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CD8$^+$T-cells Co-expressing *PD-1* and *TIGIT* Are Highly Frequent in Chronic Lymphocytic Leukemia

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ABSTRACT

The role of immune checkpoint receptors in T-cell exhaustion has been demonstrated in several cancers. We investigated the co-expression of *TIGIT/PD-1* and *LAG-3/PD-1* cells in patients with chronic lymphocytic leukemia (CLL).

The frequencies of TIGIT$^+$PD-1$^+$CD8$^+$and LAG-3$^+$PD-1$^+$CD8$^+$cells and relative mRNA expression of *LSECtin* and *CD155* were examined in PBMCs from 33 CLL patients and 20 controls.

The percentage of TIGIT$^+$PD-1$^+$CD8$^+$cells was significantly higher in CLL patients than in control subjects, with the preference in advanced-stage patients. However, LAG-3$^+$PD-1$^+$CD8$^+$cell percentage was significantly lower in CLL patients than in the control subjects, and no significant differences were found between the early and advanced stages of the disease. An increase in the mRNA expression level of *LSECtin*, but not that of *CD155*, was observed in CLL patients compared to the control subjects.

Collectively, a higher co-expression of *PD-1* and *TIGIT* on CD8$^+$ T-cells in CLL compared to control subjects suggests an important role of TIGIT in T-cell exhaustion in CLL patients especially those with advanced disease.

Keywords: Chronic lymphocytic leukemia; TIGIT protein

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy that is characterized by the accumulation of mature B-cells, expressing CD5, CD23, CD19, and CD27, and low levels of surface Immunoglobulin, in peripheral blood, secondary lymphoid organs, and bone marrow.[1,2] Many CLL patients never need treatment or are controlled by temporary chemotherapy, while others have worse clinical courses and suffer from a rapid progression of the disease.[3] Conventional chemotherapy for CLL has several side effects, as chemotherapy seems to exacerbate this problem by inducing T-cell lymphopenia. Therefore, immunotherapeutic strategies against malignant

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B-cells have been of great importance over the past years.[4,5]

Like several other cancers, CLL is believed to be associated with a defect in T-cell function.[6] Indeed, it is believed that during the development and progression of several cancers, tumor microenvironment causes effector T-cells to gradually lose their effector functions, a process called "T-cell exhaustion".[7,8] Several changes occur in exhaustion, including, altered expression of key transcription factors, metabolic derangements, impaired immune synapse formation, increased expression of inhibitory receptors, and finally, defects in proliferation, cytokine production, and cytotoxicity of T-cells.[9-11] In this regard, overexpression of immune checkpoint receptors on the surface of T-cells plays important role in "T-cell exhaustion" via the regulation of T cell-driven immune responses.[12-14]

Programmed cell death protein 1 (PD-1, CD279) is one of the main immune checkpoint receptors which is expressed on activated T-cells, Tregs, B-cells, and NK cells.[15] Ligands for PD-1 include PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC). Several studies have pointed to the central role of the PD-1/PD-L1 pathway for inhibiting T-cell responses leading to T-cell exhaustion, and blockade of this pathway has been reported to restore immune responses in many solid tumors, such as pancreatic carcinoma and melanoma, as well as hematological malignancies, including acute myeloid leukemia, Hodgkin lymphoma, and CLL.[13,15,16]

Lymphocyte activation gene 3 (LAG-3) is another well-known immune checkpoint receptor that structurally resembles the CD4 co-receptor and can bind to MHCII, as well as two other ligands, including *LSECtin* and *Galectin-3*. *LAG-3* is expressed on activated $CD4^+$ and $CD8^+$T-cells, Tregs, plasmacytoid dendritic cells, and NK cells. Several studies have reported that binding of LAG-3 to its ligands, could play a role in T-cell exhaustion.[17-19]

T-cell immunoglobulin and ITIM domain (TIGIT) is another important immune checkpoint receptor that is expressed on effector $CD4^+$ and $CD8^+$ T-cells, Tregs, and follicular T helper cells, and also found on NK and memory T-cells. TIGIT binds to three ligands, including CD155 (PVR), CD112 (PVRL2, nectin-2), and CD113 (PVRL3) which are also parts of the PVR/NECTIN family.[15,20] Some studies have shown that co-expression of *TIGIT* and *PD-1* could lead to impaired protective anti-tumor responses, and therefore, antibody co-blockade of *TIGIT* and *PD-1* could enhance $CD8^+$ T-cell effector function, resulting in significant tumor clearance.[21,22]

In this regard, it was shown that the use of anti-PD-1, -PD-L1, or -TIGIT increased proliferation and IFN-γ production from$CD8^+$ tumor-infiltrating lymphocytes (TILs) in patients with melanoma.[17,21-24]

Moreover, we previously showed that both $CD4^+$ and $CD8^+$ T-cells are functionally exhausted in CLL and that co-expression of *PD-1* and *Tim-3* on both T-cell groups was higher in the advanced stages of CLL.[25,26] In the current study, the frequency of exhausted $CD8^+$T-cells based on co-expression of immune checkpoint receptors, *PD-1*, *LAG-3*, and *TIGIT*, as well as their ligands, including *LSECtin*, and *CD155*, were evaluated in patients with CLL.

