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PD-1 AND TIM-3 BLOCKING CANNOT ENHANCE APOPTOSIS OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS INDUCED BY PERIPHERAL BLOOD CD8$^+$ T CELLS

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***Aim*: Given the invaluable success of immune checkpoint inhibitors for tumor immunotherapy, in this study, the effect of programmed cell death 1 (PD-1) and T cell immunoglobulin-3 (TIM-3) blocking was investigated to induce apoptosis of leukemic cells by exhausted CD8$^+$ T cells in patients with chronic lymphocytic leukemia (CLL). *Materials and Methods*: Peripheral blood CD8$^+$ T cells were positively isolated from 16 CLL patients using magnetic beads separation method. Isolated CD8$^+$ T cells were treated with either blocking anti-PD-1, anti-TIM-3 and isotype-matched control antibodies and then co-cultured with CLL leukemic cells as target. The percentage of apoptotic leukemic cells and the expression of apoptosis-related genes were evaluated by flow cytometry and real-time polymerase chain reaction methods, respectively. Interferon gamma and tumor necrosis factor alpha concentration was also measured by ELISA. *Results*: Flow cytometric analysis of apoptotic leukemic cells indicated that the blockade of PD-1 and TIM-3 did not significantly enhance the apoptosis of CLL cells by CD8$^+$ T cells, which then were confirmed by analysis of *BAX*, *BCL2* and *CASP3* gene expression, which was similar in blocked and control groups. No significant difference was found between blocked and control groups in terms of interferon gamma and tumor necrosis factor alpha production by CD8$^+$ T cells. *Conclusion*: We concluded that the blockade of PD-1 and TIM-3 is not an effective strategy to restore the function of CD8$^+$ T cells in CLL patients at the early clinical stages of the disease. Further *in vitro* and *in vivo* studies are needed to more address the application of immune checkpoint blockade in CLL patients.**

***Key Words*: CLL, anti-PD-1, anti-TIM-3, CD8$^+$ T cells, apoptosis.**

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Chronic lymphocytic leukemia (CLL), the most common type of adult leukemia in the Western world, is characterized by the proliferation of monoclonal and mature B cells that express CD5, CD19 and CD23 and accumulate in peripheral blood, secondary lymphoid tissues and bone marrow [1]. The average age at initial diagnosis of CLL is between 60–70 years and the incidence risk increases with age [2]. CLL patients display significant heterogeneity in terms of clinical presentation, disease progression and response to therapy. Some patients live for long periods without any treatments, but the disease progresses rapidly in some cases and these patients need early therapy [3]. The treatment options for patients with CLL are single agents or combination therapy and include chemotherapy (purine analogues and alkylating agents), a combination of chemotherapy and immunotherapy (anti-CD20 with chemotherapy), or drugs that affect signaling pathways involving in growth and/or survival of CLL cells (inhibitors of BCR signaling and BCL2 protein) [2]. However, treatment outcomes are often not satisfactory and CLL remains incurable because of some increasing medical problems such as requirement for lifelong therapy, acquired resistance mechanisms in leukemic cells, toxicity of drugs and significant short- and long-term morbidity and mortality [4, 5]. Due to these complications, introducing new therapeutic approaches is mandatory. Immunosuppression and immune defects caused by intricate and bidirectional interactions between malignant cells and various cell types of the tumor microenvironment, particularly T cells, lead to the lack of the adequate antitumor immune responses in CLL patients [6, 7]. Recently, attention has shifted to a subset of T cells named "exhausted T cells" which arise during chronic infections and cancers in animal models and humans. Exhausted T cells show diminished proliferative capacity, reduced cytotoxic ability and impaired production of effector cytokines. A main hallmark of T cell exhaustion is high level expression of inhibitory receptors such as programmed cell death 1 (PD-1), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), T cell immunoglobulin-3 (TIM-3), lymphocyte

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Abbreviations used: CLL – chronic lymphocytic leukemia; CTLA-4 – cytotoxic T lymphocyte-associated antigen 4; FDA – Food and Drug Administration; ICB – immune checkpoint blockade; IFN-γ – interferon gamma; MACS – magnetic-activated cell separation; PBMCs – peripheral blood mononuclear cells; PCR – polymerase chain reaction; PD-1 – programmed cell death 1; PD-L1 – programmed death-ligand 1; TIM-3 – T cell immunoglobulin-3; TNF-α – tumor necrosis factor alpha.

