ORIGINAL ARTICLE

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

Hadiseh Rezazadeh[1] · Mojgan Astaneh[1] · Mohsen Tehrani[1,2] · Hadi Hossein-Nataj[1] · Ehsan Zaboli[2,3] · Ramin Shekarriz[2,3] · Hossein Asgarian-Omran[1,2,4]

Abstract

Blocking antibodies targeting immune checkpoint molecules achieved invaluable success in tumor therapy and amazing clinical responses in a variety of cancers. Although common treatment protocols have improved overall survival in patients with chronic lymphocytic leukemia (CLL), they continue to relapse and progress. In the present in vitro study, the application of anti-PD-1 and anti-TIM-3 blocking antibodies was studied to restore the function of exhausted CD8+ T cells in CLL. CD8+ T cells were isolated from peripheral blood of 20 patients with CLL, treated with blocking antibodies, and cocultured with mitomycin-frozen non-CD8+ T cell fraction as target cells. Cultures were stimulated with anti-CD3/CD28 antibodies to assess the proliferation of CD8+ T cells by MTT and stimulated with PMA/ionomycin to measure the levels of CD107a expression and cytokine production by flow cytometry and ELISA, respectively. Our results showed that the blockade of PD-1 and TIM-3 does not improve the proliferation of CD8+ T cells in CLL patients. No significant difference was found between control and blocked groups in terms of degranulation properties and production of IFN-γ, TNF-α, IL-2, and IL-10 by CD8+ T cells. We observed that pre-treatment of CD8+ T cells with blocking antibodies in CLL patients at early clinical stages had no effects on restoring their functional properties. Further in vitro and in vivo complementary studies are required to more explore the utility of checkpoint inhibitors for CLL patients.

Keywords Chronic lymphocytic leukemia · Immunotherapy · Immune checkpoints · Anti-TIM-3 · Anti-PD-1

Introduction

Chronic lymphocytic leukemia (CLL) is markedly defined by localization of proliferative mature B cells in peripheral blood and lymphoid organs including lymph nodes, spleen, and bone marrow [1]. Based on the statistics provided by the American Cancer Society in 2019, CLL is the fourth prevalent cancer among all types of leukemia and men are more susceptible than women [2]. Some diagnosed patients with CLL do not need any treatments, while others who are represented with an aggressive state of the disease need frequent follow-up and treatments. At the present time, the standard first-line therapy for CLL patients depends on some factors; patients in early-stage CLL without active disease should be watched and wait until they become symptomatic, and in symptomatic or advanced-stage CLL, there are three categories: (1) in patients with TP53 dysfunction, treatment starts with ibrutinib or venetoclax or idelalisib plus rituximab; (2) in patients with unmutated IGHV and no del(17p) or TP53, if the patient is physically fit, ibrutinib or chemoimmunotherapy (CIT) is prescribed, and if the patient is unfit, venetoclax plus obinutuzumab or ibrutinib or CIT is suggested; (3) in patients with mutated IGHV and no del(17p) or TP53, if the patient is physically fit, CIT or ibrutinib, and if the patient is unfit, CIT or venetoclax plus obinutuzumab or ibrutinib is suggested [3].

✉ Hossein Asgarian-Omran
asgarianhossein@yahoo.com; hasgarian@mazums.ac.ir

[1] Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

[2] Gastrointestinal Cancer Research Center, Non-Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran

[3] Department of Hematology and Oncology, Imam Khomeini Hospital, Mazandaran University of Medical Sciences, Sari, Iran

[4] Immunogenetics Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

Nevertheless, researches are now focused on other options rather than those mentioned above, especially employing the innate nature of the host immune system to eradicate the leukemic cells. Tumor cells escape from body immune defense by means of their specific microenvironment and also inhibitory surface receptors of which ligands are expressed on immune cells [4]. Some of the well-known immune inhibitory checkpoint receptors are PD-1, CTLA-4, TIM-3, LAG-3, and TIGIT, which have been attracting more attention in the last decade for targeted tumor immunotherapy [5–7].

Ipilimumab (anti-CTLA-4 antibody) is the first FDA-approved antibody against an inhibitory receptor that is being applied in the treatment of advanced metastatic melanoma [8]. Furthermore, emerging data showed that PD-1 is the main axis of the T cell inhibitory system [9], which its expression has been indicated in the microenvironment of different tumors [5]. Promising clinical responses obtained from the anti-PD-1 antibody and its major ligands, anti-PD-L1 antibody, in reactivation of specific T cells against many tumor cell types resulted in FDA approval of several antibodies against these molecules in the treatment of different solid and hematologic tumors [10–15]. During the exhaustion process, TIM-3 is also expressed as a negative regulator on lymphocytes, and its overexpression is seen both in exhausted CD4 and CD8 T cells [16]. In our previous studies, we have shown that TIM-3 and PD-1 are more expressed on both CD4$^+$ and CD8$^+$ T cells of CLL patients contributing to their dysfunction in comparison with healthy donors [17, 18]. Later on, we observed that Gal-9 and PD-L1, as the main ligands of TIM-3 and PD-1, are more expressed in CLL patients [19]. Accordingly, we decided to conduct an in vitro study to evaluate the blockade of PD-1 and TIM-3 receptors on the restoration of the exhausted state of CD8$^+$ T cells and to improve their functional properties in CLL patients.

Materials and methods

Patients and controls

A total number of 20 CLL patients (8 females and 12 males; mean age of 63.8 years) who were admitted at Hematology and Oncology Clinic of Imam Khomeini Hospital, affiliated to Mazandaran University of Medical Sciences, were included in this study. CLL was diagnosed according to the WHO criteria, clinical examinations, and full blood count together with the morphological evaluation of peripheral blood and flow cytometric immunophenotyping. All patients were confirmed not to be infected with any of the chronic viral diseases, including HIV, HBV, HCV, or any type of congenital or acquired immunodeficiency. In addition, patients with a history of other cancers or any auto-immune diseases were excluded from the study. None of the patients received chemotherapy or other immunosuppressive medications for 6 months prior to sample collection. Written informed consent letters were obtained from all participants in accordance with the Declaration of Helsinki. Clinical and hematological characteristics of CLL patients are presented in Table 1.

Isolation of CD8$^+$ T lymphocytes using magnetic-activated cell-sorting method

Heparinized peripheral blood samples were collected from CLL patients, and peripheral blood mononuclear cells (PBMCs) were immediately isolated by density gradient centrifugation on Ficoll–Histopaque (Biosera, Nuaille, 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 were initially removed using a 70-μm pre-separation filter (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8$^+$ T cells were positively isolated from the peripheral blood lymphocytes using a magnetic bead–conjugated anti-CD8 mAbs kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Cell purity was analyzed by dual-color flow cytometry staining using anti-CD8 FITC (clone SK-1 CF) and anti-CD3PE (clone UCHT1) (both from eBioscience, San Diego, CA, USA). Briefly, isolated cells were washed and resuspended in 100-μl washing buffer (PBS 0.15 M pH: 7.4 with 0.5% BSA) and stained with appropriate amounts of fluorochrome-conjugated mAbs and incubated for 45 min at 4 °C in dark. Samples were then analyzed on the Partec PAS flow cytometer system (Partec GmBH, Munster, Germany) using the FloMax software. As expected from positive selection, the purity of isolated T CD8$^+$ cells was more than 97%.

