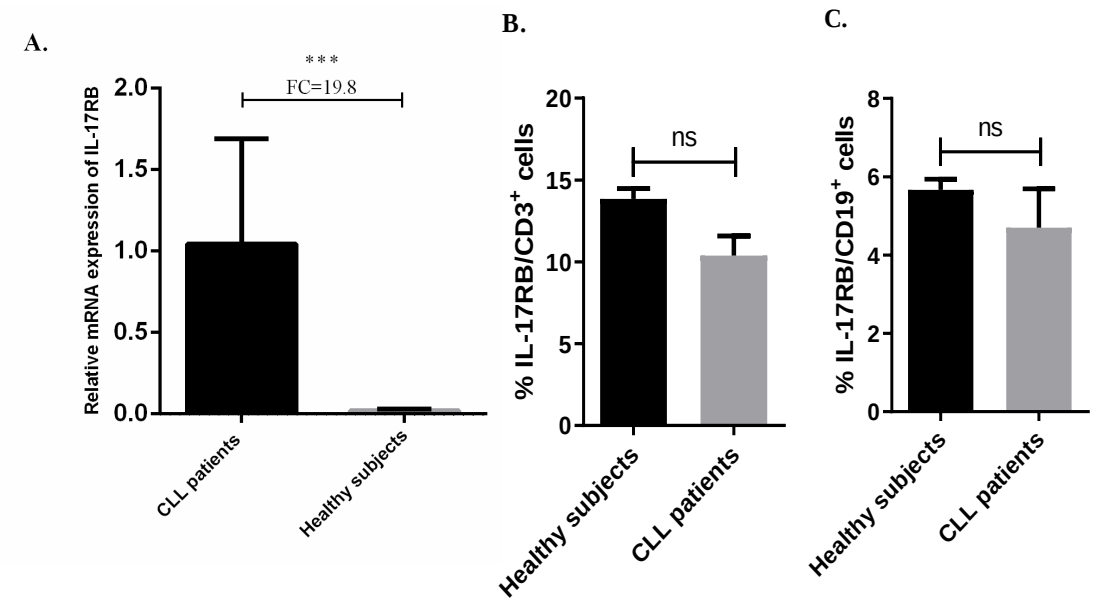

Figure 1. Evaluation of IL-17RB expression in chronic lymphocytic leukemia (CLL) patients and healthy subjects. A. Baseline IL-17RB mRNA expression levels in freshly isolated PBMCs in CLL patients (n=9) were significantly higher than the healthy donors' group (n=9) (p value=*0.0002*, FC: fold change). B. The percentage of CD3/IL-17RB positive cells in CLL patients vs. healthy subjects (n=5) by flow cytometry. The percentage of CD3/IL-17RB positive cells was not differ between patients with CLL and healthy subjects. C. The percentage of CD19/IL-17RB positive cells in CLL patients vs. healthy subjects (n=5) by flow-cytometry. The percentage of CD19/IL-17RB positive cells did not different between patients with CLL and healthy subjects. Data are expressed as mean±SEM. ***p<0.001

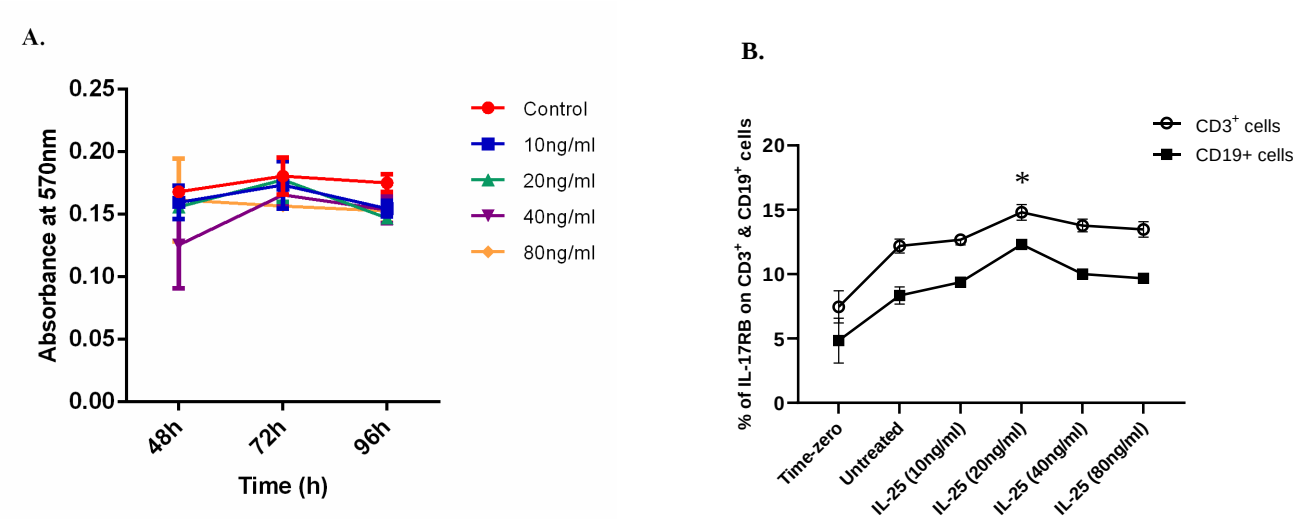

Figure 2. The effects of Interleukin (IL)-25 on the viability of peripheral blood mononuclear cells (PBMCs) and IL-17RB expression on CD3$^+$ and CD19$^+$ cells in chronic lymphocytic leukemia patients. A. The effect of IL-25 on CLL cells viability was evaluated using MTT assay after exposure to different concentrations of IL-25 (10, 20, 40, and 80 ng/ml) for 48, 72, and 96 hours. In addition, an untreated CLL cells group was used as a control group. The data are expressed as mean ± SEM. B. The percentages of IL-17RB on CD3$^+$ and CD19$^+$ after exposure to IL-25 with different doses (10-80 ng/ml) for 72 hours incubation (n=3) were analyzed by flow cytometry.

