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Scrutinizing the Expression and Blockade of Inhibitory Molecules Expressed on T Cells from Acute Myeloid Leukemia Patients

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ABSTRACT

T cell exhaustion is an immunosuppressive mechanism which occurs in chronic viral infections, solid tumors and hematologic malignancies. Exhausted T cell has increased the expression of inhibitory receptors, and functional impairment. In this study, we investigated the expression from some of those inhibitory receptors being Programmed death 1 (PD-1), T cell immunoglobulin and mucin domain containing molecules 3 (TIM-3) and CD244 on T cells from Iranian acute myeloid leukemia (AML) patients.

Peripheral blood samples were collected from Iranian newly diagnosed AML patients and flow cytometric analysis was accomplished for cell surface expression of PD-1, TIM-3, and CD244 on T lymphocytes. Functionality and proliferation assay were done in the presence of anti-PD-1 and anti-CD244 blocking antibodies.

Immunophenotyping of T cells showed a significant increase of PD-1 and CD244 expression on CD4+ and CD8+ T cells of AML patients. Whereas blockade of PD-1 and CD244 increased the proliferation of CD4+ and CD8+ T lymphocytes of AML patients but IFN-γ production was not significantly increased.

In conclusion, our data indicate that CD4+ and CD8+ T cells from AML patients appeared to be exhausted and blockade of some immune checkpoints can improve the proliferation of those cells.

Keywords: Acute myeloid leukemia; CD244; PD-1; T cell exhaustion; TIM-3

INTRODUCTION

T cell exhaustion is a phenomenon that occurs during chronic antigen exposure to viral infections and cancers. Exhausted T cells indicate impaired effector function and proliferative capacity, decreased production of inflammatory cytokines and overexpression of inhibitory receptors. Exhausted
T cells are defective in controlling tumors or infections. There are numbers of cell surface inhibitory receptors which modulate T cell exhaustion including Programmed death 1 (PD-1), T cell immunoglobulin and mucin domain containing molecules 3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), CD160, CD244, Lymphocyte activating gene 3 (LAG-3) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT).\(^1\)\(^2\) T cell exhaustion for the first time was described in chronic lymphocytic choriomeningitis virus (LCMV) infection.\(^3\) T cell dysregulation and overexpression of inhibitory receptors also occur in hematologic malignancies including myeloid leukemia, lymphoid neoplasms and also in solid tumors such as melanoma.\(^4\)\(^6\) Immune suppression and T cell dysfunction are one of the important mechanisms of tumor escape in malignancies.\(^7\)

PD-1 is one of the first inhibitory receptors that have been described in association with exhaustion. It belongs to B7: 28 families and has an inhibitory function. PD-1 is expressed on CD4\(^+\) T cells, CD8\(^+\) T cells, B cells, NK cells and monocytes.\(^8\)\(^11\)

PD-1 signaling is one of the most important mechanisms of tumor escape.\(^12\) TIM-3 is another member of inhibitory receptors which was first defined in mouse T helper1 cells. TIM-3 is expressed on T cells, macrophages and dendritic cells.\(^8\) Further CD244 belongs to signaling lymphocyte activation molecule (SLAM) family which expresses on a variety of immune cells including NK cell, T cell, monocyte, eosinophil, and basophil. The High expression level of CD244 triggers inhibitory signal through recruitment of phosphatases like SHP1, SHP2, and SHIP.\(^13\)\(^14\)

Acute myeloid leukemia (AML) is a hematologic disorder with overgrowth of leukemic clones and increase of the hematopoietic precursor in bone marrow and peripheral blood which occurs in all ages but its incidence is higher in aged people. The symptom of the disease includes anemia, fatigue, and dyspnea, thrombocytopenia and neutropenia. Treatment of AML is not prosperous. Some leukemic cells are resistant to chemotherapy and recurrences of AML after complete remission is very common.\(^15\) Whereas, allogeneic hematopoietic stem cell transplantation (HSCT) can be a good choice for treatment and causes graft-versus-leukemia (GVL) however, AML specific allogenic T cells become non-responsive within the tumor microenvironment. Over expression of inhibitory receptors on AML-specific T cell has been demonstrated to reduce GVL response and blockade of these receptors can restore functionality of effector T cells.\(^16\)\(^18\)

In this study we analyzed the expression of inhibitory receptors PD-1, TIM-3 and CD244 on T cells of peripheral blood from newly diagnosed AML patients and scrutinized the proliferation potency and cytokine production of those exhausted T cells in the presence of some inhibitory receptors blockade.

