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Higher PD-1/Tim-3 expression on IFN-γ+ T cells is associated with poor prognosis in patients with acute myeloid leukemia

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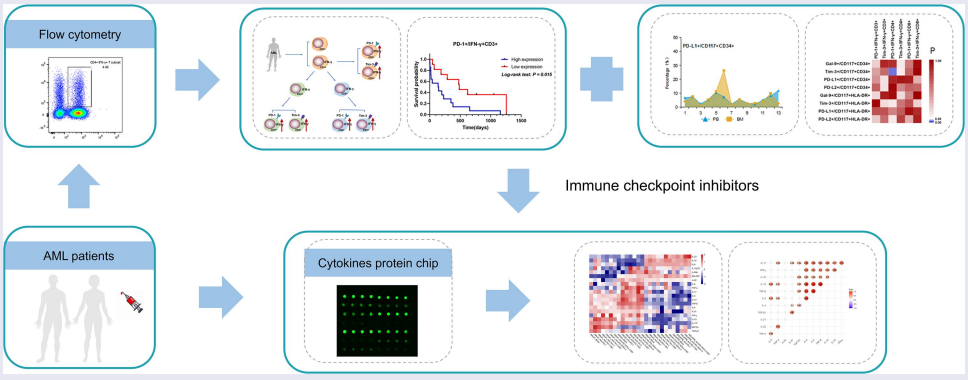
ABSTRACT

With the success of immune checkpoint inhibitors (ICI), such as anti- programmed death-1 (PD-1) antibody for solid tumors and lymphoma immunotherapy, a number of clinical trials with ICIs have been attempted for acute myeloid leukemia (AML) immunotherapy; however, limited clinical efficacy has been reported. This may be due to the heterogeneity of immune microenvironments and various degrees of T cell exhaustion in patients and may be involved in the IFN-γ pathway. In this study, we first characterized the percentage of PD-1+ and T cell immunoglobulin mucin-domain-containing-3 (Tim-3) +IFN-γ+ T cells in peripheral blood (PB) in AML compared with healthy individuals (HIs) by flow cytometry and further discussed the possibility of the reversal of T cell exhaustion to restore the secretion capacity of cytokines in T cells in AML based on blockade of PD-1 or Tim-3 (anti-PD-1 and anti-Tim-3 antibody) in vitro using a cytokine protein chip. A significantly increased percentage of PD-1+, Tim-3+, and PD-1 +Tim-3+ IFN-γ+ T cells was observed in PB from patients with AML in comparison with HIs. Moreover, higher PD-1+IFN-γ+CD3+/CD8+ T cell levels were associated with poor overall survival in AML patients. Regarding leukemia cells, the percentage of Tim-3 in CD117+CD34+ AML cells was positively correlated with PD-1 in IFN-γ+CD4+ T cells. Furthermore, blocking PD-1 and Tim-3 may involve multiple cytokines and helper T cell subsets, mainly Th1 and Treg cells. Blockade of PD-1 or Tim-3 tends to restore cytokine secretion to a certain extent, a synergistic effect shown by the co-blockade of PD-1 and Tim-3. However, we also demonstrated the heterogeneity of secretory cytokines in ICI-treated T cells in AML patients.



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AML; PD-1; Tim-3; IFN-γ; T cell exhaustion; immune checkpoint inhibitors

Introduction

T-cell dysfunction is regarded as an important cause of immune escape in acute myeloid leukemia (AML) and is closely associated with disease progression and prognosis.[1,2] The former is mainly reflected in abnormal expression of T cell receptor (TCR), dysregulation of cytokine secretion, imbalance of T cell subsets, aberrant T cell activation, and proliferation, etc.[3] It is reported that higher numbers of Treg cells were observed in AML,[4] and may suppress cytotoxic T cells through interleukeukin-10 (IL-10), transforming growth factor-β (TGF-β), and IL-35.[5] In addition, decreasing IL-2, IL-1β, tumor necrosis factor-α (TNF-α) and IL-6 levels may significantly contribute to T cell exhaustion, the presence of functional T helper (Th) cell populations and the growth and survival of AML cells, which suggests that an imbalance in the cytokine network may be involved in immune escape in AML.[6,7] Our previous findings have shown increasing immune checkpoints (IC) such as programmed death-1 (PD-1),