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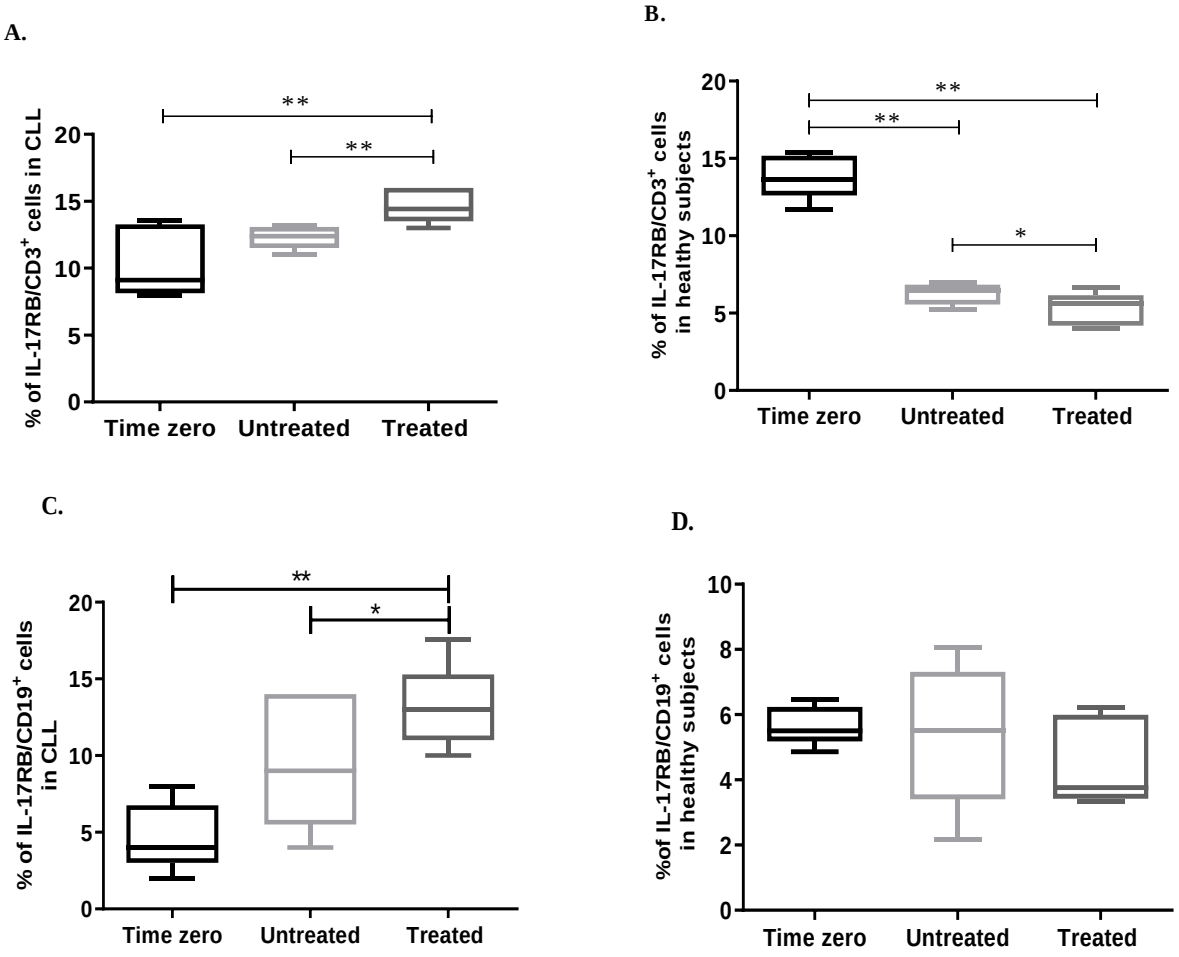
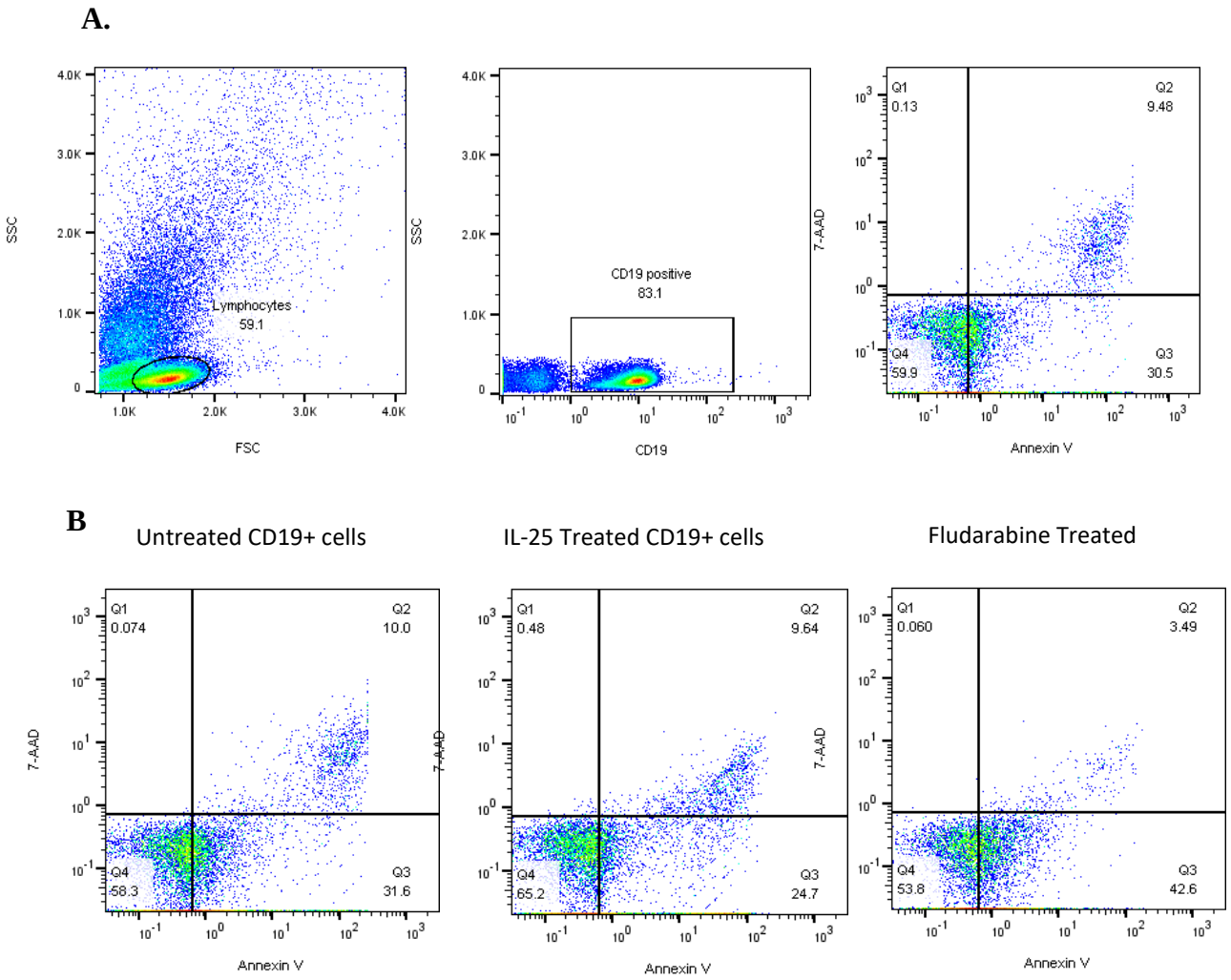


