Combined analysis of Regulatory T- cell and ZAP-70 expression in patients with B-chronic lmphocytic leukemia

Wafaa Ahmed EL- neanaey[1] and Dalia Ahmed Nafea[2]

[1] Clinical Pathology Department, Faculty of Medicine, Alexandria University, Egypt.
[2] Internal Medicine Department, Faculty of Medicine, Alexandria University, Egypt.
wafaan62@hotmail.com

Abstract: Background: Evidence is accumulating on the role of T- cells in the pathogenesis and development of chronic lymphocytic leukemia .Attention has been recently focused on regulatory T-cells (T-reg), which are considered to play a key role in the regulation of immune responses to cancer. This study aimed to define the role of regulatory T- cells and ZAP-70 expression in disease progression and to evaluate their expression in response to therapy of CLL patients**. Methods**: 58 CLL patients (37 untreated and 21 CLL patients that were assessed before and after 4 cycles of treatment with fludarabine and cyclophosphamide) and 28 age and sex matched healthy controls were enrolled in this study. T-reg cells (CD4+CD25+) and ZAP-70expression were detected by flowcytometer .**Results:** T-reg % was significantly lower in CLL patients than controls (p=0.001) while absolute T reg count was significantly higher in CLL patients than controls (p=0.001). After treatment, absolute T-reg count was significantly decreased (p=0.001) and non-significant decrease was found in T-reg %(p= 0.582). For disease progression, absolute T-reg count was significantly increased in patients with Rai stages III-IV than the patients with Rai stages 0-II (p= <0.001).while no significant difference was found with T-reg % (p =0.697). In addition, there was an association between ZAP and staging of CLL (p=0.045).However, there was no significant difference in T-reg% and absolute T-reg count for ZAP -70 positive and ZAP-70 negative CLL patients. **Conclusion:** absolute T- reg count rather than T-reg % can be used as predictive marker for disease progression in CLL patients. The mechanism of T-reg expansion as well as the interaction between CLL cells and T-reg needs further studies before developing innovative immune approaches targeting T-reg cells in CLL.

[WafaaAhmed EL- neanaey and Dalia Ahmed Nafea. **Combined analysis of Regulatory T- cell and ZAP-70 expression in patients with B-chronic lmphocytic leukemia.** *Life Sci J* 2013;10(1):3323-3328]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 421

Key words: B-CLL, T-Reg, flowcytometry.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoid malignancy characterized by the accumulation of monoclonal B- lymphocytes in bone marrow, peripheral lymphoid organs and peripheral blood (1).

Evidence is accumulating on the role of T- cells in the pathogenesis and development of CLL .Attention has been recently focused on a subset of T-cells, which are generally termed regulatory T- cells (T- reg).This naturally occurring regulatory CD4+CD25+

T cells originate from the thymus and play a central role in the maintenance of peripheral tolerance by suppression of autoreactive T-cells (2,3).

Few studies showed that this subset is increased in CLL patients. Moreover, the data obtained from solid tumors proved that increased numbers of T- reg cells are usually found as the disease progresses. (4,5).

Although a truly unique marker for T- reg cells is still not available, several molecules have been associated with these cells including cytotoxic T lymphocytes associated protein 4(CTLA4), fork head boxP3(FOXP3),L-selectinCD62) and OX40 (CD134) antigen(6-8).

ZAP-70 is a 70 KD protein tyrosine kinase associated with zeta chain of the T cell receptor. It is normally expressed in T- lymphocytes and critical for their activation by antigen.ZAP-70 expression is an unexpected finding in B-cell tumors, since the protein has not been reported in normal circulating B cells. Recent data showed that the expression of ZAP-70 protein is limited to CLL cells with unmutated immunoglobulin heavy chain genes. (9)

The present study aimed to define the role of regulatory T- cells and ZAP-70 expression in disease progression and to evaluate their expression in response to therapy of CLL patients**.**

2. Material and Methods:

The present study was conducted on 58 patients who were admitted to the hematology unit of Internal Medicine department in Alexandria Main University Hospital. They are meeting the diagnostic criteria for CLL and classified according to Rai staging. In addition , 28 age and sex matched healthy controls were also included. 37 of all patients Were untreated

(stage 0, I and II) and the other 21 patients were indicated for treatment (Stage II, III and IV).They were assessed before and after 4 cycles of fludarabine (25mg/m^2 /day) and cyclophosphamide (250 mg/m^2/day).