MATERIALS AND METHODS

Study Subjects

A total of 33 CLL patients attending the Hematology and Oncology Clinic of Imam Khomeini Hospital, affiliated to Mazandaran University of Medical Sciences, and 20 age- and sex-matched control subjects were enrolled in the study. The sample size was selected based on the previous studies.[21,27-30]CLL was diagnosed based on the clinical evaluation, blood cell count, cell morphology, and immunophenotyping analysis according to the World Health Organization criteria (NCI-WG).[31] None of the patients had a history of chronic viral infections, like HIV, HBV, HCV, or any other types of congenital or acquired immunodeficiency. In addition, patients with a history of other cancers or any auto-immune disease were ruled out from this study. None of the patients received chemotherapy or other immunosuppressive medications for 6 months before sampling. Based on the Rai staging system, given by the National Cancer Institute Working Group criteria,[32] patients were divided into two groups of early stages (stage 0-I) and advanced stages (stage II-IV). The control group was healthy volunteers, age- and sex-matched with the patients. Eight to ten milliliter of heparinized peripheral blood samples were taken from each study subject after obtaining written informed consents. The study protocol was approved by the Ethical Committee of Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1396.3068).

Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood using Ficoll-Histopaque (Biosera, Nuaille, France) density-gradient centrifugation. PBMCs were washed twice with RPMI-1640 culture medium (Biosera) and resuspended in the same medium supplemented with 10% (v/v)fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 µg/mL) (Biosera). Next, 1×10^5 isolated cells were cultured in flat-bottomed 96-well microplates in 200 µL RPMI-1640 medium, supplemented with penicillin, streptomycin, and 10% FBS, and incubated at 37°C with 5% CO_2 (GIBCO, Life Technologies, Carlsbad, CA) in the presence of 2 µg/Ml phytohemagglutinin (10 µg/mL) (PHA-L, Sigma Aldrich, St. Louis, MO, USA) for 48 hours. The viability of isolated cells was more than 95 % as assessed by the trypan blue staining viability test.

Flow Cytometric Analysis

A three-color flow cytometry method was applied to determine the frequency of exhausted $CD8^+$T-cells. PBMCs were stained with fluorochrome-conjugated monoclonal antibodies (mAbs) against human CD8a-FITC (clone SK1), LAG-3-PE (clone 3DS223H), TIGIT-PE (clone MBSA43), all obtained from eBioscience (San Diego, CA, USA), PD-1-PerCP/Cy5.5 (Clone EH12.2H7) from Biolegend (San Diego, CA, USA), and CD38-PE (clone HB-7) from BD (San Diego, CA, USA) as well as related isotype controls including FITC-mouse IgG1κ (eBioscience), PE-mouse IgG1κ (eBioscience), and PerCP/Cy5.5-mouse IgG1κ (Biolegend). After washing PBMCs with phosphate buffered-saline (PBS, 0.15M, pH:7.4) with 0.5 % BSA, 2×10^5 cells were resuspended in 200 µL of the same buffer and incubated with appropriate specific mAbs for 45 min at 4°C in the dark and then washed to eliminate nonspecific bindings of Abs. Stained cells were then analyzed on a Partec PAS flow cytometer system (PartecGmBH, Munster, Germany); using the Flow-Max software. To analyze the flow cytometry plots, $CD8^+$cells in the lymphocyte population were initially gated, and then, TIGIT, LAG-3, and PD-1 positive cells out of $CD8^+$ T-cells were determined. For compensation analysis, the spillover amount of each fluorochrome into other channels was determined using so-called single-color compensation controls.

Quantitative Reverse Transcriptase PCR

Total RNA was extracted from 7×10^6 of PBMCs, using a commercial RNA extraction kit (Yektatajhizazma Super RNA Extraction for tissue and cells, Tehran, Iran), according to the manufacturer's instructions. The quantity and quality of extracted RNAs were assessed by a nanodrop spectrophotometer (Thermo Fisher Scientific Inc, Massachusetts, USA) and agarose gel electrophoresis, respectively. Complementary DNA (cDNA) was reverse-transcribed using the Revert Aid first-strand cDNA synthesis kit (Thermo Fisher Scientific). Quantitative Reverse Transcriptase PCR (qRT-PCR) was performed using Real Q Plus 2x Master Mix for Probe (High Rox, Amplicon, Denmark) reagent on an ABI Step one plus Real-Time system (Thermo Scientific) with Taqman specific primers and probes for *CD155* and *LSECtin*, as well as *GAPDH*, as a housekeeping gene (Table 1).