**MATERIALS AND METHODS**

**Patients**

Heparinized Peripheral blood samples were collected from 30 newly diagnosed AML patients from Hematology and Oncology Clinics of Shariati Hospital (Tehran University of Medical Sciences) before therapeutic intervention. The mean age of AML patients was 36.5 years (range, 17-59 years) and 15 Peripheral blood samples from age-matched healthy volunteers (mean age of 32.1 years) were included in this study as a control group. Informed consent was obtained from participants in conformity with Ethical Committee of Tehran University of Medical Sciences (N. 93-01-30-24991-261406). The major clinical characteristics of patients are summarized in Table 1.

**Isolation of PBMCs**

Peripheral blood obtained from patients and healthy controls were diluted 1:1 with phosphate buffered saline (PBS) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on ficoll-Hypaque (lymphodex, Inno-Train, Germany). Isolated cells were washed twice with PBS. The viability of isolated cells was over 95% as checked by trypan blue staining.

**Immunofluorescence Staining of Lymphocytes**

Immunophenotyping of cell surface antigens was accomplished using fluorescence-conjugated monoclonal antibodies against CD3 (PE-CY7), CD8(PE), CD4(FITC), CD244 (APC), PD-1 (APC) and TIM-3(APC), all antibodies were purchased from BioLegend, San Diego, CA, USA. Related isotype control antibodies were also used. Using BD FACSCalibur flow cytometer (Becton-Dickinson, CA, USA) a four-color flow cytometry panel was carried out to determine the expression of inhibitory receptors on gated CD4\(^+\) and CD8\(^+\) T cells populations. Acquired
data were analyzed using FlowJo Version 7.6.1 software (Tree Star).

**In Vitro Stimulation and Intracellular Cytokine Assay**

\[2 \times 10^5\] PBMCs were cultured (complete medium of RPMI-1640 with 10% FBS) with 2 μg/mL anti-CD3 and 5 μg/mL anti-CD28 antibodies in the presence or absence of 10 μg/mL anti-PD-1 and anti-CD244 blocking antibodies for 48 hours followed by 4-hour incubation with Brefeldin A (BioLegend, San Diego, CA, USA) at 10 μg/mL concentration. After incubation, the cells were collected and stained with anti CD4-PE and anti CD8-FITC, then fixed, permeabilized and intracellularly stained with anti-IFN-γ-APC (BioLegend, USA). Appropriate isotype controls were also included in all reactions; In vitro experiments were done for 4 separate samples in duplicates.

**CFSE Proliferation Assay**

In order to evaluate the effect of PD-1 and CD244 blockade on T cell proliferation, cells were stained with 5M carboxyfluorescein succinimidyl ester (CFSE, Biolegend, USA). After quenching and washing, 10 μg/mL anti-PD-1 and anti-CD244 blocking antibodies were added to each well and cells were stimulated with 2 μg/mL anti-CD3 and 5 μg/mL anti-CD28 antibodies for 5 days. Cultured cells were then flowcytometric analyzed for T cell surface markers.

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**Figure 1.** Flow cytometric analysis of inhibitory receptors expression in CD4+ T and CD8+ T cells from acute myeloid leukemia (AML) patients and normal controls. (A) Flow cytometric analysis of PBMC from AML patients directly stained against CD3, CD4, CD8. (B-D) Cumulative data (left panel) and representative dot plots (right panel) of PD-1(B), CD244(C) and TIM-3(D) expression on CD4+ and CD8+ T lymphocytes from AML patients. Error bars indicate SEM. Significant differences are showed as * \(p<0.05\), ** \(p<0.001\).
Statistical Analysis

The comparison of different groups was done by independent-sample t-tests and ANOVA analysis using SPSS 20 software IBM, USA. For all analyses, p-values<0.05 were considered as statistically significant.

