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Blimp-1 Expression as an Exhaustion Transcription Factor in Chronic Lymphocytic Leukemia

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Abstract

Background: Previously, it was shown that exhausted CD4+ and CD8+ T cells in chronic lymphocytic leukemia (CLL) co-express the two immune-inhibitory receptors, Tim-3 and PD-1. Present study investigated the expression of Blimp-1, a transcription factor involved in T cell exhaustion, in patients with CLL.

Materials and Methods: Peripheral blood mononuclear cells were collected from 25 untreated CLL patients and 15 sex- and age-matched normal subjects. CLL patients were clinically classified according to the Rai staging system. The relative expression of Blimp-1 mRNA was determined by quantitative Real Time Polymerase Chain Reaction (qRT-PCR) after normalization with β-actin.

Results: Expression of Blimp-1 mRNA was much higher in CLL patients than in normal controls (p=0.001). Moreover, Blimp-1 was more expressed in patients with advanced clinical stages of CLL compared to those with early stages of the disease (p=0.01). Interestingly, the Blimp-1 expression was correlated with the frequencies of exhausted Tim-3+/PD-1+/CD4+ and Tim-3+/PD-1+/CD8+ T cells in CLL patients.

Conclusion: Our results highlight the role of Blimp-1 transcription factor in T cell exhaustion of CLL.

Keywords: Exhausted T cell; Blimp-1; Chronic Lymphocytic Leukemia

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Introduction

Based on the tumor immune-surveillance theory, various components of the immune system constantly survey the body for any malignant cell proliferation and eliminate or slow their growth (1). This theory suggests that both innate and adaptive immunity can respond, recognize, and destroy the malignant cells. However, to escape from the immune system mechanisms, cancerous cells induce an immunosuppressive state in the host (2, 3). One of the well-known immunosuppressive mechanisms engaged by tumor cells is induction of exhaustion phenotype in effector cells of the host immune system, such as T lymphocytes, natural killer cells, and macrophages (4). Chronic lymphocytic leukemia (CLL) is the most frequent type of leukemia recognized in western countries, accounting for about one third of all cases of adult leukemias (5-7). The immune deficiency seen in CLL is comprehensive, leading in higher vulnerability to bacterial, viral and fungal infections, as well as deficiencies in response to tumors (8, 9). During chronic infections and cancers, which involve persistent antigen exposure, the memory T cell differentiation is markedly altered and the number of exhausted T cells is increased (10). These cells exhibit common characteristic features such as loss of effector functions, sustained upregulation and co-expression of inhibitory receptors (11, 12). Some of the immune inhibitory receptors expressed by exhausted T cells are programmed death-1 (PD-1, CD279), T cell immunoglobulin domain and mucin domain-containing protein 3 (Tim-3), cytotoxic T lymphocyte

associated protein-4 (CTLA-4), lymphocyte activation gene 3 protein (LAG-3), 2B4 (also known as CD244), and CD160 (13).

Recent studies have applied genomic approaches to investigate the transcriptional factors underlying T cell exhaustion. Both CD4+ and CD8+ exhausted T cells have a distinct transcriptional profile that is markedly different from their effector and memory counterparts, including major changes in transcription factors, the expression of inhibitory and co-stimulatory receptors, signaling molecules, cytokine and chemokine receptors, and also genes involved in metabolism (11, 14, 15). Previous studies revealed the role of B lymphocyte–induced maturation protein 1 (Blimp-1) as a transcriptional regulator of CD8+ T cell exhaustion during chronic viral infections and proposed Blimp-1 as a transcriptional regulator balancing effector function and T cell exhaustion (10, 15-17). Blimp-1 is a zinc finger-containing transcriptional repressor encoded by the prdm-1 gene and is required for terminal differentiation of B cells into plasma cells (18-20). It has been found that Blimp-1 is induced during the later stages of CD8+ T cell activation and is required for terminal differentiation of effector CD8+ T cells (21, 22).