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cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), and T cell immunoglobulin mucin-domain-containing-3 (Tim-3) in T cell subsets in AML, concurrent with exhaustion phenotypes CD57 and CD244, and mainly in CD8+ T cells.[8–12] Moreover, it has been demonstrated that increased PD-1+Vβ+T cells is a common characteristic of AML and related to poor prognosis in AML.[13] Thus, blockage of immunosuppressive receptors and ligands may make the possibility to reverse T cell exhaustion and enhance anti-leukemic function in AML.[14–16] Nevertheless, immune checkpoint inhibitors (ICI), such as anti-PD-1 antibodies, for AML immunotherapy have been reported to have limited clinical efficacies.[17,18] This may be due to the heterogeneity of immune microenvironments and various degrees of T cell exhaustion in different patients.[19,20] Clinical trial data have indicated that combined blockage tends to be more effective in reversing T cell exhaustion than single blockage of the PD-1/PD-ligand 1(PD-L1) pathway.[21–23] In particular, decreased PD-1 and Tim-3 double-positive T cells were observed in AML-complete remission (CR) patients compared with AML-non-CR (NCR) patients, indicating that Tim-3 may participate in drug resistance to anti-PD-1.[24,25] Further verification has demonstrated synergistic anti-leukemia function in the co-blockage of PD-1 and Tim-3 in clinical trials for solid tumors and AML mouse models.[26,27] Furthermore, current findings have indicated that the drug resistance resulting from ICIs is related to the IFN-γ pathway.[28] On the one hand, IFN-γ plays a positive immunomodulatory role in the upregulation of major histocompatibility complex I (MHC-I) and antigen presentation by dendritic cells (DC) through PI3K-AKT and JAK2-STAT1 pathways.[28] On the other hand, IFN-γ takes part in the antitumor process by downregulation of PD-L1 in AML cells.[29] In general, the IFN-γ pathway plays a critical role in the leukemia effect.[30] However, little is known about the exhaustion status and distribution of IFN-γ+ T cells and their role in AML. In this study, we first characterized the percentage of PD-1+ and Tim-3+ IFN-γ+ T cells and further discussed the T cell exhaustion reversal to restore the secretion capacity of cytokines in T cells by blocking PD-1 and Tim-3 in vitro in AML.

Results

Increasing PD-1 and Tim-3 expression on IFN-γ+ T cell subsets in AML

Previous studies have reported higher expression of PD-1 and Tim-3 in T cells from AML patients with an exhausted phenotype.[8,9] In this study, we first analyzed CD3, CD4, CD8, IFN-γ, PD-1, and Tim-3 expression in total CD3+ T cells in peripheral blood (PB) from newly diagnosed AML patients and found an obvious increasing trend toward PD-1+ and Tim-3+ T cells in AML in comparison with HIs, with decreasing IFN-γ+ T cells in AML (Figure 1b). Simultaneously, we detected the frequencies of PD-1 and Tim-3 expression and co-expression in IFN-γ+ T cell subsets (Figure 1a). Significantly, a higher percentage of PD-1+IFN-γ+CD3+ (median: 24.9 vs 10.5, $P < .001$), Tim-3+IFN-γ+CD3+ (median: 4.94 vs 1.69, $P < .001$), and PD-1+Tim-3+IFN-γ+CD3+ T cells (median: 3.23 vs 0.33, $P = .001$) was found in patients with AML than in those with HIs (Figure 1d). Similarly, increasing percentage of PD-1+, Tim-3 and PD-1+Tim-3+ T cells were shown in IFN-γ+CD4+ T (Th1) cells (median: 34.9 vs 12.4, $P < .001$) (median: 5.24 vs 1.43, $P < .001$) (median: 3.21 vs 0.45, $P < .001$) and IFN-γ+CD8+ T (Tc1) cells (median: 21.9 vs 13.0, $P = .004$) (median: 3.11 vs 1.34, $P < .001$) (median: 0.91 vs 0.18, $P = .001$) (Figure 1e, f). In addition, comparing the different patterns of PD-1 and Tim-

