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Research Article
Prognostic Role of T Helper 9 Cells and Interleukin-9 in Chronic Lymphocytic Leukemia

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Abstract

Background and Objectives: Chronic lymphocytic leukemia (CLL) is a cancer of B cell lymphocytes, originating in the bone marrow and affecting most body tissues. The objective of this study was to estimate the level of T helper 9 (Th9) cells and interleukin-9(IL-9) in CLL patients and the determination of their association with different clinical and prognostic parameters of CLL and correlation with clinical and laboratory measurements in CLL patients. **Materials and Methods:** This study included 70 Egyptian patients with CLL in addition to 15 healthy controls. The number of Th9 cells was determined by flow cytometry, serum concentration of IL-9 determined by enzyme-linked immunosorbent assay (ELISA) technique and IL-9 mRNA expression evaluated by qRT-PCR. **Results:** IL-9 level was significantly higher in CLL patients when compared to normal subjects. Higher levels of both Th9 cells and IL-9 in CLL patients were associated with fever, skin involvement, lymphadenopathy, chemotherapy treatment, lymphocyte doubling time (LDT) less than 1 year, TP53 Del (17p) and higher Rai stages. Serum IL-9 level, unlike circulating Th9 cells, was higher significantly in CLL patients positive for BCL2. Positive cases for CD38 and ZAP70 were strongly associated with higher levels of both Th9 cells and IL-9 levels. **Conclusion:** The current study indicated that Th9 cells and IL-9 contribute to the pathogenesis of CLL and are correlated with TP53 Del (17p), BCL2 expression and the more complex form of disease and worse prognosis. These results may allow one to speculate a potential therapeutic target of Th9 cells and IL-9 in CLL.

Key words: Chronic lymphocytic leukemia, T helper 9 cells, interleukin-9, prognosis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a type of blood cancer that affects B cell lymphocytes. B cell lymphocytes originate in the bone marrow, develop in the lymph nodes and normally fight infection by producing antibodies[1]. In United States in 2018, about 20,940 new cases were diagnosed with CLL and lead to 4,510 deaths. The 5 years survival rate is 81.7%. More than 75% of newly diagnosed CLL patients are over the age of 50 and the majority is men. However teenagers and children can be affected in rare cases[2]. Leukemia in Egypt has an incidence of 6.0 per 100,000 (6.7 among males and 5.3 among females) and the most common type is CLL, with an incidence of 1.3 per 100,000[3]. First-degree relatives of CLL patients have more than twice the risk for this disease[4].

The diagnosis of CLL is based on the demonstration of peripheral blood or bone marrow monoclonal B lymphocytes co-expressing both cluster of differentiation (CD)5 and B-cell surface antigens CD19, CD20 and CD23. Peripheral blood monoclonal B lymphocytes are more than 5000 cells μL^{-1} for at least 3 months. Nodular or interstitial pattern of spread of CLL cells in the bone marrow usually indicates a better outlook for CLL than a diffuse pattern. Lymphocyte doubling time (LDT), the time needed for blood lymphocytes to duplicate its count, less than one year indicates higher risk. Serum levels of β2 microglobulin and lactate dehydrogenase (LDH) may estimate tumor burden, since high levels indicate a poorer prognosis[5]. Veldhoen *et al.*[6] discovered a new subset of $CD4^+$ T cells, Th9 cells, that its signature cytokine is interleukin-9 (IL-9). The Th9 cell can contribute to both protective immunity and immunopathological disease. However, Th9 cells aren't the sole source of IL-9 during an immune response and, accordingly, the relative importance of Th9 cells *in vivo* has been difficult to define[7]. Previous studies showed that Th9 cells can promote allergic disease[8], autoimmune disease[9], immunity against parasites[10], antitumor activity[11] and transplant rejection[12]. Interleukin-9 is a pleiotropic cytokine, which affects multiple cell types, produced in different amounts by a wide variety of immune cells. This cytokine belongs to the family of 4-helix bundle cytokines that also includes IL-2, IL-3, IL-4, IL-6, IL-7 and IL-15[13]. Interleukin-9 activates the heterodimeric IL-9 receptor (IL-9R) and promotes the cross-phosphorylation of Janus kinase 1 (JAK1) and JAK3 leading to the activation of signal transducer and activator of transcription-1 (STAT-1), STAT-3 and STAT-5 and inducing IL-9 gene expression[14]. The effect of IL-9 in neoplasia may depend whether the tumor is solid or not. In solid tumors, specifically in melanoma, it has been demonstrated that Th9 and IL-9 have an important antitumor effect favoring the recruitment of both innate and adaptive immune cells which reduce tumor burden[11]. On the other hand, ectopic expression of IL-9 *in vitro* induces the proliferation of mouse thymic lymphomas. In humans, *in vitro* studies have observed an increase in IL-9 production in cells of Hodgkin's lymphoma causing growth of cultured cells[15]. Aforementioned data indicates that the exact role of IL-9 in tumor is not clear. So in this study, the aim was to evaluate the level of IL-9 gene expression by qRT-PCR and its protein level by ELISA and the level of Th9 cells by flow cytometry in patients with CLL in order to understand the prognostic role of IL-9 and Th9 in CLL.

MATERIALS AND METHODS

Selection of patients: This study was conducted on 70 Egyptian patients with CLL who were admitted to the Oncology Center of Mansoura University, Mansoura, Egypt, between December 2016 and July 2017 in addition to 15 apparently healthy individuals were selected as a control reference group. All subjects were notified that blood samples will be used for research purposes and all gave consent to their participation in this study. Out of CLL patients (n = 70), 36 patients were males and 34 were females and their age ranged from 34-87 years. The control group included 8 males and 7 females in the same age range of patients (40-72 years). All CLL patients were subjected to the following clinical and laboratory investigations; detailed medical history including disease duration, type and duration of previous or current therapies and significant past or current diseases, infections or surgery; clinical examination and abdominal ultrasonography.