activation gene-3 and T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain [8, 9]. Blockade of inhibitory receptors has led to the development of a promising approach for the treatment of cancers and has demonstrated clinical efficacy in some kinds of tumors [10]. In this regard, monoclonal antibodies against CTLA-4 (ipilimumab), PD-1 (nivolumab and pembrolizumab), and programmed death-ligand 1 (PD-L1) (atezolizumab, durvalumab, and avelumab) have been approved for the treatment of melanoma, non-small cell lung carcinoma, head and neck cancer, urothelial carcinoma, and many other kinds of cancers. Besides these approved antibodies, many other checkpoint blockade molecules are in clinical trials for monotherapy or combination therapy of a variety of malignancies [8]. Despite solid tumors, little success was achieved about application of immune checkpoint inhibitors for treatment of hematological malignancies. Since in patients with CLL, $CD8^+$ T cells are exhausted, expressed checkpoint receptors like PD-1 and TIM-3 and have functional defects [11], we have designed an *in vitro* study to block the PD-1 and TIM-3 molecules to enhance the apoptosis of CLL leukemic cells by $CD8^+$ T cells.

MATERIALS AND METHODS

Patients. A total of 16 untreated CLL patients (12 males and 4 females; mean age of 69.6 years), referred to the Hematology and Oncology Clinic of Imam Khomeini Hospital affiliated to Mazandaran University of Medical Sciences, were enrolled in this study. The purpose of the research was explained to all participants who took part in the study. A written consent letter was achieved from all patients and the study was approved by the Ethical Committee of Mazandaran University of Medical Sciences. The diagnosis of CLL was done based on the blood cell count, cell morphology, immunophenotyping analysis and clinical symptoms according to the WHO criteria. Rai staging system was used to classify the patients into early (stages 0–II) and advanced stages (stages III and IV). The main clinical and hematological characteristics of the patients are summarized in the Table.

Table. Major clinical and laboratory characteristics of CLL patients

No	Age (year)	Sex	WBC $*10^3/mm^3$	Lym (%)	PLT $*10^3/mm^3$	Hb (g/dl)	CD38 (%)	Rai stage
P1	66	M	17.5	34	150	14.2	6.5	0
P2	78	M	63.9	86.5	118	15.6	21	2
P3	70	M	32.8	78.5	147	13.9	1.3	1
P4	80	M	68.2	NA	133	11.6	1.4	2
P5	71	F	23.9	NA	153	11.5	0.2	1
P6	64	F	9.6	61.5	237	10.6	14	0
P7	83	F	6.1	43.6	140	15.1	3.6	0
P8	77	M	45.5	61.9	176	13.6	7.5	1
P9	83	M	44.2	87.6	228	14.8	2.8	1
P10	47	M	37	89.1	166	15.3	3.1	1
P11	70	M	111.1	NA	125	9.5	22	2
P12	79	M	2.3	29.9	121	8.7	0.8	NA
P13	53	M	64.9	NA	170	11.3	NA	2
P14	74	F	59.6	NA	141	10.8	2.1	2
P15	71	M	5.3	30	30	11.5	5.8	NA
P16	48	M	24.7	19.2	143	15.7	3	0

Notes: M – male; F – female; WBC – white blood cell count; Lym – lymphocytes percent in peripheral blood; PLT – platelet count; Hb – hemoglobin; NA – not available.

Isolation of $CD8^+$ T lymphocytes by magnetic-activated cell separation (MACS) method. Heparinized peripheral blood samples were taken from all patients and fresh peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque density gradient centrifugation according to the manufacturer's instructions (Biosera, France). Isolated PBMCs were washed twice with RPMI-1640 culture medium (Biosera, France) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Biosera, France). The viability of isolated cells was > 98% as determined by trypan blue staining. To isolate the $CD8^+$ T cells from PBMCs, any cell clamps and possible debris were removed using a 70 µm pre-separation filter (Miltenyi Biotec, Germany). Positive isolation of $CD8^+$ T cells was done using magnetic beads-conjugated anti-CD8 mAbs kit (Miltenyi Biotec, Germany). The purity of isolated cells was analyzed by dual color flow cytometry staining using anti-CD8-FITC and anti-CD3-PE (eBioscience, USA). As expected from positive selection, the purity of isolated $CD8^+$ T cells was more than 98%.