RESULTS

CD4+ and CD8+ T Cells from Peripheral Blood of AML Patients Showed Increased Expression of PD-1 and CD244

By conducting multicolor flow cytometry and sequent gating, expression of inhibitory receptors PD-1, TIM-3 and CD244 were assessed on CD3+CD4+ and CD3+CD8+ T lymphocytes from peripheral blood of AML patient and healthy controls. Immunophenotyping of T cells showed significant increase of PD-1 expression on CD4+ T and CD8+ T cells from AML patients in comparison to normal controls (p=0.001, p=0.009) (Figure 1B). CD244 level was also increased in both CD4+ T and CD8+ T cell subsets of AML patients (p=0.009, p=0.01) (Figure 1C). In contrast to PD-1 and CD244, however, there was no increase of TIM-3 expression level on CD4+ cells but it slightly increased on CD8+ T cells of AML patients in comparison to normal controls (Figure 1D).

Figure 2. Proliferation assay of CD4+ and CD8+ T cells from acute myeloid leukemia (AML) patients. (A) Representative histograms of CD4+ and CD8+ T cells proliferation after 5 days stimulation with anti-CD3/CD28 antibodies in the presence of blockers or isotype antibodies (Dot curves: carboxyfluorescein succinimidyl ester (CFSE) day 0, Solid curves: CFSE day 5). (B-C) Cumulative results from T cells proliferation index in the presence of blocker antibodies or isotype antibodies: (B) CD4+ T lymphocytes data, (C) CD8+ T lymphocytes data. Error bars indicate SEM (4 samples). Significant differences are shown as *: p<0.05, **: p<0.001.
Figure 3. Intracellular IFN-γ assay in PBMCs culture. Peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3/CD28 antibodies followed by 4 hours brefeldin A treatment. (A) Representative flow cytometric based dot plots analysis from acute myeloid leukemia (AML) patients stained for CD4, CD8 and IFN-γ. (B-D) Cumulative results of T cells IFN-γ production in the presence of inhibitory receptors blocker antibodies or isotype antibody. (B) CD4+ T cells in PBMCs of AML patients, (C) CD8+ T cells in PBMC of AML patients, (D) CD4+ T cells in PBMCs of normal controls, (D) CD8+ T cells in normal controls. Error bars indicate SEM (4 samples).

Increased Proliferation of T Cells from AML Patients in Response to PD-1 and CD244 Blockade

To determine whether blockade of PD-1/CD244 can change the proliferation of T cells we stimulated PBMCs with anti-CD3/CD28 antibodies and anti PD-1/CD244 blocking antibodies or matching isotype control antibody for 5 days. Analysis of T cell proliferation was done using CFSE and to demonstrate
the phenotype of proliferating cells they were stained with human antibody directly against CD4+ and CD8+ T cells. Data were analyzed and proliferation index was calculated using Flow Jo Version 7.6.1 software. Our results showed significant proliferation of CD4+ and CD8+ T cells from AML patients, in the presence of anti-PD-1 blocking antibody ($p=0.004$, $p=0.02$) (Figures 2B and C) or combination of anti-PD-1 and anti-CD244 blocking antibodies ($p=0.004$, $p=0.03$) (Figures 2B and C). Blocking of PD-1 and CD244 molecules in the culture of PBMCs from the healthy controls group had no effect on proliferation of CD4+ T and CD8+ T cells.

PD-1 and CD244 Blockade Do Not Changed the IFN-γ Production from T Cells of AML Patient

To assess whether blocking of inhibitory molecules can change the production of IFN-γ by T cells, PBMCs from AML patients were stimulated with anti-CD3/CD28 antibodies in the presence or absence of anti-PD-1 and anti-CD244 blocking antibodies for 48 h and intracellular IFN-γ production was measured in gated CD4+ and CD8+ T cells using flow cytometry. Our results showed that IFN-γ production from CD4+ and CD8+ T cells of AML patients was not significantly increased by PD-1 and CD244 blockade (Figure 3).

Table 1. Major clinical characteristics (age, sex, FAB classification and WBC count) of acute myeloid leukemia patients in the present study

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DISCUSSION

T cells are main cells of adaptive immune response and they play a major role in tumor immunity and immunosurveillance. T cells become exhausted in the presentation of tumor antigens or chronic viral infections and lose their functionality. Memory T cells are also affected during the chronic stress of tumor and viral infection and cannot respond properly in recurrence of antigen encountering. The most important feature of exhaustion is overexpression of inhibitory molecules on T cells such as PD-1, TIM-3, LAG-3, and CD244 which can decrease proliferation capacity, cytokine production and cytotoxicity. Further, increased expression of immune checkpoints during viral infections, solid tumors, and hematologic malignancies are reported by numerous studies.