Laboratory investigations: All CLL patients and control groups were subjected to the following laboratory investigations; complete blood count (CBC); liver function tests including glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), lactate dehydrogenase (LDH), total bilirubin, total protein and kidney function tests including creatinine and uric acid that were done according to manufacturer's protocol.

Flow cytometric immunophenotypic analysis: Expression levels of CD79b, Flinder Medical Centre (FMC)7, surface immunoglobulin M (sIgM), CD38, zeta-associated protein-70 (ZAP-70), BCL (B cell lymphoma)-2, BCL6 and cyclin D were assessed by flow cytometric immunophenotyping using a panel of antibodies designed to characterize $CD5^+$ $CD19^+$ $CD23^+$ abnormal B cell population during the routine clinical evaluation for diagnosis, staging or follow-up.

Whole blood was incubated with 2% fetal calf serum (FCS) and the antibody cocktail in the dark at room temperature for 15 min. Red blood cells were lysed by treating the sample with 3 mL of ammonium chloride for 8 min followed by centrifugation at 300×g for 5 min. After that, the cells were washed two times with 3 mL 1x PBS containing 0.1% sodium azide. The stained cells were fixed with 100 µL of 2% formaldehyde. Positive cases were selected at 20% of cut-off value as recommended by Oncology Center of Mansoura University, Mansoura, Egypt.

Cytogenetics: Interphase fluorescence in situ hybridization (FISH) studies were performed in Cytogenetics Laboratory in Oncology Center, Mansoura University on peripheral blood samples taken from CLL patients. The following probes were used; ataxia telangiectasia mutated (ATM) deletion (11q22.3), t(11;14) (q13;q32) Immunoglobulin heavy chain (IGH)/B-cell leukemia/lymphoma 1 (Bcl-1) translocation and TP53 deletion (17p13.1). Cut-off values to determine positive or negative samples for each probe were based on individual laboratory experience with clinical specimens.

Determination of Th9 cells in peripheral blood samples by flow cytometry: After isolation of PBMCs by standard Ficoll-Hypaque density-gradient centrifugation from the EDTA blood samples of healthy control subjects as well as CLL patients, 1-2×10^6 cells mL^{-1} was incubated with fluorescently-labeled antibodies for CD5 and CD19 (BD Biosciences). Intracellular IL-9 level was determined after fixation and permeabilization by using fluorescently-labeled anti IL-9 (BD Biosciences). Then stained cells were re-suspended in 1x PBS and analyzed. Data acquisition was performed by using FACS Canto II (BD Biosciences, San Jose). Appropriate isotype-matched controls IgG (BD Pharmingen, San Diego, CA, USA) were used to determine specific binding for each fluorescent channel. Th9 cells level were defined as the percentage of $IL\text{-}9^+$ cells among $CD5^-$ $CD19^-$ T cells.

Quantification of gene expression of IL-9 by qRT-PCR: The RT-PCR analysis for determination of IL-9 gene expression was performed as described previously by Lv *et al.*[16]. Whole blood samples were withdrawn from CLL patients and controls. Then Total RNA was extracted from whole blood using QIAamp® RNA blood Mini kit (Qiagen, USA). About 200 ng RNA was used to synthesize the first strand by Verso SYBR Green 1-Step qRT-PCR Kit (ThermoScientific™) and ABI 7500 Sequence Detection System (Applied Biosystems, Foster city, CA) was used to detect the level of mRNA. The level of gene expression was normalized to the level of housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Specific primers for IL-9 and *GAPDH* were used as previously described by Gu *et al.*[17]; IL-9 Forward: 5-CCATGGTCCTTA CCTCTGCC-3 , IL-9 Reverse: 5 -AGCTGGATCTTCCTGCATCTT-3 , GAPDH Forward: 5 -GAAGGTGAAGGTCGGAGTC-3 , GAPDH Reverse: 5 -GAAGATGGTGATGGGATTTC-3 and primers were obtained from Biosearch Technologies, Inc. (Petaluma, CA, USA). PCR reaction set up was done in 20 µL and the synthesis of first strand cDNA was done at 50°C for 15 min (1 cycle) followed by inactivation by heating at 95°C for 15 min (1 cycle). Denaturation step (at 95°C for 15 sec), annealing step (at 52°C for 30 sec) and extension step (at 72°C for 30 sec) were repeated 40 times. The results were analyzed by $2^{\wedge\text{-}\Delta\Delta Ct}$ method.

Estimation of serum interleukin-9 (IL-9): Serum samples from CLL patients and healthy controls were collected and stored at -80°C till analysis. Estimation of serum level of IL-9 was done using Human IL-9 DuoSet ELISA (R and D Systems) according to the manufacturer's instructions. The sensitivity of ELISA kits for IL-9 was 5 pg mL^{-1}, without detecting of any cross-reactivity. All samples were measured in triplicates. The colorimetric changes in the wells were read at a wavelength of 450 nm in a microplate reader (ELx800, BioTek Instruments, Inc., Winooski, Vermont).

Statistical analysis: Data were analyzed using the SPSS (statistical package for social science) program (SPSS Inc., Chicago, IL) version 20 and with GraphPad Prism 3.0 program (GraphPad® Software Inc.). Chi-square and Fisher's exact tests were used to compare groups. Comparisons between two groups were done using t-test or Man Whitney (for non-parametric). Comparisons between multiple groups were done by one-way ANOVA test. The ROC curves were conducted for discrimination between CLL and control subjects. Kaplan–Meier test was used for survival analysis and the statistical significance of differences among curves was determined by Log-Rank test. Pearson correlation is used for parametric correlations and Spearman's for non-parametric correlations. A probable value of $p<0.05$ was considered to be statistically significant at confidence interval 95%.

