

T-Cell Immunoglobulin and ITIM Domain (TIGIT) Associates with CD8⁺ T-Cell Exhaustion and Poor Clinical Outcome in AML Patients

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Abstract

Purpose: T-cell immunoglobulin and immunoreceptor tyrosine–based inhibitory motif (ITIM) domain (TIGIT) is a recently identified T-cell coinhibitory receptor. In this study, we aimed to determine the clinical impact of TIGIT in patients with acute myelogenous leukemia (AML) and dissect the role of TIGIT in the pathogenesis of leukemia progression.

Experimental Design: TIGIT expression on T cells from peripheral blood collected from patients with AML was examined by flow cytometry. The correlation of TIGIT expression to clinical outcomes, including rate of complete remission and relapse post-allogeneic stem cell transplantation (alloSCT) in AML patients, was analyzed. Phenotypic and functional study (cytokine release, proliferation, killing, and apoptosis) of TIGIT-expressing T cells were performed. Using siRNA to silence TIGIT, we further elucidated the regulatory role of TIGIT in the

T-cell immune response by dissecting the effect of TIGIT knockdown on cytokine release and apoptosis of T cells from AML patients.

Results: TIGIT expression on CD8⁺ T cells is elevated in AML patients and high-TIGIT correlates with primary refractory disease and leukemia relapse post-alloSCT. TIGIT⁺ CD8⁺ T cells display phenotypic features of exhaustion and exhibit functional impairment manifested by low production of cytokines and high susceptibility to apoptosis. Importantly, their functional defects are reversed by TIGIT knockdown.

Conclusions: TIGIT contributes to functional T-cell impairment and associates with poor clinical outcome in AML. Our study suggests that blockade of TIGIT to restore T-cell function and antitumor immunity may represent a novel effective leukemia therapeutic. *Clin Cancer Res*; 22(12); 3057–66. ©2016 AACR.

Introduction

Successful treatment of acute myelogenous leukemia (AML) is challenging. General management involves induction chemotherapy followed by post-remission consolidation (1). Allogeneic hematopoietic stem cell transplantation (AlloSCT) in many situations significantly improves survival. Despite considerable effort, only approximately 35% and 10% of young adult and elderly patients, respectively, are cured from their disease (2–4). Primary refractory disease (failure to induction therapy) and leukemia relapse post-alloSCT represent two major challenging scenarios

that correlate with poor clinical outcomes. Novel effective leukemia therapeutics is urgently needed because treatment options are extremely limited for this patient population.

The delicate balance between immune stimulatory and inhibitory signals determines the effectiveness of antitumor responses (5, 6). Recent success using reagents targeting negative regulators (so-called "immune checkpoints") offers great promise for effective cancer therapy (7–12). These approaches target T-cell exhaustion; a unique immune inhibitory mechanism involving a state of T-cell dysfunction that develops in response to persistent antigen stimulation (13). Inhibitory pathways, including programmed cell death protein 1 (PD-1), T-cell immunoglobulin domain, and mucin domain 3 (TIM-3), 2B4, CD160, B- and T-lymphocyte attenuator (BTLA), and lymphocyte-activation gene 3 (LAG-3), contribute to the development of T-cell exhaustion (14–20). Several studies, including ours, have demonstrated an involvement of inhibitory pathways in AML progression (21–25). Strategies for blocking immune suppression are attractive due to their relatively simple administration and the resulting increased T-cell activity and enhanced tumor killing. In fact, agents blocking the PD-1 pathway, nivolumab, and pembrolizumab, have been recently approved by the FDA in treating several solid tumors. Early-phase clinical trials testing the safety and efficacy of nivolumab in AML are currently ongoing (NCT02275533, NCT02464657, and NCT02397720). Although promising, it should be noted that single blockade of one inhibitory pathway is unlikely to accomplish an optimal anti-leukemia immune response. Leukemia may use a variety of inhibitory pathways to evade immune attack (21, 22, 26, 27) and inhibition

