

Higher PD-1 expression concurrent with exhausted CD8+ T cells in patients with *de novo* acute myeloid leukemia

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Abstract

Objective: To investigate the association between the T cell inhibitory receptor programmed death 1 (PD-1) and T cell exhaustion status in T cells from patients with *de novo* acute myeloid leukemia (AML) and AML in complete remission (CR).

Methods: Surface expression of PD-1 and the exhaustion and immunosenescence markers CD244 and CD57 on CD3+, CD4+ and CD8+ T cells from peripheral blood samples from 20 newly diagnosed, untreated AML patients and 10 cases with AML in CR was analyzed by flow cytometry. Twenty-three healthy individuals served as control.

Results: A significantly higher percentage of PD-1+ cells were found for CD3+ T cells in the *de novo* AML group compared with healthy controls. In addition, an increased level of PD-1+CD8+ T cells, but not PD-1+CD4+, was found for CD3+ T cells in the *de novo* AML and AML-CR samples. A higher percentage of CD244+CD4+, CD244+CD8+, CD57+CD4+ and CD57+CD8+ T cells was found in CD3+ T cells in samples from those with *de novo* AML compared with those from healthy controls. Strong increased PD-1+CD244+ and PD-1+CD57+ co-expression was found for CD4+ and CD8+ T cells in the *de novo* AML group compared with healthy controls.

Conclusions: We characterized the major T cell defects, including co-expression of PD-1 and CD244, CD57-exhausted T cells in patients with *de novo* AML, and found a particular influence on CD8+ T cells, suggesting a poor anti-leukemia immune response in these patients.

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

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Introduction

Acute myeloid leukemia (AML) is an aggressive malignant disease with unfavorable prognosis for most subtypes. Moreover, the prognosis of this disease is related to the T cell immune status of patients (1). Increasing data have revealed that T cell immunodeficiency is a common characteristic of patients with leukemia that plays an important role in leukemia progression and promotes the

expansion of malignant clones (1-3). However, different degrees of T cell dysfunctionality have been described for different leukemia subtypes and different cases (1). Previous studies have shown that such T cell immunodeficiencies are characterized by peripheral T cells that are incapable of interacting with blasts, reduced thymic output function, and oligoclonally restricted T cell repertoires, and led to low activation and low antigen responses. Moreover, the immunosuppressive microenvironment, including

dysregulation of Th subset cytokines in the bone marrow, where both the innate and adaptive immune responses are profoundly deregulated, sustains and modulates the proliferation, survival, and drug resistance of AML (4-10).

Based on the T cell immune status of AML patients, targeted molecular therapies and immune-based therapies, such as a leukemia-associated antigen (LAA) vaccine and antigen-specific cytotoxic T cells, are under investigation for managing high-risk AML (2,11). Long-term complete remission (CR) was demonstrated for some AML cases with cellular cytotoxicity against myeloid leukemia cells (3). However, the effects of such immunotherapies vary for different cases, which may be due to the T cell suppression and exhaustion that is facilitated by the T cell inhibitory receptor. For example, upregulating of programmed death 1 (PD-1) and its ligand PD-L1 mediates CD8+ T cell dysfunction in chronic lymphocytic leukemia (CLL) (12).

PD-1 is a co-receptor that is expressed on T cells and interacts with its ligands upon TCR ligation, resulting in modulation of the T cell response. The PD-1 ligands PDL1 and PDL2 are expressed on antigen presenting cells (APCs) and many tumor cells. Significantly increased PD-1/PDL1 expression is related to immunosuppression in cancer and the enhancement of resistance to immunotherapy in cancer (13,14). Recently, researchers have focused on the context of T cell exhaustion, a state of T cell dysfunction defined by the increased expression of several inhibitory receptors in combination with poor effector function, which is a unique immune inhibitory mechanism that was first demonstrated for chronic viral infections in mice and recently reported in several cancers in humans. Such T cells lose their capacity to produce cytokines, such as interleukin 2 (IL-2), tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), as well as the ability to proliferate and produce cytotoxicity, ultimately undergoing apoptosis (15). It was demonstrated that leukemia cells such as CLL can induce an exhausted T cell phenotype in a CLL mouse model, and T cell exhaustion contributes to CLL pathogenesis (16). Moreover, the exhausted T cell phenotype could be identified as co-expressing PD-1, T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) on CD8+ T cells in mice with advanced AML (17). Furthermore, it was also reported that increased PD-1^{hi}Tim-3+ T cells could predict leukemia relapse in AML patients after allogeneic stem cell transplantation (allo-HSCT) (15). However, there were different findings regarding the role of such T cell inhibitory receptors in AML. For example, it was suggested that increased PD-1 expression is only observed at the time of relapse, and T cell exhaustion does not play a major role