Blocking of PD-1 and TIM-3 receptors

In order to block the PD-1 and TIM-3 inhibitory receptors, 10^5 of magnetic-activated cell sorting (MACS)–isolated CD8$^+$ T cells were incubated with 10 μg/ml of anti-human PD-1 (clone EH12.2H7, BioLegend, San Diego, CA, USA) and anti-human TIM-3 (clone F38-2E2, BioLegend, San Diego, CA, USA) at 4 °C for 2 h. Corresponding isotype-matched antibodies were also used as the control group (BioLegend, San Diego, CA, USA). Non-CD8$^+$ cell fraction of PBMCs from MACS isolation, which was considered target cells for isolated CD8$^+$ T cells, was treated with 10 μg/ml of mitomycin (SPAL, Gujarat, India) at 37 °C for 40 min to cease their proliferation activity. After incubation with mitomycin, freshly isolated target cells were immediately washed 4 times with a sterile PBS solution and kept at 4 °C for 2 h till the effector cells were incubated with anti-PD-1 and anti-TIM-3 antibodies and prepared for co-culture. To activate the isolated CD8$^+$ T cells, a 96-well culture plate was coated with 2 μg/ml of purified anti-human CD3 antibody (Clone OKT3, BioLegend,

Table 1 Major clinical and laboratory characteristics of CLL patients

No	Age	Sex	WBC $\times 10^3/mm^3$	Lym (%)	PLT $\times 10^3/mm^3$	Hb (g/dL)	LDH (IU/L)	Rai stage	Organomegaly	CD5$^+$/CD19$^+$ percentage*
1	80	F	9.1	38	425	12.1	NA	NA	NA	70
2	50	F	4.2	88	157	12.5	NA	0	-	65
3	64	M	17.9	79	111	13.3	401	I	LAP	80
4	61	M	104.7	90	226	16.6	354	0	-	82
5	46	M	10.1	57	134	13	281	0	-	78
6	NA	F	18.0	74	306	13.1	NA	NA	NA	71
7	78	M	42.2	86	231	NA	348	0	-	88
8	61	F	48.0	NA	NA	13.1	NA	NA	NA	74
9	63	F	17.2	60	176	12.7	534	0	-	62
10	58	M	46.2	77	160	10.5	796	0	-	76
11	78	M	49.2	92	164	14	1008	0	-	77
12	60	F	82.0	57	510	11.9	510	0	-	91
13	76	F	78.2	91	139	13.7	599	II	LAP/SPM	90
14	65	M	35.4	80	150	12.6	422	0	-	74
15	64	F	21.4	77	220	14.1	387	0	-	69
16	57	M	25.5	76	188	14.4	272	0	-	64
17	61	M	28.5	86	126	14.0	339	I	LAP	87
18	62	M	43.6	88	91	13.6	1380	I	LAP	82
19	53	M	28.8	79	223	11.8	452	I	LAP	55
20	77	M	94.7	91	209	12.9	384	0	-	76

CLL, chronic lymphocytic leukemia; *M*, male; *F*, female; *WBC*, white blood cell count; *Lym*, lymphocyte percent in peripheral blood; *PLT*, platelet count; *Hb*, hemoglobin; *LDH*, serum lactate dehydrogenase; *LAP*, lymphadenopathy; *SPM*, splenomegaly; *NA*, not available

*Percentage of CD5$^+$/CD19$^+$ double-positive cells in lymphoid gate obtained from flow cytometry

San Diego, CA, USA) at 4 °C overnight. Finally, PD-1- and TIM-3-blocked CD8$^+$ T cells and mitomycin-frozen target cells (1:1 ratio) together with 1 μg/ml of purified anti-human CD28 antibody (BioLegend, San Diego, CA, USA) were added to the coated plate in 200 μl RPMI-1640 medium; supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum (Biosera, Nuaille, France); and then incubated at 37 °C with 5% CO_2 for 72 h to assess the proliferation activity of blocked CD8$^+$ T cells. For degranulation and cytokine production assays, the same procedure was applied except that the stimulation was performed with 2 μl/ml of cell stimulation cocktail, PMA/ionomycin (eBioscience, San Diego, CA, USA), in 200 μl RPMI-1640 medium at 37 °C with 5% CO_2 overnight. All tests were run in duplicate.

MTT assay for the proliferation of CD8$^+$ T lymphocytes

After 72 h of stimulation with anti-CD3/CD28 antibodies, the proliferation of blocked CD8$^+$ T cells was measured by MTT assay (Sigma-Aldrich, MO, USA) according to the manufacturer's protocol. Briefly, MTT reagent was added to each well at a final concentration of 0.5 mg/ml. Following incubation for 4 h, the flat-bottomed 96-well microplates were centrifuged at

300g for 10 min, the supernatants were discarded, and 150 μl of DMSO was added to each well. Purple crystals of formazan were dissolved by gently shaking of microplates, and absorbance was measured using a microplate spectrophotometer (Synergy H1 BioTek, Winooski, USA) at 570 nm. Stimulation index (SI) was calculated by dividing the mean ratio of optical density (OD) values of stimulated cells treated with anti-CD3/CD28 antibodies by that of unstimulated cells.

Evaluation of degranulation activity by CD107a expression assay

To evaluate the degranulation activity of isolated T-CD8$^+$ lymphocytes, CD107a degranulation assay was applied. Isolated CD8$^+$ T cells (10^5 cells) were initially treated with blocking anti-PD-1 and anti-TIM-3 antibodies for 2 h and then cocultured with mitomycin-frozen non-CD8$^+$ cell fraction of PBMCs as target cells (1:1 ratio). Thirty minutes after stimulation with PMA/ionomycin, anti-CD107a mAbs-PE (clone H4A3, eBioscience, San Diego, CA, USA) and isotype control antibody (clone P3.6.2.8.1, eBioscience, San Diego, CA, USA) were added to the corresponding wells. Following overnight stimulation, cells were harvested and washed with a washing buffer (PBS 0.15 M, pH: 7.4, BSA 0.5%) and then

were incubated with anti-CD8 FITC (clone SK-1.CF, eBioscience, San Diego, CA, USA) at 4 °C in dark for 45 min. Afterward, cells were washed with a washing buffer and fixed in 1% paraformaldehyde (Merck, Darmstadt, Germany). Samples were then analyzed on the Partec PAS flow cytometer system (Partec GmBH, Munster, Germany) using the FloMax software. Following gating on the lymphocyte population, CD8$^+$ T cells were selected, and then the expression of surface CD107a on CD8$^+$ T cells was determined.