Table 1. Primers and Probes

Gene	Primers and Probes (5'-3')*	Product Size (bps)
LSECtin	F: GAG GCT CCA GAA CAA CTC CT	243 bps
	R:CCC TGA ACC TTG CCC AGA T	
	P:TCT CTG TGC CAA AGA CGA CG	
CD155	F:CAA CGT CAC CAA TGC CCT AG	194 bps
	R:CTC CAG TCT CTC TCC CCT CT	
	P:GAT CCA CAG ACA GAG GGC AC	
GAPDH	F:GGG TGT GAA CCA TGA GAA GT	228 bps
	R:GCA GGG ATG ATG TTC TGG AG	
	P: TCG TGG AAG GAC TCA TGA CC	

* F, Forward primer; R, reverse primer; P, Probe

FAM or HEX was selected for 5' end of reporters and NFQ-MGB quencher for 3' ends of the probes. qRT-PCR was performed using a volume of primer and probe: 1.5 µL, cDNA: 1 µL, Master mix: 7.5 µL, and PCR grade water: 5 µL, and amplified at 95°C for initial denaturation followed by 45 cycles at, 95°C for 30 seconds, 60°C for 30 seconds, and extension at 72°C for 30 seconds. After normalized to *GAPDH*, relative expression levels of *CD155* and *LSECtin* were determined; using the $2^{-\Delta\Delta Ct}$method.[33]

Statistical Analysis

Data were expressed as means±standard error of the mean (SEM). All statistical analyses were performed using the SPSS 20 statistical package (SPSS, Chicago, USA). The results were evaluated by Mann–Whitney U test and Spearman correlation tests as appropriate. P values of less than 0.05 were considered significant. All graphs were designed; using the Graph pad Prism 6 software.

RESULTS

Study Population

A total of 33 CLL patients (19 males and 14 females, mean age: 61.5 years) and 20 age- and sex-matched control subjects (14 males and 6 females, mean age: 56.8 years) participated in the study. According to the Rai staging system, 28 patients were in the early stages and 5 in the advanced stages. Serum LDH concentration and CD38 percentage were not significant between patients with early and advanced stages of CLL (p=0.590 and p=0.722, respectively). Major clinical and laboratory characteristics of patients and controls are summarized in Table 2.

***TIGIT* Expression in CLL Patients and Controls**

It is known that chronic T-cell stimulation leads to upregulation of immune checkpoints on the cell surface.[11] To evaluate *TIGIT* and *PD-1* expression on $CD8^+$ T-cells, we first stimulated T-cells from CLL patients and controls with PHA for 48 hours and then performed phenotypic characterization of T-cells using a three-color flow cytometry method. The results showed that the percentage of $TIGIT^+CD8^+$cells was significantly higher in CLL patients than in control subjects (55.82±4.17 and 39.14±3.28, respectively, p=0.003, Figure 1A and B). In addition, $TIGIT^+PD\text{-}1^+CD8^+$ cell percentage was significantly higher in CLL patients than in control subjects (17.63±3.29 and 9.40±1.47, respectively, p=0.028, Figure 1C). To investigate the mRNA expression profile of *CD155*, as a corresponding ligand for TIGIT, PBMCs from CLL patients and control subjects were examined, using qRT-PCR. The results showed that the expression of *CD155* was not significantly different between CLL patients and control subjects (p=0.322, Figure 1D).

***TIGIT* Expression on $CD8^+$T-cells in Early and Advanced Stages of CLL**

We next evaluated the frequency of $CD8^+$ T-cells in CLL patients with different clinical stages. The results showed that $TIGIT^+CD8^+$cell percentage was significantly higher in CLL patients with advanced stages than in those with early stages (74.81±7.25 and 52.37±4.56, respectively, p=0.041, Figure 2A, B, and C). Concurrently, $TIGIT^+PD\text{-}1^+CD8^+$cell percentage was significantly higher in CLL patients with advanced stages than in those with early stages (34.13±14.38 and 15.09±2.95, respectively, p=0.047, Figure 2D). We also examined the expression of *CD155*, as a TIGIT ligand, in CLL patients with early and advanced stages. As shown in Figure 2E, no significant difference was observed in CLL patients with advanced clinical stages when compared to patients with early stages of the disease (p=0.462).