in AML (18). In addition, no significant impact of PD-1/PD-L1 expression on the survival of AML patients was observed in a study of a cohort of 90 patients (19). One reason explaining this result may be the immune heterogeneity found in different cohorts of patients and different leukemia subtypes.

In this study, we investigated the characteristics of the distribution of PD-1, CD244 and CD57 on CD3+, CD4+ and CD8+ T cells from patients with *de novo* AML and AML in CR. In our previous studies showing the cases in which there were low T cell activation and a significant difference in T cell dysfunction in AML (1,20), T cell exhaustion may have been a significant cause of this phenotype. There are few data regarding alterations in T cell exhaustion and T cell senescence in AML, particularly for Chinese leukemia cases.

Materials and methods

Samples

The peripheral blood samples used in this study were derived from 20 newly diagnosed, untreated AML patients including 10 males and 10 females (median age: 46 years, range: 11–81 years) and 10 cases with AML in CR including 4 males and 6 females (median age: 31.5 years, range: 20–59 years). The clinical data of the patients are listed in *Table 1*. Twenty-three healthy individuals including 10 males and 13 females (median age: 40 years, range: 19–78 years) served as controls. All human peripheral blood samples were obtained with informed consent. All procedures were conducted according to the guidelines of the Medical Ethics Committees of the Health Bureau of the Guangdong Province in China, and ethical approval was obtained from the Ethics Committee of the Medical School of Jinan University.

Sample preparation for flow cytometry

Cell surface staining analysis by flow cytometry was performed using the following antibodies (21): CD45-BV510 (clone HI30), CD3-FITC (clone HIT3a), CD8-PerCP/Cy5.5 (clone SK1), CD244 (2B4)-PE (clone C1.7), CD57-Pacific Blue (clone HCD57) (Biolegend, San Diego, USA), CD4-APC-H7 (clone RPA-T4), PD-1 (CD279)-PE-Cy7 (clone EH12.1), and a PE-Cy7 isotype control (clone MOPC-21) (BD Biosciences, San Jose, USA). Extracellular staining was performed according to the manufacturer's instructions. First, 7 μ L of conjugated fluorescent antibodies (different surface marker

Table 1 Clinical information for AML patients

No.	Subtype	Sex	Age (year)	WBC ($\times 10^9/L$)	Blast+promyelocyte cell (%)	Platelets ($\times 10^9/L$)
1	M2	M	20	9.68	88.7	14.0
2	M2	F	47	6.90	47.0	268.0
3	M2	M	20	1.58	79.0	85.0
4	M2	F	64	22.88	80.5	17.3
5	M2	M	63	7.26	46.0	17.0
6	M2	M	35	4.32	50.0	52.0
7	M2	M	81	2.06	31.0	44.0
8	M2	F	12	6.12	65.0	47.0
9	M3	F	59	4.33	83.0	4.0
10	M3	F	37	11.24	94.0	8.0
11	M3	F	38	23.18	93.5	35.0
12	M5	F	61	30.34	82.0	44.1
13	M3	M	45	20.00	70.0	42.0
14	M3	M	27	1.38	82.0	24.0
15	M3	F	47	11.12	76.0	35.0
16	M3	M	32	7.20	52.0	32.0
17	M5	F	70	16.27	75.0	24.0
18	M5	M	60	10.24	70.0	44.0
19	M6	F	67	38.50	42.0	12.0
20	M3	M	29	13.37	96.0	41.4