In this study, we scrutinized the expression of inhibitory receptors on PBMCs of Iranian AML patients in comparison to normal controls. We demonstrate that expression of PD-1 and CD244 are significantly increased in both CD4+ and CD8+ T cells from patients. Similar data have been reported in other hematologic malignancies such as CLL, Hodgkin lymphoma, diffuse large B-cell and primary mediastinal B-cell lymphoma, AML and multiple myeloma. Concordance to our results, Tan et al showed that PD-1 and CD244 were increased on T cells of AML patients, also CD244, PD-1, and CD160 were increased in T cells of CLL patients. Lichtenberger and colleagues indicated the rise of PD-1 receptor on CD4+ and CD8+ T cells of AML patients during disease relapse after allogeneic stem cell transplantation, whereas this receptor didn’t increase in newly diagnosed patients. In addition, they indicated that CD244 were increased only in bone marrow T cells in transplanted patients, which seems that these inhibitory molecules need longer to be expressed on peripheral T cells. Taghiloo et al showed that coexpression of TIM-3 and PD-1 increased significantly on CD8+ cells of patients in advanced stages of CLL. Other reports demonstrated that HTLV specific CD8+ T cells from adult T cell lymphoma/leukemia (ATLL) patients express PD-1 and CD244.

We observed no significant surface expression of TIM-3 levels on T cells of AML patients. Similar to our results, Riches et al detected no increase of TIM-3 expression on T cells from CLL patients. Although, after allogeneic stem cell transplantation in AML patients the level of PD-1 and TIM-3 on T cells increased and PD-1 TIM-3+ T cells were directly associated with disease relapse after transplantation. To our knowledge, based on our results and other comparable studies it seems that increased level of TIM-3 accompanied with PD-1 may only detectable in advanced stages or relapse of AML disease.

Our finding showed that PD-1 and CD244 blocking increased the proliferation level of T cells from AML patients but not on healthy people, to our knowledge until now no proliferation results were obtained after the blocked of two mentioned inhibitory receptors. Whereas, Schonorfeil et al reported no defect of proliferation from PD-1+ T cell in AML patients, PD-1+ 244+ and CD160+ CD8+ T cells from CLL patients were defective in T cell function.

Since the blocking of PD-1 and CD244 did not change the level of IFN-γ production from T cells in AML patients in our study which is in line with Schonorfeil et al study. PD-1+ TIM-3+ CD8+ and CD4+ T cells in AML relapse patients showed no defect in IFN-γ production. In addition, the exhaustion phenotype of T cells in CLL did not affect the IFN-γ production but affected the cytotoxicity of CD8 T cells. However, in HIV+ PBMCs, the in IFN-γ production was dramatically decreased.

Moreover, immune checkpoint blockade revealed that there are heterogeneous exhausted T cell populations which respond differently to anti-PD-1/PDL-1 pathway blockade, a subset of T cells may rescue through anti PD-1 antibody and other subsets would be more prone to differentiate into exhausted phenotype and have poor response to PD-1/PDL-1 blockade. T-bethi Eomeslo PD-1int cells produce more IFN-γ and TNF-α than Eomeshi T-bethi PD-1hi cells and last population had higher expression of other inhibitory receptors.

It seems that TIM-3 is a key molecule in fully exhausted T cells and the extent of T cell dysfunction is associated with degrees of exhaustion development and disease stage. Other investigation confirmed that CD4+ T lymphocytes have higher PD-1 level in more advanced stages of CLL and MM patients. Sakuishi et al indicated increased frequency of PD-1+Tim-3+ cells in melanoma mouse model and PD-1+Tim-3+ TILs (tumor infiltrating lymphocytes) were more exhausted than PD-1+Tim-3- cell and had defect in functionality. Taken together from above mentioned
studies we can conclude that stage and severity of disease over time cause the co-expression of inhibitory molecules and attenuate the T cell functionality.

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Scruburizing of Inhibitory Molecules in Acute Myeloid Leukemia