Table 2. Major clinical and laboratory characteristics of CLL patients

No	Age (y)	Sex	WBC×10^3/ mm^3	Lym (%)	PLT×10^3/ mm^3	Hb (g/dL)	LDH (IU/L)	Rai stage	CD38 (%)	Organomegaly
1	50	F	4.2	87.7	157	11.6	308	0	NA	Negative
2	64	M	17.85	79.0	111	12.5	401	I	NA	LAP+
3	61	M	104.67	90.1	226	13.3	354	0	NA	Negative
4	46	M	10.03	57.08	134	16.6	281	0	1.1	Negative
5	54	F	170.09	90.4	42	8.4	709	I	6	LAP+
6	80	M	60.7	92.2	71	8.1	566	IV	NA	LAP+
7	63	F	17.15	60.3	176	13.1	534	0	NA	Negative
8	64	M	18.1	75	16	14.1	630	0	NA	Negative
9	58	M	46.2	76.56	160	12.7	796	0	NA	Negative
10	80	M	71	89	119	11.9	NA	0	1.4	Negative
11	78	M	49.15	92	164	10.5	1008	0	NA	Negative
12	78	M	42.16	85.8	231	13.1	348	0	0.6	Negative
13	75	F	46.56	55.9	191	9.1	356	0	3.57	Negative
14	52	F	84.50	88.15	173	12	232	II	23.01	LAP+SPM+
15	72	F	53.10	84.82	147	10.6	284	0	NA	Negative
16	77	M	49.40	88.15	180	8.5	562	III	6.7	Negative
17	61	F	17.2	70.88	127	12.7	NA	0	NA	Negative
18	78	M	36.0	84	104	15	NA	0	21	Negative
19	60	F	82.0	56.9	139	14	510	0	1.1	Negative
20	76	F	78.2	91.2	139	11.9	599	II	0.58	LAP+HSP+
21	84	F	51.3	85.1	231	11	892	II	1.16	SPM+
22	61	M	55.06	89.8	148	12.6	333	0	4.5	Negative
23	70	M	17.0	72	216	13.5	450	0	5.84	Negative
24	62	F	17.3	65	220	14.5	NA	0	5.0	Negative
25	65	M	35.4	80	150	13.7	422	0	2.54	Negative
26	64	F	21.38	76.5	220	12.6	387	0	4.72	Negative
27	57	M	25.46	76.0	188	14.4	272	0	6.94	Negative
28	61	M	28.5	86	126	14.0	339	I	4.01	LAP+
29	62	M	43.6	88	91	13.6	1380	1	4.70	LAP+
30	53	M	28.8	78.8	223	11.8	452	1	20	LAP+
31	77	M	94.7	90.5	209	12.9	384	0	2.46	Negative
32	44	F	99.50	93.27	139	10.5	600	0	4.90	Negative
33	63	F	47.5	85	188	13.3	548	0	9.44	Negative

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 and; LDH, serum lactate dehydrogenase; LAP, lymphadenopathy; SPM, splenomegaly; HPM, hepatomegaly; NA, not available.