Figure 3. Effects of Interleukin (IL)-25 on the IL-17RB expression on $CD3^+$ and $CD19^+$ cells in chronic lymphocytic leukemia (CLL) patients versus healthy subjects. A. There was a significant increase in the percentage of IL-17RB on $CD3^+$ cells in CLL patients (n=5) after IL-25 (20ng/ml) treatment for 72 hours compared with the time-zero and untreated groups (*p* value=0.0074, *p* value=0.0060, respectively). B. There was a significant reduction in the percentage of IL-17RB on $CD3^+$ cells in healthy subjects (n=5) after IL-25 (20ng/ml) treatment for 72 hours compared with the time-zero and untreated group (*p* value=0.0040 and p value=0.0089, respectively). C. There was a significant increase in the percentages of IL-17RB on $CD19^+$ in CLL patients (n=5) after IL-25 (20ng/ml) treatment for 72 hours compared with time-zero and untreated groups (*p* value=0.0040 and p value=0.026, respectively). D. There was no significant difference in the percentages of IL-17RB on $CD19^+$ in healthy subjects (n=5) after IL-25 (20ng/ml) treatment for 72 hours compared with the time-zero and untreated group. The data are analyzed by flow cytometry and expressed as boxplots (Min to Max values). *p<0.05, **p<0.01

IL-25 Restricts Apoptosis of CLL Cells

To determine the role of IL-25 in CLL progression, viability and apoptosis assays were performed. Figure 4A shows a summary of the gating strategy for apoptosis assay in $CD19^+$ B cells population. The results of the Annexin V/7-AAD apoptosis assay showed that IL-25 significantly decreased the percentage of $CD19^+$ apoptotic cells in PBMCs of CLL patients compared with the untreated group (25.6±2.9% vs 35.5±4.2%, mean±SEM, n=9; respectively) and the fludarabine group as a positive control (25.6±2.9% vs. 74.3±2.9%, mean± SEM, n=9; respectively) (Figure 4B, and Figure 4C). Subsequently, to eliminate the protective effect of T cells and investigate the direct effect of IL-25 on the survival of purified $CD19^+$ leukemic cells, the apoptosis of purified B cells was assessed. The results demonstrated that IL-25 significantly reduced apoptosis of purified leukemic B cells compared to the untreated

