RESEARCH ARTICLE

Editorial Process: Submission:03/13/2021 Acceptance:07/11/2022

Combined Blockade Of PD-1 and TIGIT is not Sufficient to Improve the Function Of CD8+ T-Cells in Chronic Lymphocytic Leukemia

Fatemeh Hatefi[1], Hossein Asgarian-Omran[1,2], Hadi Hossein-Nattaj[1], Armin Akbar[1], Ramin Shekarriz[2,3], Ehsan Zaboli[2,3], Ghasem Janbabaei[2], Mohsen Tehrani[1,2]*

Abstract

Background and Objective: Blockade of immune checkpoint receptors in the treatment of cancers has been mentioned in several studies. Here, we investigated the efficacy of combined blockade of two inhibitory receptors, PD-1 and TIGIT, in restoring functional features of CD8+ T-cells in CLL. **Methods:** CD8+ T-cells were separated from the peripheral blood of 11 CLL patients and targeted with malignant B-cells isolated from the same patients. Cells were then stimulated with anti-CD3/CD28 and PMA/ionomycin to assess their proliferative response and cytotoxic activity using MTT and CD107a degranulation assays, respectively. Cytokine production of isolated CD8+ T-cells was also determined using ELISA. **Results:** There were no significant differences in proliferation and cytotoxic activity of CD8+ T-cells co-blocked with anti-PD-1/TIGIT compared to those single blocked with anti-PD-1, anti-TIGIT, or the control antibody. There was no significant difference in cytokine production of mentioned groups, either. **Conclusions**: Collectively, combined blockade of PD-1 and TIGIT failed to restore the proliferation and function of CD8+ T-cells isolated from CLL patients.

Keywords: Chronic lymphocytic leukemia- PD-1- TIGIT- T-cell exhaustion- immune checkpoint blockade

Asian Pac J Cancer Prev, **23 (7)**, 2225-2231

Introduction

Chronic lymphocytic leukemia (CLL) is a chronic malignancy characterized by clonal proliferation and accumulation of mature CD5+, CD10-, CD19+, CD20 weakly positive, and CD23+ B-cells within the blood stream, bone marrow, lymph nodes, and spleen (Yano, 2017). CLL is the most common leukemia in western adults and includes 25% of all types of leukemia, but less than 5% in eastern countries(Kalil and Cheson, 1999). Median age at diagnosis is 60-74 years(Redaelli et al., 2004). Conventional treatments are chemotherapy drugs, such as Fludarabine and Cyclosporine, and in higher stages radiotherapy, is currently administered to CLL patients (O'Brien, 2008; Freeman and Gribben, 2016); however, there is often a recurrence in symptoms after a period of remission. Higher doses of chemotherapy can strongly destroy malignant cells, but could damage the bone marrow, which causes severe adverse effects (Schwarzbich et al., 2016). Allogeneic hematopoietic stem cell transplantation (HSCT) is a most promising approach and has less side effects, but it is not a definitive treatment, and has the risk of GVHD (Xu et al., 2018).

Immunotherapeutic methods against cancers are of great importance over the past few years. Immune checkpoint receptors play important roles in homeostasis and self-tolerance. The most defined immune checkpoint receptors include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death-1 (PD-1), T-cell immunoglobulin and ITIM domains (TIGIT), and T-cell immunoglobulin and mucin domain-3 (Tim-3) (Anderson et al., 2016). Overexpression of immune checkpoint receptors on T-cells in some chronic infections and cancers can lead to dysfunction of T-cells in proliferation, cytokine production, and cytotoxicity, what is commonly called as T-cell exhaustion. Moreover, several studies have shown that blockade of immune checkpoint receptors could restore T-cell function (Nguyen and Ohashi, 2015).

PD-1 is one of the most important immune checkpoint receptors expressed on B, T, and NK cells as well as some myeloid cells. PD-1 plays an important role in

*[1]Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran. [2]Gastrointestinal Cancer Research Center, Mazandaran University of Medical Sciences, Sari, Iran. [3]Department of Hematology and Oncology, Imam Khomeini hospital, Mazandaran university of Medical sciences, Sari, Iran. *For Correspondence: drmtehrani@gmail.com*

self-tolerance and also in regulation of T-cell function (Nguyen and Ohashi, 2015; Anderson et al., 2016; Shapiro et al., 2017). TIGIT is a member of immunoglobulin super family expressed on follicular T helper cells, memory T-cells, NK cells, subtypes of regulatory T-cell, and tumor cells(Anderson et al., 2016). Co-blockade of PD-1 and TIGIT have been used to treat some cancers, such as glioblastoma and melanoma (Hung et al., 2018), suggesting that co-blockade of PD-1 and TIGIT could be more effective in cancer immunotherapy than single blockade.