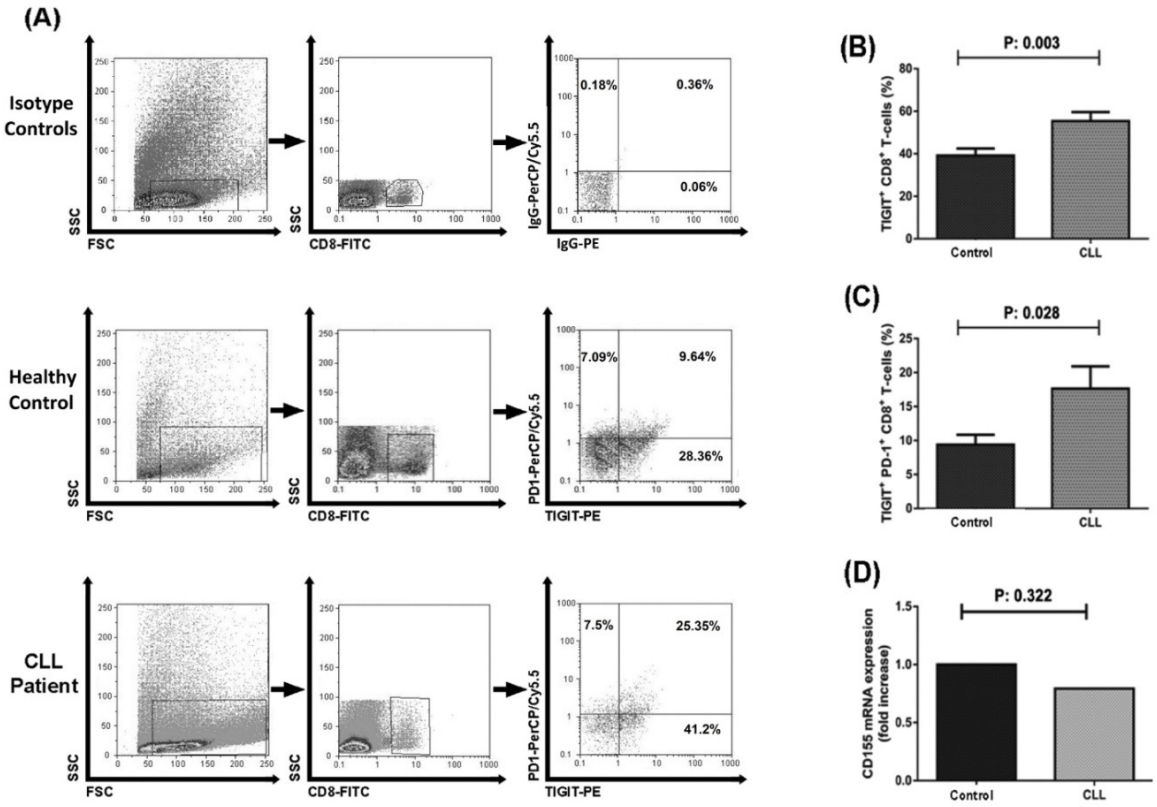

Figure 1. ***TIGIT*** **expression on CD8$^+$T-cells in chronic lymphocytic leukemia (CLL) patients and control subjects. (A) Representative flow cytometric dot plots from a CLL patient and a control subject are shown. To analyze the obtained graphs, CD8$^+$ cells were initially gated from the lymphocyte population, and then, the TIGIT and PD-1 positive cells from CD8$^+$ lymphocytes were determined. (B, C) The percentage of TIGIT$^+$CD8$^+$(B) and TIGIT$^+$PD-1$^+$CD8$^+$ T-cells (C) from CLL patients and controls are represented. Horizontal bars represent mean±SEM. *p* values<0.05 were considered significant. (D)** ***CD155*** **mRNA expression was first normalized to that of** ***glyceraldehyde 3-phosphate dehydrogenase*** **(*GAPDH)* and then expressed as a fold change as the average expression level of the patient samples dividing by that of the control samples. The value of 1 indicates the level of gene expression of control samples.**

***LAG-3* Expression on CD8$^+$T-cells in CLL Patients and Controls**

We next evaluated LAG-3$^+$PD-1$^+$CD8$^+$ T-cells, defined as exhausted CD8$^+$ T-cells, in CLL patients and controls. LAG-3$^+$PD-1$^+$CD8$^+$cell percentage was significantly lower in CLL patients than in the control group (20.13±3.78 and 54.00±4.56, respectively, p<0.0001, Figure 3A and B). Concurrently, LAG-3$^+$PD-1$^+$CD8$^+$cell percentage was significantly lower in CLL patients than in control subjects (8.33±2.11 and 21.53±4.58, respectively, p<0.0001, Figure 3C). We next investigated the expression of *LSECtin*, as a ligand for LAG-3, and showed that the expression of *LSECtin* was significantly higher in CLL patients than in control subjects (p<0.0001, Figure 3D). Further analysis showed that CD8$^+$LAG-3$^+$cell percentage was not significantly different between CLL patients with early and advanced stages (p=0.288, Figure 4 A-C). Also, LAG-3$^+$PD-1$^+$CD8$^+$cell percentage was not significantly different between CLL patients with early and advanced stages (p=0.605, Figure 4D). In addition, the expression of *LSECtin*, as a ligand for LAG-3, was not significantly different between CLL patients with early and advanced stages (p=0.393, Figure 4E).

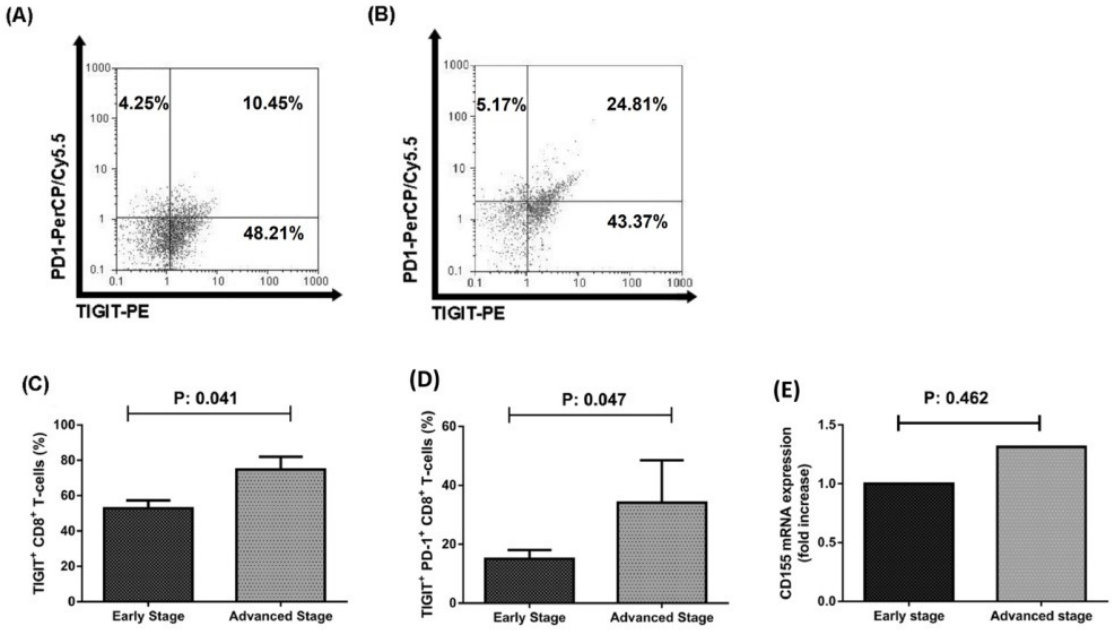

Figure 2. *TIGIT* expression on CD8$^+$T-cells in chronic lymphocytic leukemia (CLL) patients with early and advanced clinical stages (A, B) Representative flow cytometric dot plots from a CLL patient with early (A) and advanced (B) Stages are shown. To analyze the flow cytometry graphs, CD8$^+$ cells were initially gated from the lymphocyte population, and then, the TIGIT and PD-1 positive cells from CD8$^+$ lymphocytes were determined. (C, D) The percentage of TIGIT$^+$CD8$^+$(C) and TIGIT$^+$PD-1$^+$CD8$^+$(D) T-cells from CLL patients with early and advanced stages are represented. Horizontal bars represent mean±SEM. p values<0.05 were considered significant. (E) *CD155* mRNA expression was first normalized to that of *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH)* and then expressed as a fold change as the average expression level of the CLL samples with advanced stages dividing by that with early stages. The value of 1 indicates the level of gene expression of CLL samples with early stages.