Cytokine production by ELISA

As described in degranulation assay, isolated CD8$^+$ T cells (10^5 cells) were treated with blocking anti-PD-1 and anti-TIM-3 antibodies for 2 h and then cocultured with mitomycin-frozen non-CD8$^+$ cell fraction of PBMCs as target cells (1:1 ratio). After overnight stimulation with PMA/ionomycin, culture supernatants were collected and stored at −20 °C for cytokine assays. Concentrations of IFN-γ (sensitivity, 4 pg/ml), TNF-α (sensitivity, 4 pg/ml), IL-10 (sensitivity, 2 pg/ml), and IL-2 (sensitivity, 2 pg/ml) were measured in supernatants by ELISA according to the manufacturer's protocol (all from eBioscience, San Diego, CA, USA).

Statistical analyses

Statistical analyses were performed with the GraphPad Prism 6 and SPSS20 software. All data are expressed as mean ± SD. Normal distribution of data was tested with the Kolmogorov–Smirnov test. Non-parametric Mann–Whitney U and Kruskal–Wallis tests were appropriately used to calculate the mean difference between two or more groups. The significance was set at $p < 0.05$.

Results

Flow cytometric analysis of MACS-isolated CD8$^+$ T cells from CLL patients

Following MACS isolation, dual-color flow cytometric analysis with anti-CD8 and anti-CD3 was performed on positively selected CD8$^+$ T cells from CLL patients. As shown in Fig. 1, the purity of isolated CD8$^+$ T cells was always more than 97% in all patients.

Blockade of PD-1 and TIM-3 receptors did not improve the proliferation of CD8$^+$ T cells in CLL patients

Purified CD8$^+$ T cells from 20 CLL patients were treated with anti-TIM-3 and anti-PD-1 as well as with isotype-matched control antibodies and then cocultured with/without

mitomycin-frozen target cells (non-CD8$^+$ T cell fraction of PBMCs). Treated cells were further stimulated with anti-CD3/CD28 antibodies for 72 h. The proliferation capacity of CD8$^+$ T cells was determined by MTT assay. Obtained results showed that CD8$^+$ T cells from both control and blocked groups showed similar proliferation responses either in the presence or absence of target cells (Fig. 2). Representative histograms show stimulation indices between control and blocked groups both among cultures with/without target cells ($p > 0.05$).

Isolated CD8$^+$ T cells from CLL patients showed similar degranulation properties following blocking with anti-TIM-3 and anti-PD-1 antibodies

Since blockade of TIM-3 and PD-1 did not enhance the proliferation of CD8$^+$ T cells, we next addressed whether the same would be true in the degranulation activity of these cells after blockade of TIM-3 and PD-1 axes. To do so, MACS-purified CD8$^+$ T cells from CLL patients were treated with anti-PD-1, anti-TIM-3, and corresponding isotype-matched control antibodies, then cocultured with/without mitomycin-frozen target cells (non-CD8$^+$ T cell fraction of PBMCs). Cell cultures were then stimulated with PMA/ionomycin cocktail and, finally, the expression of CD107a was measured by flow cytometry. Our data showed no significant difference between control and blocked groups in terms of CD107a expression ($p > 0.05$, Fig. 3).

Blockade of TIM-3 and PD-1 signaling in isolated CD8$^+$ T cells did not improve the production of pro-inflammatory cytokines

To confirm the consistency of previous results regarding the functional assay of isolated CD8$^+$ T cells, we next sought to determine the cytokine production profile of these cells. Thus, positively selected CD8$^+$ T cells from CLL patients were treated with anti-TIM-3, anti-PD-1, and isotype-matched control antibodies, then cocultured with/without mitomycin-frozen target cells (non-CD8$^+$ T cell fraction of PBMC). Cell cultures were stimulated with PMA/ionomycin cocktail overnight, and culture supernatants were collected to measure the levels of IFN-γ, IL-2, IL-10, and TNF-α. As shown in Fig. 4, the cytokine production level between control and blocked groups was not significantly different ($p > 0.05$).

Discussion

Although blocking antibodies against inhibitory immune checkpoint receptors have shown promising treatment modalities for multiple cancers, many challenges and milestones are on the road to a definite advisable conclusion. In our previous

Fig. 1 Purity analysis of isolated CD8^{+} T cells from CLL patients. Dual-color (anti-CD8-FITC and anti-CD3-PE) flow cytometric analysis was performed on MACS-isolated CD8^{+} T cells from CLL patients. A representative dot plot obtained from one patient is shown
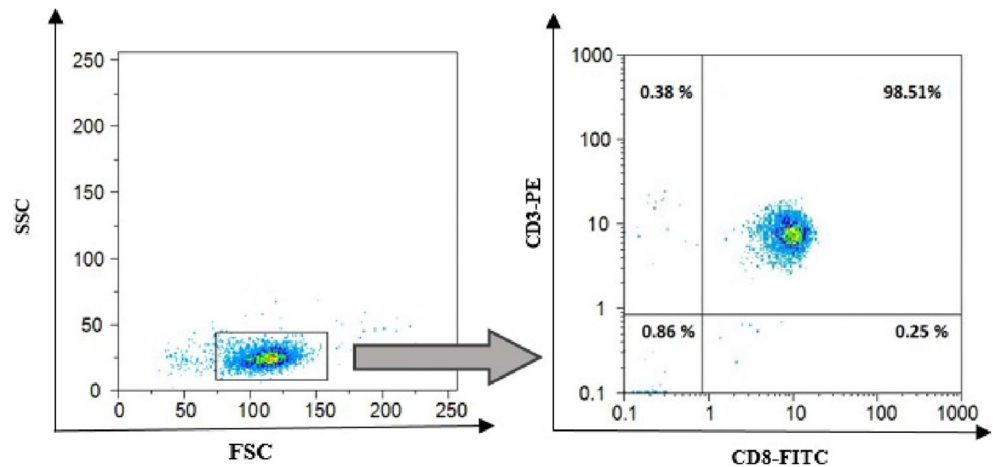
studies, we observed higher expression of PD-1 and TIM-3 molecules on both CD8^{+} and CD4^{+} T cells of CLL patients and also the more expression of Gal-9 and PD-L1 mRNA in these patients which corresponding functional studies confirmed the significant state of exhaustion on both T cells [17–19]. Moreover, we observed that TIM-3 is significantly upregulated on natural killer (NK) cells of CLL patients which was accompanied by lower expression of NKp30-activating receptor, confirming the low functional activities of NK cells in these patients [20]. In our current in vitro study, we decided to investigate whether blockade of PD-1 and TIM-3 inhibitory molecules would reverse the exhaustion state of peripheral CD8^{+} T cells in CLL patients or not. It has been shown that blockade of PD-1/PD-L1 axis with specific antibodies, such as nivolumab, has a promising therapeutic effect in many cancers including melanoma [10], head and neck squamous cell cancer [15], non-small cell lung cancer [12], renal cell cancer [13],