Treatment of MACS-isolated $CD8^+$ T cells with anti-PD-1, anti-TIM-3 and isotype-matched control antibodies. For the blocking experiments, $5 \cdot 10^5$ of MACS-isolated $CD8^+$ T cells were incubated with 5 µg ml^{-1} of anti-PD-1, anti-TIM-3 and their isotype-matched control antibody (all from Biolegend, USA) at 37 °C with 5% CO_2 overnight. Following incubation with blocking and control antibodies, $CD8^+$ T cells were transferred to 96-well culture plate pre-coated with anti-CD3 (1 µg ml^{-1}) (Biolegend, USA) and then co-cultured with non-$CD8^+$ cell fraction of PBMCs from MACS isolation which was considered as target cells in 500 µl RPMI-1640 medium, supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10% heat-inactivated fetal bovine serum (Biosera, France) in the presence of soluble anti-CD28 antibody (0.5 µg ml^{-1}) (Biolegend, USA) and recombinant human interleukin-2 (rhIL-2; 10 ng ml^{-1}) (Biolegend, USA) for 48 h. Target cells and $CD8^+$ T cells were cultured in 1:1/5 ratio. All cultures were run in duplicate.

Flow cytometric analysis of apoptosis in CLL leukemic cells. Based on previously reported data [12], the 48 h time point was selected for the analysis. Apoptosis was determined using the Annexin-V-FITC apoptosis detection kit, according to the manufacturer's instructions (eBioscience, USA). Briefly, after 48 h culture, cells were harvested and resuspended in 100 µl of binding buffer, incubated with 5 µl anti-CD19-PE (eBioscience, USA) for 45 min and then with 5 µl Annexin V-FITC (eBioscience, USA) for 10 min. For flow cytometry analysis, $CD19^+$ cells were initially gated and the binding of Annexin-V was determined in the gated cells. Ir-

relevant isotype-matched PE control antibody was also applied to subtract the background staining.

Quantitative real-time reverse transcription polymerase chain reaction (PCR). Total RNA was extracted from co-culture using an RNA isolation kit (DenaZist Asia, Iran) according to the manufacturer's instruction and cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Yekta Tajhiz, Iran). The isolated RNA quality was confirmed by nano-spectrophotometer (WPA, England) and electrophoresis. Real-time PCR was performed using Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) reagent in StepOnePlus Real-Time PCR system (Applied Biosystems, USA) with the following primers: *BAX* (forward): TGT TTT CTG ACG GCA ACT, *BAX* (reverse): CGG AGG AAG TCC AAT GTC; *CASP3* (forward): GAT TAT CCT GAG ATG GGT TTA TG, *CASP3* (reverse): GTT TCT GAA TGT TTC CCT GAG; *BCL2* (forward): GGG AGA ACA GGG TAC GAT AA, *BCL2* (reverse): GGC TGG GAG GAG AAG ATG; β-actin (forward): CCT TCC TGG GCA TGG AGT CCT, β-actin (reverse): TGG GTG CCA GGG CAG TGA T. PCR was performed as initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58.5 °C (*BAX* and β-actin), 60 °C (*CASP3* and *BCL2*) for 30 s and extension at 72 °C for 30 s. Melting curve analysis was carried out for every run to verify the specificity of the amplification and the lack of the primer dimers. The expression level of *BAX*, *CASP3* and *BCL2* mRNA was identified with $2^{-\Delta Ct}$ value using β-actin as an internal housekeeping gene.

Cytokine measurement by ELISA. After 48 h of co-culture of CLL leukemic target cells with isolated $CD8^+$ T cells, the culture supernatants were collected for measurement of tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) by ELISA according to the manufacturer's protocol (Thermo Fisher Scientific, USA). Detection ranges were 4–500 pg/mL for both IFN-γ and TNF-α.

Statistical analysis. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc.). Kolmogorov — Smirnov test was performed on the obtained data for determination of normality distribution. ANOVA test was used for mean comparison between groups. All data are expressed as mean ± SEM. Statistical significance was set at *p*-values less than 0.05.