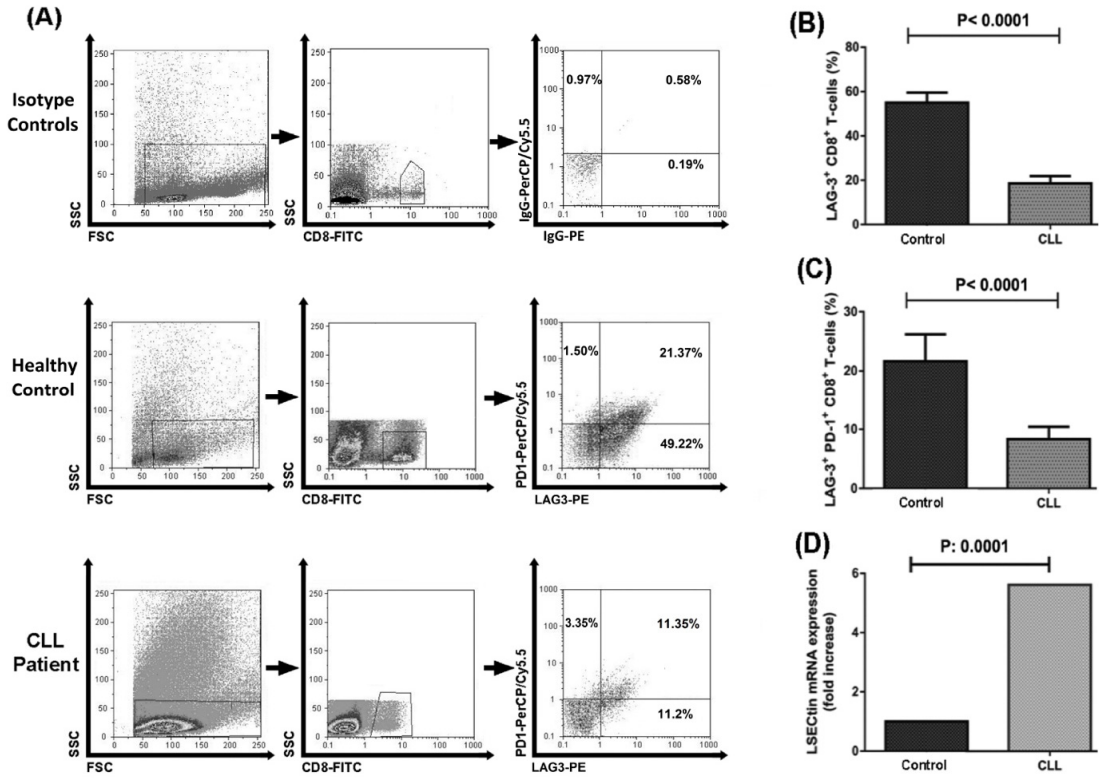

Figure 3. ***LAG-3*** **expression on $CD8^+$ T-cells in chronic lymphocytic leukemia (CLL) patients and control subjects (A) Representative flow cytometric dot plots from a CLL patient and a control subject are shown. To analyze the obtained graphs, $CD8^+$ cells were initially gated from the lymphocyte population, and then, the LAG-3 and PD-1 positive cells from $CD8^+$ lymphocytes were determined. (B, C) The percentage of $LAG\text{-}3^+CD8^+$(B) and $LAG\text{-}3^+PD\text{-}1^+CD8^+$ T-cells (C) from CLL patients and controls are represented. Horizontal bars represent mean±SEM. *p* values<0.05 were considered significant. (D)** ***LSECtin*** **mRNA expression was first normalized to that of** ***glyceraldehyde 3-phosphate dehydrogenase*** **(*GAPDH)* and then expressed as a fold change as the average expression level of the patient samples dividing by that of the control samples. The value of 1 indicates the level of gene expression of control samples.**