RESULTS

Th9 cell frequencies and IL-9 level in control and CLL patients groups: Th9 cell levels in peripheral blood samples were detected by flow cytometry as well as serum level of IL-9 was measured by ELISA in CLL patients and healthy controls to investigate the possible involvement of Th9 cells and IL-9 in

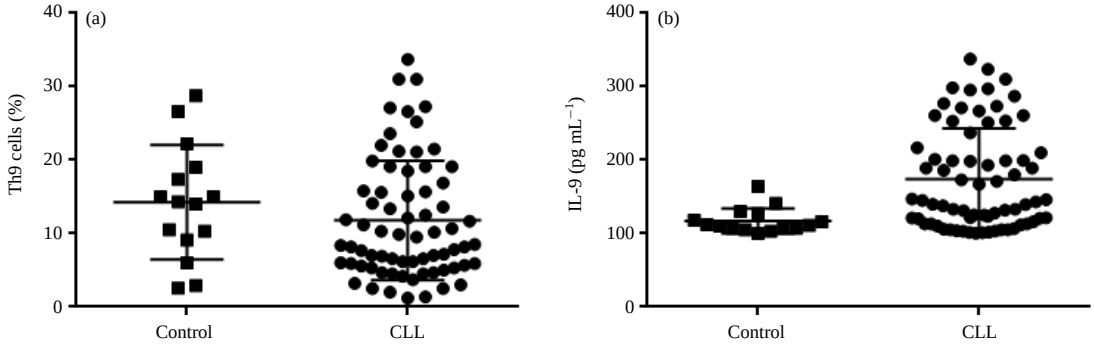

Fig. 1(a-b): Th9 cell frequencies and IL-9 level in control and CLL patients groups. (a) No statistical significant difference was found between CLL and controls regarding Th9 cells level (p = 0.207) and (b) Serum IL-9 level was significantly (p<0.001) elevated in CLL patients more than healthy controls

CLL. No statistical significant difference was observed between the two groups regarding Th9 cells level. The data showed that serum IL-9 level in CLL patients was significantly higher (p<0.001) than its level in healthy controls (Fig. 1).

Association of Th9 cells and IL-9 level with TP53 Del (17p), advanced Rai stages and different clinical and prognostic parameters in CLL patients: Both Th9 cells and IL-9 level were observed to be higher in patients with fever (p = 0.022, p = 0.004, respectively), skin involvement (p = 0.029, p = 0.046, respectively), lymphadenopathy (p = 0.004, p = 0.012, respectively) (Fig. 2), chemotherapy treatment (p = 0.001, p = 0.014, respectively), LDT less than one year (p = 0.023, p = 0.006, respectively) (Fig. 3), TP53 Del(17p) (p = 0.016, p = 0.026, respectively) and higher Rai stages (p = 0.022, p<0.001, respectively) (Fig. 4). In *post-hoc* comparisons, both Th9 cell frequency and serum IL-9 levels were higher in Rai stage IV than Rai stages I and II (p = 0.001, p<0.001 respectively). Again, Th9 cells and IL-9 level were higher in Rai stage III than their levels in Rai stages I (p = 0.005, p = 0.009 respectively) and II (p = 0.002, p = 0.004 respectively) (Fig. 4).

Circulating Th9 cells were significantly associated with positivity for BCL2 in CLL patients: Patients with CLL positive for CD38 and ZAP70 were strongly associated with higher levels of both Th9 cells (p = 0.044, p = 0.001, respectively) and IL-9 level (p = 0.026, p = 0.005, respectively). Circulating level of Th9 cells, unlike IL-9 level, was higher significantly in CLL patients positive for BCL2 (p = 0.038) (Fig. 5).

Significant negative correlation of both Th9 and IL-9 with time to start treatment (TST): The clinical and laboratory features in 70 CLL patients were analyzed, to examine the correlation between Th9 cells, the gene expression and serum level of IL-9 and the clinical or laboratory parameters. There were significant negative correlations between time to start treatment (TST) and all of Th9 cells, serum level and gene expression of IL-9 (r = -0.397, p = 0.003; r = -0.373, p = 0.005; r = -0.900, p = 0.037, respectively). Furthermore, there were significant negative correlations between serum IL-9 level and serum level of both GPT and total bilirubin (r = -0.274, p = 0.022; r = -0.307, p = 0.010, respectively). On the other hand, there were significant positive correlations between circulating level of Th9 cells and both serum IL-9 level and its mRNA level in peripheral blood leucocytes (r = 0.742, p<0.001; r = 0.905, p = 0.002, respectively) in CLL patients. Serum protein and mRNA level of IL-9 in peripheral blood leucocytes were positively correlated in CLL group (r = 0.929, p = 0.001) (Fig. 6).

Serum IL-9 discriminated between CLL cases and control groups: The ROC curves of circulating Th9 cells and serum IL-9 levels were conducted for discrimination between CLL and control subjects (Fig. 7) in order to evaluate the specificity and sensitivity. The cut-off level of circulating Th9 cells 5.0%in CLL patients (AUC = 0.396, 95% CI = 0.224-0.549, p = 0.207) were decided by using ROC Curve analysis. At this cut-off level of Th9 cells, a sensitivity of 81.4% and specificity of 13.3% were achieved. On the other hand, serum IL-9 levels showed significant AUC (p = 0.001) that could discriminate between CLL cases and control groups. The cut–off level of serum IL-9 131.1 pg mL^{-1} (AUC = 0.396, 95% CI = 0.658-0.870, p = 0.001) were decided by using ROC Curve analysis. At this cut-off level of IL-9, a sensitivity of 61.4% and specificity of 86.7% were reached.