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Translational Relevance

Overall prognosis of acute myelogenous leukemia (AML) remains poor. Primary refractory AML (failure to induction chemotherapy) and leukemia relapse post-allogeneic stem cell transplantation (alloSCT) are among the most challenging clinical scenarios; treatment options are extremely limited. In the present study, we demonstrate that T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT), a recently identified T-cell inhibitory receptor, is upregulated on CD8⁺ T cells from AML patients. High expression of TIGIT associates with primary refractory disease and leukemia relapse post-alloSCT. Furthermore, TIGIT⁺ CD8⁺ T cells exhibit features of exhaustion and show reduced capacity to produce cytokines and high susceptibility to apoptosis. Importantly, their functional defects are reversed by TIGIT knockdown. This is the first study to uncover a critical role of TIGIT in leukemia pathogenesis and its significant clinical impact. Our data provide a strong rationale of targeting TIGIT for novel leukemia therapeutics.

of one negative receptor may cause upregulation of others. Strategies combining blockade of multiple immune regulators are appealing. To this aim, it is crucial to identify additional inhibitory pathways as potential targets for effective leukemia therapeutics.

T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) is a recently identified coinhibitory receptor that is expressed on activated T cells, Tregs, and NK cells (28–31). Similar to CTLA-4 and CD28, TIGIT shares ligands (CD155 and CD112) with its costimulatory counterpart, CD226 (32, 33). Emerging data suggest an important inhibitory role of TIGIT in tumor immunity (34–36). However, whether TIGIT regulates anti-leukemia immune responses in patients with AML is unknown. Here, we performed a phenotypic and functional study on T cells isolated from the peripheral blood of AML patients. We report that TIGIT expression is upregulated on CD8⁺ T cells in AML. Importantly, a high frequency of TIGIT-expressing cells associates with primary refractory disease and leukemia relapse post alloSCT, both of which correlate with poor clinical outcome in AML. On further dissection of the immune regulatory role of TIGIT in AML, we observed that TIGIT⁺ CD8⁺ T cells exhibit features of exhaustion manifested by their preferential expression on the T_{EMRA} subset, co-expression of other inhibitory receptors, decreased capacity of producing cytokines, and high susceptibility to apoptosis. The functional defects can be reversed by knockdown of TIGIT gene expression. In addition, downregulation of CD226 likely contributes to TIGIT-mediated immune suppression.

Materials and Methods

Patients

Peripheral blood samples of AML patients were from the tissue bank maintained by the Penn State Hershey Cancer Institute of Penn State University College of Medicine (Hershey, PA). The study was approved by the Institutional Review Board of Penn State University College of Medicine. Full informed consent was obtained from all patients. Samples from 25 patients diagnosed with AML per WHO classification (10 males and 15 females, age:

60 ± 16 years, range, 23–76 years) were used in the initial study. Samples of 18 healthy volunteers (10 males and 8 females, age: 51 ± 15 years, range, 21–65 years) were obtained as controls. Clinical characteristics of post-transplant patient cohorts were described previously (22). Briefly, samples from 11 AML patients who received alloSCT from 2013 to 2015 were selected, five of whom had leukemia relapse at 2 to 6 months posttransplantation; the other 6 patients remained in remission at the time of blood collection (3–6 months). Age and gender were evenly distributed in relapse versus remission patients. Most patients received 8/8 HLA-matched transplantations from filgrastim-mobilized peripheral blood stem cells. One patient received 7 of 8 matched donor cells. One patient received bone marrow-derived stem cells. Seven patients (3 from each relapse, 4 from remission group) underwent ablative conditioning, the other 4 patients (two from each group) received nonablative conditioning regimens due to age or comorbidity.