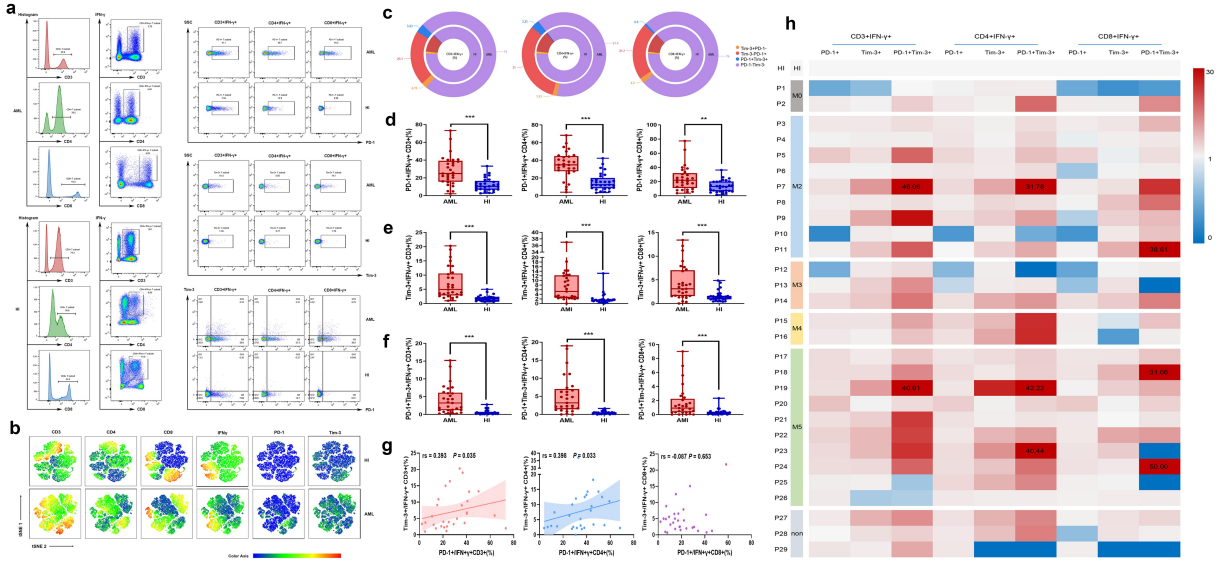

Figure 1. Increasing PD-1 and Tim-3 on IFN-γ+ T cell subsets in PB in AML. a Detection of PD-1+, Tim-3+, and PD-1+Tim-3+ on IFN-γ+ T cell subsets in a patient with *de novo* AML and healthy individuals by flow cytometry. b tSNE clusters of the global distribution and frequency of different T cell phenotypes in patients with *de novo* AML and healthy individuals. c Different patterns of PD-1 and Tim-3 expression in IFN-γ+ T cell subsets in the *de novo* AML and HI groups. d-f Frequency of PD-1 (d), Tim-3 (e) expression, and co-expression (f) in the IFN-γ+CD3+/CD4+/CD8+ T cells in the *de novo* AML and HI groups. g Correlation between the percentages of PD-1 and Tim-3 in IFN-γ+ T cell subsets in *de novo* AML patients. h Heat map representing the frequency of PD-1+, Tim-3+, and PD-1+Tim-3+ cells on IFN-γ+ T cell subsets from patients with *de novo* AML compared with HIs.

3 expression in Th1 and Tc1 cells, we found that the percentage of PD-1+Tim-3+ and Tim-3-PD-1+ T cells tended to be higher in Th1 cells than in Tc1 cells (Figure 1c). Furthermore, we analyzed the correlation between the percentages of PD-1 and Tim-3 in IFN-γ+ T cell subsets in AML (Figure 1g). The results demonstrated that PD-1 was positively correlated with Tim-3 expression in IFN-γ+CD3+ T (rs = 0.393, $P = .035$) and IFN-γ+CD4+ T cells ($rs = 0.398$, $P = .033$), whereas there was no statistically significant difference in IFN-γ+CD8+ cells ($rs = 0.087$, $P = .653$).

Our previous studies have found that varying degrees of T cell immunodeficiency exist in patients with AML of different subtypes.[4] In this study, we characterized the distribution of PD-1 and Tim-3 on IFN-γ+ T cells in 29 patients with AML. According to the heatmap made by taking the median of the HI group as the standard and then standardizing the patient data, a higher tendency of PD-1+, Tim-3+, and PD-1+Tim-3+ on IFN-γ+ T cells, especially Th1 cells, was detected in the M2 and M5 groups, followed by the M0 and M3 groups in comparison with HIs (Figure 1h), indicating the heterogeneity of PD-1 and Tim-3 expression on IFN-γ+ T cell subsets in AML.

Higher expression of PD-1 and Tim-3 on IFN-γ+ T cells resulting in inferior prognosis in AML

To evaluate the relationship between PD-1 and Tim-3 expression and AML prognosis, we compared the differences in the distribution of IFN-γ+ T cell subsets between the AML-CR and AML-NCR groups after chemotherapy. A persistent, lower skewed PD-1+ IFN-γ+CD3+, Tim-3+ IFN-γ+CD3+ and PD-1+Tim-3+IFN-γ+CD3+ T cell distributions were demonstrated for AML-CR patients (median: 13.6 vs. 35.9, $P = .012$) (median: 2.18 vs. 6.48, $P = .036$) (median: 1.22 vs. 4.55, $P = .046$) (Figure 2a). Interestingly, we found a significantly decreased PD-1+ T cell percentage in IFN-γ+CD4+ T cells in the AML-CR group compared to the AML-NCR group (median: 10.3 vs. 25.3, $P = .022$) (Figure 2b), while the differences were not statistically significant in PD-1+IFN-γ+CD8+ T cells (median: 21.2 vs. 43.5, $P = .072$) (Figure 2c), which may be related to the immune suppression of chemotherapy. We further analyzed the relationship between the expression of PD-1/Tim-3 and achieving CR after first treatment through a receiver operating characteristic (ROC) curve and observed that increasing expression of PD-1+IFN-γ+CD3+/CD4+/CD8+ T cells showed a high probability for *de novo* AML patients to achieve CR after the first chemotherapy treatment (Area Under Curve (AUC) = 0.786, $P = .043$) (AUC = 0.833, $P = .018$) (AUC = 0.798, $P = .035$) (Figure 2f–h).

We have gone a step further to follow up the survival status of 29 newly diagnosed patients with AML since their diagnosis, with four cases lost. Subsequently, the Kaplan–Meier survival curve and log-rank analysis showed that a higher number of PD-1+IFN-γ+CD3+ ($X^2 = 5.892$, $P = .015$) (Figure 2d) and PD-1+IFN-γ+CD8+ T cells ($X^2 = 6.623$, $P = .015$) (Figure 2e) indicated an inferior prognosis.