In CLL, combination of Ibrutinib (a Bruton's tyrosine kinase inhibitor) with anti-PD-1 or anti-PD-L1 was used in an animal model of CLL and showed increased function of CD8+ T-cells (Hanna et al., 2021). Also, combination of ibrutinib and nivolumab (an anti-PD-1) showed promising results in a phase II trial for patients with relapsed or refractory CLL or Richter's transformation (Jain et al., 2016b). However, combination blockade of immune checkpoint receptors in CLL patients has never carried out, yet. In this study, we used combination blockade of PD-1 and TIGIT on CD8+ T-cells from CLL patients to restore effector functions of the cells.

Materials and Methods

Sample collection

CLL patients attending the Hematology and Oncology Clinic of Imam Khomeini Hospital, affiliated to Mazandaran University of Medical Sciences (Sari, Iran), were enrolled in the study. Diagnosis of patients was based on the clinical and laboratory evaluations, according to the World Health Organization criteria (Swerdlow et al., 2008). Patients with a history of any type of cancers other than CLL, auto-immune diseases, chronic viral infections, like HIV, HBV, and HCV, or any other types of congenital or acquired immune deficiencies were ruled out from the study. Also, none of the patients received chemotherapy or other immunosuppressive medications for at least three months before sampling. Major clinical and laboratory characteristics patients and controls are summarized in Table 1. Ten milliliters of heparinized peripheral blood were taken from each study subject after obtaining written informed consents, according to the Ethical Committee of Mazandaran University of Medical Sciences.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood using Lymphodex (Inno-Train, Germany) density centrifugation according to the manufacturer's instructions. In continuation, CD8+ T-cells were purified, using MACS Column Technology (Miltenyi Biotec, Germany). Unbound cells were also collected in another tube which later treated with mitomycin and used as target cells. Purity of MACS separated CD8+ T-cells was measured using a dual-color flow cytometric analysis with anti-CD3-PE and anti-CD8-FITC (both from Biolegend, USA). CD8+ T-cells were then treated with monoclonal antibodies in four sets, including anti-PD-1 antibody (10 µg/mL, LEAFTM Purified anti-human CD279 (PD-1) antibody, Biolegend), anti-TIGIT antibody (10 µg/mL, LEAFTM Purified anti-human TIGIT antibody, Biolegend), anti-PD-1 and anti-TIGIT antibodies (10 µg/mL from each antibody), and isotype control antibody; then incubated with mitomycin-treated unbound cells as target cells (Effector to target ratio, 1:1), in a 96-well plate for 48 hours. One set of mentioned wells was used for the MTT assay, and another one for degranulation assay and ELISA.

MTT assay

After 48-hour incubation, cells were transferred to new wells pre-coated with anti-CD3 antibody (10 µg/mL, Biolegend). Next, anti-CD28 antibody (2 µg/mL, Biolegend) was added to each well and incubated for 72 hours at 37°C. Then, 20 µL (5 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye were added to each well and three more hours of incubation was considered. Once purple crystals were clearly visible, 150 µL of DMSO, as a solvent, was added to each well to solve the crystals and the plate was shacked for 20 minutes. Optical density (OD) of the cells was read by a plate reader in 570 nanometer wavelength.

Degranulation assay using CD107a

Another set of wells containing cells, pre-treated with blocking antibodies and incubated with mitomycin treated target cells, was considered for degranulation assay, as described above. PMA-ionomycin (0.4 µL/well, Thermo Fisher scientific, USA) was added to the cells in each well. After 30 minutes, appropriate amounts of anti-CD107a-PE and anti-CD8-FITC (both from Biolegend) were added to each well. Another well was considered for corresponding isotype controls. Cells were then incubated for more five and half hours at 37°C. Degranulation of CD8+ T-cells was assessed, using flow cytometry.

ELISA

Following 6-hour stimulation with PMA/ionomycin, culture supernatants were collected and stored at -20°C for cytokine assays. Concentrations of IFN-γ and TNF-α in the cultured supernatants were measured using commercial ELISA kits (eBioscience, California, USA), according to the manufacturer's protocol. All samples were measured in duplicate.