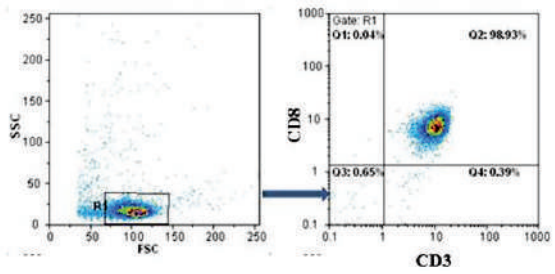

Fig. 1. Purity analysis of MACS-isolated $CD8^+$ T cells from CLL patients. Dual color flow cytometry analysis (anti-CD8-FITC and anti-CD3-PE) was done on MACS-isolated $CD8^+$ T cells from CLL patients. Cells were initially gated based on their forward and side scatter properties and then the co-expression of CD3 and CD8 was evaluated. A representative dot-plot obtained from a CLL patient is represented

RESULTS

Purity assessment by flow cytometry for MACS-isolated $CD8^+$ T cells from CLL patients. Following MACS isolation of $CD8^+$ T cells from all enrolled CLL patients, flow cytometric analysis with anti-CD8 and anti-CD3 monoclonal antibodies was performed on positively isolated $CD8^+$ T cells. The purity of isolated cells was always more than 98% as represented in Fig. 1.

Blockade of PD-1 and TIM-3 receptors could not enhance the apoptosis of leukemic cells by $CD8^+$ T cells in CLL patients. Following MACS isolation of $CD8^+$ T cells from all enrolled CLL patients, the cells were treated with blocking anti-PD-1, anti-TIM-3 and isotype-matched control antibodies overnight and then co-cultured with leukemic CLL cells as target in the presence of anti-CD3/CD28 antibodies and rhIL-2. After 48 h, the cells were stained with anti-CD19-PE and Annexin V-FITC to monitor the apoptosis pattern of $CD19^+$ leukemic cells by flow cytometry. Although a slight increase in apoptosis was observed in blocked groups, statistical analysis showed no significant difference between blocked and unblocked groups in terms of apoptosis pattern of leukemic cells ($p > 0.05$; Fig. 2).

Blocking of $CD8^+$ T cells with anti PD-1 and TIM-3 did not alter the expression of pro- and anti-apoptotic genes in leukemic CLL cells. After apoptosis results regarding no improvement of cell death by TIM-3/PD-1 blocking, we then measured the mRNA expression of some pro- and anti-apoptotic genes to confirm the apoptosis data. MACS-isolated $CD8^+$ T cells were treated with anti-PD-1, anti-TIM-3 and isotype-matched control antibodies and then co-cultured with leukemic CLL cells in the presence of anti-CD3/CD28 antibodies and rhIL-2. Following 48 h of culture, total RNA was extracted from all samples and converted to cDNA. Real-time PCR was performed for *BAX, CASP3, BCL2* and β-actin genes. As shown in Fig. 3, no significant difference was found in expression profile of *BAX*, *CASP3* and *BCL2* genes between blocked and unblocked groups ($p > 0.05$).

Blockade of PD-1 and TIM-3 receptors did not affect the production of cytokines by $CD8^+$ T cells in CLL patients. To explore whether the blockade of TIM-3 and PD-1 receptors might change the cytokine production profile of $CD8^+$ T cells, MACS-isolated $CD8^+$ T cells from all patients were treated with anti-PD-1, anti-TIM-3 and isotype-matched control antibodies and then co-cultured with leukemic CLL cells in the presence of anti-CD3/CD28 antibodies and rhIL-2 for 48 h. After that, the culture supernatants were collected and the concentration of TNF-α and IFN-γ was