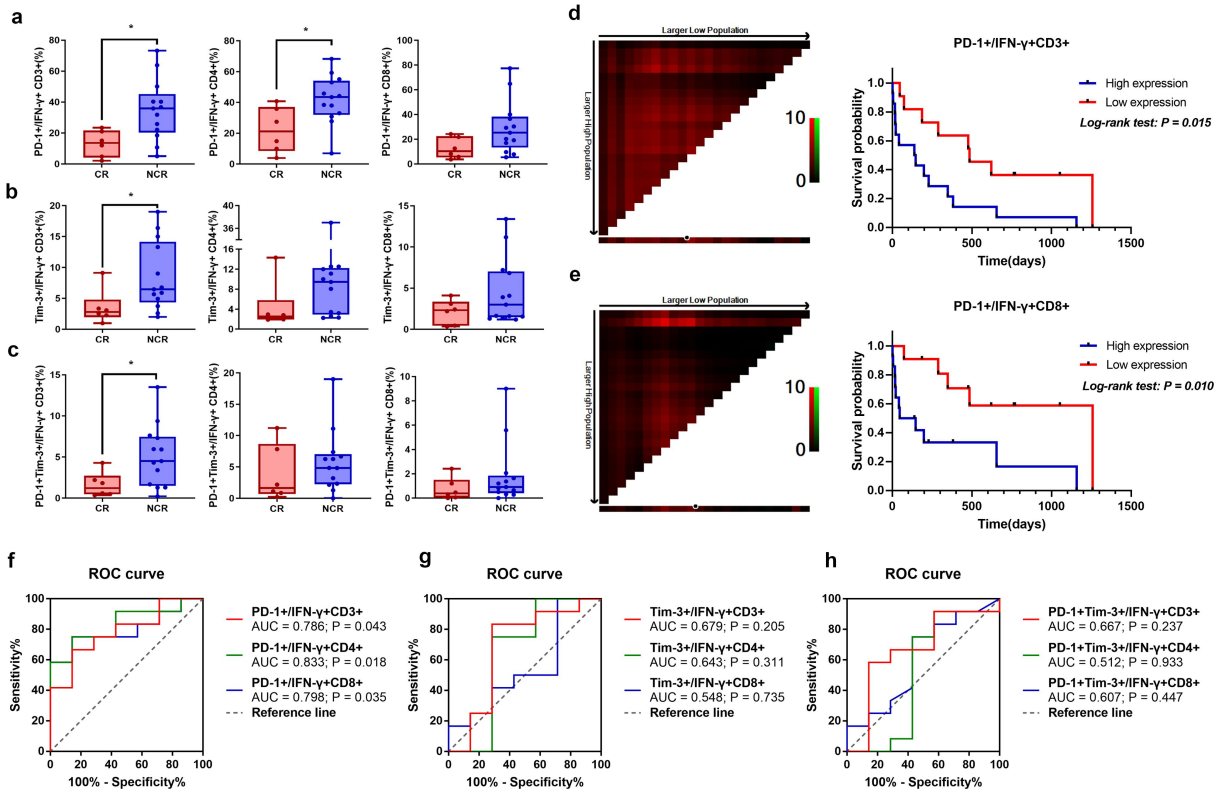

Figure 2. Higher PD-1 and Tim-3 on IFN-γ+ T cell subsets are related with poor prognosis in AML. a-c Comparison of the percentages of PD-1+ (A), Tim-3+ (B), and PD-1+Tim-3+ cells (C) in the IFN-γ+ T cell subsets in the *de novo* AML and HI groups. d-e Overall survival (OS) analysis of PD-1+IFN-γ+CD3+ T cells and PD-1+IFN-γ+CD8+ T cells in *de novo* AML cohort. f-h ROC curve predicting the relationship between PD-1+ (f), Tim-3+ (g), and PD-1+Tim-3+ cells (h) on IFN-γ+ T cell subsets and whether they achieved CR after the first course of treatment.