AML, acute myeloid leukemia; WBC, white blood cell; M, male; F, female.

combinations) was added to 100 μ L of each peripheral blood sample without plasma and incubated at room temperature for 15 min in the dark. Next, 2 mL 1 \times red blood cell (RBC) lysis buffer (BD) was then added to resuspend the stained sample, which was then incubated at room temperature for 20 min. Afterward, the mixture was centrifuged for 5 min at 350 \times g, and the supernatant was discarded to stop cell lysis. Finally, the cells were washed twice with 2 mL cell staining buffer by centrifugation at 350 \times g for 5 min and discarding the supernatant. The samples were resuspended with 0.5 mL staining buffer to prepare for flow cytometry analysis. A total of 30,000 CD3 $^+$ cells were analyzed with a BD FACS VERSE flow cytometer (BD Biosciences, San Jose, USA), and data analysis was performed with Flowjo software (Flowjo LLC, USA).

Statistical analyses

The frequency of PD-1 $^+$, CD57 $^+$, PD-1+CD57 $^+$ in CD3 $^+$, CD4 $^+$ or CD8 $^+$ T cells and CD244+CD8 $^+$ T cells was expressed as $\bar{x}\pm s$, whereas medians were used for presentation the frequency of CD244+CD4 $^+$, PD-1+CD244+CD4 $^+$ T cells and PD-1+CD244+CD8 $^+$ T cells.

Statistical analyses were performed using independent-sample *t*-test and Mann-Whitney test by SPSS software (Version 13.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Increased PD-1+CD3 $^+$ and PD-1+CD8 $^+$ T cells in AML patients

We first detected the percentage of PD-1 $^+$ cells in the CD3 $^+$ T cell population, and a significantly higher PD-1 $^+$ cell percentage was found in peripheral blood from 20 patients with *de novo* AML (33.83% \pm 10.30%) compared with 23 healthy individuals (25.16% \pm 7.88%) ($P=0.003$). Therefore, we further analyzed the distribution of PD-1 $^+$ T cells in the CD4 $^+$ and CD8 $^+$ T cell subtypes, and an increased level of PD-1+CD8 $^+$ T cells in the CD3 $^+$ T cell population was found for *de novo* AML (11.81% \pm 5.93%) and AML-CR (10.01% \pm 3.14%) patients compared with healthy individuals (6.62% \pm 3.10%) ($P=0.001$ and 0.007, respectively). For PD-1+CD4 $^+$ T cells, there was no significant difference between *de novo* AML

(19.48%±6.41%) and AML-CR samples (23.16%±8.76%) ($P=0.201$) or healthy controls (18.04%±6.92%) ($P=0.485$) (Figure 1). We also compared the PD-1 frequency on peripheral blood CD3+, CD4+ and CD8+ T cells between 8 cases with AML-M2 and 8 cases with AML-M3. The percentage of PD-1+CD3+, PD1+CD4+ and PD-1+CD8+ T cells in the M2 group (36.43%±7.65%, 21.53%±4.41%, and 12.46%±9.63%, respectively) appeared to be high compared with the M3 group (29.60%±9.56%, $P=0.137$; 16.21%±6.97%, $P=0.090$; and 9.63%±4.51%, $P=0.215$, respectively); however, the differences were not statistically significant.

We also analyzed the association between the PD-1+, CD57+ or CD244+ T cell numbers and the leukemia blast percentage in peripheral blood from AML patients, but the differences were not statistically significant ($P=0.891$, $P=0.787$ and $P=0.121$, respectively).