group (28.57±1.85% vs 34.59±1.78%, mean± SEM, n=5), whereas the percent of purified B apoptotic cells significantly increased following *in vitro* treatment by fludarabine compared with the untreated group (66.72±3.65% vs 34.59±1.78%, mean± SEM, n=5) (Figure 4D). On the other hand, the effect of IL-25 on the apoptosis of B cells in healthy subjects did not show any significant difference compared to the untreated group (29.98± 1.499% vs 29.52± 1.067, mean± SEM, n=9). However, there was a slightly significant increase in the percentage of apoptotic cells after treatment with fludarabine compared with the control group (35.71±2.468 vs 29.52± 1.067, mean± SEM, n=9) (Figure 4E). In addition, the results of cell viability using MTT assay indicated that incubation of PBMCs with IL-25 did not show a significant difference compared to control groups; conversely, following *in vitro* treatment with IL-4 (20ng/ml) after 72 hours of incubation, as a cultured B-CLL cell protector from death by apoptosis without stimulating cell proliferation [17], did not display any significant effect on PBMCs cells of CLL patients in comparison with the untreated group (*p value*>0.05). Fludarabine showed significant cytotoxic effects on PBMCs of CLL patients compared with the untreated group, IL-25 treated group and IL-4 treated group (*p value*<0.001, *p value*<0.001, and *p value*<0.0001, respectively; Figure 4F). In healthy subjects, no significant difference was observed in the treated groups in MTT assay (data not shown). Given these results, it can be concluded that IL-25 exerts anti-apoptotic activity and promotes the survival of CLL cells, which may further play a positive role in the development of this disease.



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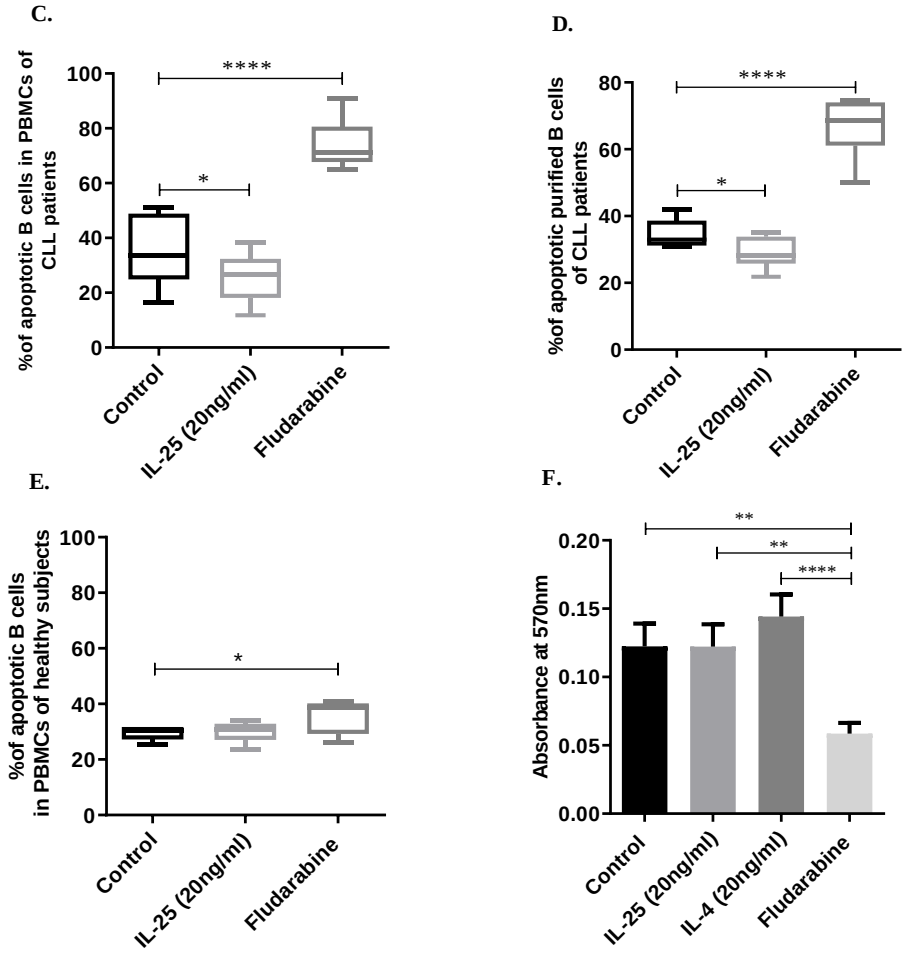
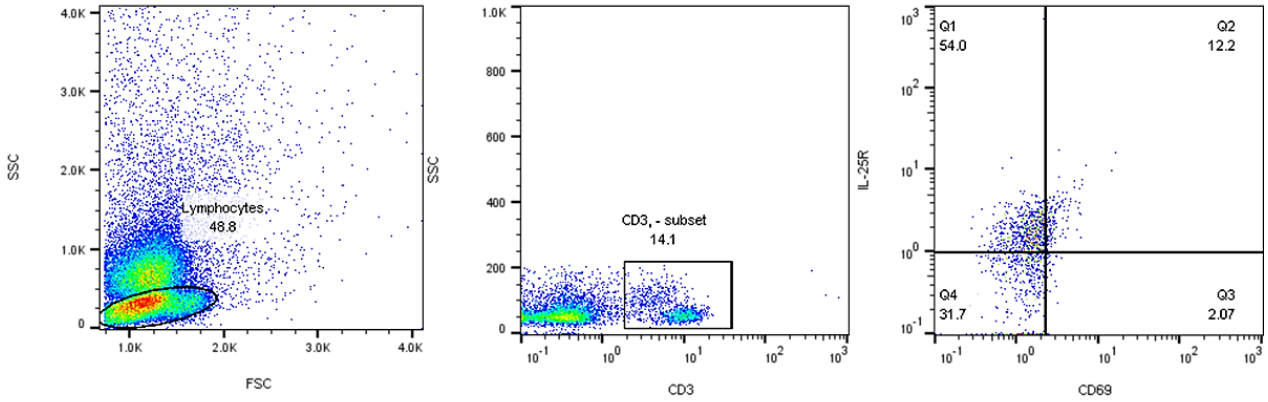