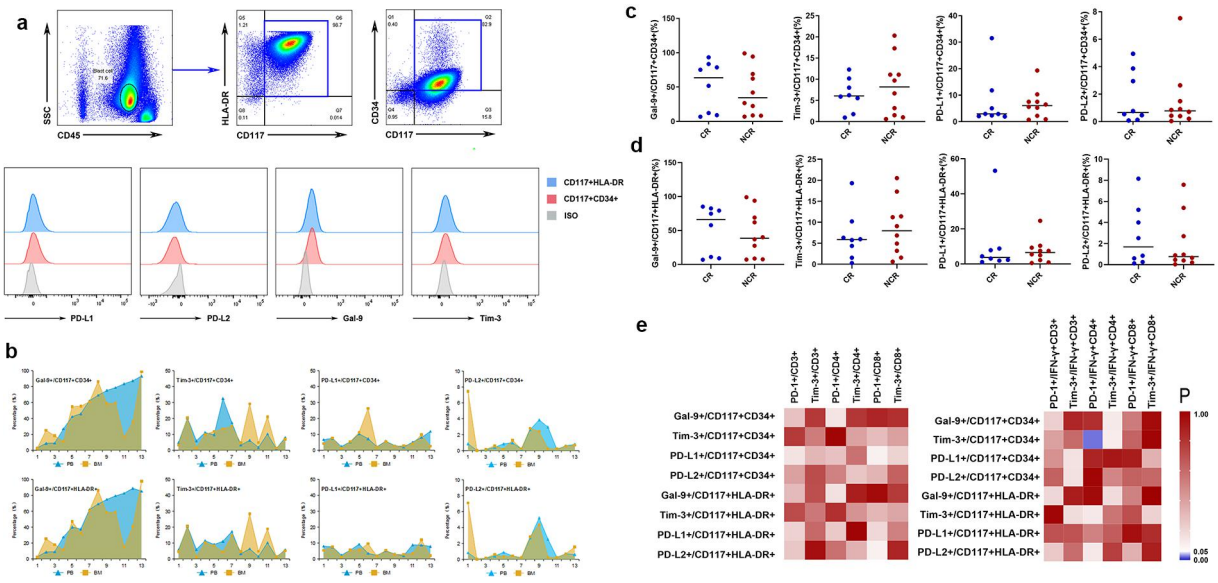

Figure 3. Distribution and frequency of PD-L1/PD-L2/Gal-9/Tim-3 in AML cells in PB and BM. a Detection of PD-L1+, PD-L2+, Gal-9+, and Tim-3+ in CD117+HLA-DR+/CD117+CD34+ AML cells in a patient with *de novo* AML using flow cytometry. b Comparison of the percentages of PD-L1+, PD-L2+, Gal-9+, and Tim-3+ in CD117+HLA-DR+/CD117+CD34+ AML cells in PB and BM. c-d Comparison of the percentages of PD-L1+, PD-L2+, Gal-9+, and Tim-3+ in CD117+HLA-DR+/CD117+CD34+ AML cells in PB between the CR and NCR groups. e Correlation between the percentages of PD-L1+, PD-L2+, Gal-9+, and Tim-3+ in AML cells and PD-1 and Tim-3 in IFN-γ+ T cells in PB in *de novo* AML.

Tim-3 in AML cells positively correlated with PD-1 in IFN-γ+CD4+ T cells in AML

It is well known that the ligands of PD-1 are PD-L1 and PD-ligand 1 (PD-L2) and the ligand of Tim-3 is Gal-9. To discuss the characteristics of immunosuppressive ligands in AML tumor cells, we determined the percentage of PD-L1, PD-L2, Gal-9, and Tim-3 in CD117+CD34+ and CD117+HLA-DR+ AML cells (Figure 3a). The frequency of PD-L1+/PD-L2+/Gal-9+/Tim-3+CD117+CD34+ AML cells in PB was relatively different, ranging from 0.85% to 32.5% for PD-L1, 0.21%–7.53% for PD-L2, 2.94%–99.1% for Gal-9, and 0.61%–17.3% for Tim-3. In this study, we had not found the significant difference of ligand expression in AML cells in PB and BM (Figure 3b). We further followed up patients who achieved CR after the first treatment and analyzed the difference between the AML-CR and NCR groups in PB. However, there was no significant difference in the expression of PD-L1/PD-L2/Gal-9/Tim-3 between the CR and NCR groups (Figure 3c–d). In addition, we obtained a step further to establish correlations between ligands in AML cells and receptors in T cells. Interestingly, a positive correlation was found between Tim-3 in CD117+CD34+ AML cells and PD-1 in IFN-γ+CD4+ T cells ($rs = 0.572$, $P = .022$), while no correlation was found between Tim-3 in CD117+CD34+ AML cells and PD-1 in CD4+ T cells ($rs = 0.056$, $P = .819$) (Figure 3e).

Blockade of PD-1 or Tim-3 tends to enhance the secretion of cytokines in vitro in AML

As described above, the higher expression of PD-1 and Tim-3 in T cell subsets may be associated with T cell exhaustion and disease prognosis. It is unclear whether PD-1 and Tim-3 inhibitors can restore anti-leukemia function in T cells. We further performed a cytokine protein chip analysis of the supernatant of T cells and the AML cell co-culture system treated with or without anti-PD-1 or anti-Tim-3 in three samples from AML patients (AML#1 BM, AML#2 BM, AML#3 BM, and AML#3 PB). The characteristics of the 24 cytokines in the three BM samples are shown in Figure 4a. It was also found that blockade of PD-1 or Tim-3 may bring about a change in different types of cytokines or variable quantities, which demonstrated heterogeneity of the secretory cytokines in ICI-treated T cells in AML (Figure 4a). We found correlations among cytokines in groups treated with ICI and observed that IL-10 was positively correlated with TGF-β, which was reported to be negatively regulated by cytokines. Moreover, strong positive correlations were observed between IL-17, TNF-β, IL-13, IL-2, IL-4, and IFN-γ (Figure 4b). In addition, we divided T cell subsets into Th1 (IL-2, IFN-γ, TNF-α, and TNF-β), Th2 (IL-4, IL-5, and IL-13), Th17 (IL-17 and IL-22), follicular helper (Tfh) cells (IL-21), and Treg (IL-10 and TGF-β) according to the cytokines and found that anti-PD-1 and anti-Tim-3 inhibitors may be mainly affected by Th1 and Treg cell functions (Figure 4c). We further performed an individual analysis and found that blocking PD-1 or Tim-3 can enhance the secretion of cytokines to a certain extent (Figure 4d–e). As for AML#1, increased secretion levels of IFN-γ, IL-1β, IL-6, and macrophage inflammatory protein 3α (MIP-3α) were observed in the anti-PD-1 group compared to those in the anti-PD-1-ISO group ($P = .008$; $P = .001$; $P = .001$; $P < .001$); increased secretion levels of IFN-γ, IL-6, IL-17, TNF-α, and MIP-3α were observed in the anti-Tim-3 group compared to those in the anti-Tim-3-ISO group ($P < .001$; $P = .003$; $P = .001$; $P = .034$; $P = .017$). Interestingly, the co-blockade of PD-1 and Tim-3 synergis-