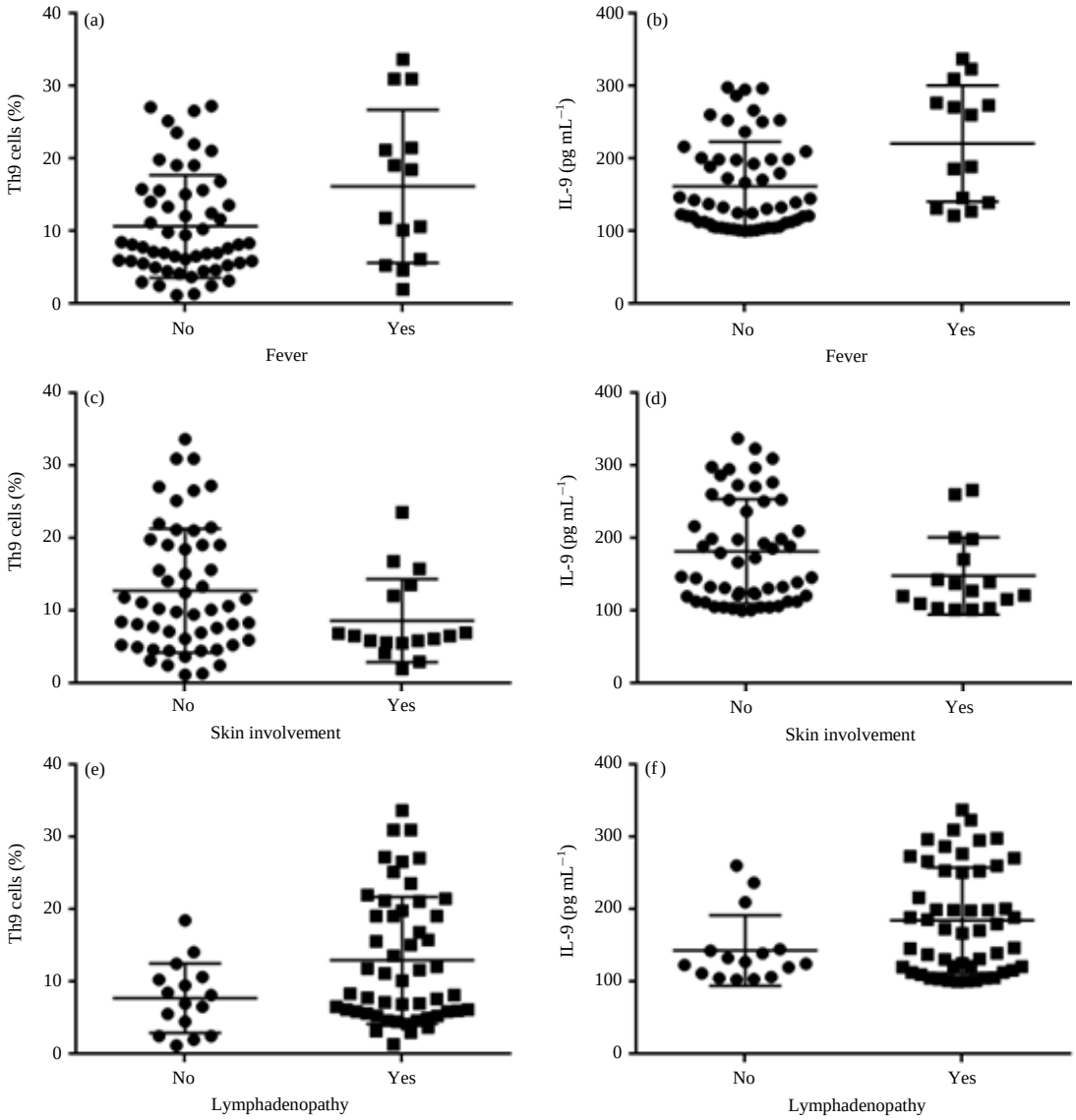

Fig. 2(a-f): Association of Th9 cells and IL-9 levels with fever, skin involvement and lymphadenopathy in CLL patients. Higher levels of both Th9 cells and IL-9 were observed in CLL patients with fever (p = 0.022, p = 0.004, respectively), skin involvement (p = 0.029, p = 0.046, respectively) and lymphadenopathy (p = 0.004, p = 0.012, respectively)

TST was longer in CLL patients with low serum IL-9 level: There was no significant difference regarding TST between CLL patients with low and high levels of Th9 cells. It was observed that TST associated with low serum IL-9 level was significantly (p = 0.010) longer than that associated with high IL-9 level (Fig. 8).

DISCUSSION

The B-cell type chronic lymphocytic leukemia (CLL) is a heterogeneous disease with some patients surviving for years or even decades with minimal or no need for treatment with a "watch and wait" strategy especially those who are in earlier Rai or Binet stages[18]. The present study aimed to evaluate the level of IL-9 gene expression by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and its protein level by ELISA and the level of Th9 cells by flow cytometry in blood samples taken from CLL's patients followed by assessment of the relation of the aforementioned parameters with each other and with available prognostic markers for CLL patients. Circulating Th9 cells were evaluated as the percentage of IL-9$^+$ cells among CD5$^-$ CD19$^-$ T cells.