Frequency of CD244+ and CD57+ cells in CD3+, CD4+ and CD8+ T cell populations in AML

To evaluate the T cell status of patients with AML, we

detected the exhaustion and immunosenescence markers CD244 and CD57 by flow cytometry analysis of T cells. A higher percentage of CD244+CD4+, CD244+CD8+, CD57+CD4+, and CD57+CD8+ T cells in the CD3+ T cell population was found for *de novo* AML samples compared with those from healthy individuals (median: CD244+CD4+ T cells: 7.70% vs. 4.31%, $P=0.001$; CD244+CD8+ T cells: 29.28%±8.03% vs. 18.52%±7.31%, $P=0.000$; CD57+CD4+ T cells: 6.15%±4.11%, $P=0.011$; CD57+CD8+ T cells: 15.3%±6.74% vs. 8.59%±4.76%, $P=0.001$). A higher percentage of CD244+CD4+ T cells was found for the *de novo* AML group compared with AML-CR group (median: 7.70% vs. 3.80%, $P=0.003$), and there was a similar trend demonstrating an increasing level of CD57+CD4+ T cells in the AML group compared with AML-CR group (6.15%±4.11% vs. 3.56%±1.58%, $P=0.065$); it is interesting that in CD4+ T cells, there was no significant difference in the CD244 and CD57 distribution between the AML-CR and healthy control groups, whereas in CD8+ T cells, an increasing level of CD244 and CD57 was found in AML-CR group compared with healthy control groups (Figure 2).

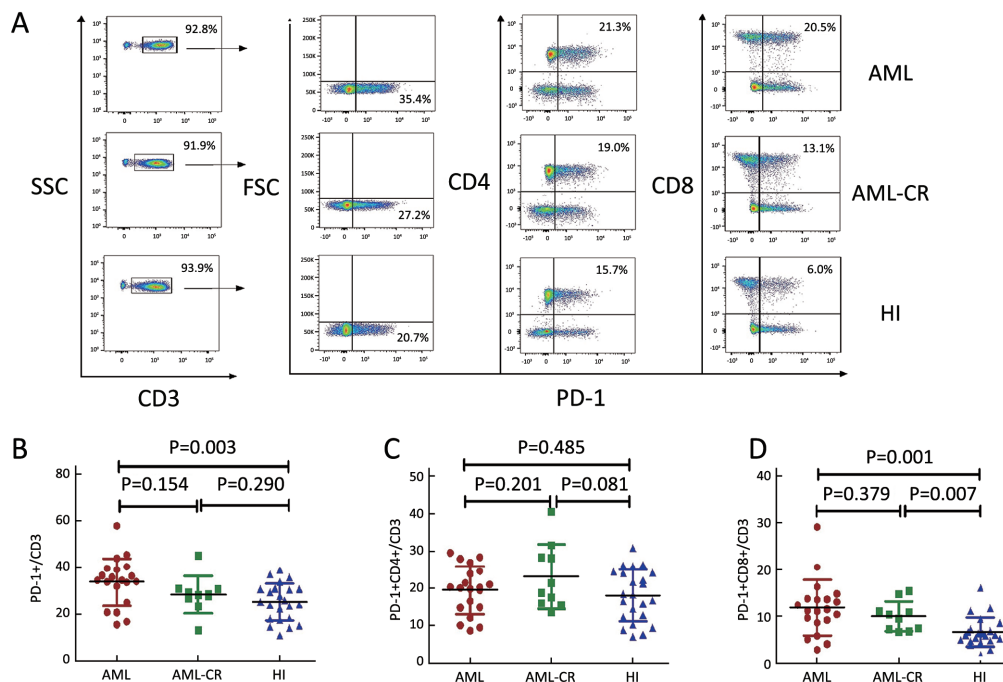


Figure 1 The percentage of PD-1+CD3+, PD-1+CD4+ and PD-1+CD8+ cells in the CD3+ T cell population from patients with *de novo* AML and AML-CR and healthy individuals. (A) Detection of PD-1/CD3+, PD-1+CD4+ and PD-1+CD8+ T cells in one case with AML, one case with AML in CR (AML-CR), and one healthy individual (HI) by flow cytometry; (B–D) The percentage of PD-1+CD3+ (B), PD-1+CD4+ (C), and PD-1+CD8+ (D) cells in the CD3+ T cell population from 20 patients with *de novo* AML, 10 cases with AML-CR, and 23 healthy individuals. Increased PD-1+CD3+ and PD-1+CD8+ T cells were found in the AML groups. PD-1, programmed death 1; AML, acute myeloid leukemia; CR, complete remission.