Figure 4. Effects of Interleukin (IL)-25 on chronic lymphocytic leukemia (CLL) cell survival. A. Showed gating strategy for apoptosis assay in CD19$^+$ B cells population. B. Representative Flow cytometric plots of apoptosis in CD19$^+$ B cells. C. Flow cytometric analysis of apoptosis in CD19$^+$ B cells in PBMCs of CLL patients (n=9) after 72 hours of treatment with and without IL-25 (20 ng/mL) and fludarabine (1 µM/mL), as determined by Annexin V/7AAD staining. There was a significant reduction in the percentage of apoptotic cells after treatment with IL-25 compared with in the control and fludarabine groups (*p* value=0.0467 and *p* value<0.0001, respectively). D. Flow cytometric analysis of apoptosis in CD19$^+$ B cells in purified CD19$^+$ B cells from CLL patients (n=9) after 72 hours treatment with and without IL-25 (20 ng/mL) and fludarabine (1 µM/mL), as determined by Annexin V/7AAD staining. There was a significant reduction in the percentage of apoptotic cells after treatment with IL-25 compared with that in the control and fludarabine groups (*p* value=0.032 and *p* value<0.0001, respectively). E. Flow cytometric analysis of apoptosis in CD19$^+$ B cells in PBMCs of healthy subjects (n=9) after 72 hours of treatment with and without IL-25 (20ng/ml) and fludarabine (1 µM/mL), determined by Annexin V/7AAD staining. There was a slightly significant increase in the percentage of apoptotic cells after treatment with fludarabine compared to that in the control group (p value=0.0418). F. MTT results showing the viability percentage of PBMCs of CLL patients (n=9) treated with IL-25 (20 ng/mL), IL-4(20 ng/mL), fludarabine (1 µM/mL) and medium after 72 hours incubation. Data are expressed as boxplots (Min to Max values) and bar plots (mean ± SEM). Statistical significance was determined using the Mann-Whitney U test. **p* value <0.05, ***p* value < 0.01, ****p* value< 0.001, *****p* value< 0.0001.

IL-25 Induces T Cells Activation

To elucidate the role of IL-25 in T cell activation, the mean fluorescence intensity (MFI) of CD69 on $CD3^+$ cells was assessed by flow cytometry. The gating strategy for CD3/CD69/IL-25R positive cells is shown (Figure 5A and 5B). Our data indicated lower MFI of CD69 on $CD3^+$ cells in PBMCs of untreated with IL-25 of CLL patients and healthy subjects (7.658±0.81 and 7.765±0.05, respectively, mean± SEM, n=5); hence, there was no difference in the MFI of CD69 of $CD3^+$ cells between CLL patients and healthy subjects before *in vitro* IL-25 treatment (*p value*>0.05, Figure 5C). Following IL-25 (20ng/ml) treatment for 72 hours, the MFI of CD69 on $CD3^+$ cells of CLL patients increased (from 7.658±0.81 to 12.29±1.3, (mean± SEM, n=5), *p* value=0.0079, Figure 5C); however, IL-25 had no significant effect on the MFI of CD69 on $CD3^+$ cells from healthy subjects after 72 hours (from 7.765±0.05, 7.54±0.2, (mean± SEM, n=5), *p value*>0.05, Figure 5C). The results demonstrate that IL-25 induces an activation phenotype in T cells of CLL patients through induction of increased IL-17RB presence on T cells which is probably associated with the survival of tumor B cells.

A.



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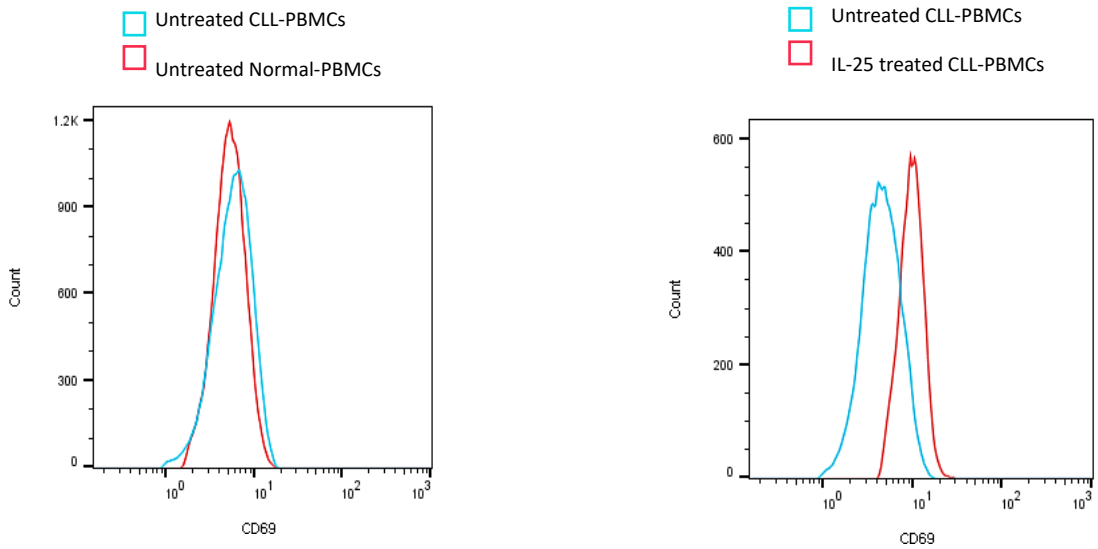