CLL patients with autoimmune hemolytic anemia and autoimmune thrombocytopenia were excluded from the study. Informed consent was obtained from patients and the study was approved by the Ethics Committee of Faculty of Medicine, Alexandria University.

Laboratory investigations:

All patients& controls were subjected to:

(a)- Full history taking and complete clinical examination.

(b)- Complete blood picture on Sysmex XT-1800 automated blood cell counter(Siemens Health care Diagnostics Inc, USA).

(c) - Coomb's test and antiplatelet antibodies to exclude autoimmune conditions.

(d)- Imunophenotyping to confirm diagnosis of CLL patients using the following panel: CD5, 19, CD22, CD23, FMC7, Kappa and Lambda.

(e)- Flowcytometric measurement of regulatory T-cells expression by monoclonal antibodies PE-CD4, clone: SK3 and FITC-CD25, clone: BC96 (Biolegend, San Diego,CA.) using Miltenyi Biotec, MACSQuant flowcytometer Analyzer equipped with MACSQuantify software version 2.3, (USA).(10,11)

(f)-Flowcytometric measurement of PE- ZAP -70expression, clone: IE7.2 (Biolegend, San Diego,CA.)On CD19-FITC, Clone: HD37 (DAKO, Denmark) using Miltenyi Biotec, MACSQuant flowcytometer Analyzer equipped with MACS Quantify software version 2.3,(USA).(12,13)

Flowcytometric analysis of T- reg: 100µl of whole EDTA blood was mixed with 10 ul of each CD4+ andCD25+ MoAb in a single tube and incubated for 10 minutes at room temp. After red cell lysis and repeated washing, the cells were analyzed by the flow cytometer. Absolute CD4 count was calculated from the percentage of CD4 subsets measured by flowcytometer and absolute lymphocyte count measured by well calibrated automated cell counter. T-reg% was evaluated according to gating strategy protocol previously described by Beacher Allan *et al.* (14)

Absolute T-reg count was calculated from the T- reg % and absolute CD4 count. (15)

Flowcytometric analysis of ZAP-70:100µlof whole EDTA blood was mixed with 2ml 4% paraformaldehyde in PBS and incubated for 10 minutes at room temp. After repeated washing, the cells were permeabilized by using 0.2% Tween 20.Then; they were incubated with 10 ul of ZAP-70 MoAb for 10 minutes at room temp. After repeated washing with 0.2% Tween 20 followed by PBS, the cells were analyzed by flowcytometer. During analysis a gate was set around the required cell population (positive for CD19).The cut-off point of positivity for ZAP-70 was considered when more than 20% of the cells were stained with a particular antibody in excess of the background fluorescence in the negative controls. (9)

Statistical analysis:

Data were analyzed using the Predictive Analytics Software *(***PASW Statistics 18)** (16)**.**

3.Results :

According to the diagnostic criteria of CLL, our patients showed significantly higher values of white cell count (WBCs) and lymphocyte count while the median of hemoglobin and platelet count were significantly lower than control (Table 1). T-reg % was significantly lower in CLL patients than the controls (median=1.52% vs.4.15%, p=0.001).However, absolute T reg count was significantly higher in CLL patients than the controls (median=529 vs.75.5, p=0.001). Figure (1,2) &Table (2). The absolute T-reg count was significantly decreased after 4 cycles of treatment with fludarabine and cyclophosphamide (median=529.0 vs 33.0, p=0.001). Figure (3).while non-significant change was found in T-reg %(p= 0.582) and ZAP-70 expression(p= 1.000).Table (2)

Regarding disease progression, absolute T-reg count was significantly higher in patients with stages III-IV than patients with stages 0-II (median=729.0 vs.173.0, p=<0.001). (Figure 4). Non-significant change was found with T-reg % ((median= 0.971 % vs. 0.964 %, p =0.697).Moreover, we observed that absolute T-reg count was highly correlated with absolute lymphocyte count for both untreated patients and those who were assessed before and after treatment (r =0.584,p =<0.001,r =0.841= p=<0.001 and r =0.482,p =0.027 respectively).

There was an association between ZAP-70 and staging of CLL (p=0.045).However, there was no significant difference in T-reg% and absolute T-reg count for ZAP -70 positive and ZAP-70 negative CLL patients(1.67% vs.1.52%,p = 0.667for T-reg % and497.0vs.743.0 p = 0.868 for absolute T-reg count).