A higher percentage of PD-1+CD244+ and PD-1+CD57+ in CD4+ and CD8+ T cells in AML

To investigate the association between PD-1 and the T cell exhaustion status of T cells, we analyzed co-expression of PD-1 and CD244 or CD57 in CD4+ and CD8+ T cells. A strong upregulation of PD-1+CD244+ and PD-1+CD57+ cells was found for CD4+ and CD8+ T cells in the *de novo* AML group compared with the healthy control group (PD-1+CD244+/CD4: 8.25% vs. 3.62%, P=0.000; PD-1+CD57+/CD4: 7.02%±3.53% vs. 4.12%±3.01%, P=0.006; PD-1+CD244+/CD8: 29.30% vs. 21.60%, P=0.017; PD-1+CD57+/CD8: 16.21%±8.93% vs. 9.81%±4.30%, P=0.007). There was no significant difference between PD-1+CD244+ and PD-1+CD57+ cells in CD4+ and CD8+ cells in the AML-CR group compared with healthy individuals (5.35%±3.17% vs. 4.70%±3.56%, P=0.273;

5.25%±2.13% vs. 4.12%±3.01%, P=0.295; 24.82%±8.54% vs. 21.50%±7.07%, P=0.253; 12.06%±4.91% vs. 9.81%±4.30%, P=0.195). Interestingly, there was a significant difference between the PD-1+CD244+ populations for CD4+ cells from *de novo* AML compared with AML-CR (10.63%±6.89% vs. 5.35%±3.17%, P=0.022), whereas for PD-1+CD244+/CD8, PD-1+CD57+/CD4+ or PD-1+CD57+/CD8+ T cells, this was not observed (Figure 3).

Discussion

Increasing data have indicated that T cell immunosuppression in cancer patients is primarily mediated by T cell inhibitory receptors, a so-called immune checkpoint, which may be reversed by checkpoint inhibitors. Most of the immune checkpoint blockade studies have focused on solid tumors, and few clinical attempts have been reported for leukemia. Recently, PD-1/PDL-1 have been extensively investigated in leukemia, and clinical trials with PD-1 inhibitors for patients with hematological malignancies are ongoing with promising clinical responses (22), while the failure of PD-1 blockade in multiple myeloma (MM) was reported in a phase I study (23). Moreover, most studies have been performed using leukemia mouse models or *in vitro* analyses (24,25), and most have focused on lymphocytic malignancies such as CLL and diffuse large B-cell lymphoma (DLBCL) (26,27). Little is known about the characteristics of the PD-1 expression level and T cell exhaustion status of patients with *de novo* AML. In this study, we firstly analyzed the number of PD-1+ T cells in the CD3+, CD4+ and CD8+ T cell populations from patients with *de novo* AML. The most striking finding was an increased number of PD-1+ CD3 cells, and most of these PD-1+ cells were CD8+ cells, while the number of PD1+CD4+ cells was not increased in the AML group, which may be related to a dysfunction in the cytotoxicity of T cells in AML.

To evaluate whether these T cells displayed the exhausted phenotype, we examined the number of CD244+ and CD57+ T cells in patient blood. CD244 and CD57 are commonly used to evaluate T cell exhaustion and immunosenescence (28-31). The numbers of the CD244+CD4+, CD244+CD8, CD57+CD4+, and CD57+CD8+ T cells were significantly increased in the AML group. Therefore, we further analyzed co-expression of PD-1 and CD244 or CD57 on the CD4+ and CD8+ T cell subsets. There was a parallel increase in the number of all four T cell subsets. This result is different from an *in vitro* study that showed that T cells co-cultured with