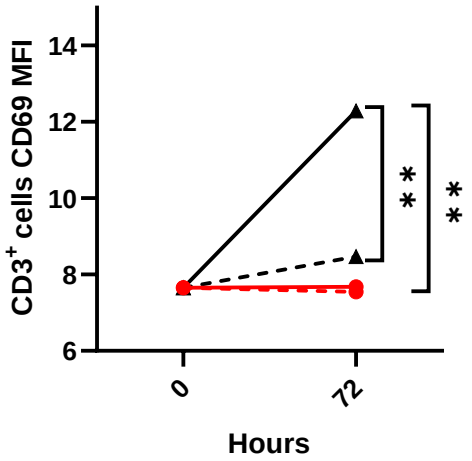

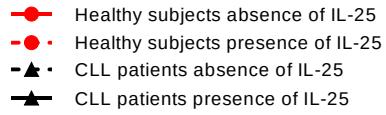

CD3+ cells CD69	Healthy subjec		CLL patients	
Time point	Untreat	IL-25 (20ng/m	Untreat	IL-25 (20ng/m
0 hours	7.665	-	7.658	-
72 hours	7.682	7.544	8.472	12.29

Figure 5. Effect of IL-25 on CD3⁺ cell surface expression of the activation marker (CD69). A. Demonstration of the gating strategy for CD3/CD69/IL-25R positive cells. B. Representative histogram demonstrating mean fluorescence intensity of CD69. C. PBMCs from CLL patients and controls were cultured for 72 hours in the medium in the absence or presence of IL25. Color lines represent median values from the mean fluorescence intensity from healthy subjects (n=5, red) and CLL patients (n=5, black) in the absence (dashed line) or presence (unbroken line) of IL25 (20ng/ml) in the culture medium. CD69 expression was analyzed using flow cytometry. **p<0.01

DISCUSSION

The CLL microenvironment includes non-leukemic cells, chemokines, and cytokines, which greatly influence the growth and survival of leukemic B cells.[4] In this study, we demonstrated that IL-17RB mRNA expression was significantly higher in patients with CLL than in healthy subjects. The expression of this receptor at the protein level on the surface of T cells is higher than that on B cells, and it is interesting to note that in a dose- and time-dependent manner, IL-25 induces the expression of its receptor in the T and B cells of patients with CLL compared to healthy subjects as positive feedback. In addition, the treatment of tumor cells with IL-25 induced the survival of these cells. We determined that following treatment with IL-25, in addition to inducing the expression of IL-17RB, it induces the expression of activation markers, such as CD69, in the T cells of patients with CLL compared to healthy individuals.

Perturbation of T cell function is a common feature of CLL. Autologous T cells through direct cell-to-cell contact, overexpression of anti-apoptotic factors such as BCL-XL and MCL-1, and production of soluble factors such as IL-4 have a supportive effect on tumor cell survival in CLL.[18] Panayiotidis et al, demonstrated that IL-4 in CLL plays a crucial role in the maintenance of the anti-apoptotic protein Bcl-2 and induces the survival of leukemic CLL cells.[17]

Proinflammatory cytokines are the major mediators that link inflammation and cancer.[19]

IL-17E (IL-25) is a distinct member of the interleukin 17 family that induces Th2 immune responses and plays a critical role in the initiation of Th2 cell-mediated immunopathogenesis, along with an increase in IL-4, IL-13, and IL-5.[20] Therefore, we propose that IL-25 in the tumor microenvironment may play an important role in leukemic B cell survival. It has been reported that Overexpression of the IL-25 receptor, IL-17RB, in tumor cells of gastric, pancreatic, and breast cancer is significantly higher than that in normal samples and is associated with poor prognosis.[21-24] Hence, based on the high affinity between IL-25 and IL-17RB, we analyzed the expression of IL-17RB in PBMCs from CLL patients and healthy subjects. We found that IL-17RB expression significantly increased in CLL cells. Recent studies have indicated that IL-17RB in PBMCs of healthy subjects is expressed on CD3⁺ cells (approximately 23%) and CD19⁺ cells (approximately 8%).[25] In this regard, our results also confirmed the higher expression of IL-17RB in T cells than in B cells in CLL and normal subjects. It has been shown that IL-25 elevates IL-17RB on Naïve T cells and promotes Th2 differentiation through GATA3

enhancement.[26] Furthermore, in earlier studies, overexpression of IL-25 in transgenic mice was associated with the upregulated expression of IL-17RB.[27] The highest IL-17RB expression levels were obtained after treatment with 20ng/ml IL-25 for 72 hours. Our results indicated that IL-17RB is upregulated after treatment with IL-25 in $CD19^+$ and $CD3^+$ cells in CLL. However, according to this study upregulation of IL-17RB is very slight in B cells and significantly reduced in $CD3^+$ cells in healthy subjects. This significant decrease in IL-17RB on normal T cells in contrast to T cells in CLL patients is further evidence of the supportive roles of T cells in CLL, which is in line with previous studies by our group.[18]