In our previous studies on CLL patients, we have shown that both CD4+ (23) and CD8+ T cells (24) are exhausted and show functional defects. These exhausted T cells are characterized by overexpression of Tim-3 and PD-1 immune inhibitory receptors. We have also indicated the upregulation of Gal-9 and PD-L1 immune checkpoint molecules in CLL patients as the main ligands of Tim-3 and PD-1, respectively (25). Notwithstanding our increasing knowledge of the molecules involved in immune regulation and T cell exhaustion in CLL, the role of their related transcription factors remained unclear. In order to address this question, we surveyed the expression profile of Blimp-1 as an exhaustion transcriptional factor in CLL patients.

Materials and Methods

Patients and controls

Peripheral blood was collected from 25 CLL patients who had not received any chemotherapy regimens, including 13 males and 12 females with the age range of 48–84 years, attending the Hematology and Oncology Clinic of Imam Khomeini Hospital, affiliated to Mazandaran University of Medical Sciences, and 15 normal subjects, who were age- and sex-matched with CLL patients, including 9 males and 6 females with the age range of 35-77 years. A consent letter was taken from all participants and the study was approved by the Ethical Committee of Mazandaran University of Medical Sciences. CLL was diagnosed based on blood cell count, cell morphology, immunophenotyping analysis, and clinical symptoms. The patients were clinically classified according to the Rai staging system and National Cancer Institute Working Group (NCI-WG) criteria. Stages 0 and I were then defined as early clinical stage (n=16) and stages II, III and IV as advanced clinical stages (n=9). The major clinical and laboratory characteristics of our CLL patients and normal subjects are summarized in Table I.

Table 1. Major clinical and laboratory characteristics of CLL patients and normal controls.

Characteristics		CLL Patients (n=25)	Healthy Controls (n=15)	p-value
Sex	Male Female	13 12	9 4	0.24
Age (years)	Median Range	62.24 48 – 84	58.13 35 -77	0.34
WBC×10^3/mm^3	Median Range	39.79 13.48 – 112.0	7.50 4.20 – 9.79	< 0.0001
Lym (%)	Median Range	80.61 56 – 95	35.83 27 – 40	< 0.0001
PLT×10^3/mm^3	Median Range	173.9 38 – 365	214.6 131 – 270	0.04
Hb (g/dl)	Median Range	12.19 6 – 15	13.47 11 – 15	0.04

CLL: chronic lymphocytic leukemia, WBC: white blood cell count, Lym: lymphocytes percent in peripheral blood, PLT: platelet count, Hb: hemoglobin. P-values < 0.05 were considered significant.

RNA extraction and cDNA synthesis

Peripheral blood mononuclear cells (PBMCs) were isolated from all samples using Ficoll Histopaque density gradient centrifugation, according to the manufacturer's instructions (Biosera, Nuaille, France). The viability of isolated cells was > 95% as determined by trypan blue staining. Total RNA was extracted from 8×10^6 PBMCs using RNeasy kit (CinnaGen, Tehran, Iran) based on the manufacturer's protocol. The quality of isolated RNA was confirmed by a Nano-spectrophotometer (WPA, England) and electrophoresis. Complementary DNA (cDNA) was reverse-transcribed from 1 microgram of total RNA in a 20µl reaction mixture containing 1µl random hexamer primer, 4µl of 5x reaction buffer, 1µl RNase inhibitor, 2 µl dNTP 10mM, 200 unit RevertAid M-MuLV reverse transcriptase enzyme and appropriate RNase/DNase free water. The mixture was then incubated at 25 °C for 5 min, 42 °C for 1 hour and 70 °C for 5 min using the Thermo Scientific RevertAid first strand cDNA synthesis kit (Thermo Scientific, Massachusetts, USA).