Table (1): Clinical and Hematologic Profile of CLL Patients and healthy Controls.

	Group I(37) (CLL untreated cases)	Group II (treated cases)(21)		Group III (28) (control)
		Before treatment	**After treatment**	
Age:	**64.57 ± 9.06**	**65.0 ± 9.58**		**63.10 ± 6.46**
Sex :	**Male: 20 Female: 17**	**Male : 12 Female : 9**		**Male :17 Female : 11**
Stage				
0	**9 (24.3%)**	**0 (0.0%)**	**0 (0.0%)**	
I	**10 (27.0%)**	**0 (0.0%)**	**0 (0.0%)**	
II	**18 (48.6%)**	**4 (19.0%)**	**4 (19.0%)**	
III	**0 (0.0%)**	**10 (47.6%)**	**10 (47.6%)**	
IV	**0 (0.0%)**	**7 (33.3%)**	**7 (33.3%)**	
Hb (g/dl) median (min. – max.)	**12.0 (11-12.9)**	**9.2(8.10-12.50)**	**11.3 (11.0-13.30)**	**14.1(13.0-15.5)**
$^{MW}p_1$	**<0.001***	**<0.001***	**<0.001***	
$^{WSRT}p_2$		**<0.001***		
Platelet(X 10^9/L) median (min. – max.)	**207.0 (173-246)**	**205.0 (17-224)**	**209.0 (110 -283)**	**304.5 (219-434)**
$^{MW}p_1$	**<0.001***	**<0.001***	**<0.001***	
$^{WSRT}p_2$		**0.001***		
WBCs (X 10^9/L) median (min. – max.)	**28.0 (23.6- 52.0)**	**43.1 (25.9-182.0)**	**8.7 (3.2-10.7)**	**6.3 (4.8-8.3)**
$^{MW}p_1$	**<0.001***	**<0.001***	**0.251**	
$^{WSRT}p_2$		**0.001***		
Lymphocytosis (%) median (min. – max.)	**83.0 (78-88)**	**85.0 (77- 99)**	**34.0 (25- 37)**	**32.0 (25-38)**
$^{MW}p_1$	**<0.001***	**<0.001***	**0.200**	
p_2		**0.001***		
Lymphocyte number/µL median (min. – max.)	**24.20(19.6-44.26)**	**34.85(22.02-180.2)**	**1.76 (1.184 – 4.212)**	**2.10 (1.25-3.14)**
$^{MW}p_1$	**<0.001***	**<0.001***	**<0.001***	
$^{WSRT}p_2$		**0.001***		

^{MW}p: p value for Mann Whitney test
^{WSRT}p: p value for Wilcoxon signed ranks test
p_1: p value for comparison between control and each other group
p_2: p value for comparison between before and after
*: Statistically significant at p ≤0.05

Table (2): flowcytometric studies in CLL patients and healthy controls.

	Group I(37) (CLL untreated cases)	Group II (treated cases)(21)		Group III (28) (control)
		Before treatment	**After treatment**	
T-Reg% median (min. – max.)	**0.53 (0.21-1.3)**	**1.60 (0.52-2.2)**	**1.52 (0.51-2.18)**	**4.15 (2.5-5.7)**
$^{MW}p_1$	**<0.001***	**<0.001***	**<0.001***	
$^{WSRT}p_2$		**0.582**		
T-Reg number/µL median (min. – max.)	**155.0 (47-307)**	**529.0 (412- 2162)**	**33.0 (17-58)**	**75.5 (45-158)**
$^{MW}p_1$	**0.002***	**<0.001***	**<0.001***	
$^{WSRT}p_2$		**0.001***		
ZAP-70				
-ve	**37 (100.0%)**	**15 (71.4%)**	**15 (71.4%)**	**28 (100.0%)**
+ve	**0 (0.0%)**	**6 (28.6%)**	**6 (28.6%)**	**0 (0.0%)**
$^{MC}p_1$	**-**	**FEp =0.021***	**FEp =0.021***	
p_2		**1.000**		