Overexpression of IL-17RB has been reported in AML cells, and IL-17RB signaling induces upregulation of phosphorylation of the ERK and NF-κB pathways and overexpression of Bcl-2. Thus, IL-17RB promotes AML cell survival-mediated Bcl-2 expression and is associated with resistance to chemotherapy.[28] In another study, it was shown that high expression of IL-17RB in cancer cells induced tumorigenic potential, IL-17RB overexpression in adult T-cell leukemia caused tumor cell survival and IL-25 positive feedback stimulated tumor cell proliferation through NF-KB activation.[8, 29] In addition, it has been recognized that breast cancer cells use c-RAF/S6 kinase pathway for IL-25 signal transmission. This process ultimately produces low-molecular-weight forms of cyclin E (LMW-E), which increase the survival of tumor cells.[21] In contrast, some studies have shown that IL-25 exerts antitumor activity through several pathways, including the recruitment of eosinophils and B cells into tumors.[30] Further analysis in this study showed that IL-25 had a pro-tumor effect, probably by reducing apoptosis of CLL cells *in vitro*. The viability of PBMCs was similar in the IL-25 and IL-4 treated groups compared with the untreated group, whereas fludarabine significantly reduced cell viability. Previous studies have shown that the MTT assay on PBMCs and T cells of atopic subjects treated with IL-25 (20-100ng/ml) for 72 hours did not show any significant effect on cell proliferation.[31] T cells support CLL cell survival in vitro. IL-4 contributes to tumor cell survival when added to the tumor cell medium[18], and it has been suggested that IL-25 acts similarly to IL-4 in the tumor microenvironment of CLL. Recent studies reported that IL-25 had no significant effect on the apoptosis of isolated follicular lymphoma cells that were incubated with hrIL-25 compared to that in the medium.[32] Chronic overexpression of IL-25 in animal models induces survival of $CD19^+$ B lymphocytes and eosinophils in peripheral blood. IL-25 induces overexpression of CD5 on $CD19^+$ B lymphocytes and overexpression of CD5 causes survival of malignant B cells in patients with CLL.[27]

According to our findings, IL-25 leads to T cell activation and overexpression of CD69 in patients with CLL compared to the control group. In previous studies, it has been shown that CD69 expression level on T cells from healthy controls and non-progressive CLL was similar; on the contrary, the CD69 expression level on T cells of PBMCs in progressive CLL patients was higher than that in non-progressive patients. Based on these results, activated T cells in progressive CLL are significantly correlated with poor prognosis.[14] Taken together, these results indicate that IL-25 induces T cell activation and Th2 polarization. Therefore, it may promote tumor cell survival and CLL progression by providing supportive microenvironment conditions for tumor cells growth.

In conclusion, this study explored the role of IL-25 in the CLL microenvironment plays a role in proinflammatory response. This response results in an increase in its receptor (IL-17RB) as positive feedback in CLL cells and induces the viability of B CLL cells. Moreover, IL-25 leads to the activity of $CD3^+$ cells and CD69 overexpression in CLL patients, which enhances the enhancement of the supportive role of T cells in the tumor microenvironment. Our and other similar studies imply that the high expression of IL-17RB could be a potential mechanism for the survival and progression of tumor cells. It is suggested that evaluation of the level of IL-25 and its receptor (IL17RB) in the tumor microenvironment can help diagnose the prognosis of CLL patients.

The limitations of our study include the lack of technical facilities to evaluate live imaging of B cell apoptosis. Future studies should focus on the effects of IL-25, along with the inhibition of IL-25 and its receptors. In addition, further studies are required to identify the specific role of IL-25 and IL-17RB receptor signaling in dysregulated T cells in the CLL microenvironment, which may lead to new therapeutic strategies.

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STATEMENT OF ETHICS

The study was approved by the ethical committee of the Semnan University of Medical Sciences, Semnan, Iran (ethical code: 93/551669).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Khan M, Saif A, Sandler S, Mirrakhimov AE. Idelalisib for the treatment of chronic lymphocytic leukemia. ISRN oncology. 2014;2014:931858. PubMed PMID: 25093123. Pubmed Central PMCID: PMC4003830. Epub 2014/08/06. eng.
2. Goldin LR, Slager SL. Familial CLL: genes and environment. Hematology Am Soc Hematol Educ Program. 2007;2007(1):339-45.
3. Collins RJ, Verschuer LA, Harmon BV, Prentice RL, Pope JH, Kerr JF. Spontaneous programmed death (apoptosis) of B-chronic lymphocytic leukaemia cells following their culture in vitro. Br J Haematol. 1989;71(3):343-50.
4. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. Blood. 2009 Oct 15;114(16):3367-75. PubMed PMID: 19636060. Epub 2009/07/29. eng.
5. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001;15(6):985-95.
6. Deng C, Peng N, Tang Y, Yu N, Wang C, Cai X, et al. Roles of IL-25 in type 2 inflammation and autoimmune pathogenesis. Frontiers in Immunology. 2021;12:691559.
7. Tian E, Sawyer JR, Largaespada DA, Jenkins NA, Copeland NG, Shaughnessy JD. Evi27 encodes a novel membrane protein with homology to the IL17 receptor. Oncogene. 2000;19(17):2098.
8. Lavorgna A, Matsuoka M, Harhaj EW. A critical role for IL-17RB signaling in HTLV-1 tax-induced NF-κB activation and T-cell transformation. PLoS Pathog. 2014;10(10):e1004418.
9. Bastid J, Dejou C, Docquier A, Bonnefoy N. The emerging role of the IL-17B/IL-17RB pathway in cancer. Frontiers in immunology. 2020;11:718.
10. Song X, Qian Y. IL-17 family cytokines mediated signaling in the pathogenesis of inflammatory diseases. Cellular signalling. 2013 Dec;25(12):2335-47. PubMed PMID: 23917206. Epub 2013/08/07. eng.
11. Sancho D, Gómez M, Sánchez-Madrid F. CD69 is an immunoregulatory molecule induced following activation. Trends in immunology. 2005;26(3):136-40.
12. Scrivener S, Kaminski ER, Demaine A, Prentice AG. Analysis of the expression of critical activation/interaction markers on peripheral blood T cells in B-cell chronic lymphocytic leukaemia: evidence of immune dysregulation. British journal of haematology. 2001;112(4):959-64.
13. Podhorecka M, Dmoszynska A, Rolinski J, Wasik E. T type 1/type 2 subsets balance in B-cell chronic lymphocytic leukemia—the three-color flow cytometry analysis. Leukemia research. 2002;26(7):657-60.
14. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Näsman-Glaser B, Young E, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. haematologica. 2017;102(3):562-72.
15. Wang Y-H, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC–activated Th2 memory cells. The Journal of experimental medicine. 2007;204(8):1837-47.
16. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute–Working Group 1996 guidelines. Blood. 2008;111(12):5446-56.
17. Panayiotidis P, Ganeshaguru K, Jabbar SA, Hoffbrand AV. Interleukin-4 inhibits apoptotic cell death and loss of the bcl-2 protein in B-chronic lymphocytic leukaemia cells