Quantitative Reverse-Transcriptase PCR

The mRNA level of Blimp-1 was measured by quantitative Real Time PCR (qRT-PCR) based on 2X Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Massachusetts, USA) reagent in an iCycler iQ5 Real-Time PCR system (Bio-Rad, California, USA). In addition, the mRNA level of β-actin, as an internal control, was measured and used to normalize the Blimp-1 gene expression. PCR was performed using the following primers: Blimp-1, forward: TAC ATA CCA AAG GGC ACA CGT, reverse: GAT TCA CAT AGC GCA TCC AGT; β-actin, forward: CCT TCC TGG GCA TGG AGT CCT, reverse: TGG GTG CCA GGG CAG TGA T. The PCR reactions were amplified at 95 °C for initial denaturation followed by 40 cycles at 94 °C for 30 seconds, 60 °C for Blimp-1 and 57 °C for β-actin for 30 seconds, and 72 °C for 30 seconds. The PCR amplicon sizes were 175 bp and 174 bp for Blimp-1 and β-actin, respectively. Each run was completed with a melting curve analysis to confirm the specificity of the amplification and the absence of the primer dimers. Expression levels of Blimp-1 mRNA were determined by $2^{-\Delta Ct}$ value. The relative quantification system was used for data analysis based on the relative expression of Blimp-1 mRNA level to β-actin mRNA level as a housekeeping gene.

Ethics Statement

A consent letter was taken from all participants and the study was approved by the Ethical Committee of Mazandaran University of Medical Sciences.

Statistical analysis

Data analysis was performed using SPSS 20. Normality distribution of the obtained data was determined by Kolmogorov-Smirnov test. For comparing quantitative differences of Blimp-1 expression in patients and normal controls, the Mann-Whitney U test was used. For correlation analysis, Spearman rank correlation test was applied. Data are expressed as median (interquartile range) and p-values less than 0.05 were considered significant. All graphs were prepared using the GraphPad Prism 6 software.

Results

Blimp-1 mRNA was highly expressed in CLL patients

Our previous findings indicated that $CD4^+$ and $CD8^+$ T cells (23, 24) from CLL patients are exhausted and show functional defects. To investigate the profile of Blimp-1 expression as an important transcription factor in T cell exhaustion, mRNA expression of Blimp-1 was measured in peripheral blood of CLL patients. The relative expression of Blimp-1 mRNA was evaluated in all samples by a qRT-PCR method using β-actin as an internal control. The primer efficiency was defined as 102% for Blimp-1. Our results demonstrated that Blimp-1 was highly upregulated in CLL patients compared to normal subjects (p = 0.001, Figures 1A and B). Indeed, Blimp-1 mRNA level measured in CLL patients was six times higher than that of normal controls.

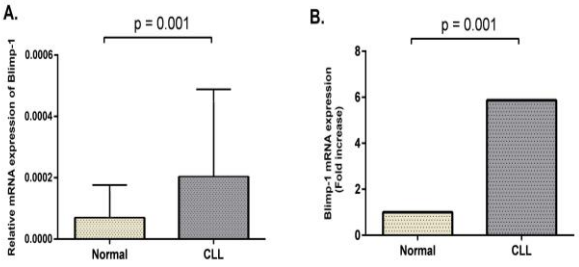

Figure 1. Blimp-1 mRNA expression profile in CLL patients and normal controls.
(A) Relative mRNA transcript levels of Blimp-1 from CLL patients and normal controls. (B) Fold increase of Blimp-1 in CLL patients compared to normal controls. Fold increase was calculated by dividing the mean ratio of Blimp-1 relative expression value obtained from CLL patients to normal controls. The results are represented as median ± interquartile range of $2^{-\Delta Ct}$ after normalization with β-actin as an internal control. P-values less than 0.05 were considered significant.

Blimp-1 was more expressed in CLL patients at advanced clinical stages

In our previous studies, we have indicated higher frequencies of exhausted $CD4^+$ and $CD8^+$ T cells in CLL patients in advanced clinical stages of the disease compared to those in early clinical stages (23, 24). Also, the frequency of exhausted $CD4^+$ and $CD8^+$ T

cells was correlated with poor prognosis of CLL patients. To confirm our previous findings, in this study, we analyzed the correlation of Blimp-1 expression with clinical stages and disease severity of the CLL patients. As shown in Figures 2A and B, our results indicated that the relative expression level of Blimp-1 was significantly higher in advanced clinical stages of CLL patients than that of early stages (p = 0.01).