Results

Purity analysis of MACS-isolated CD8+ T-cells from CLL patients

Isolated CD8+ T-cells from CLL patients were evaluated using a two-color flow cytometric analysis with anti-CD3-PE and anti-CD8-FITC antibodies. The purity of isolated CD8+ T-cells was shown to be more than 97% for all samples (Figure 1).

PD-1 and TIGIT blockade could not restore the proliferation of CD8+ T-cells from CLL patients

The effect of immune checkpoint blockade on proliferation of CD8+ T-cells was measured following blocking of CD8+ T-cells with anti PD-1 and anti TIGIT

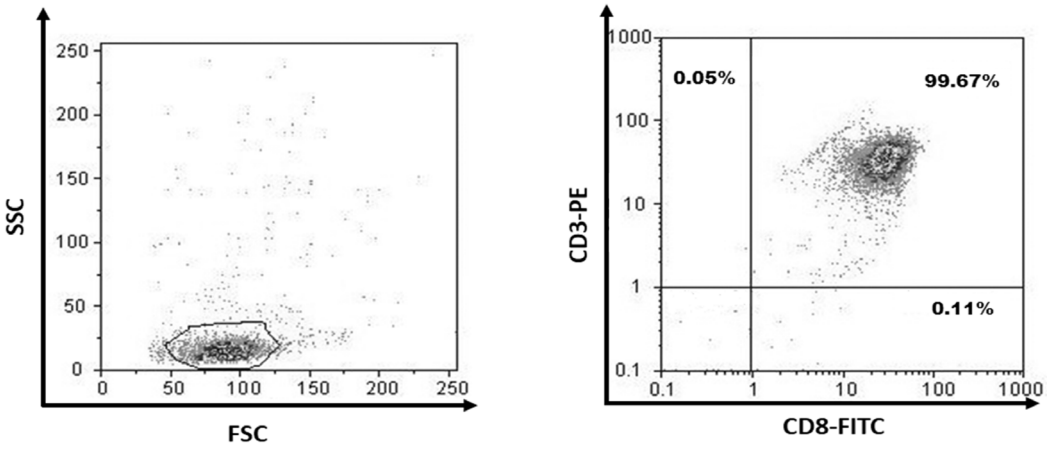

Figure 1. Purity of MACS Separated CD8+ T-cells from CLL Patients. A two-color flow cytometry was performed on CD8+ T-cells separated from MACS column, using anti CD3-PE and anti CD8-FITC antibodies. Here is a dot plot chart of a sample which shows a purity of more than 99%.

antibodies, using the MTT assay. After OD values, stimulation index (SI) for each well was calculated by dividing the OD of the wells treated with anti-PD-1, anti-TIGIT, or their combination, minus the OD of background, over the OD of corresponding wells treated with isotype control, minus the OD of background:

$$SI = \frac{\text{OD of test} - \text{OD of background}}{\text{OD of isotype} - \text{OD of background}}$$

SIs showed no significant differences among cells treated with PD-1/TIGIT, PD-1, TIGIT, and the isotype control antibody.

Degranulation was not improved in CD8+ T-cells from CLL patients following PD-1 and TIGIT blockade

To evaluate the effect of immune checkpoint blockade on degranulation of CD8+ T-cells, we made a flow cytometric examination on CD107a protein. Following 6-hour stimulation with PMA/ionomycin, cultured cells were harvested and subjected to two-color flow cytometry for CD8 and CD107a markers. The results showed no significant differences in degranulation in PD-1/TIGIT-, PD-1-, TIGIT-, and isotype control antibody-treated cells.

PD-1 and TIGIT blockade could not increase the cytokine production by CD8+ T-cells from CLL patients

The effect of immune checkpoint blockade on cytokine production by of CD8+ T-cells was measured after blocking of CD8+ T-cells with anti PD-1 and anti TIGIT antibodies, using ELISA. TNF-α and IFN-γ concentrations in culture supernatants showed no significant differences among cells treated with PD-1/TIGIT, PD-1, TIGIT, and the isotype control antibody.