urothelial cancer [11], and relapsed or refractory Hodgkin's lymphoma [14]. Although treatment with ipilimumab (anti-CTLA-4 antibody and the first FDA-approved antibody for the treatment of advanced melanoma), nivolumab, and pembrolizumab has proved to be relatively effective, there are large numbers of patients with different types of cancer who do not respond to these drugs. Therefore, further studies in this area are required and more strategies for blocking of other inhibitory receptors, such as the TIM-3 molecule, need to be considered [21]. Monoclonal antibodies against TIM-3 restored T cell function and cytokine production in HIV-1-specific T cells [22] and antigen-specific CD8^{+} T cells from patients with gastric cancer [23] and melanoma [24]. However, despite the upregulation of TIM-3 and PD-1 on T cells from CLL subjects, our in vitro results showed that blocking of these receptors does not generally have effective impacts on restoring the functional activities of CD8^{+} T cells. Various functional properties of
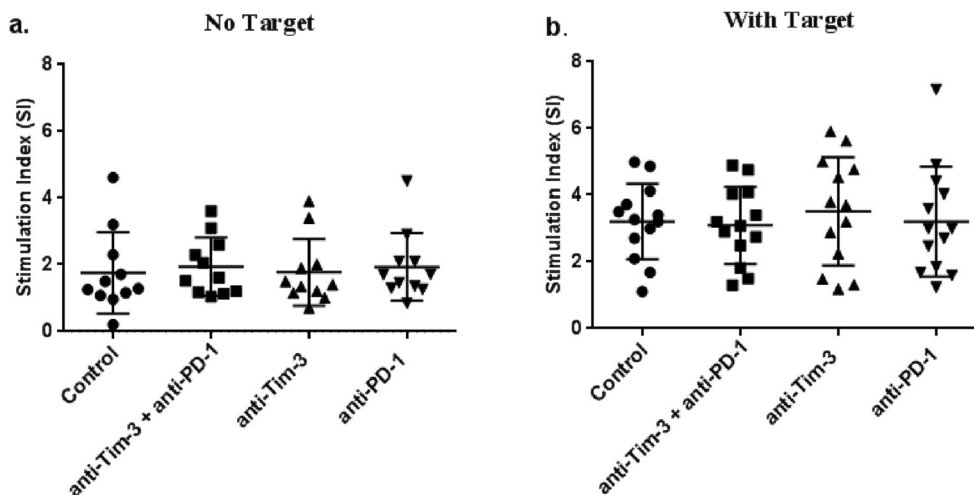

Fig. 2 Proliferation responses of isolated CD8^{+} T cells after blocking with anti-PD-1 and anti-TIM-3 antibodies. Isolated CD8^{+} T cells from all CLL patients were treated with anti-PD-1 and anti-TIM-3 as well as corresponding isotype antibodies and then cocultured with/without mitomycin-frozen target cells in one series. Treated cells were further stimulated with anti-CD3/CD28 antibodies, and cell proliferation was determined by MTT assay. Stimulation index (SI) was calculated by dividing the OD values of stimulated cells treated with blocking/isotype antibodies by that of unstimulated cells with no treatments. Representative histograms show no significant difference between control and blocked groups ($p > 0.05$). Both graphs present the mean ± SD
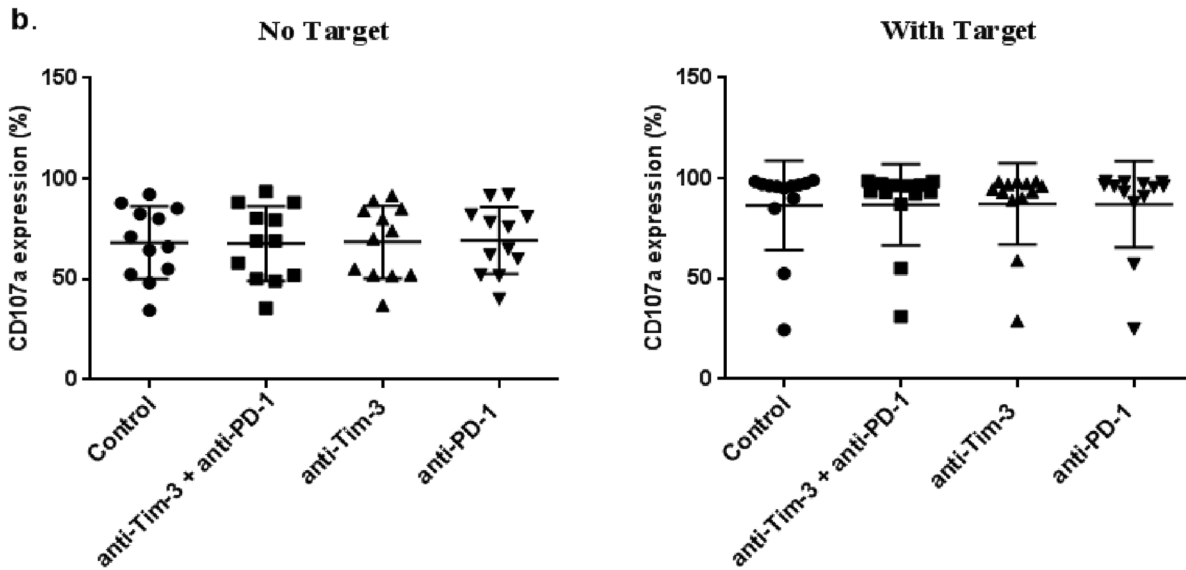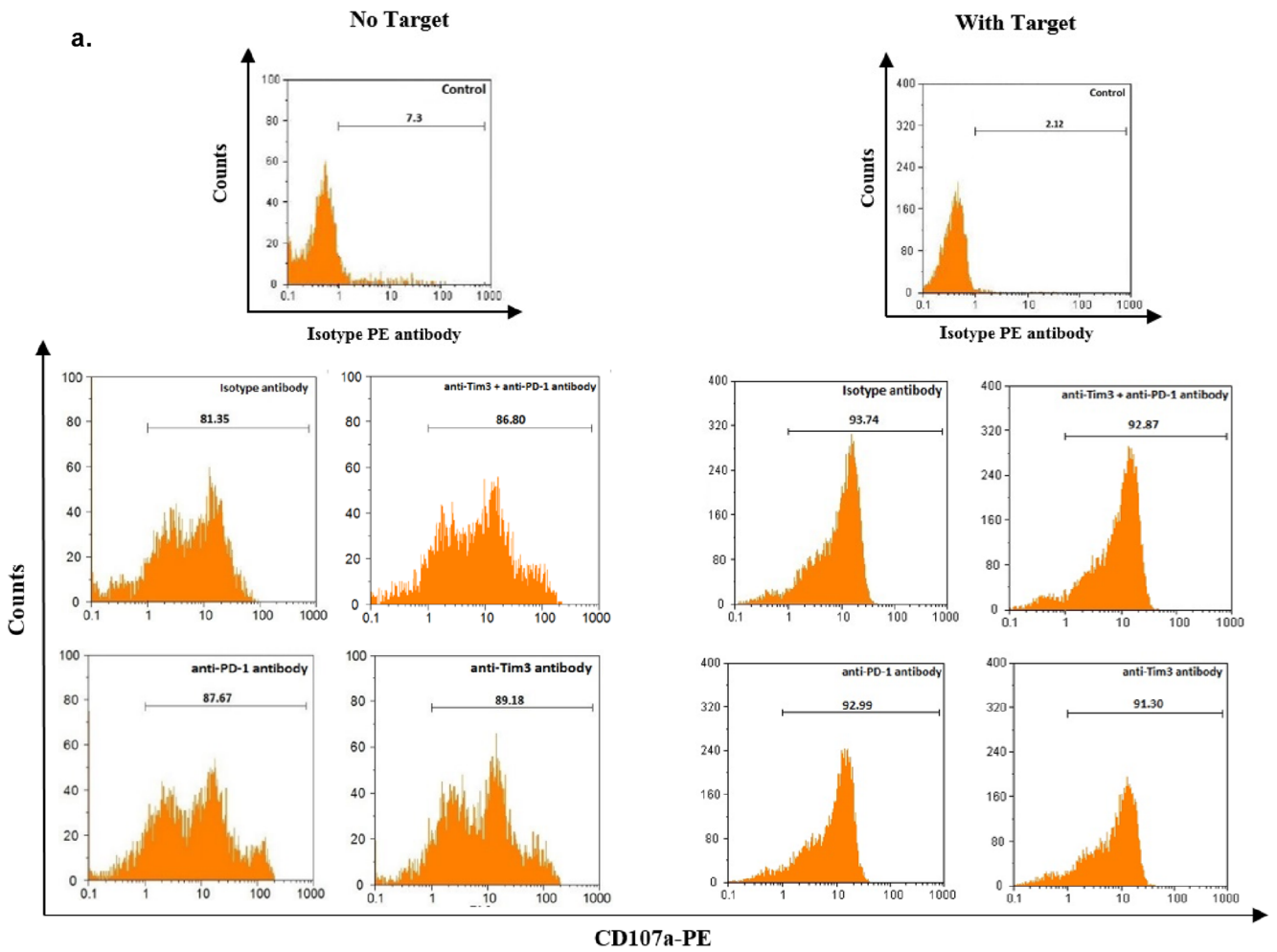
a.