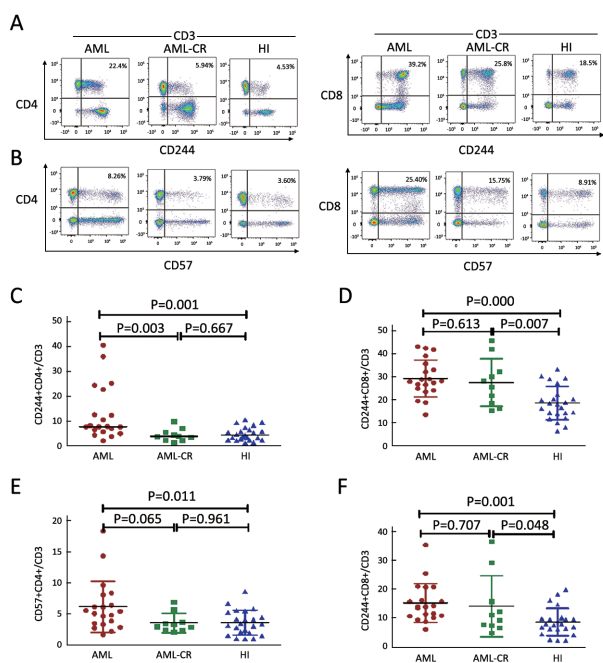


Figure 2 The percentage of CD244+ and CD57+ T cells in the CD4+ and CD8+ T cell populations of patients with *de novo* AML or AML-CR and healthy individuals. (A) CD244+CD4+ and CD244+CD8+ T cells; (B) CD57+CD4+ and CD57+CD8+ T cells within the CD3+ T cell population from one case with AML, one case with AML-CR and one healthy individual (HI); (C-F) CD244+CD4+, CD244+CD8+, CD57+CD4+ and CD57+CD8+ T cells within the CD3+ T cell populations from 20 cases with *de novo* AML, 10 cases with AML-CR and 23 healthy individuals. Increased CD244+CD4+, CD244+CD8+, CD57+CD4+ and CD57+CD8+ T cells were found in the AML group compared with HI group. AML, acute myeloid leukemia; CR, complete remission.