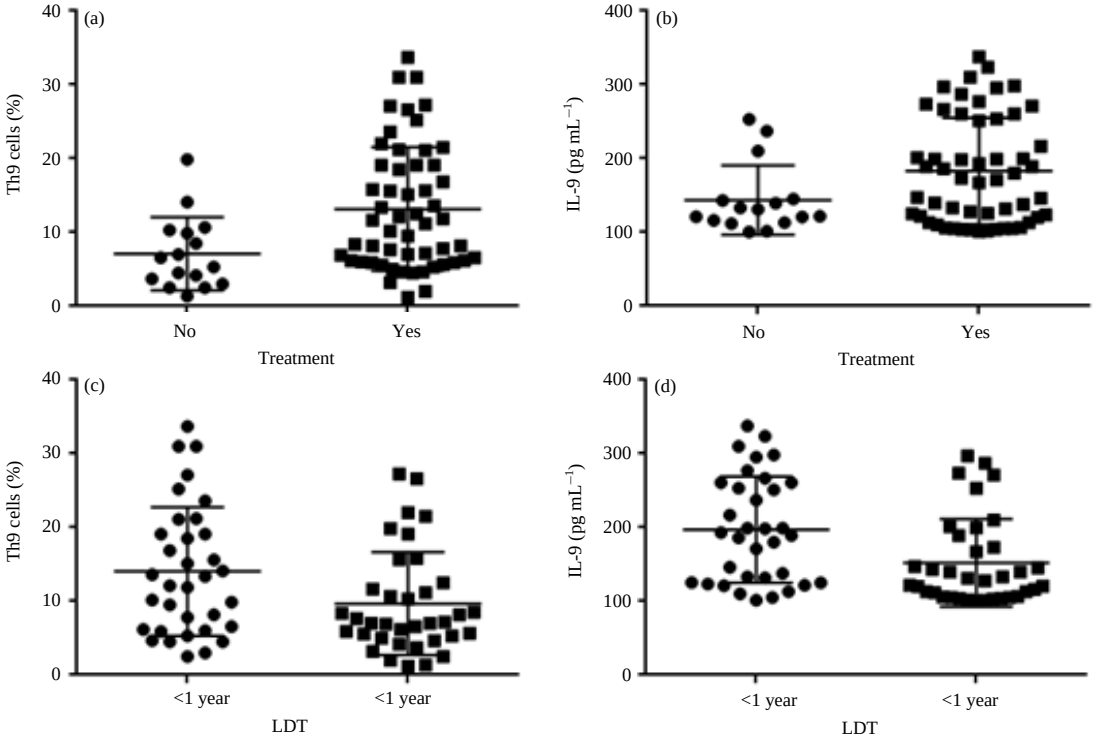

Fig. 3(a-d): Association of Th9 cells and IL-9 levels with treatment and LDT in CLL patients. Higher levels of both Th9 cells and IL-9 were observed in CLL patients treated with chemotherapy (p = 0.001, p = 0.014, respectively) and CLL patients with LDT less than one year (p = 0.023, p = 0.006, respectively)

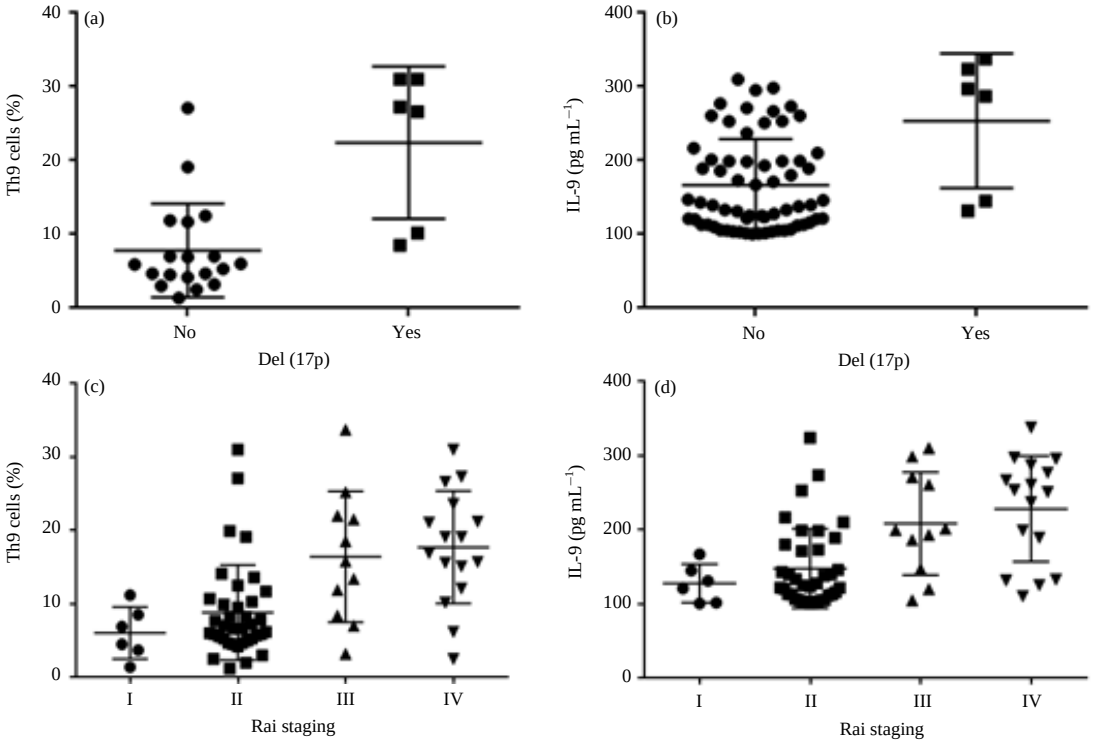

Fig. 4(a-d): Association of Th9 cells and IL-9 levels with Del (17p) and Rai staging in CLL patients. Higher levels of both Th9 cells and IL-9 were observed in CLL patients with Del (17p) (p = 0.016, p = 0.026 respectively) and higher Rai stages (p = 0.022, p<0.001 respectively)