^{WSRT}p: p value for Wilcoxon signed ranks test ^{MC}p: p value for McNemar test ^{MW}p: p value for Mann Whitney test
FEp: p value for Fisher Exact test p_1: p value for comparison between control and each other group ZAP-70 expression (cut-off 20%)
p_2: p value for comparison between before and after *: Statistically significant at p ≤0.05

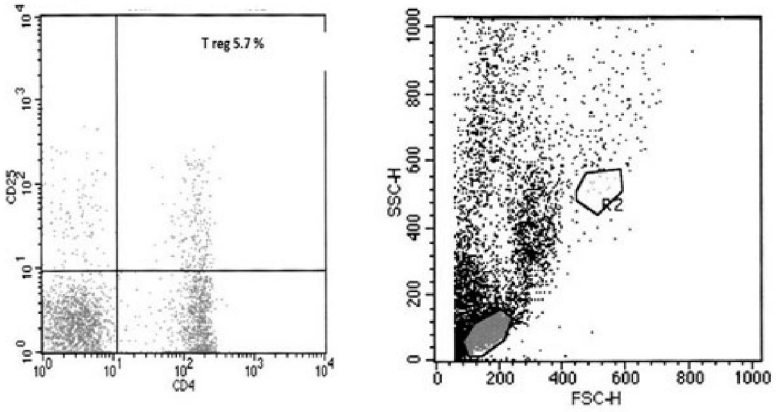

Figure (1): regulatory T-cell expression in control

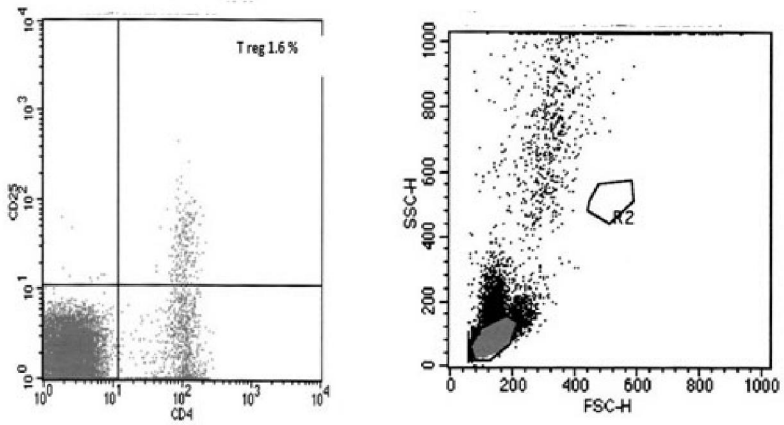

Figure (2): Regulatory T cell expression in CLL patient

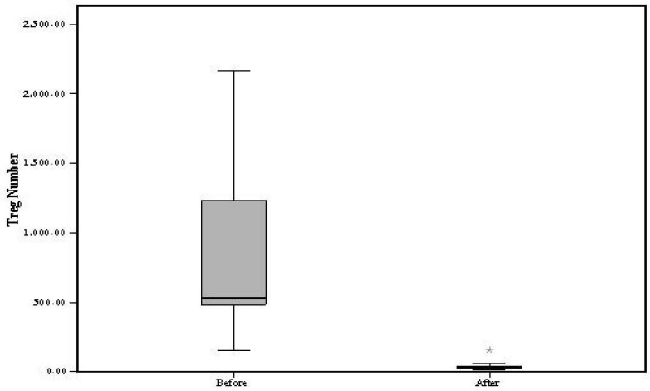

Figure (3): TREG number before and after treatment in CLL patients

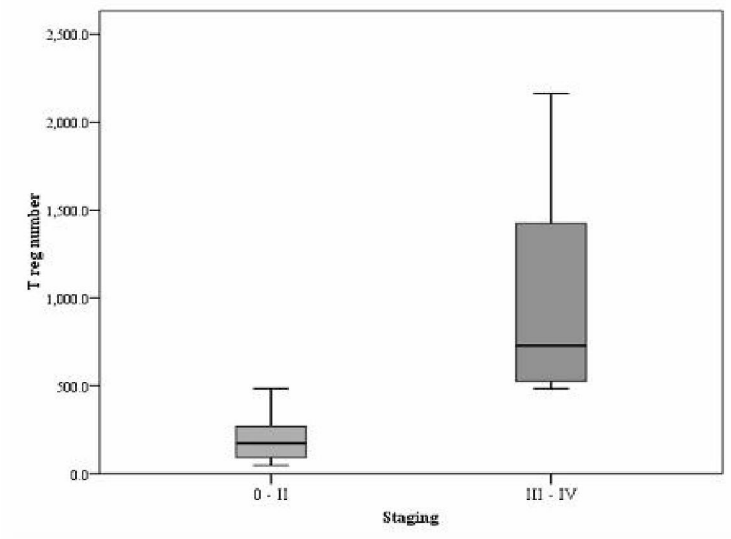

Figure (4): T- reg number and staging in CLL patients

4. Discussion

T –reg cells are a subset of CD4+ lymphocytes playing a key role in the modulation of immune response. They preferentially move and accumulate at tumor sites (17). T-reg

cells exist in markedly higher proportions within tumor infiltrating lymphocytes (TIL), peripheral blood lymphocytes (PBL) and or regional lymph node lymphocytes of patients with cancer including B-CLL (18).

In agreement with Arena GD et al and Varma S et al, we observed that T-reg % was significantly lower in CLL patients than controls. However, the absolute count of T-reg was significantly higher in CLL patients than controls. They concluded that absolute T-teg count may be important than T-reg % in the pathogenesis of CLL (19, 20). Neoplastic cells also directly recruit T-reg cells by producing chemoattractants with the consequence of creating a growth –favorable environment where the immune response is efficiently suppressed (21, 22).Also, there was a significant decrease in absolute count of T-reg after therapy with fludarabine and cyclophosphamide. These drugs augment the efficacy of antitumor immune response by decreasing the frequencies of T-reg cells and