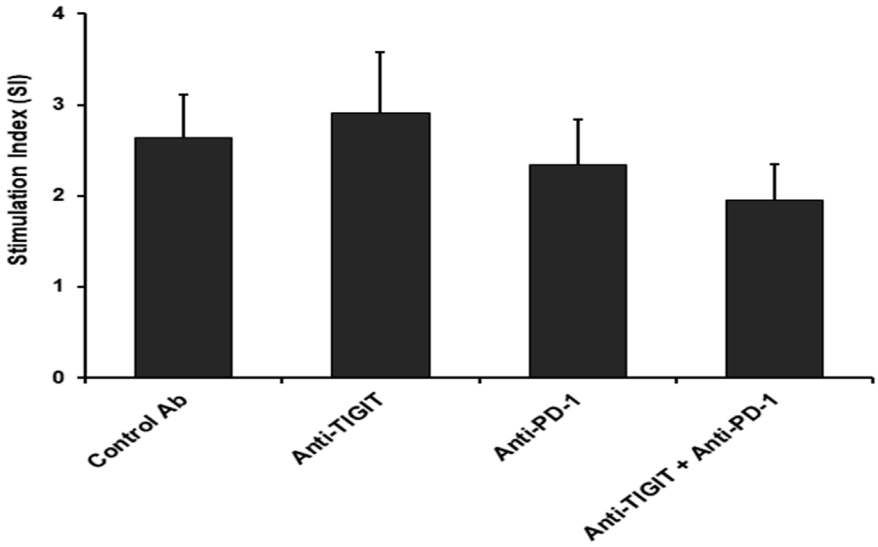

Figure 2. Proliferation of CD8+ T-cells Isolated from CLL Patients after PD-1 and TIGIT Blockade. CD8+ T-cells separated from CLL patients were treated with anti-PD-1 and anti-TIGIT blocking antibodies and then co-cultured with malignant B-cells as target cells. Cells were then stimulated with anti-CD3/CD28 antibodies and cell proliferation was evaluated using the MTT assay. Stimulation index (SI) was calculated by dividing the OD values of cells treated with blocking antibodies or their isotype controls into the OD value of blank.

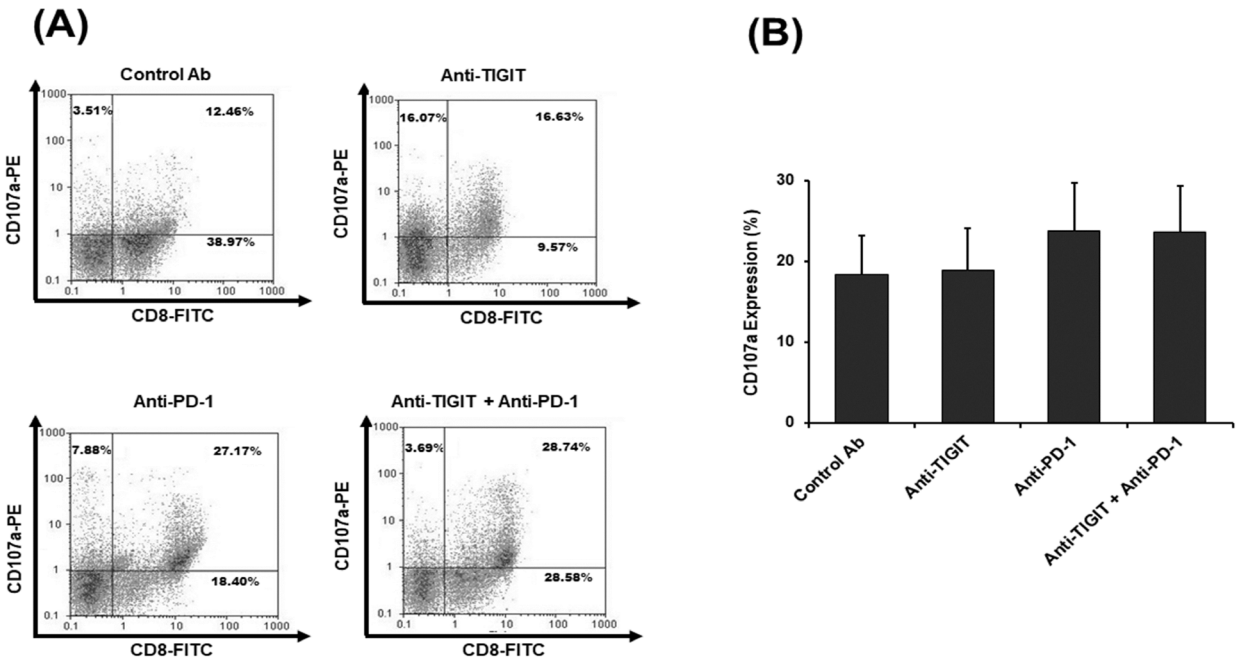

Figure 3. Degranulation of T-CD8 + T-cells Isolated from CLL Patients after PD-1 and TIGIT Blockade. CD8+ T-cells separated from CLL patients were treated with anti-PD-1 and anti-TIGIT blocking antibodies and then co-cultured with malignant B-cells as target cells. Cells were then stimulated with PMA/ ionomycin and evaluated using a two-color flow cytometric analysis with anti-CD107a-PE and anti-CD8-FITC antibodies. A) Representative graphs showing the percentage of CD8+CD107a+ cells obtained from a CLL patient. B) CD107a expression using a combined blockade of PD-1/TIGIT, single blockade of PD-1, single blockade of TIGIT, and the isotype control antibody