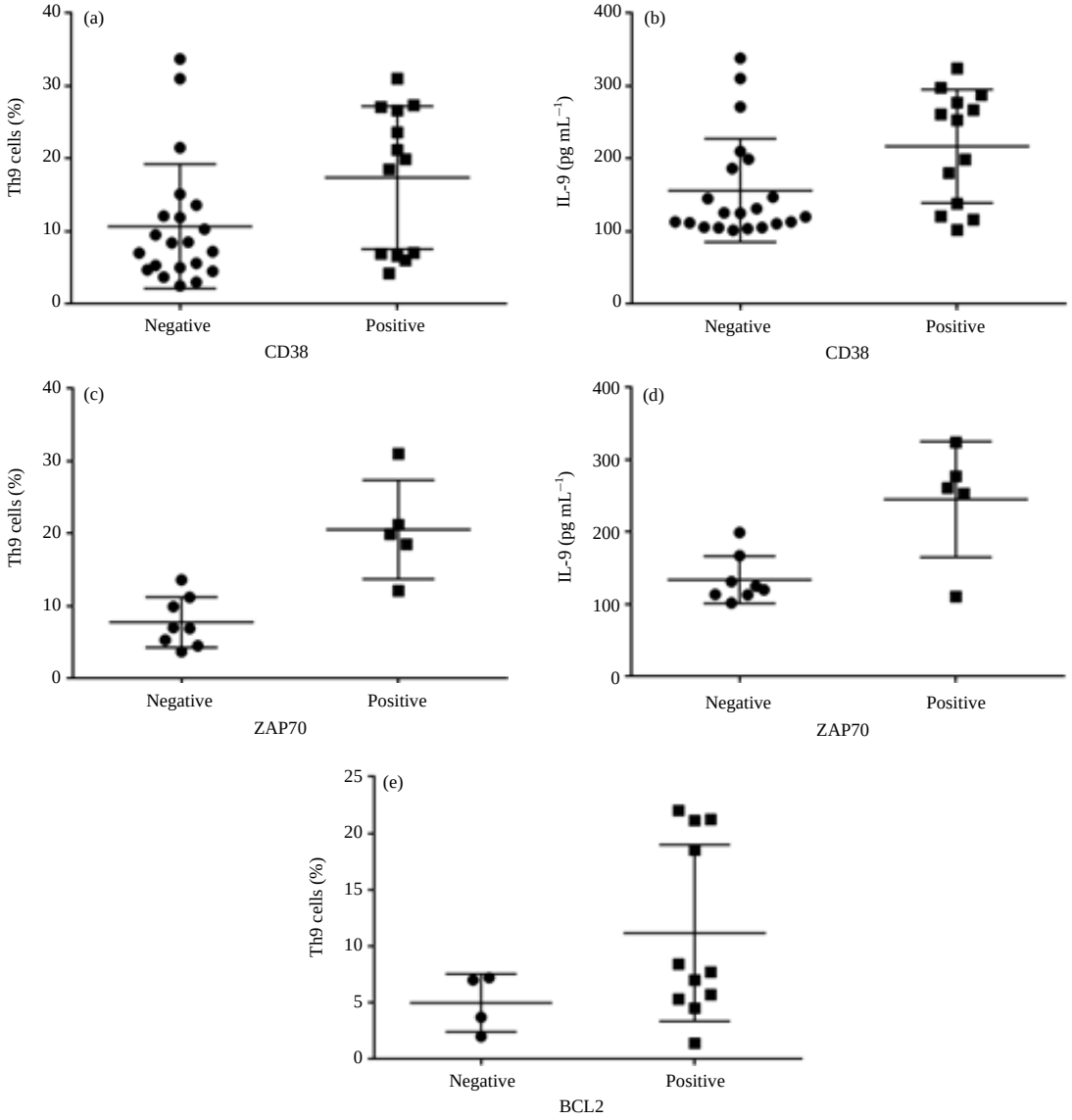

Fig. 5(a-e): Association of Th9 cells and IL-9 levels with CD38 and ZAP70 as well as association of IL-9 with BCL2 in CLL patients. Positive cases for CD38 and ZAP70 were strongly associated with higher levels of both Th9 cells ($p = 0.044$, $p = 0.001$, respectively) and IL-9 levels ($p = 0.026$, $p = 0.005$, respectively). Circulating level of Th9 cells was increased significantly ($p = 0.038$) in CLL patients positive for BCL2

No significant change in Th9 cell frequency was observed between CLL patients and controls. On the other hand, serum level of IL-9 was significantly ($p<0.001$) elevated in CLL patients when compared with the control group.

Malignant CLL cells secrete IL-4 that induces STAT6 phosphorylation and activation to promote IL-9 upregulation and secretion[19]. Chen *et al.* [19] have detected higher levels of IL-9 at both protein and mRNA levels in CLL patients. The upregulated IL-9 was correlated to the advanced stages, β2 microglobulin expression, ZAP-70 expression and IgVH status of the patients[19]. Interleukin-9 participates in the pathogenesis of CLL by both enhancing proliferation and inhibiting apoptosis of cancer cells[7]. Increased level of IL-9 along with no significant change in Th9 cell frequency, as detected in this study, may allow one to speculate other sources for IL-9 other than Th9 cells during CLL development.

In the current study, both Th9 cells and IL-9 level were observed to be higher in patients with fever, skin involvement, chemotherapy treatment, LDT less than one year, TP53 Del (17p) and higher Rai stages. The current results are consistent with that of Chen *et al.*[19], who have described higher levels of IL-9 and its mRNA expression in CLL patients that correlated