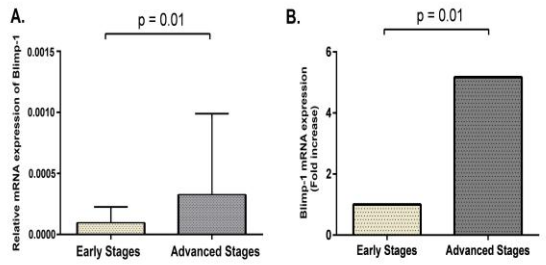

Figure 2. Higher expression of Blimp-1 in advanced clinical stages CLL patients.
CLL patients were clinically classified based on the Rai staging system. Stages 0 and I were defined as early clinical stage (n=16) and stages II, III and IV as advanced clinical stages (n=9). (A) Relative mRNA transcript levels of Blimp-1 from early and advanced clinical stages of CLL patients. (B) Fold increase of Blimp-1 in advanced clinical stages of CLL patients compared to early stages. Fold increase was calculated by dividing the mean ratio of Blimp-1 relative expression value obtained from advanced clinical stages of CLL patients to early stages. The results are represented as median ± interquartile range of $2^{-\Delta Ct}$ after normalization with β-actin as an internal control. P-values less than 0.05 were considered significant.

Blimp-1 expression was positively correlated with the subsets of exhausted T cells from CLL patients

To find any correlations between Blimp-1 mRNA expression and the frequency of exhausted T-CD4$^+$ and T-CD8$^+$ cells in CLL patients, the results of this study were analyzed with the frequencies of Tim-3$^+$/PD-1$^+$/CD4$^+$ and Tim-3$^+$/PD-1$^+$/CD8$^+$ T cells of CLL patients from our previous findings.

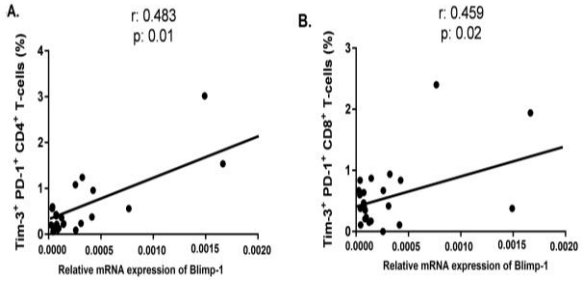

Figure 3. Correlation of Blimp-1 mRNA expression with the frequency of exhausted T-CD4+ and T-CD8+ cells in CLL patients.
Blimp-1 mRNA expression was significantly associated with the frequency of Tim-3+/PD-1+/CD4+ T-cells (r = 0.483, P = 0.01) (A) and Tim-3+/PD-1+/CD8+ T-cells of CLL patients (r = 0.459, P = 0.02) (B). Statistical comparisons were done by Spearman's rank correlation test.

As shown in Figure 3, the Blimp-1 mRNA expression was significantly correlated, not only with the frequency of Tim-3$^+$/PD-1$^+$/CD4$^+$ T cells (r = 0.483, p = 0.01), but also with the frequency of Tim-3$^+$/PD-1$^+$/CD8$^+$ T cells in CLL patients (r = 0.459, p = 0.02). These results suggest a potential role for Blimp-1 in the regulation of Tim-3 and PD-1 expression as an important inhibitory receptor in T cell exhaustion.

Discussion

T cell exhaustion is a common feature of chronic inflammatory conditions, including chronic infections and tumors, leading to poor control of the pathogens by the host immune system (26, 27). Multiple immune inhibitory pathways are involved in T cell exhaustion, and blocking these pathways can reinvigorate the immune responses during chronic complications (27-29). Although several transcription factors have been shown to regulate effector and memory T cell differentiation following acute infections, the transcriptional mechanisms of T cell exhaustion are still unclear. In our previous studies, we found out that both CD4$^+$ and CD8$^+$ T cells in patients with CLL overexpressed Tim-3 and PD-1 (23, 24). Later on, we indicated the upregulation of Gal-9 and PD-L1, main ligands of Tim-3 and PD-1, respectively, in CLL patients (25). To further explore the immune regulatory pathways in CLL, we examined the expression of Blimp-1, as a potential transcription factor regulating exhaustion processes. Our results showed a higher expression of Blimp-1 mRNA in CLL patients compared to normal individuals. Moreover, Blimp-1 mRNA was expressed higher in advanced clinical stages of CLL patients in comparison with early stages of the disease. To our knowledge, this is the first study which shows the expression of Blimp-1 in CLL.