Discussion

Blockade of PD-1/PD-L1 inhibitory axis using monoclonal antibodies has shown hopeful therapeutic effects in many cancers, such as melanoma, head and neck squamous cell carcinoma (HNSCC), non-small-cell lung carcinoma (NSCLC), renal cell carcinoma (RCC), urinary tract cancer, and resistant and recurrent Hodgkin's lymphoma (Hamid et al., 2013; Ansell et al., 2015; Balar et al., 2016; Gadgeel et al., 2016; Motzer et al., 2016; Seiwert et al., 2016), while it was not effective in some other cancers. Combination therapy with Nivolumab (an anti-PD-1 antibody) and Ipilimumab (an anti-CTLA-4 antibody) has been the first co-blockade of immune checkpoint receptors which has been FDA-approved for treatment of advanced melanoma and RCC (Vaddepally et al., 2020). TIGIT overexpression on CD4+ and CD8+ T-cells has also been mentioned in some cancers, such as glioblastoma and melanoma (Swerdlow et al., 2008; Hamid et al., 2013; Ansell et al., 2015; Balar et al., 2016; Gadgeel et al., 2016; Jain et al., 2016a; Motzer et al., 2016; Seiwert et al., 2016; Hung et al., 2018; Vaddepally et al., 2020; Hanna et al., 2021).

Our previous study demonstrated elevated expression of PD-1 and TIGIT on CD8+ T-cells in patients with CLL(Hajiasghar-Sharbaf et al., 2021). In the present study, we performed an in vitro co-blockade of two inhibitory molecules, PD-1 and TIGIT, in CD8+ T-cells from CLL

Table 1. Major Clinical and Laboratory Characteristics of CLL Patients

No	Age (year)	Sex	WBC × 10^3/mm^3	Lym (%)	Hb (g/dL)	HCT	PLT × 10^3/mm^3	Rai stage	Organomegaly
1	58	M	24,400	53.0	14.7	41.8	191,000	0	None
2	58	M	31,000	83.0	11.4	36.5	196,000	I	None
3	65	F	24,000	81.0	13.8	43.6	211,000	0	None
4	60	M	8,900	48.7	14.3	43.6	158,000	0	None
5	54	M	119,500	29.0	11.5	36.6	183,000	II	SPM
6	64	F	47,000	85.0	13.7	41.0	213,000	0	None
7	61	M	37,600	76.9	15.5	45.0	183,000	I	LAP
8	65	M	39,100	84.0	11.4	34.6	128,000	I	None
9	65	M	21,600	77.5	14.0	42.7	139,000	0	None
10	87	M	12,400	56.4	9.7	31.3	145,000	0	None
11	85	F	62,800	87.9	11.8	37.9	268,000	II	SPM

CLL, chronic lymphocytic leukemia; M, Male; F, Female; LAP, lymphadenopathy; SPM, splenomegaly; HPM, hepatomegaly; WBC, white blood cell count; Lym, lymphocytes percent in peripheral blood; PLT, platelet count; Hb, hemoglobin.