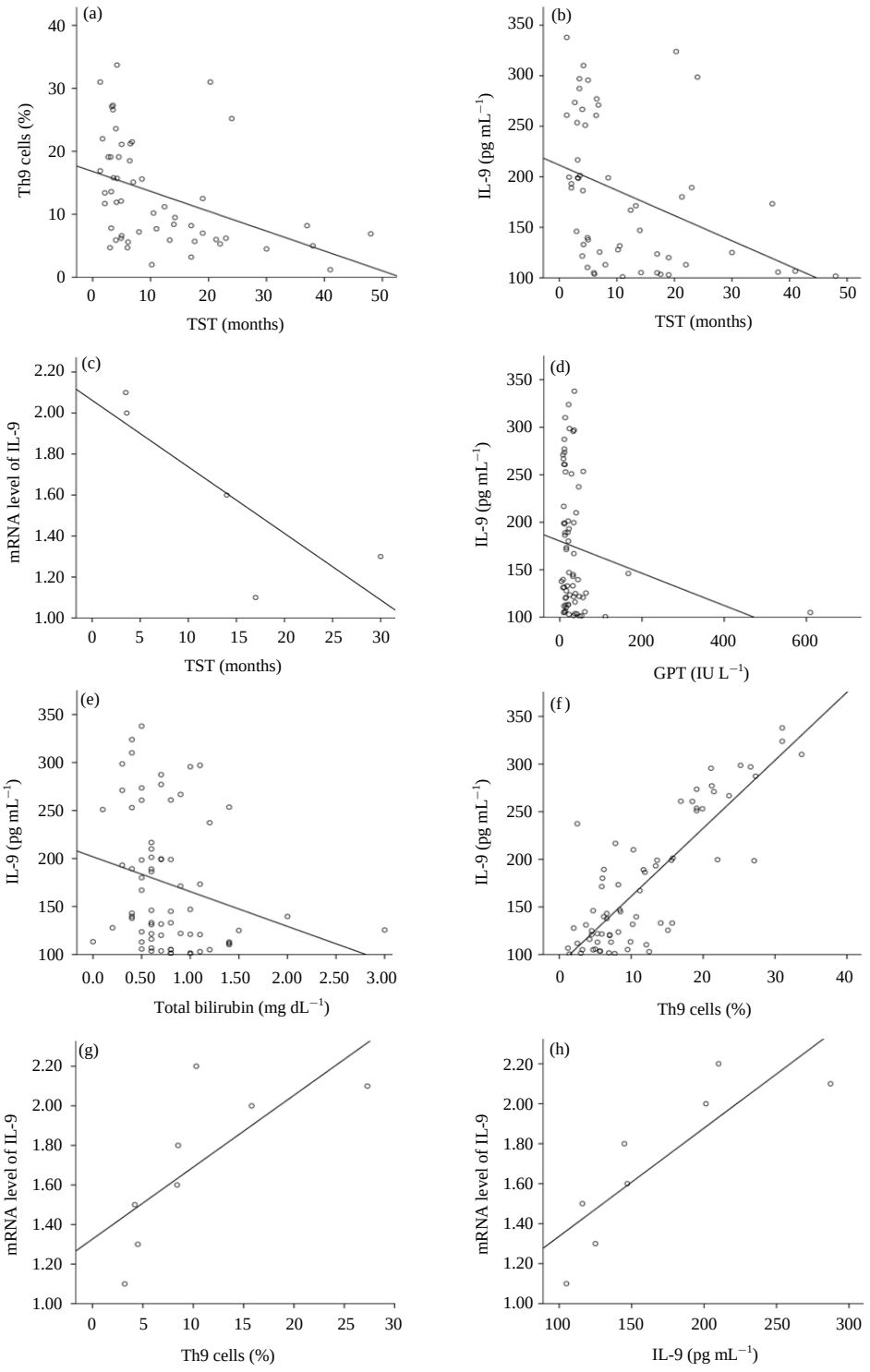

Fig. 6(a-h): Correlations between Th9 cells, IL-9 expression and serum levels, clinical and laboratory measurements in CLL patients. Time to start treatment (TST) in CLL patients was negatively correlated with all of Th9 cells, serum levels and expression of IL-9 ($p = 0.003$, $p = 0.005$, $p = 0.037$, respectively). Significant negative correlations between serum IL-9 level and serum levels of both GPT and total bilirubin ($p = 0.022$, $p = 0.010$, respectively) were found. Significant positive correlations between circulating level of Th9 cells and each of serum IL-9 level and its mRNA level in peripheral blood leucocytes ($p<0.001$, $p = 0.002$, respectively) in CLL patients were observed. Serum protein and mRNA levels of IL-9 in peripheral blood leucocytes were positively correlated in CLL group ($p = 0.001$)

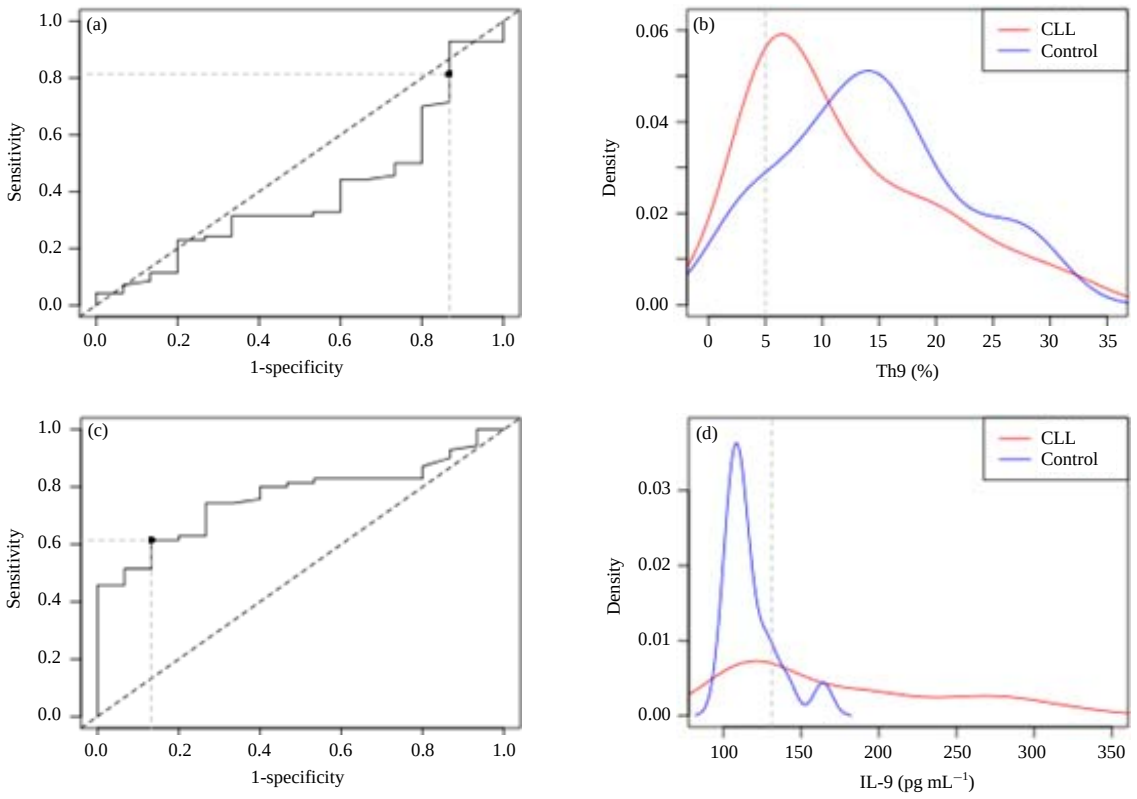

Fig. 7(a-d): ROC curves and distribution graphs of Th9 and IL-9 levels for discrimination between CLL and control groups. Serum IL-9 levels showed significant AUC (p = 0.001) that could discriminate between CLL cases and control groups, while Th9 cells showed non-significant AUC (p = 0.207) that couldn't discriminate between CLL and control cases