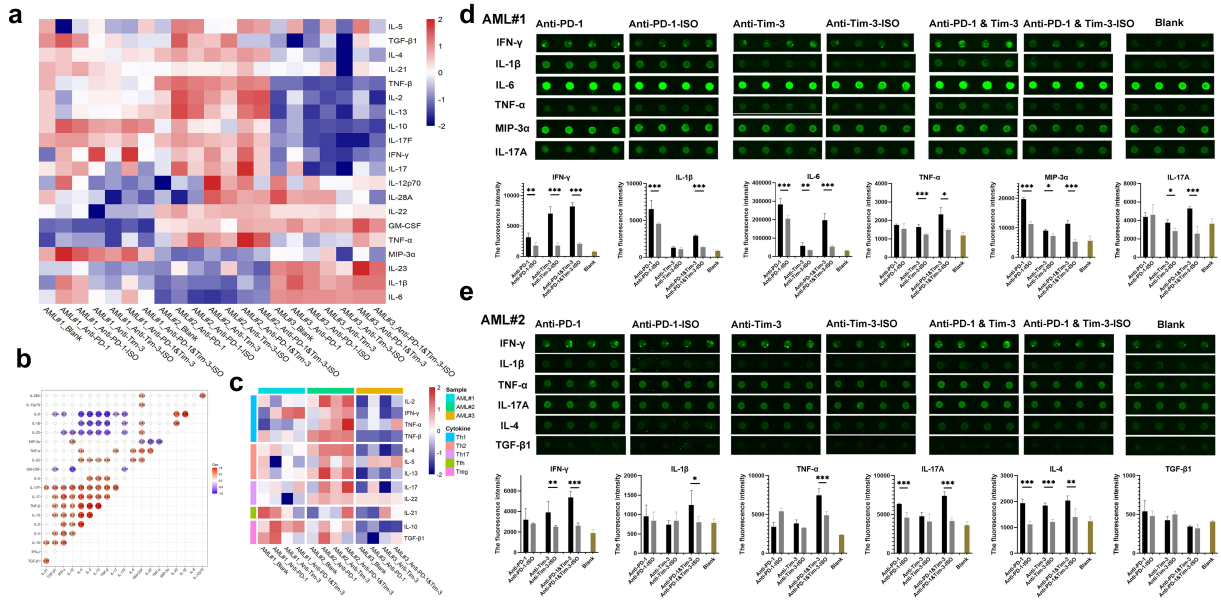

Figure 4. The characteristics of the secretory cytokines in ICI-treated T cells in vitro detected by protein chip. a Heatmap representing the mean fluorescence intensity of 20 cytokines in the BM ($n = 3$) of AML patients (AML#1, AML#2, AML#3) treated with ICI compared with the isotype. b Correlation between secretory cytokines in T cells co-cultured with AML cells in vitro in BM ($n = 3$). All points shown in the figure are significantly correlated ($P < .05$). Blue indicates a negative correlation and red indicates a positive correlation. c Heatmap representing the characteristics of the main cytokines in different secretory T cell subsets treated with ICI in the BM ($n = 3$) in *de novo* AML. d-e Comparison of the secretory cytokines (mean fluorescence intensity) in T cells treated with anti-PD-1, anti-Tim-3, and ICI combination in AML#1 patients and AML#2 patients detected by protein chip

tically enhanced the cytokine secretion of IFN-γ, TNF-α, and IL-17 ($P < .001$; $P = .011$; $P < .001$) (Figure 4d). A similar trend was observed for the secretory cytokines in AML#2 (Figure 4e). In addition, higher levels of IL-21 ($P = .003$), IL-2 ($P < .001$), TNF-β ($P = .007$), IL-5 ($P < .001$), and TNF-α ($P = .004$), as well as lower levels of IL-10 ($P < .001$) were observed in AML#3-BM than in AML#3-PB co-cultured with AML cells and treated with ICI in vitro (Supplementary Figure S1A-B).