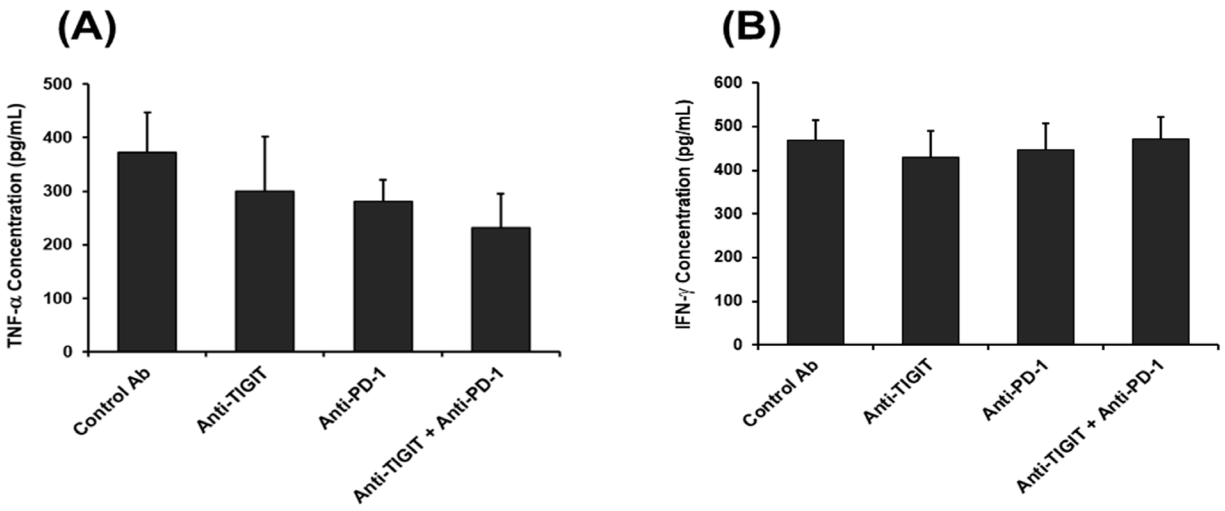

Figure 4. Cytokine Production by CD8 + T-cells after PD-1 and TIGIT Blockade. CD8+ T-cells separated from CLL patients were treated with anti PD-1 and anti TIGIT blocking antibodies and then co-cultured with malignant B-cells as target cells. Cells were then stimulated with PMA/ionomycin and then their supernatants were collected to evaluate cytokine production levels. TNF-α (A) and IFN-γ (B) concentrations were measured using ELISA.

patients. Our results showed that treatment of CD8+ T-cells from CLL patients with 10 µg/mL of anti-PD-1 and anti-TIGIT had no impact on proliferation, degranulation, or cytokine production by CD8+ T-cells. Concurrently, our recent study showed that blockade of CD8+ T-cells from CLL patients with anti-PD-1 and anti-Tim-3 failed to restore proliferation, degranulation, or cytokine production by CD8+ T-cells (Rezazadeh et al., 2020). In contrast with our results, in a study by (Chauvin et al., 2015), on melanoma patients, co-blockade of PD-1 and TIGIT significantly increased the proliferation of CD4+ and CD8+ T-cells compared to single blockade or the control antibody. In another study by (Duraiswamy et al., 2013) dual blockade of PD-1 and CTLA-4 increased the proliferation of CD4+ and CD8+ T-cells and the release of inflammatory cytokines in murine models of colon and ovarian cancers. On the other hand, some studies showed the upregulation of other immune checkpoint receptors, like CD244 and CD160, on CD8+ T -cells in CLL patients (Chauvin et al., 2015). Therefore, it can be assumed that in CLL, immune checkpoint receptors other than PD-1 and TIGIT could play an important role in exhaustion of CD8+ T-cells.

In CLL, malignant B-cells are present in the peripheral blood, as well as in the bone marrow and secondary lymphoid organs, like the spleen and lymph nodes. Therefore, responding CD8+ T-cells are assumed to be infiltrated to those organs. However, a study by (Hanna et al., 2019), on a mouse model of CLL showed that of exhausted CD8+ T-cells specifically accumulate in lymph nodes, but not in the peripheral blood. They also indicated that CD8+ T-cells from lymph nodes expressed higher levels of PD-1 and lower levels of TNF-α compared to those from the peripheral blood. Concurrently, another study by (de Weerdt et al., 2019), using peripheral blood and fine-needle lymph node biopsies collected from CLL patients, showed that the expression of PD-1 by CD8+ T-cells was significantly higher in lymph nodes than in the peripheral blood. In our study, we worked on CD8+ T-cells collected from the peripheral blood, but not from the lymph nodes. Therefore, the percentage of exhausted CD8+ T-cells might be too low to be impacted by anti-PD-1 and anti-TIGIT treatment.

Most CLL patients in our study were at early stages of the disease. According to several studies on CLL and other malignancies, it is likely that blockade of inhibitory receptors in patients with early stages might not be as effective as in patients with advanced stages of the disease (Riches et al., 2013; Hanna et al., 2019). In particular, our previous study showed higher expression of PD-1 and TIGIT on CD8+ T-cells from patients with advanced stages of CLL compared to those with early stages(Hajiasghar-Sharbaf et al., 2021). Since in the current study, most CLL patients were in early stages, we assume that immune checkpoint blockade might be more effective in advanced stages of CLL than in early stages.