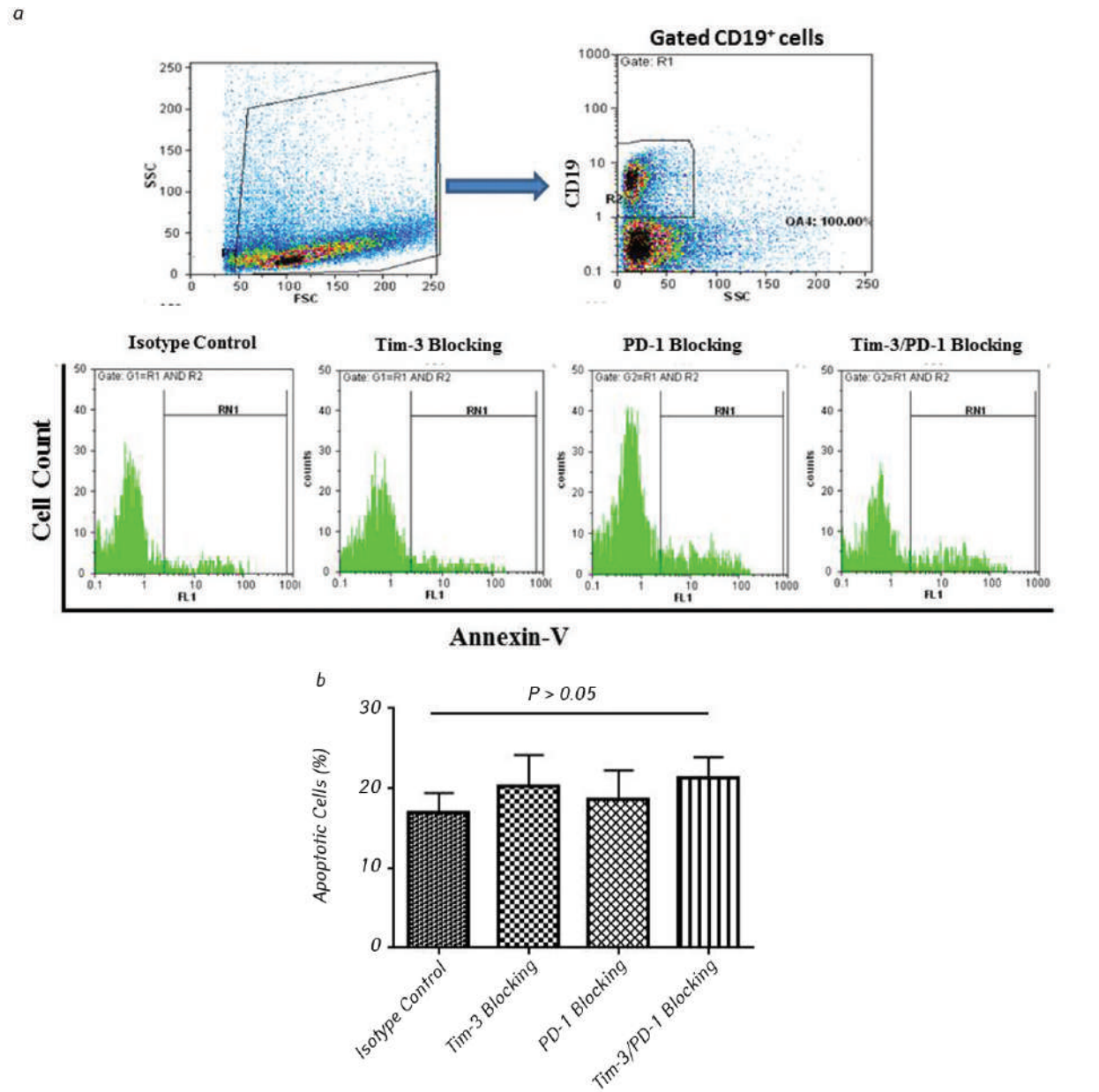

Fig. 2. Flow cytometric analysis of apoptosis induction in leukemic CLL cells by $CD8^+$ T cells in the presence of anti-TIM-3 and anti-PD-1 blocking antibodies. MACS-isolated $CD8^+$ T cells from 16 CLL patients were treated with anti-PD-1, anti-TIM-3 and isotype-matched control antibodies overnight and then co-cultured with leukemic CLL cells as target in the presence of anti-CD3/CD28 antibodies and rhIL-2. After 48 h, cells were stained with anti-CD19-PE and Annexin V-FITC and then analyzed by flow cytometry. $CD19^+$ cells were initially gated and the expression of Annexin-V was then measured: *a* — gating strategy and representative flow cytometry histograms obtained from a CLL patient are shown; *b*— percent of apoptotic cells in all CLL patients are shown. Apoptotic cells were determined as Annexin V^+ cells out of $CD19^+$ cells. No significant difference was found between blocked and unblocked groups ($p > 0.05$). Graph represents the mean ± SEM

measured by ELISA. Our data showed no significant difference between blocked and unblocked groups in terms of TNF-α and IFN-γ production profile (shown in Fig. 4)