Discussion

Helper T cell function may be compromised in AML, and several observational studies have found that Th1 populations have a decreased frequency and lower expression of IFN-γ in both the PB and BM of AML patients.[3,31] In this study, we presented t-SNE visualizations that illustrated generally lower IFN-γ expression and higher PD-1 and Tim-3 expression in AML than in HIs, which was consistent with previous reports.[32] In order to better evaluate the immune function status of T cells in AML, we characterized PD-1 and Tim-3 expression on IFN-γ + T cell subsets and found increasing PD-1+, Tim-3+, PD-1, and Tim-3 double positive T cells in IFN-γ+CD3+/CD4+/CD8 + T cells in AML, illustrating that T cell exhaustion is obviously present in both Th1 and Tc1 cells and is associated with leukemia. Our previous findings have reported an increasing trend toward PD-1+CD4+ T cells in PB in AML.[8] Interestingly, a higher percentage of PD-1+IFN-γ+CD4+ T cells was found, indicating that PD-1/PD-L1 pathways may impact CD4+ T cell function through the tumor-infiltrating inflammatory cytokines IFN-γ.[33] Further comparative analysis of different patterns of PD-1 and Tim-3 on IFN-γ+ T cells found that PD-1 and Tim-3-mediated T cell exhaustion were principally involved in Th1 rather than Tc1, with Tim-3 mainly in the form of co-expression with PD-1. The former was once more time to be attested in the positive relationship between PD-1 and Tim-3 in Th1, but not in Tc1, suggesting that PD-1 and Tim-3 are involved in the negative immunoregulation of IFN-γ+ T cells, especially Th1 cells in AML. In addition, the distribution of PD-1 and Tim-3 on IFN-γ+ T cells varied in different AML subsets, emphasizing the phenotypic heterogeneity of T cell exhaustion in AML patients.[3] It is well known that one of the important aspects of clinical practice is to evaluate the risk of disease. In this study, according the follow-up data, we have pointed out that PD-1/Tim-3 on IFN-γ+ T cells may be an independent risk factor for AML patients whether they achieved CR after chemotherapy. Higher PD-1 expression in IFN-γ+ T cell subsets is associated with poor overall survival in patients with AML, providing evidence for the possibility of reserving T cell exhaustion based on blockage of PD-1 and Tim-3, which may restore the secretion capacity of cytokines in Th cells.

It has been reported that Tim-3 is not only expressed on immune cells, particularly T cells and NK cells, but is also expressed on leukemic stem cells (LSCs) in AML, which plays a double immunosuppressive role in the anti-tumor response.[34,35] In this study, we also detected PD-L1/PD-L2 /Gal-9/Tim-3 expression in primary AML cells; however, we had not found the significant difference of ligand expression in AML cells in PB, and there was no significant difference of IC ligand expression between samples from PB and BM, even we know that leukemia bone marrow as tumor microenvironment may play immune suppression which may enhance the IC and ligand expression. In this study, we are unable to evaluate the result, one of the reasons may be due to the limit sample size to

make effective comparison, we will continue to collect more AML samples to confirm the result.[24] Overall, on the other hand, the positive correlation between PD-1+IFN-γ+CD4+ T cells and Tim-3+CD117+CD34+ AML cells demonstrated that PD-1 in Th1 cells may synergistically promote T cell exhaustion with Tim-3 in AML cells, which was indirectly supported by Silva et al.[36]

Thus, we tried to reverse T cell exhaustion by blocking or co-blocking PD-1 and Tim-3 in primary AML patients in vitro. The protein chip demonstrated the heterogeneity of secretory cytokines in ICI-treated T cells in AML, which may depend on the expression of ICs and individualized tumor immune microenvironments.[1,20] We make inferences based on changes in cytokines and find that blocking PD-1 and Tim-3 may involve numerous T cell subsets, mainly on Th1 and Tregs, which, respectively, play a positive and negative role in the regulation of immunity. Binder et al. illustrated that anti-inflammatory mediators such as TGF-β and IL-10 appear to impede AML progression, while pro-inflammatory mediators such as IL-1β, TNF-α, and IL-6 tend to increase AML aggressiveness,[6] which is consistent with the results reported in this study. Moreover, it is an indirect proof that the reversal of PD-1-mediated T-cell exhaustion in AML helps restore the secretory capacity of IL-2, IFN-γ, and TNF-α.[37] In this study, the Blockade of PD-1 or Tim-3 tended to enhance cytokine secretion to a certain extent, with a synergistic effect (generally TNF-α, IFN-γ, and IL-17A) shown in the co-blockade of PD-1 and Tim-3. Nevertheless, the limitations of the synergistic effects resulting from co-blockage, such as IL-1β, require a large number of investigations to explore the underlying mechanism. At present, the preclinical experiments and clinical trials of ICI for solid tumors and hematological malignancies have emerged drug resistance and the clinical efficiency seemed relative heterogeneity in different individuals from the same disease, such as AML and MDS, the main reason may be related to different immune suppression status in patients.[38] It is reported that dysregulated cytokine expression, which is a general hallmark of chronic inflammation and auto-inflammatory diseases, may also promote the development of hematological malignancies.[6] Current findings have revealed that the drug resistance resulting from ICIs is related to the IFN-γ pathway that IFN-γ takes part in the anti-tumor process by downregulation of PD-L1 in AML cells.[30] Moreover, Tim-3 is a molecule originally identified as being selectively expressed on IFN-γ–secreting Th1 and Tc1 cells.[39] These indicated that the possibility of combinational approaches to enhancing PD-L1/PD-1 pathways blockade efficacy with several cytokines such as IL-2, IL-10, and IFN-α may result in additional benefits for AML patient immunotherapy based on IC blockade.[40]

Conclusion

In conclusion, we first made a novel observation that increased PD-1 and Tim-3 expression on IFN-γ+ T cells may contribute to T cell exhaustion and impair anti-leukemia function and is associated with poor prognosis in AML. Co-blockade of PD-1 and Tim-3 synergistically enhanced cytokine secretion based on the distribution of ICs.