The results of our study showed that PD-1 and TIGIT blockade had no impact on cytokine production by CD8+ T-cells. Several studies have indicated the increased production of inflammatory cytokines from CD8+ T-cells, following blockade of inhibitory receptors, such as PD-L1, LAG3, and CTLA-4 (Day et al., 2006; Trautmann et al., 2006; McClanahan et al., 2015). In this regard, a study by (Hung et al., 2018), showed that combined blockade of PD-1 and TIGIT enhanced survival, production of IFN-γ and TNF-α, and reduced the number of tumor infiltrating DCs in a murine model of glioblastoma. In another study by (Inozume et al., 2016) there was a significant increase in production of IFN-γ following combined blockade of PD-1 and TIGIT in melanoma. Under normal conditions, IFN-γ, IL-2, and TNF-α are the main cytokines produced by effector CD8+ T-cells responding tumor cells. In chronic infections and cancers, however, chronic stimulation of T-cells results in overexpression of PD-1 and simultaneous production of IFN-γ in the tumor microenvironment which finally leads to T-cell exhaustion. In this regard, a study by (Riches et al., 2013)confirmed that although

CD8+ T-cells are exhausted in CLL, they are still able to produce inflammatory cytokines. Moreover, (Hanna et al., 2019) showed a higher percentage of peripheral blood CD8+ T-cells producing IFN-γ and TNF-α, but not IL-2, in CLL patients compared to those from healthy individuals. Further studies are needed to elucidate the cytokine production by exhausted CD8+ T-cells in CLL.

In conclusion, the present study is the first in vitro study used co-blockade of two immune checkpoint receptors, PD-1 and TIGIT, on CD8 + T-cells in CLL patients. Our results showed that blockade of these two molecules had no effect on restoring the function of CD8 + T-cells isolated from these patients. Further investigations, such as in vitro blockade of PD-1 and TIGIT in CD8+ T-cells from CLL patients with advanced stages, or combined blockade of immune checkpoint receptors in animal models of CLL could elucidate how to restore functions of exhausted CD8+ T-cells in CLL.

Author Contribution Statement

All authors contributed to the study. MT and HAO designed and conducted the research. FH and HHN carried out the assays. FH contributed to data collection and analysis, and prepared the manuscript. RS, GJ, and EZ provided the samples. FH, HAO, AA, and MT prepared the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors thank the patients and their families for their support, cooperation, and patience. We would like to thank the staff of the Gastrointestinal Cancer Research Center, Comprehensive Cancer Center, Imam Khomeini hospital of Sari, Mazandaran University of Medical Sciences.

Funding/Support

This work was supported by the Gastrointestinal Cancer Research Center, Comprehensive Cancer Center, Imam Khomeini hospital of Sari, Mazandaran University of Medical Sciences; grant number 1282. This article has been extracted from FH. thesis.

Ethics approval

The authors state that written informed consent was obtained from all participants and the study has been approved by the Ethical Committee of Mazandaran University of Medical Sciences according to the Declaration of Helsinki.

Availability of data

The data that supported the findings of this study are available on request from the corresponding author, MT. The data are not publicly available since it contains information that can compromise the privacy of research participants.

Conflict of Interests

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

References

Anderson AC, Joller N, Kuchroo VK (2016). Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity*, **44**, 989-1004.

Ansell SM, Lesokhin AM, Borrello I, et al (2015). PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*, **372**, 311-9.

Balar A, Bellmunt J, O'donnell P, et al (2016). Pembrolizumab (pembro) as first-line therapy for advanced/unresectable or metastatic urothelial cancer: preliminary results from the phase 2 KEYNOTE-052 study. *Ann Oncol*, **27**, vi567.

Chauvin JM, Pagliano O, Fourcade J, et al (2015). TIGIT and PD-1 impair tumor antigen-specific CD8- T cells in melanoma patients. *J Clin Invest*, **125**, 2046-58.

Day CL, Kaufmann DE, Kiepiela P, et al (2006). PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature*, **443**, 350-4.

de Weerdt I, Hofland T, de Boer R, et al (2019). Distinct immune composition in lymph node and peripheral blood of CLL patients is reshaped during venetoclax treatment. *Blood Adv*, **3**, 2642-52.

Duraiswamy J, Kaluza KM, Freeman GJ, et al (2013). Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. *Cancer Res*, **73**, 3591-603.

Freeman CL, Gribben JG (2016). Immunotherapy in Chronic Lymphocytic Leukaemia (CLL). *Curr Hematol Malig Rep*, **11**, 29-36.