No Target   **With Target**

CD107a-PE

b.

Fig. 3 Degranulation assay of isolated CD8$^+$ T cells from CLL patients by flow cytometric analysis of CD107a expression after blocking with anti-PD-1 and anti-TIM-3 antibodies. MACS-isolated CD8$^+$ T cells from CLL patients were treated with anti-TIM-3, anti-PD-1, and corresponding isotype antibodies, and then cocultured with/without mitomycin-frozen target cells. Cell cultures were stimulated with PMA/ionomycin cocktail, and the expression of CD107a was measured on CD8$^+$ T cells by flow cytometry. No significant differences were observed among control and blocked groups ($p > 0.05$). **a** A representative flow cytometry histogram for CD107a expression obtained from a CLL patient is shown. **b** CD107 expression on CD8$^+$ T cells from all CLL patients is shown. Both graphs present the mean ± SD

CD8$^+$ T cells including proliferation, degranulation, and cytokine production were investigated, and obtained data from all experiments showed no improvements after blocking with anti-PD-1 and anti-TIM-3 antibodies. By working on the Eμ-TCL1 CLL mouse model, McClanahan et al. have indicated the functional changes of T cells during CLL development, high PD-

L1/PD-L2 expression in leukemic cells and low functional activity of PD-1$^+$ T cells, confirming the potential role of this pathway in the progression of CLL [25]. Later on, the first clinical trial conducted on CLL patients demonstrated the clinical efficacy of pembrolizumab in CLL with Richter transformation and not relapsing CLL patients [26]. A preclinical study on a CLL mouse model reported that while mice receiving dual blockade of PD-1 and LAG-3 show promising results and successful control of disease development, single anti-PD-1 therapy has no clinical effects [27]. No clinical effects of single anti-PD-1 therapy on CLL development have been recently confirmed by Hanna et al., showing that a combination of ibrutinib with PD-1 blockade improved the effector function of CD8$^+$ T cells and control of CLL [28]. Taking these considerations into account, no improvement in the function of CD8$^+$ T cells after blockade of the PD-1 pathway could be addressed in our CLL patients. On the other hand, we have isolated peripheral blood
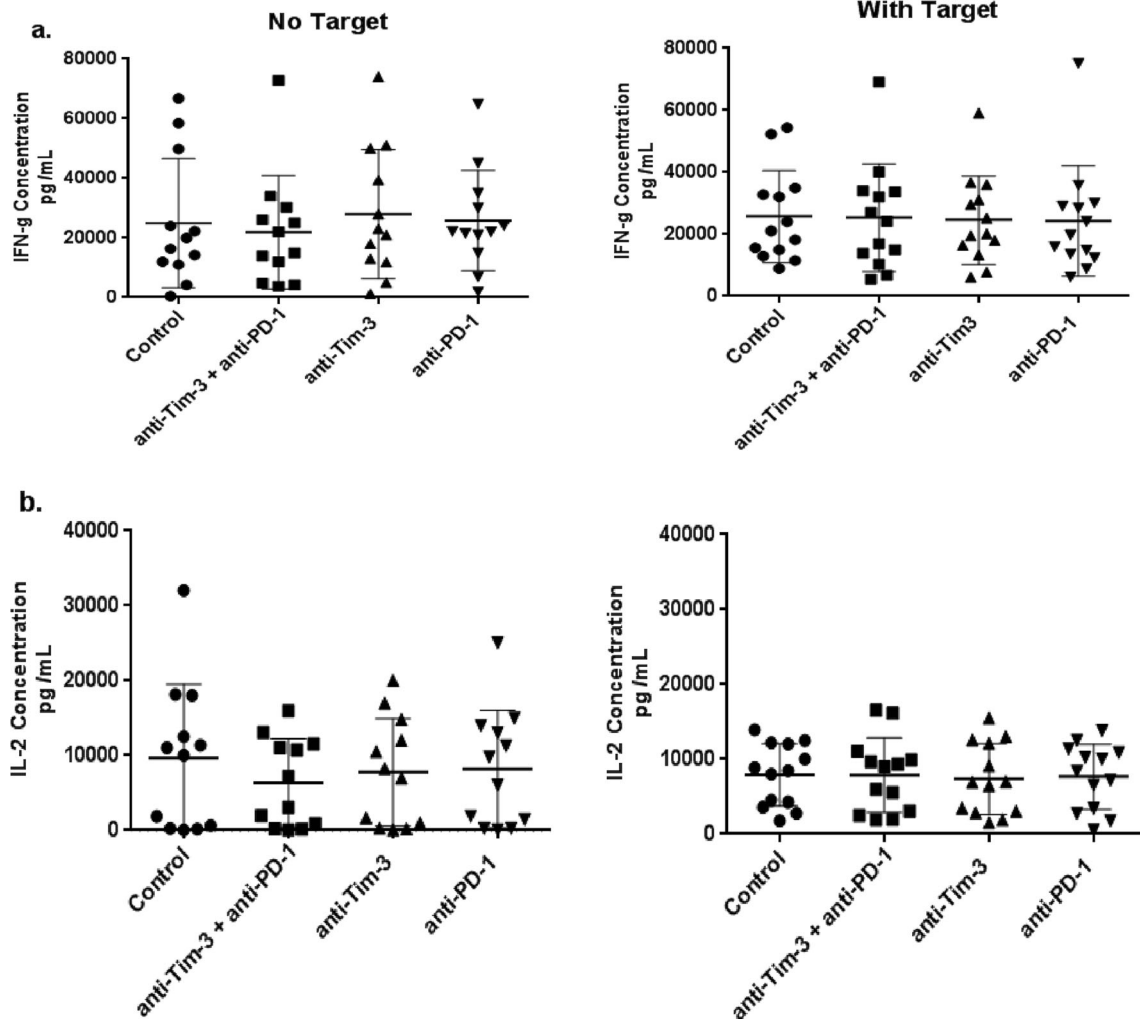