DISCUSSION

In the last decade, a novel strategy for immunotherapy called immune checkpoint blockade (ICB) has been introduced as one of the most promising and powerful treatment options for many types of cancers. Anti-CTLA-4 antibody (ipilimumab) was the first approved monoclonal antibody by the Food and Drug Administration (FDA) for treatment of metastatic melanoma in 2011 [13]. After invaluable clinical success, anti-PD-1 antibody (nivolumab) has been approved for a variety in cancers including metastatic melanoma [14], metastatic squamous non-small-cell lung carcinoma [15], advanced renal cell carcinoma [16], classical Hodgkin lymphoma [17], recurrent or metastatic squamous cell carcinoma of the head and neck [18], metastatic urothelial carcinoma [19], metastatic colorectal cancer [20] and hepatocellular carcinoma [21] between 2014–2017. Together with nivolumab, another anti-PD-1 immune checkpoint inhibitor, pembrolizumab, has also obtained FDA approval for some malignan-

cies in 2017 [22–25]. Finally, anti-PD-L1 antibodies (atezolizumab, avelumab and durvalumab) have also received FDA approval for metastatic urothelial carcinoma [26, 27], metastatic non-small-cell lung carcinoma [28, 29] and metastatic Merkel cell carcinoma [30] in 2016–2018. Given the incredible clinical success of these FDA-approved immune checkpoint inhibitors in solid tumors, many ongoing researches are focused for application of this strategy in hematopoietic malignancies.

With this aim, we decided to explore the effects of immune checkpoint inhibitors on restoration of $CD8^+$ T cells function in CLL patients. Although blockade of CTLA-4, PD-1 and PD-L1 has revealed highly impressive results in treatment of many cancers, many patients with various kinds of tumors fail to respond to ICB strategy. One possible reason could be the compensatory immune escape mechanisms applied by cancer cells to deviate the host immune system. Therefore, intense investigations in targeting other inhibitory receptors, such as the TIM-3, T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain and lymphocyte activation gene-3 are required [31]. Despite up-regulation of PD-1 and TIM-3 and their ligands in CLL patients, which was also indicated in our previous studies [11, 32, 33], our primary *in vitro* results did not show effective improvements in function of $CD8^+$ T cells after blocking with anti-PD-1 and/or anti-TIM-3 [34]. In previous studies, anti-TIM-3 blocking antibody restored T cell proliferation and cytokines production in HIV-1-specific T cells [35], antigen-specific $CD8^+$ T cells in gastric cancer [36] and melanoma patients [37]. Regarding chronic viral infections, blockade of PD-1/PD-L1 interaction resulted in restoration of antigen-specific T cell responses in LCMV-infected mice [38] and humans infected with HIV [39]. In addition, interference in PD-1–PD-L signaling with blockade antibody restored T cell function in murine acute myeloid leukemia model [40], Hodgkin lymphoma [41] and chronic myeloid leukemia [42]. Simultaneous targeting of the TIM-3 and PD-1 pathways improved $CD8^+$ T cell functional properties in a model of chronic viral infection [43] and in mice bearing solid tumors [44]. Combined blockade of PD-1 and CTLA-4 increased $CD8^+$ and $CD4^+$ T cells proliferation and cytokines production in a murine model of colon carcinoma [45].

In contrast, our current results with cells from CLL patients are inconsistent with data mentioned above. This discrepancy may be interpreted in several ways. First, T cells from CLL patients express other inhibitory receptors besides PD-1 and TIM-3, which could be responsible for their unresponsiveness. Riches *et al.* [46] showed that other inhibitory receptors such as CD244 and CD160 are more highly expressed checkpoint receptors in CLL patients and they may overcome the effects of PD-1 or TIM-3 blocking. However, the main reason for no effects of TIM-3 and PD-1 blocking on function of $CD8^+$ may