Hajiasghar-Sharbaf R, Asgarian-Omran H, Valadan R, et al (2021). CD8+ T-cells Co-expressing PD-1 and TIGIT Are Highly Frequent in Chronic Lymphocytic Leukemia. *Iran J Allergy Asthma Immunol*, **20**, 751-63.

Hamid O, Robert C, Daud A, et al (2013). Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med*, **369**, 134-44.

Hanna BS, Roessner PM, Yazdanparast H, et al (2019). Control of chronic lymphocytic leukemia development by clonally-expanded CD8(+) T-cells that undergo functional exhaustion in secondary lymphoid tissues. *Leukemia*, **33**, 625-37.

Hanna BS, Yazdanparast H, Demerdash Y, et al (2021). Combining ibrutinib and checkpoint blockade improves CD8+ T-cell function and control of chronic lymphocytic leukemia in Em-TCL1 mice. *Haematologica*, **106**, 968-77.

Hung AL, Maxwell R, Theodros D, et al (2018). TIGIT and PD-1 dual checkpoint blockade enhances antitumor immunity and survival in GBM. *Oncoimmunology*, **7**, e1466769.

Inozume T, Yaguchi T, Furuta J, et al (2016). Melanoma Cells Control Antimelanoma CTL Responses via Interaction between TIGIT and CD155 in the Effector Phase. *J Invest Dermatol*, **136**, 255-63.

Jain N, Basu S, Thompson PA, et al (2016a). Nivolumab combined with ibrutinib for CLL and Richter transformation: a phase II trial. *Blood*, **128**, 59.

Jain N, Basu S, Thompson PA, et al (2016b). Nivolumab Combined with Ibrutinib for CLL and Richter Transformation: A Phase II Trial. *Blood*, **128**, 59.

Kalil N, Cheson BD (1999). Chronic lymphocytic leukemia. *Oncologist*, **4**, 352-69.

McClanahan F, Hanna B, Miller S, et al (2015). PD-L1 checkpoint blockade prevents immune dysfunction and leukemia development in a mouse model of chronic lymphocytic leukemia. *Blood*, **126**, 203-11.

Nguyen LT, Ohashi PS (2015). Clinical blockade of PD1 and

LAG3--potential mechanisms of action. *Nat Rev Immunol*, **15**, 45-56.
O'Brien S (2008). New agents in the treatment of CLL. Hematology Am Soc Hematol Educ Program, 457-64.
Redaelli A, Laskin BL, Stephens JM, et al (2004). The clinical and epidemiological burden of chronic lymphocytic leukaemia. Eur J Cancer Care (Engl), 13, 279-87.
Rezazadeh H, Astaneh M, Tehrani M, et al (2020). Blockade of PD-1 and TIM-3 immune checkpoints fails to restore the function of exhausted CD8(+) T cells in early clinical stages of chronic lymphocytic leukemia. Immunol Res, 68, 269-79.
Riches JC, Davies JK, McClanahan F, et al (2013). T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. *Blood*, **121**, 1612-21.
Schwarzbich MA, McClanahan F, Gribben J (2016). Allogeneic Transplantation for Chronic Lymphocytic Leukemia in the Age of Novel Treatment Strategies. *Oncology* (Williston Park), **30**, 526-33, 40.
Seiwert TY, Burtness B, Mehra R, et al (2016). Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. *Lancet Oncol*, **17**, 956-65.
Shapiro M, Herishanu Y, Katz BZ, et al (2017). Lymphocyte activation gene 3: a novel therapeutic target in chronic lymphocytic leukemia. *Haematologica*, **102**, 874-82.
Swerdlow SH, Campo E, Harris NL, et al (2008). WHO classification of tumours of haematopoietic and lymphoid tissues, International agency for research on cancer Lyon.
Trautmann L, Janbazian L, Chomont N, et al (2006). Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med*, **12**, 1198-202.
Vaddepally RK, Kharel P, Pandey R, et al (2020). Review of Indications of FDA-Approved Immune Checkpoint Inhibitors per NCCN Guidelines with the Level of Evidence. *Cancers (Basel)*, **12**.
Xu L, Chen H, Chen J, et al (2018). The consensus on indications, conditioning regimen, and donor selection of allogeneic hematopoietic cell transplantation for hematological diseases in China-recommendations from the Chinese Society of Hematology. *J Hematol Oncol*, **11**, 33.
Yano T (2017). Chronic lymphocytic leukemia: biology, disease progression, and current treatment strategies. *Rinsho Ketsueki*, **58**, 1960-72.