Fig. 4 The cytokine production profile of isolated CD8$^+$ T cells from CLL patients after blocking with anti-PD-1 and anti-TIM-3 antibodies. Positively selected CD8$^+$ T cells from CLL patients were treated with anti-TIM-3, anti-PD-1, and isotype-matched control antibodies, then cocultured with/without mitomycin-frozen target cells. Cell cultures were then stimulated with PMA/ionomycin cocktail, and culture supernatants were collected to measure the levels of IFN-γ (**a**), IL-2 (**b**), IL-10 (**c**), and TNF-α (**d**) by ELISA. Produced cytokine levels between control and blocked groups were not significantly different ($p > 0.05$). All graphs present the mean ± SD
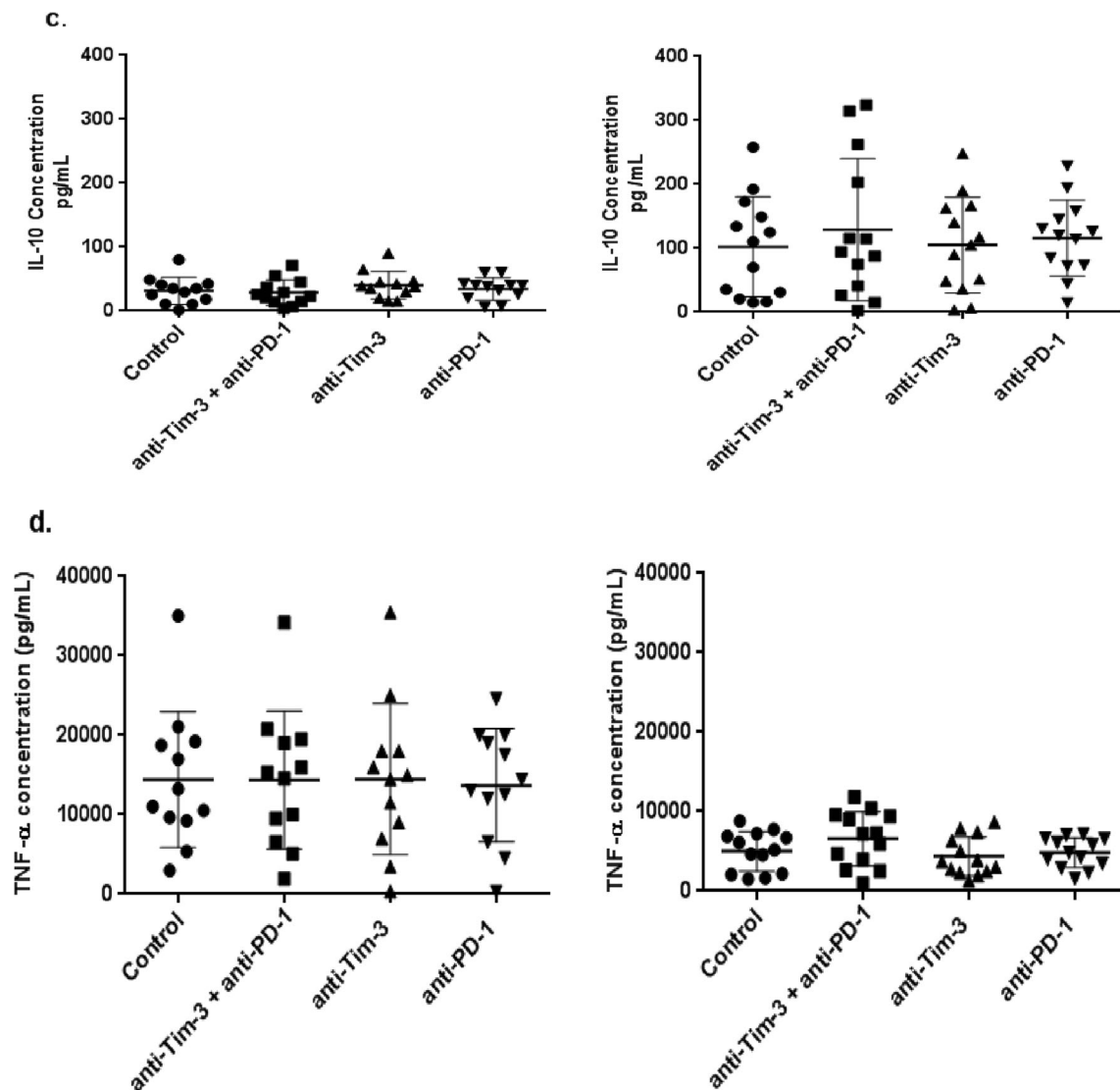
c.

d.

Fig. 4 (continued)

CD8^{+} T cells and evaluated their functional restoration after blocking with anti-PD-1 and anti-TIM-3. As reported recently by Hanna et al. [29] and Weerdt et al. [30], lymphocytes in peripheral lymphoid organs of CLL patients and the mouse model show more features of exhaustion than those of peripheral blood. So, the obtained blocking results might be different if it was possible to apply the lymphocytes from peripheral lymph nodes in our experiments. But, when we look at the clinical trial results regarding no response of CLL patients to anti-PD-1 therapy, more confirmatory studies are needed to address these issues. In HIV-1-specific T cells, blocking of the TIM-3 pathway restored proliferation capacity and improved cytokine production [22]. Similarly, in a murine model of colon carcinoma, dual blockade of PD-1 and CTLA-4 increased proliferation of CD8^{+} and CD4^{+} T cells and cytokine release [31]. Our results present some contradiction with these studies, and this might be due to the fact that PD-1 and TIM-3

are not the only inhibitory receptors expressed on T cells in CLL patients. Riches et al. demonstrated that TIM-3 is not upregulated on CLL cells, but there are other inhibitory receptors including CD244, CD160, and PD-1 which are overexpressed in CLL patients, and consequently, they may prevail the effect of the PD-1 or TIM-3 blockade [32]. The same has been seen in preclinical models that single blockade of CTLA-4 or PD-1 has led to the upregulation of the other immune inhibitory pathways which is not blocked [33].