Methods

Samples

PB samples for detecting PD-1 and Tim-3 on IFN-γ+ T cells were collected from 29 *de novo* AML patients, including 17 males and 12 females (median age: 52 years, range: 23–81 years) named P1 to P29, as well as 29 PB in healthy individuals (HIs), including 17 males and 12 females (median age: 44 years, range: 22–81 years) served as controls. In addition, 23 PB samples (male: female = 12:11, median age: 44 years, range: 14–83 years) from *de novo* AML patients were collected to detect the expression of PD-L1/programmed death-ligand 2 (PD-L2)/Gal-9/Tim-3 from AML cells, named P1, P2, P9-P13, P16, and P22–P36, as well as 13 BM samples from matched AML patients. Finally, three samples, P2, P37, and P38, were used to perform cytokine protein chip analysis after treatment with ICI in vitro. Clinical data of the patients are presented in Supplementary Table S1. All human samples were obtained with informed consent, and ethical approval was obtained from the Ethics Committee of the Medical School of Jinan University.

Flow cytometry analysis

First, mononuclear cells were separated from fresh blood samples. Cells were stimulated with PMA/ionomycin (CS1002, Multisciences, China) and Brefeldin A (BFA) (00–4975, eBioscience, USA) and cultured at 37°C in 5% CO_2 for 5 h. The cells were then stained with CD45, CD3, CD4, CD8, PD-1, and Tim-3 antibodies for 15 min at room temperature in the dark. The cells were washed twice with Phosphate Buffered Saline (PBS), fixed with Fixation Buffer (420801, BioLegend, USA) in the dark for 15 min, permeabilized with 1X Intracellular Staining Perm Wash Buffer (421002, BioLegend, USA) twice, and then intracellularly stained for IFN-γ in the dark for 15 min. Finally, 30,000 CD3+ cells were acquired for analysis with a BD FACS Canto flow cytometer (BD Biosciences, San Jose, USA) and subsequent analysis using Flowjo software (Flowjo LLC, USA).

Meanwhile, 100 μL of BM or PB blood samples were collected and mixed with Red Blood Cell Lysis Buffer in the dark at room temperature for 8 min. Cell surface staining analysis for CD45, HLA-DR, CD117, CD34, PD-L1, PD-L2 Tim-3, and corresponding isotypes (ISO) and intracellular staining analysis for Gal-9 were performed. The expression was detected and analyzed using flow cytometry and FlowJo software, as mentioned above. The antibodies used in this study are listed in Supplementary Table S2.

Protein chip

CD3+ T cells were obtained by magnetic bead sorting and co-cultured with AML (CD3-) cells at a ratio of 1:10. The cells were divided into groups and treated with Anti-PD-1 (A2002, Selleck, USA), Anti-PD-1-ISO (403702, BioLegend, USA), Anti-Tim-3 (345004, BioLegend, USA), Anti-Tim-3-ISO (400124, BioLegend, USA), Anti-PD-1/Tim-3 and Anti-PD-1/Tim-3-ISO at 37°C in 5% CO2 for 48 h. The cellular supernatant was collected and 24 cytokines were detected using a RayBiotech human GSH-TH17-1 antibody array.

Statistical analysis

Data analyses were performed using the Mann–Whitney test with SPSS software (version 22.0, IBM, Armonk, NY, USA), GraphPad Prism (version 8.4.2, CA, USA), and R (version 3.6.1, https://www.r-project.org/). Correlations were analyzed using Spearman's correlation analysis. The frequencies of different T-cell subsets are presented as medians. According to optimal cutoff values, PD-1/Tim-3 expression was divided into high and low expression groups. Kaplan–Meier analysis was used to analyze the expression of ICs and the survival of patients with AML. Moreover, the heatmap was using "Pheatmap" in R software and a correlation heatmap was generated using the "ggcorrplot" package. Differences were considered statistically significant at $P < .05$.

List of abbreviations

AML	acute myeloid leukemia
AUC	Area Under Curve
BM	bone marrow
CR	complete remission
CTLA-4	cytotoxic T lymphocyte-associated molecule-4
DC	dendritic cell
Gal-9	galectin 9
His	healthy individuals
IC	Immune checkpoint
ICI	Immune checkpoint inhibitor
IFN-γ	interferon-γ
IL-10	interleukin 10
ISO	Isotypes
PB	peripheral blood
PBS	Phosphate Buffered Saline
PD-1	programmed cell death receptor-1
PD-L1	programmed death-ligand 1
PD-L2	programmed death-ligand 2
LAG-3	T cell lymphocyte activation gene-3
MHC-I	major histocompatibility complex I
MIP-3α	macrophage inflammatory protein 3 α
NCR	non complete remission
ROC	receiver operating characteristic
TCR	T cell receptor
Tfh	Follicular helper cells
TGF-β	transforming growth factor-β
Th cells	T helper cells
Tim-3	T cell immunoglobulin mucin-domain-containing-3
TNF-α	tumor necrosis factor-α
Treg	regulatory T.

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Availability of data and materials

The materials supporting the conclusions of this research article are included within the article.

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