Recent reports have demonstrated that several transcription factors regulate the immune exhaustion processes in chronic inflammatory conditions (15, 30, 31). It has been found that NFAT family of transcription factors, in the absence of AP-1 cooperation, induce a state of reduced responsiveness to subsequent stimulation both in CD4$^+$ and CD8$^+$ T cells, and this process may be a common initiating stimulus in the induction of exhaustion (31, 32). Further studies revealed the importance of T-bet and Eomes in subsets determination of terminally exhausted cells (33, 34). It has also been reported that CD8$^+$ T cells expressing TCF-1 display hallmarks of the exhausted phenotype via the expression of PD-1 and LAG-3, during chronic infections (35). Other transcription factors involved in CD4$^+$ and CD8$^+$ T cell exhaustion include Blimp-1, BATF, FoxO1, FoxO3, Zeb2, Bach2, NFIL-3, Prdm-1, Egr2, and Helios (35, 36), among which, Blimp-1 has attracted

more attention and has been introduced as an exhaustion transcription factor in various chronic pathological conditions (15, 26, 30). It has been shown that high levels of Blimp-1 is correlated with the maintenance of the exhausted cells and appeared to control the expression of PD-1 and also regulate the expression of other inhibitory receptors, including Tim-3, LAG3, CD160, and 2B4 (9, 13, 37). Consistent with these reports, conditional deletion of Blimp-1 resulted in reduced inhibitory receptor expression by exhausted T cells during chronic LCMV infection (36). Most recently, Hwang et al. reported that Blimp-1 was upregulated and acted as a critical regulator for $CD4^+$ T cell exhaustion during chronic toxoplasmosis and that conditional deletion of Blimp-1 in $CD4^+$ T cells regained $CD8^+$ T cell function and improved infection control (38). On the other hand, despite lower expression of inhibitory receptors by $CD8^+$ T cells in the absence of Blimp-1, these cells remained defective in terms of cytokine production. Therefore, it seems that other pathways may also regulate the cytokine production by the exhausted cells (10, 37, 39). These findings suggest a complex role for Blimp-1 in regulating T cell responses.

Although well studied in viral infections, few studies addressed the regulatory role of Blimp-1 in immune response to tumors, such as leukemias (40). Zhu et al. demonstrated more expression of Blimp-1 in both $CD4^+$ and $CD8^+$ T cells of AML patients, and that Blimp-1^+ T cells express high levels of co-inhibitory receptors, such as PD-1 and TIGIT (40). The mechanisms by which Blimp-1 regulates T cell responses are not fully understood. We found a correlation between Blimp-1 expression and co-expression of inhibitory receptors, Tim-3 and PD-1, on both $CD4^+$ and $CD8^+$ T cells. Some other studies have demonstrated the potential roles of Tim-3 and PD-1 in inhibition of anti-leukemia T cell responses (9, 24, 41). We assume that in CLL, Blimp-1 suppresses T cell function through the positive regulation of Tim-3/Gal-9 and PD-1/PD-L1 pathways and facilitates evasion of tumor cells from immune effector mechanisms. Therefore, these findings demonstrated that Blimp-1 is a transcriptional regulator of immune inhibitory receptors.

To our knowledge, this is the first study indicating Blimp-1 overexpression in CLL and its association with T cell exhaustion. Although other transcription factors and pathways contributing to T cell exhaustion remained to be understood, this study introduces Blimp-1 as a potential transcription regulator of T cell exhaustion in CLL. This leads to a better understanding of underlying mechanisms of T cell exhaustion which could be helpful in finding new therapeutic strategies for CLL.

Acknowledgements

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Conflict of Interest

The authors declare that the current research was conducted in the absence of any commercial or financial relationships that could be considered as conflict of interest.

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