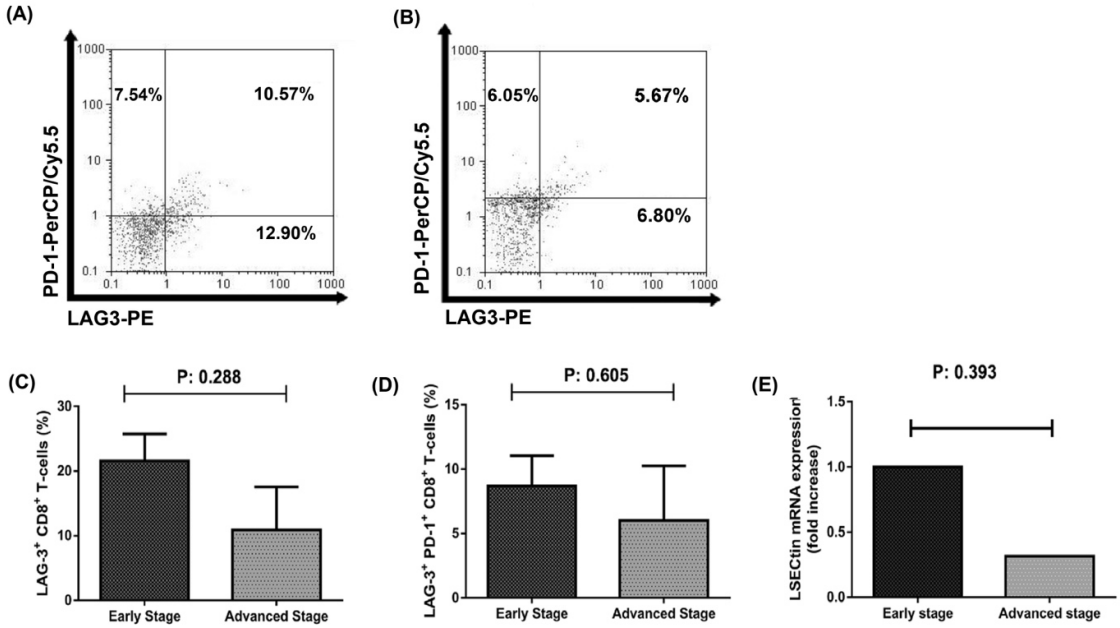

Figure 4. ***LAG-3*** **expression on CD8$^+$ T-cells in chronic lymphocytic leukemia (CLL) patients with early and advanced clinical stages (A, B) Representative flow cytometric dot plots from a CLL patient with early (A) and advanced (B) stages are shown. To analyze the flow cytometry graphs, CD8$^+$ cells were initially gated from the lymphocyte population, and then, the LAG-3 and PD-1 positive cells from CD8$^+$ lymphocytes were determined. (C, D) The percentage of LAG-3$^+$CD8$^+$(C) and LAG-3$^+$PD-1$^+$CD8$^+$(D) T-cells from CLL patients with early and advanced stages are represented. Horizontal bars represent mean ± SEM.** ***p*** **values<0.05 were considered significant. (E)** ***LSECtin*** **mRNA expression was first normalized to that of** ***glyceraldehyde 3-phosphate dehydrogenase*** **(*GAPDH)* and then expressed as a fold change as the average expression level of the CLL samples with advanced stages dividing by that with early stages. The value of 1 indicates the level of gene expression of CLL samples with early stages.**

DISCUSSION

Immune checkpoint receptors are expressed on various immune cells, particularly onCD4$^+$ and CD8$^+$ T-cells. Under physiologic circumstances, their expression on effector CD4$^+$ and CD8$^+$T-cells leads to modulate effector T-cell responses.[15] Within the tumor microenvironment, however, both CD4$^+$ and CD8$^+$ T-cells differentiate into the state of T-cell exhaustion, in which they upregulate immune-checkpoint receptors and lose their functional capacities, including proliferation, cytokine production, and cytotoxicity.[9] Also in some tumors, Tregs and MDSCs upregulate immune checkpoints and exert more robust immunosuppressive functions.[15] In this sense, targeting immune-checkpoint receptors has had a revolutionary impact on cancer treatment via restoring the immune system.[34] Therefore, exploring the role of these receptors in different cancers could help plan new therapeutic strategies. Previously, we showed that both CD4$^+$and CD8$^+$T-cells are functionally exhausted in CLL and that co-expression of *PD-1* and *Tim-3* on both cells was higher in advanced stages of CLL compared to early stages.[25,26] In the present study, we focused on two other important immune checkpoint receptors, LAG-3 and TIGIT, and their ligands. Also, given the crucial role of CD8$^+$ T-cells in tumor immunity, we studied the frequency of LAG-3$^+$PD-1$^+$CD8$^+$ and TIGIT$^+$PD-1$^+$CD8$^+$ T-cells in CLL.