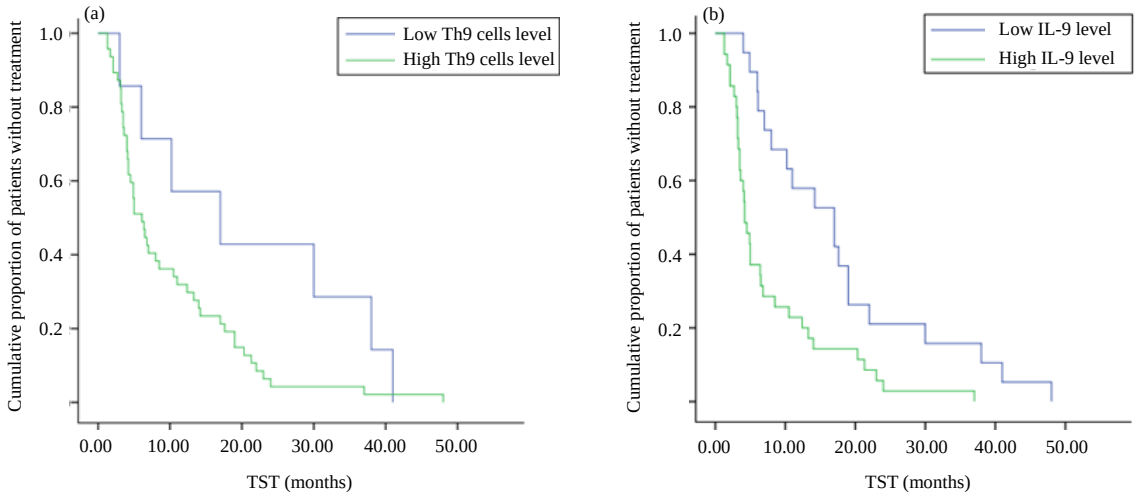

Fig. 8(a-b): TST according to Th9 cell and IL-9 levels in studied CLL patients. A significantly (p = 0.010) longer TST was associated with low serum IL-9 level than that associated with high IL-9 level. There was no significant difference regarding TST between CLL patients with low and high levels of Th9 cells (p = 0.192)

with the advanced stages and ZAP-70 expression. In line with this study's findings, Abbassy *et al.*[20] demonstrated higher levels of IL-9 protein and mRNA levels in CLL patients with advanced Rai stages, positive cases for ZAP-70 and CD38. These results indicated that the overexpression of IL-9 was associated with CLL clinical progression. According to this

knowledge, this is the first report to demonstrate association between higher level of Th9 cells and IL-9 in CLL patients with fever, skin involvement, LDT less than 1 year, TP53 Del (17p) and those who received chemotherapy treatment which predict unfavorable progression and overall survival of patients. These results may allow us to speculate its contribution in the pathogenesis and progression of CLL and its importance as a future immunotherapeutic target.

In the present study, IL-9 was elevated in positive cases for BCL2 ($p = 0.038$). Expression of BCL-2 on CLL cells favors inhibition of apoptosis which explains the long life span and accumulation of CLL cells in many organs and indicates bad prognosis[21]. In addition, IL-9 induces STAT-3 leading to induction of the anti-apoptotic gene BCL2[22]. During this study, significant negative correlations between TST and all of Th9 cells, IL-9 protein and mRNA level were observed. These results provided clinical evidence for their contribution to the pathogenesis of CLL. Moreover, serum IL-9 level is negatively correlated with serum levels of both GPT and total bilirubin ($p = 0.022$, $p = 0.010$ respectively). Previous study of Yu *et al.*[23] showed that Th9 cells and IL-9 are not related to extent of liver injury and the impact on liver functions in patients infected with HBV. They found no obvious correlation between the percentage of Th9 cells, as well as IL-9 level with serum levels of GPT, albumin and total bilirubin. The relation of IL-9 with liver function needs further research.

Here, significant positive correlations were also shown between circulating level of Th9 cells and both serum IL-9 level ($p<0.001$) and its mRNA level ($p = 0.002$) in CLL patients. Previous studies have demonstrated that IL-9 is the signature cytokine of Th9 cells[6]. In accordance with a previous study[20], a positive correlation ($p = 0.001$) between level of IL-9 gene expression and its protein level was found. These results indicated that Th9 cells are a source of IL-9 in CLL, so these cells may participate in the pathogenesis of the disease.

CONCLUSION

Higher levels of IL-9 protein and mRNA contribute to the pathogenesis of CLL and are associated with more complex form of disease and worse prognosis. Th9 cells didn't differ significantly in CLL patients when compared to controls but may have effects in the process of CLL as they were associated with complex form of the disease. Exploring the mechanisms among these effects of Th9 cells and IL-9 needs further investigation steps and directions.

SIGNIFICANCE STATEMENT

This study signifies the role of Th9 cells and IL-9 in CLL. To the best knowledge, this is the first study to provide a preliminary insight into evaluation and effects of Th9 cells in the process of CLL and to study its relation with IL-9 in CLL patients. Higher levels of Th9 cells and IL-9 were associated with more complex form of CLL and worse prognosis. These findings suggest that determination of Th9 cells and IL-9 could be functional as prognostic factors for prediction of clinical disease outcomes and progression of CLL disease and could be used as a potential therapeutic target.

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