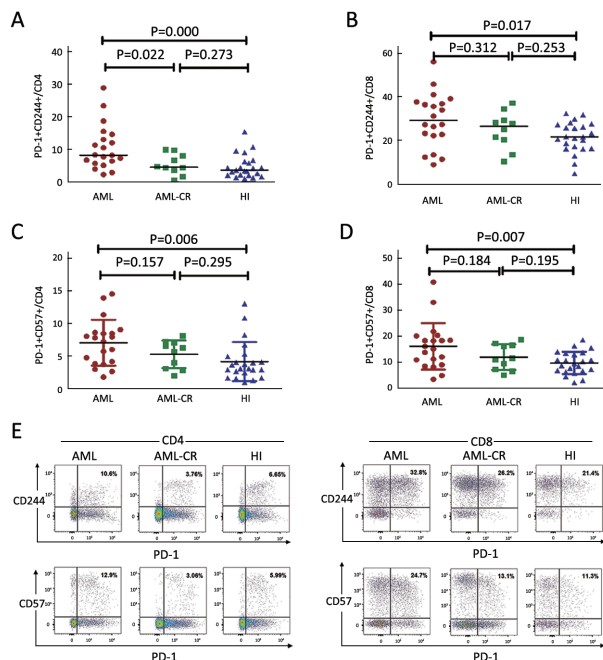


Figure 3 The percentage of PD-1+CD244+ and PD-1+CD57+ T cells in the CD4+ and CD8+ T cell populations from patients with *de novo* AML and AML-CR and healthy individuals. (A) The percentage of PD-1+CD244+ cells in the CD4+ T cell population in patients with AML and AML-CR and healthy individuals (HI); (B) The percentage of PD-1+CD244+ cells in the CD8+ T cell population in patients with AML and AML-CR and HI; (C) The percentage of PD-1+CD57+ cells in the CD4+ T cell population in the AML, AML-CR and HI groups; (D) Flow cytometry analysis demonstrates the percentage of PD-1+CD57+ cells in the CD8+ T cell population in the AML, AML-CR and HI groups; (E) The percentage of PD-1+CD244+ and PD-1+CD57+ cells in the CD4+ and CD8+ T cell populations in one case with AML, one case with AML-CR and a healthy individual (HI), respectively. Increased PD-1+CD244+ and PD-1+CD57+ cells in both CD4+ and CD8+ T cells were found in the AML group compared with HI group. PD-1, programmed death 1; AML, acute myeloid leukemia; CR, complete remission.

leukemia cells could generate only exhausted Th cells, which are defined by upregulation of the PD-1, cytotoxic T-lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG3) and Tim-3 inhibitory receptors (32). Overall, it may be summarized that exhausted T cells are increased in AML patients, and CD8+ T cells are mainly involved, while CD4+ T cells appear to have less impact (Figure 4). This result is consistent with the finding of CLL with increased T cell exhaustion (16,33), the finding further supports a study that reported that the exhausted T cell phenotype could be identified in AML mouse models (17). Moreover, we found that increased exhausted T cells, particularly CD8+ T cells, began at the time of diagnosis,

and most of these cells could be decreased to a normal level at the time of AML in CR. No reports have demonstrated reversal of the exhausted T cell status in AML-CR. Recently, data from CML analysis have demonstrated that maximal restoration of immune responses by increased effector T cell cytolytic function and reduced PD-1+ T cells occur in CML with molecular remission, which is a similar observation as our results (34). Although there was no significant difference in the numbers of PD-1+CD244+, PD-1+CD57+ T cells between *de novo* AML and AML-CR groups in this study, the decreased tendency was observed in AML-CR group, further investigation will be performed in a large cohort samples to confirm the results. We found that the level of exhausted T cells at the time of diagnosis and in CR is relatively different for different patients with AML, which may be related to different statuses of T cell dysfunction, and whether it is associated with clinical outcome requires follow up. At a minimum, this phenotype may provide a biomarker for patients who are considering adding immune checkpoint blockade to achieve more effective therapy (21,35).

Conclusions

We characterized one of the major T cell defects, co-expression of PD-1, CD244, and CD57 exhausted T cells in patients with *de novo* AML, and found a particular influence of CD8+ T cells, suggesting that there may be a reason of poor anti-leukemia immune response in patients with AML. Moreover, heterogeneous alterations in the exhaustion status of different cases may provide information for selecting checkpoint blockade as a follow-up therapy in different cases. However, further investigation is needed to characterize alterations in multiple immune checkpoint proteins, such as CTLA-4, Tim-3, and LAG-3, to provide a global view of T cell immune suppression in different T cell subtypes in AML.

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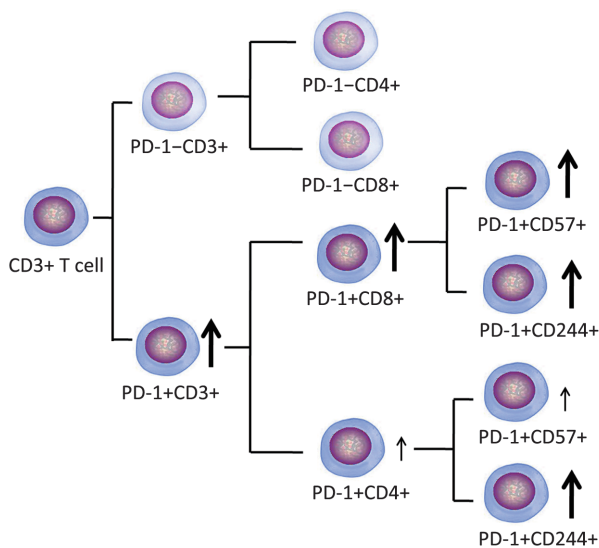


Figure 4 Overview of alteration in T cell phenotypes in patients with acute myeloid leukemia (AML).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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