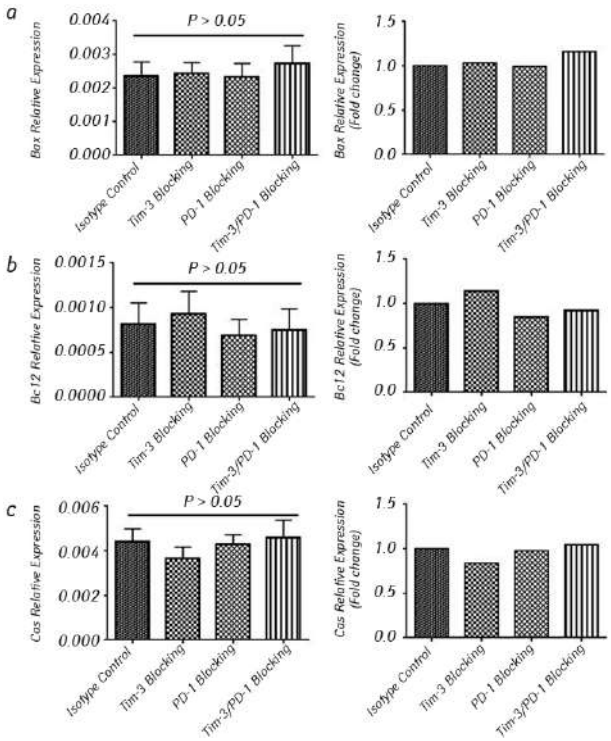

Fig. 3. Expression profile of *BAX*, *CASP3* and *BCL2* genes after blocking of PD-1 and TIM-3 receptors in leukemic CLL cells. MACS-isolated $CD8^+$ T cells from 16 CLL patients were treated with anti-PD-1, anti-TIM-3 and isotype-matched control antibodies overnight and then co-cultured with leukemic CLL cells as target in the presence of anti-CD3/CD28 antibodies and rhIL-2. Following 48 h of culture, total RNA was extracted from cells, cDNA was synthesized and real-time PCR was performed for *BAX*, *CASP3*, *BCL2* and β-actin genes. Relative mRNA transcript level and fold change of *BAX* (*a*), *CASP3* (*b*) and *BCL2* (*c*) genes in blocked and unblocked groups are represented. No significant difference was found between blocked and unblocked groups ($p > 0.05$). Data are represented as the mean ± SEM of $2^{-\Delta Ct}$ after normalization with β-actin as an internal control

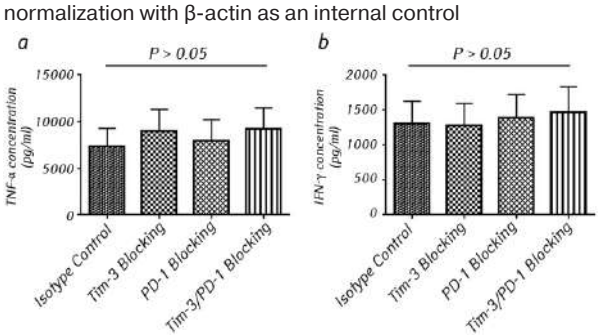

Fig. 4. Cytokine production pattern of isolated CD8+ T cells from CLL patients after blocking with anti-PD-1 and anti-TIM-3 antibodies. MACS-isolated CD8+ T cells from 16 CLL patients were treated with anti-PD-1, anti-TIM-3 and isotype-matched control antibodies overnight and then co-cultured with leukemic CLL cells in the presence of anti-CD3/CD28 antibodies and rhIL-2. After 48 h, the culture supernatants were collected for measurement of TNF-α (*a*) and IFN-γ (*b*) by ELISA. No significant difference was observed between blocked and unblocked groups ($p > 0.05$). Graph represents the mean ± SEM

be related to the clinical stages of CLL patients. Our previous study showed a higher frequency of exhausted $CD8^+$ T cells in CLL patients at advanced clinical stages compared to those in early stages of the disease, Rai stage 0–II, who do not require any therapies unless disease progresses [11]. In our