increasing both lymphocyte proliferation and memory T- cells (23,24) Therefore , Beyer et al, suggested that T-reg cells cannot be used as predictive marker in cancer patients treated with chemotherapy, particularly with drugs that influence the function and frequency of these cells. (18). CLL patients with concomitant autoimmune disorders were excluded from the study because in the context of autoimmunity, T-reg act by restraining autoreactive lymphocytes and their number is found to be decreased in autoimmune disorders (25). Some investigators reported that anti-CD20 (rituximabe) is able to restore the number of T-reg cells when used to treat idiopathic thrombocytopenic purpura (26). This appears very interesting because this drug is increasingly used to treat CLL in association with fludarabine and cyclophosphamide. Our results were matched with other investigators who found that T-reg frequencies in the peripheral blood were significantly increased in patients with advanced stages of disease (Rai staging III and IV) (18, 19).

Jak *et al.,* suggested that the role of T- reg in the pathogenesis and expansion of CLL is not due to tumor antigen stimulation. T-reg may accumulate by increased formation facilitated by CD27-CD70 interaction in the lymph node proliferation centers and decreased sensitivity to apoptosis(27).

Like Herishanu Yet al, there was an association between ZAP expression and staging of CLL (28). In addition, no significant difference in T-reg% and absolute T-reg count for ZAP positive and ZAP negative CLL patients as reported by Arena GD et al and Verma S et al(19,20).

In conclusion, absolute T- reg count rather than T-reg % can be used as predictive marker for disease progression. The mechanism of T-reg expansion as well as the interaction between CLL cells and T-reg needs further studies before developing innovative immune approaches targeting T-reg cells in CLL.