We first showed a higher co-expression of *TIGIT* and *PD-1* on CD8$^+$ T-cells, defined as exhausted CD8$^+$T-cells, in CLL patients compared to control subjects. We also showed that TIGIT$^+$PD-1$^+$CD8$^+$ cell percentage was significantly higher in CLL patients

with advanced stages than in those with early stages. Concurrently, several studies have mentioned the overexpression of *PD-1* and *TIGIT* on T-cells in different cancers. Hung et al, found high levels of *PD-1* and *TIGIT* expression on $CD4^+$and $CD8^+$TILs from glioma tumor samples. They also found that the expression of *CD155* was correlated with poor survival in patients with low-grade glioma.[35] Another study by Wang et al, observed increased frequencies of PD-1- and TIGIT-expressing $CD8^+$ T-cells in AML patients. They also showed that most leukemia blast cells from AML patients expressed the two TIGIT ligands, CD112 and CD155.[36] In addition, Kong et al showed that $CD8^+$ T-cells from AML patients had higher expression of *PD-1* and *TIGIT* leading to T-cell exhaustion. They also showed that the majority of leukemia cells in AML patients expressed *CD155* and suggested a potential role for TIGIT/CD155 signaling in AML.[23] Also recently, Johnston et al, showed that TIGIT and PD-1 were highly expressed on both human and murine tumor-infiltrating $CD8^+$T-cells in colorectal cancer. They also noted that blockade of both receptors was necessary to restore the function of $CD8^+$T-cells within a highly suppressive tumor microenvironment and their results suggested TIGIT as a critical and specific regulator of the effector functions of chronically stimulated $CD8^+$T-cells.[22] In another study, Inozume et al showed that TIGIT upregulation in melanoma-specific CTLs was induced by tumor stimulation and *CD155* was constitutively expressed on melanoma cell lines. They finally concluded that T-cell suppression via the CD155-TIGIT interaction within the melanoma tumor microenvironment appears to be a critical immune checkpoint that limits the anti-melanoma T-cell responses. They demonstrated that TIGIT blockade, alone or in combination with PD-1, enhanced the anti-melanoma CTL response in the effector phase.[24]On the contrary, one study showed that $CD8^+$T-cells in CLL patients did not upregulate TIGIT while they found an enrichment of $TIGIT^+$T-cells in the $CD4^+$ T-cell compartment in advanced stages of CLL. They also observed low but measurable levels of CD112 and CD155 on the surface of CLL cells.[28] Taken together, PD-1 and TIGIT appear to be co-expressed on $CD8^+$T-cells in CLL and several other cancers.

Regarding the role of PD-1 and TIGIT in cancer progression, Kong et al observed a significantly higher percentage of $TIGIT^+$ cells among $CD8^+$T-cells in patients who had leukemia relapse compared to that of patients who remained in remission.[23] Another report showed that TIGIT was upregulated on the majority of tumor-specific $CD8^+$T-cells in the periphery and within metastatic tumors of patients with advanced melanoma. They also noted that in metastatic melanoma tumors, the expression of *CD155* and *CD112* by monocytes and dendritic cells was substantially higher than in PBMCs from non-metastatic melanoma patients and healthy donors.[21] In CLL, however, the results of our study showed that *CD155* was not significantly expressed on malignant B-cells from CLL patients. In line with our results, Josefsson et al showed that malignant B-cells from CLL patients were negative for CD155 and CD112, although follicular dendritic cells from the same patients showed *CD155* and *CD112* expression.[37] This might be due to the downregulation of *CD155* responding T-cells, as Nishiwada et al, also showed an inverse correlation between *CD155* expression and tumor-infiltrating lymphocytes (TILs) in pancreatic cancer.[38] Overall, the expression pattern of *CD155* on malignant cells seems to be various in different tumors and requires further investigation.

We next examined the co-expression pattern of *PD-1* and *LAG-3* on $CD8^+$ T-cells in CLL. Previous studies on some non-hematological cancers have reported that *LAG-3* expression was higher on $CD8^+$ T-cells and that co-blockade of *PD-1* and *LAG-3* could enhance function and survival of $CD8^+$ T-cells.[13,17,39-42] Regarding CLL, a study on a murine model of CLL showed the expression of *PD-1* and *LAG-3* on $CD8^+$ T-cells as well as expression of *PD-L1* on malignant B-cells.[43] Moreover, two studies by Shapiro et al and Kotaskova et al suggested LAG-3 as a prognostic marker in human CLL.[1,44] In our study, $LAG\text{-}3^+PD\text{-}1^+CD8^+$ cell percentage was lower in CLL patients than in the control group, which was contrary with the results of the previous studies. This might be because most CLL patients in our study were in early stages, thus CD8+ T-cells did not upregulate LAG-3, while they upregulate PD-1 and TIGIT. Of course, further studies with a higher number of CLL patients with advanced stages would achieve more suitable results.

In conclusion, the present study showed that $CD8^+$ T-cells in CLL co-express PD-1 and TIGIT, and also this co-expression is higher in clinically advanced stages of CLL. Our results suggest *TIGIT* as an immune checkpoint receptor related to T-cell exhaustion in CLL. A limitation of our study was that we did not perform functional studies on CD8+ T-cells, including

proliferation, cytokine production, and cytotoxicity. Of course, evaluating correlations between the expression of immune-checkpoint receptors and functions of CD8+ T-cells could make the results more comprehensible. Also, further studies evaluating the therapeutic benefits of co-blockade of *PD-1* and *TIGIT* might be crucial in different cancers. We are now conducting a study on the effect of *in vitro* co-blockade *PD-1* and *TIGIT* in CLL.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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