current study, most CLL patients were at the early stages of the disease and did not indicate progression criteria. Thus, we conclude that in CLL patients at early clinical stages that show lower expression of PD-1 and TIM-3 on their $CD8^+$ T cells, blockade of PD-1 and TIM-3 is not efficient and conversely CLL patients at advanced clinical stages may respond to this therapy. In this regard, Behdad *et al*. [47] have indicated the correlation between clinical severity and higher expression of PD-1 on leukemic B cells of patients with Richter syndrome representing an aggressive transformation form of CLL. Later on, in a clinical trial on patients with Richter syndrome, the combination regimen of Ibrutinib and anti-PD-1, nivolumab, indicated 65% overall response, while in patients with relapsed/refractory CLL and follicular lymphoma, only treatment with single-agent Ibrutinib was effective [48]. In addition, two other phase II clinical trials conducted on patients with Richter syndrome demonstrated that combination therapy with Ibrutinib and PD-1 blocking monoclonal antibody show an acceptable outcome confirming the idea that clinical stage is an important issue in response of CLL patients to immune checkpoint therapy [49, 50].

According to these preclinical and clinical studies with different approaches, it seems that ICB-based therapy cannot be considered as a treatment option in CLL patients at an early stage of the disease. In conclusion, we investigated the effects of blocking antibodies against two important inhibitory receptors, PD-1 and TIM-3, on restoring the function of exhausted $CD8^+$ T cells. Our data from all experiments indicated that blocking of PD-1 and TIM-3 does not restore the function $CD8^+$ T cells in CLL patients. Together with our previous reports and related clinical studies, we conclude the immune checkpoint therapy may not be considered for CLL patients at early clinical stages.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Shakiba Jafarkhani performed the experiments and wrote the manuscript. Hadi Hossein-Nataj contributed to performing and analyzing the flow cytometry experiments. Mohammad Eslami-Jouybari and Maryam Ghoreishi consulted as clinicians to select the patients. Hossein Asgarian-Omran conceived the original idea, supervised the project and edited the final manuscript.

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БЛОКУВАННЯ PD-1 І TIM-3 НЕ ЗБІЛЬШУЄ АПОПТОЗУ ЗЛОЯКІСНИХ КЛІТИН ХРОНІЧНОЇ ЛІМФОЦИТАРНОЇ ЛЕЙКЕМІЇ, ІНДУКОВАНОГО CD8$^+$ Т-КЛІТИНАМИ ПЕРИФЕРИЧНОЇ КРОВІ

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Мета*:** Зважаючи на успіхи застосування інгібіторів імунних контрольних точок для імунотерапії в онкології, вивчали ефект блокування рецептора програмованої клітинної загибелі 1 (programmed cell death 1 — PD-1) та Т-клітинного імуноглобуліну 3 (T cell immunoglobulin-3 — TIM-3) на індукцію апоптозу лейкемічних клітин виснаженими CD8$^+$ Т-клітинами у хворих на хронічну лімфоцитарну лейкемію (ХЛЛ). ***Матеріали та методи: CD8$^+$ Т-клітини виділяли з периферичної крові 16 хворих на ХЛЛ сепаруванням на магнітних гранулах. Виділені CD8$^+$ Т-клітини обробляли або блокувальними антитілами проти PD-1 та TIM-3, або антитілами ізотипового контролю, після чого культивували їх з лейкозними клітинами ХЛЛ. Відсоток апоптотичних клітин визначали методом проточної цитометрії. Експресію генів, пов'язаних з апоптозом, визначали методом полімеразної ланцюгової реакції в реальному часі. Концентрацію інтерферону-γ та фактора некрозу пухлини-α визначали методом імуноферментного аналізу. ***Результати***: Аналіз апоптозу методом проточної цитометрії показав, що блокування PD-1 та TIM-3 не впливало суттєво на відсоток апоптозу клітин ХЛЛ, індукованого CD8$^+$ Т-клітинами, що підтверджено й відсутністю змін експресії генів *BAX*, *BCL2* та *CASP3*. Різниці в продукції інтерферону-γ та фактора некрозу пухлини-α в разі блокування PD-1 та TIM-3 також не визначали. ***Висновки***: Блокування PD-1 та TIM-3 не є ефективною стратегією для відновлення функції CD8$^+$ Т-клітин у хворих на ХЛЛ на ранніх клінічних стадіях захворювання. Подальші дослідження *in vitro* та *in vivo* необхідні для розробки більш адресного застосування блокування імунних контрольних точок у хворих на ХЛЛ.

***Ключові слова*:** ХЛЛ, анти-PD-1, анти-TIM-3, CD8$^+$ Т клітини, апоптоз.