References

1. Chiorozz N, RaiK R, Ferranini M. Chronic lymphocytic leukemia. NEngl J Med 2005;352:804-15.
2. Hamblin AD, Hamblin TZ: The immunodeficiency of chronic lymphocytic leukemia. Br Med Bull 2008;87:49-52.
3. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self- tolerance and negative control of immune response. Ann Rev Immuno 2004;22:531-562.
4. Giannopoulos K, Schwitt, Kowal M, Walasiuk P. Charachterization of regulatory T cells in patients with B- cell chronic lymphocytic leukemia.Oncol Res 2008;20:677-82.
5. Bayer M, Schultze JL. Regulatory T cells in cancer. Blood 2006; 108:804-11.
6. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor FoxP3.Science 2003;299:1057-61.
7. Szanya V, Ermann J, Taylor C, Holness C. the subpopulation of CD4+CD25 splenocytes that delays adaptive transfer of diabetes express L- selectin and high level of CCR7 .J Immunol 2002;169:2461-65.
8. Takeda I, Ine S, Killen N. Distinct roles for the OX40-OX 40 ligand interaction in regulatory and nonregulatory T cells. J Immunol 2004; 172: 3580-89.
9. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, Marce S, Lopez-Guillermo A, Campo E, Montserrat E: ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia.N Engl J Med 2003, 348:1764-1775
10. Vaickus L, Ball E D, Foon K A. Immune markers in hematologic malignancies". Critical Reviews in Oncology/Hematology 1991:11;267-97.
11. Davis B H, Foucar K, Foon KA.-Canada consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flowcytometry: Medical Indication". Cytometry 1997:30;243-53.
12. Ertault-Daneshpouy M, Noguera ME, Gisselbrecht C, Haddad A, Brice P, Marolleau JP, Soulier J, Mounier N: ZAP-70 protein expression and CD38 positivity in B-cell chronic lymphocytic leukemia. Clin Adv Hematol Oncol 2008, 6:55-63.
13. Pezzutto A, Dörken B, Rabinovitch PS, *et al.* CD19 monoclonal antibody HD37 inhibits anti immunoglobulin-induced B cell activation and proliferation. J Immunol 1987; 138:2793-9.
14. Baecher AC, Brown JA, Feerman GA. CD4+CD25 high regulatory cells in human peripheral blood .J Immunol 2001;167:1245-53
15. Christopoulos P, Pfeifer D, Bartholome K, Follo M. Definition and characterization of the systemic T-cell dysregulation in untreated indolent B-cell lymphoma and very early CLL .Blood 2011; 117: 3836-46.
16. Wallach J. (2000): Interpretation of diagnostic tests. 7th ed. Williams and wilkins. Philadelphia, USA.
17. Albers AE, Ferris RL, Kim GG, Chikamatsu K, Cikamatsu K and Whiteside TL. Immune response to p53 in patients with cancer enrichment with tetramer +p53 peptide-specific T cellsandregulatoey T6 cells at tumor sites. Cancer Immunol. Immunnther (2005): 54;1072-1081
18. Beyer M, Kochanek M, Darbi K, Jensen M, Endl E, Knolle P A, Thomas RK. Reduced frequencies and suppressive function of CD4+ CD25+ high regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. Blood 2005;106:2018-25.
19. Arena GD, Laureni L, Minervini MM, Deaglio S, Bonello L, Martino LD. Regulatory T- cell number is increased in chronic lymphocytic leukemia patients and correlates with progressive disease. Leukemia Research 2010;35(3):363-8.
20. Varma S, Lad D, Varma N, Bose P, Malhorta P. Do regulatory T cells have a role in disease progression and autoimmune cytopenia of chronic lymphocytic leukemia? 53rd ASH annual meeting and exposition, December 12, 2011.
21. Chatila TA. Role of regulatory T cells in human diseases allergy Clin Immunol 2005; 116: 949-1949.
22. Sakaguchi S, Wing K, Onishi Y, Prieto-MP. Regulatory T- cells: how do they suppress immune response? IntImmunol 2009; 21: 1105-11.
23. Loefffler M, Kruger J A, Reisfeld RA. Immuno stimulatory effect of low dose cychlophosphamide are controlled by inducible nitric oxide synthase. Cancer Res 2005; 65: 5027-30.
24. Lutisiak C, Semnani RT, Pascalis R. Kashmiri SV, Scholm J. Inhibition of CD4+CD25++ regulatory cell function implicated in enhanced immune response by low dose cyclophosphamide. Blood 2005;105:2862-68.
25. Beacher Allan C, Hafler DA. Human regulatory T-cells and their roles in autoimmune disease. Immunol Rev 2006; 212: 203-216.
26. Stase R, Cooper N, Del Poeta G, Stipo E. Analysis of regulatory T cells changes in patients with idiopathic thrombocytopenic purpura receiving B- cell depleting therapy with Rituximab. Blood 2008; 112:1147-50.
27. Dutsch V, Perry C, Polliack A. Expansion of regulatory T cells in B chronic lymphocytic leukemia: enhanced: 'brakes' on host immunity .leuk lymphoma 2009; 50-687-8.
28. Herishanu Y, Kay S, Rogowski O, Pick M, Naparstek E, Deutsch VR, Polliack A. T-cell ZAP-70 overexpression in chronic lymphocytic leukemia (CLL) correlates with CLL cell ZAP-70 levels, clinical stage and disease progression. Leukemia 2005; 19; 1289–91.

3/3/2013