in vitro. British journal of haematology. 1993;85(3):439-45.
18. Kiaii S, Kokhaei P, Mozaffari F, Rossmann E, Pak F, Moshfegh A, et al. T cells from indolent CLL patients prevent apoptosis of leukemic B cells in vitro and have altered gene expression profile. Cancer Immunology, Immunotherapy. 2013;62(1):51-63.
19. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis. 2009;30(7):1073-81.
20. Yao X, Sun Y, Wang W, Sun Y. Interleukin (IL)-25: Pleiotropic roles in asthma. Respirology. 2016;21(4):638-47.
21. Mombelli S, Cochaud S, Merrouche Y, Garbar C, Antonicelli F, Laprevotte E, et al. IL-17A and its homologs IL-25/IL-17E recruit the c-RAF/S6 kinase pathway and the generation of pro-oncogenic LMW-E in breast cancer cells. Scientific reports. 2015;5:11874.
22. Laprevotte E, Cochaud S, du Manoir S, Lapierre M, Dejou C, Philippe M, et al. The IL-17B-IL-17 receptor B pathway promotes resistance to paclitaxel in breast tumors through activation of the ERK1/2 pathway. Oncotarget. 2017;8(69):113360.
23. Wu H-H, Hwang-Verslues WW, Lee W-H, Huang C-K, Wei P-C, Chen C-L, et al. Targeting IL-17B–IL-17RB signaling with an anti–IL-17RB antibody blocks pancreatic cancer metastasis by silencing multiple chemokines. Journal of Experimental Medicine. 2015;212(3):333-49.
24. Bie Q, Sun C, Gong A, Li C, Su Z, Zheng D, et al. Non-tumor tissue derived interleukin-17B activates IL-17RB/AKT/β-catenin pathway to enhance the stemness of gastric cancer. Scientific reports. 2016;6(1):1-12.
25. Caruso R, Stolfi C, Sarra M, Rizzo A, Fantini MC, Pallone F, et al. Inhibition of monocyte-derived inflammatory cytokines by IL-25 occurs via p38 Map kinase–dependent induction of Socs-3. Blood. 2009;113(15):3512-9.
26. Bredo G, Storie J, Shrestha Palikhe N, Davidson C, Adams A, Vliagoftis H, et al. Interleukin-25 initiates Th2 differentiation of human CD4+ T cells and influences expression of its own receptor. Immunity, inflammation and disease. 2015;3(4):455-68.
27. Kim MR, Manoukian R, Yeh R, Silbiger SM, Danilenko DM, Scully S, et al. Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production. Blood. 2002;100(7):2330-40.
28. Guo H-Z, Niu L-T, Qiang W-T, Chen J, Wang J, Yang H, et al. Leukemic IL-17RB signaling regulates leukemic survival and chemoresistance. The FASEB Journal. 2019:fj. 201900099R.
29. Younesi V, Nejatollahi F. Induction of anti-proliferative and apoptotic effects by anti-IL-25 receptor single chain antibodies in breast cancer cells. International immunopharmacology. 2014;23(2):624-32.
30. Benatar T, Cao MY, Lee Y, Lightfoot J, Feng N, Gu X, et al. IL-17E, a proinflammatory cytokine, has antitumor efficacy against several tumor types in vivo. Cancer Immunology, Immunotherapy. 2010;59(6):805-17.
31. Wong CK, Li PW, Lam CWK. Intracellular JNK, p38 MAPK and NF-κB regulate IL-25 induced release of cytokines and chemokines from costimulated T helper lymphocytes. Immunology letters. 2007;112(2):82-91.
32. Ferretti E, Di Carlo E, Ognio E, Fraternali-Orcioni G, Corcione A, Belmonte B, et al. IL-25 dampens the growth of human germinal center-derived B-cell non Hodgkin Lymphoma by curtailing neoangiogenesis. OncoImmunology. 2018;7(3):e1397249.