IFN-γ, IL-2, and TNF-α are major cytokines that are produced from CD8^{+} T cells in case of recognition of the tumor cells to provide better protection by recruitment of more cells to the tumor site [34]. Again, Riches et al. showed that although T cells from CLL patients present exhaustion properties, they are able to produce various cytokines [32]. This might be one of the possible reasons why we could not observe any significant difference in cytokine production profile

among our blocked and control groups in CLL patients. Contrarily, some studies on mouse model of CLL and HIV infection have shown that cytokine release improves after blockade of the inhibitory receptors such as PD-L1, TIM-3, LAG-3, and CTLA-4 from CD8$^+$ T cells [35, 36]. Altogether, here, we studied the function of isolated CD8$^+$ T cells in different conditions to somehow simulate the CLL microenvironment in peripheral blood. The results obtained from blocked and control groups were similar, confirming that blockade of PD-1 and TIM-3 pathways has no positive effects in CLL patients. Although there were not any significant differences between blocked and control groups in terms of IL-10 and TNF-α production, we found more production of IL-10 in the "with target" compared with the "no target" group, indicating that tumoral cells produce IL-10 to evade host immune response (Fig. 4c). The scenario for TNF-α was different, in which the "no target" group showed more production of this cytokine in comparison with the "with target" group (Fig. 4d). Since TNF-α is not produced by leukemic cells, the low production of TNF-α was observed in co-culture of CD8$^+$ effector T cells with target leukemic cells.

In general, CLL patients at the early stages of the disease (Rai stages 0–II) do not need any treatments, unless the disease progresses and cytopenia occurs [37]. Following disease progression and based on molecular criteria, various chemotherapy and immunotherapy strategies are applied. Based on the data provided in this study, our CLL patients were generally at lower stages and do not present progression criteria. Therefore, we speculate that blockade of PD-1 and TIM-3 inhibitory receptors is not effective at the early stages of CLL, and it might be more applicable in patients with higher stages or more aggressive forms of the disease. In our previous study, we observed more expression of both PD-L1 and Gal-9 in CLL patients compared with controls, which was more remarkable in patients at advanced clinical stages [19]. Based on those results, we decided to conduct the current study. But, it seems that CLL leukemic cells from patients at early clinical stages do not express PD-L1 as much as other responding tumors to the PD-1/PD-L1 blockade like melanoma and lung cancer. Behdad et al. in a study on the expression of PD-1 on leukemic B cells of patients with Richter syndrome, which is defined as an aggressive transformation of CLL, showed that there is a correlation between higher PD-1 expression and clinical severity [38]. In accordance with this finding, in a cohort of patients with Richter syndrome, treatment with the combination of ibrutinib (Bruton's tyrosine kinase inhibitor) and nivolumab (anti-PD-1) showed encouraging overall response of 65%, while responses in patients with relapsed/refractory CLL and follicular lymphoma were only seen in treatment with ibrutinib alone [39]. Besides, there are two phase II clinical trials that resulted in promising effects of combination therapy of PD-1 blockade and ibrutinib in patients with Richter syndrome [40, 41], which more confirm the importance of disease stage in response to immune checkpoint therapy. Moreover, data from different preclinical and clinical studies indicated that advanced age in subjects with cancer is associated with changes in quality and quantity of immune system especially in T cell receptor repertoire and naive to memory T cell ratio, to the extent that it could decrease the response to immunotherapies [42]. Besides these interpretations, it should be noted that up to now, clinical single anti-PD-1 therapy failed both in mouse model and CLL patients. These discrepancies indicate that exhaustion in CLL needs to be completely understood, and signaling pathways down the road awaits us to be explored.

In conclusion, our study is the first in vitro study on CLL patients which applies blocking antibodies against two important inhibitory receptors, PD-1 and TIM-3, to reverse the function of exhausted CD8$^+$ T cells. Our results demonstrated that blockade of these receptors is not an effective approach toward reactivation of CD8$^+$ T cells from CLL patients at the early stages of the disease. Further studies on advanced stages of the disease as well as on the murine model of CLL are required to provide more actual conditions of the tumor microenvironment and a better understanding of the application of immune checkpoint inhibitors in targeted immunotherapy of CLL.

Compliance with ethical standards

References

1. Brusa D, Serra S, Coscia M, Rossi D, D'Arena G, Laurenti L, et al. The PD-1/PD-L1 axis contributes to T-cell dysfunction in chronic lymphocytic leukemia. Haematologica. 2013;98(6):953–63.

2. Society AC. Estimated number of deaths for selected cancers by state, US, 2019. 2019.

3. Eichhorst B, Robak T, Montserrat E, Ghia P, Hillmen P, Hallek M, et al. Chronic lymphocytic leukaemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2015;26(suppl_5):v78–84.

4. Sun C, Dotti G, Savoldo B. Utilizing cell-based therapeutics to overcome immune evasion in hematologic malignancies. Blood. 2016;127(26):3350–9.

5. Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK, et al. PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. Front Pharmacol. 2017;8:561.

6. Dougall WC, Kurtulus S, Smyth MJ, Anderson AC. TIGIT and CD 96: new checkpoint receptor targets for cancer immunotherapy. Immunol Rev. 2017;276(1):112–20.

7. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. Immunity. 2016;44(5):989–1004.

8. Das M, Zhu C, Kuchroo VK. Tim-3 and its role in regulating anti-tumor immunity. Immunol Rev. 2017;276(1):97–111.

9. Greaves P, Gribben JG. The role of B7 family molecules in hematologic malignancy. Blood. 2013;121(5):734–44.

10. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med. 2013;369(2):134–44. https://doi.org/10.1056/NEJMoa1305133.

11. Balar A, Bellmunt J, O'donnell P, Castellano D, Grivas P, Vuky J, et al. Pembrolizumab (pembro) as first-line therapy for advanced/unresectable or metastatic urothelial cancer: preliminary results from the phase 2 KEYNOTE-052 study. Eur Soc Med Oncol; 2016.

12. Gadgeel SM, Stevenson J, Langer CJ, Gandhi L, Borghaei H, Patnaik A, et al. Pembrolizumab (pembro) plus chemotherapy as front-line therapy for advanced NSCLC: KEYNOTE-021 cohorts AC. Am Soc Clin Oncol 2016.

13. Motzer RJ, Sharma P, McDermott DF, George S, Hammers HJ, Srinivas S, et al. CheckMate 025 phase III trial: outcomes by key baseline factors and prior therapy for nivolumab (NIVO) versus everolimus (EVE) in advanced renal cell carcinoma (RCC). Am Soc Clin Oncol 2016.

14. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. N Engl J Med. 2015;372(4):311–9.

15. Seiwert TY, Burtness B, Mehra R, Weiss J, Berger R, Eder JP, et al. 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. 2016;17(7):956–65.

16. Tomkowicz B, Walsh E, Cotty A, Verona R, Sabins N, Kaplan F, et al. TIM-3 suppresses anti-CD3/CD28-induced TCR activation and IL-2 expression through the NFAT signaling pathway. PLoS One. 2015;10(10):e0140694.

17. Allahmoradi E, Taghiloo S, Tehrani M, Hossein-Nattaj H, Janbabaei G, Shekarriz R, et al. CD4+ T cells are exhausted and show functional defects in chronic lymphocytic leukemia. Iran J Immunol : IJI. 2017;14(4):257–69.

18. Taghiloo S, Allahmoradi E, Tehrani M, Hossein-Nataj H, Shekarriz R, Janbabaei G, et al. Frequency and functional characterization of exhausted CD8+ T-cells in chronic lymphocytic leukemia. Eur J Haematol. 2017;98:622–31. https://doi.org/10.1111/ejh.12880.

19. Taghiloo S, Allahmoradi E, Ebadi R, Tehrani M, Hosseini-Khah Z, Janbabai G, et al. Upregulation of galectin-9 and PD-L1 immune checkpoints molecules in patients with chronic lymphocytic leukemia. Asian Pac J Cancer Prev. 2017;18(8):2269–74. https://doi.org/10.22034/apjcp.2017.18.8.2269.

20. Hadadi L, Hafezi M, Amirzargar AA, Sharifian RA, Abediankenari S, Asgarian-Omran H. Dysregulated expression of Tim-3 and NKp30 receptors on NK cells of patients with chronic lymphocytic leukemia. Oncol Res Treatment. 2019;42(4):197–203.

21. Du W, Yang M, Turner A, Xu C, Ferris R, Huang J, et al. TIM-3 as a target for cancer immunotherapy and mechanisms of action. Int J Mol Sci. 2017;18(3):645.

22. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, et al. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J Exp Med. 2008;205(12):2763–79. https://doi.org/10.1084/jem.20081398.

23. Lu X, Yang L, Yao D, Wu X, Li J, Liu X, et al. Tumor antigen-specific CD8+ T cells are negatively regulated by PD-1 and Tim-3 in human gastric cancer. Cell Immunol. 2017;313:43–51.

24. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med. 2010;207(10):2175–86. https://doi.org/10.1084/jem.20100637.

25. McClanahan F, Riches JC, Miller S, Day WP, Kotsiou E, Neuberg D, et al. Mechanisms of PD-L1/PD-1-mediated CD8 T-cell dysfunction in the context of aging-related immune defects in the Eμ-TCL1 CLL mouse model. Blood. 2015;126(2):212–21. https://doi.org/10.1182/blood-2015-02-626754.

26. Ding W, LaPlant BR, Call TG, Parikh SA, Leis JF, He R, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. Blood. 2017;129(26):3419–27. https://doi.org/10.1182/blood-2017-02-765685.

27. Wierz M, Pierson S, Guyonnet L, Viry E, Lequeux A, Oudin A, et al. Dual PD1/LAG3 immune checkpoint blockade limits tumor development in a murine model of chronic lymphocytic leukemia. Blood. 2018;131(14):1617–21. https://doi.org/10.1182/blood-2017-06-792267.

28. Hanna BS, Yazdanparast H, Demerdash Y, Roessner PM, Schulz R, Lichter P, et al. Combining ibrutinib and checkpoint blockade improves CD8+ T-cell function and control of chronic lymphocytic leukemia in Em-TCL1 mice. Haematologica. 2020: haematol.2019.238154. https://doi.org/10.3324/haematol.2019.238154.

29. Hanna BS, Roessner PM, Yazdanparast H, Colomer D, Campo E, Kugler S, et al. Control of chronic lymphocytic leukemia development by clonally-expanded CD8(+) T-cells that undergo functional exhaustion in secondary lymphoid tissues. Leukemia. 2019;33(3):625–37. https://doi.org/10.1038/s41375-018-0250-6.

30. de Weerdt I, Hofland T, de Boer R, Dobber JA, Dubois J, van Nieuwenhuize D, et al. Distinct immune composition in lymph node and peripheral blood of CLL patients is reshaped during venetoclax treatment. Blood Adv. 2019;3(17):2642–52. https://doi.org/10.1182/bloodadvances.2019000360.

31. Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. Cancer Res. 2013;73(12):3591–603. https://doi.org/10.1158/0008-5472.can-12-4100.

32. Riches JC, Davies JK, McClanahan F, Fatah R, Iqbal S, Agrawal S, et al. T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. Blood. 2013;121(9):1612–21.

33. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci. 2010;107(9):4275–80.

34. Yousefi H, Yuan J, Keshavarz-Fathi M, Murphy JF, Rezaei N. Immunotherapy of cancers comes of age. Expert Rev Clin Immunol. 2017;13(10):1001–15. https://doi.org/10.1080/1744666x.2017.1366315.

35. McClanahan F, Hanna B, Miller S, Clear AJ, Lichter P, Gribben JG, et al. PD-L1 checkpoint blockade prevents immune dysfunction and leukemia development in a mouse model of chronic lymphocytic leukemia. Blood. 2015;126(2):203–11. https://doi.org/10.1182/blood-2015-01-622936.

36. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature. 2006;443(7109):350–4. https://doi.org/10.1038/nature05115.

37. Hallek M. Chronic lymphocytic leukemia: 2015 update on diagnosis, risk stratification, and treatment. Am J Hematol. 2015;90(5):446–60.

38. Behdad A, Griffin B, Chen YH, Ma S, Kelemen K, Lu X, et al. PD-1 is highly expressed by neoplastic B-cells in Richter transformation. Br J Haematol. 2019;185(2):370–3.

39. Younes A, Brody J, Carpio C, Lopez-Guillermo A, Ben-Yehuda D, Ferhanoglu B, et al. Safety and activity of ibrutinib in combination with nivolumab in patients with relapsed non-Hodgkin lymphoma or chronic lymphocytic leukaemia: a phase 1/2a study. Lancet Haematol. 2019;6(2):e67–78.

40. Ding W, Le-Rademacher J, Call TG, Parikh SA, Leis JF, Shanafelt TD, et al. PD-1 blockade with pembrolizumab in relapsed CLL including Richter's transformation: an updated report from a phase 2 trial (MC1485). Am Soc Hematol 2016.

41. Jain N, Ferrajoli A, Basu S, Thompson PA, Burger JA, Kadia TM, et al. A phase II trial of nivolumab combined with Ibrutinib for patients with Richter transformation. Am Soc Hematol 2018.

42. Poropatich K, Fontanarosa J, Samant S, Sosman JA, Zhang B. Cancer immunotherapies: are they as effective in the elderly? Drugs Aging. 2017;34(8):567–81. https://doi.org/10.1007/s40266-017-0479-1.