Immunofluorescence staining and flow cytometric analysis

Peripheral blood mononuclear cells (PBMC) were incubated with directly conjugated mAbs for 30 minutes at 4°C. The cells were then washed before flow cytometric analysis. mAbs used were anti-human CD3-PerCp-Cy5.5 or CD3-BV421, CD4-FITC or CD4-V500, CD8-APC-H7, CD45RA-PE-Cy7, CCR7-PerCp-Cy5.5, CD95-FITC (BD Biosciences), PD-1-PE, CD226-FITC, BTLA-BV421, CD160-PE-Cy7 (BioLegend), TIGIT-APC (Ebioscience), LAG-3-AF700 (R&D Systems) antibodies, and corresponding isotype controls. Data acquisition was performed on a LSR Fortessa flow cytometer (BD Biosciences) and data analysis was performed using FlowJo Software (Tree Star).

In vitro stimulation and intracellular staining

PBMCs were cultured in RPMI-1640 medium (GIBCO) containing 10% FBS and stimulated with anti-CD3/CD28 (2 µg/mL and 5 µg/mL; Ebioscience) or PMA/ionomycin (50 ng/mL and 1 µg/mL), plus Golgiplug (BD Biosciences) for 5 hours. The cells were then surface stained with CD3-BV605, CD4-FITC, CD8-APC-H7, PD-1-PE, TIGIT-APC, and intracellularly stained with IFNγ-V500, TNFα-PerCp-Cy5.5, or IL2-PerCp-Cy5.5 (BD Biosciences) antibodies. For ki67 and perforin staining, PBMCs were surface stained with CD3-BV605, CD4-V500, CD8-APC-H7, PD-1-PE, TIGIT-APC, and intracellularly stained with perforin-FITC and ki67-PerCp-Cy5.5 (BD Biosciences) antibodies. A Fixable Viability Dye eFluor 450 (Ebioscience) was used to assess cell viability.

Apoptosis analysis

Apoptosis rates were measured using the PE Annexin V Apoptosis Detection Kit (BD Biosciences) following the manufacturer's instructions, in combination with markers for T cells. Samples were analyzed by flow cytometry.

Cell separation, culture

CD8⁺ T cells were isolated from PBMCs using positive selection with the EasySep Human CD8-Positive Selection Kit (StemCell Technologies). T cells were cultured in RPMI-1640 medium containing 10% FBS with or without anti-CD3 Ab coated.

CFSE staining and T-cell proliferation assay

CD8⁺ T cells were labeled with 1 µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen). A total of 4 × 10⁴

CFSE-labeled CD8⁺ T cells were cultured with 5 µg/mL anti-CD3-coated plates in the presence of soluble anti-CD28 (5 µg/mL) in RPMI-1640-supplemented medium for 96 hours. Proliferation of CFSE-labeled cells was accessed by flow cytometry.

siRNA transfection

Transfection of CD8⁺ T cells with siRNA was performed according to the manufacturer's instruction (GE Dharmacon). SMART-pool Accell TIGIT siRNA, Accell Non-targeting Pool and Accell siRNA delivery media were purchased from GE Dharmacon. One µmol/L siRNA was transfected in a 96-well tissue culture plate with Accell siRNA delivery media for 72 hours. TIGIT expression, Annexin V staining, and cytokine production were measured by flow cytometry.

Statistical analysis

Data are expressed as the mean ± SD. GraphPad5 (GraphPad Software) or SPSS (IBM Corporation) were used for statistical calculations. The normality of each variable was evaluated using the Kolmogorov-Smirnov test. In cases of normally distributed data, the comparison of variables was performed using unpaired or paired where specified two-tailed Student *t* tests for unpaired and paired data, respectively. When the data were not normally distributed, the comparison of variables was performed with a Mann-Whitney *U* test or a Wilcoxon matched-pairs signed rank test for unpaired and paired data, respectively. Comparisons of patient characteristics were analyzed using Fisher exact test (categorical variables) or Wilcoxon-rank sum test (continuous variables). To evaluate correlation, Pearson's correlation coefficients

were used. For all analyses, *P* values less than 0.05 were considered statistically significant.

Results

TIGIT is elevated on CD8⁺ T cells in patients with AML

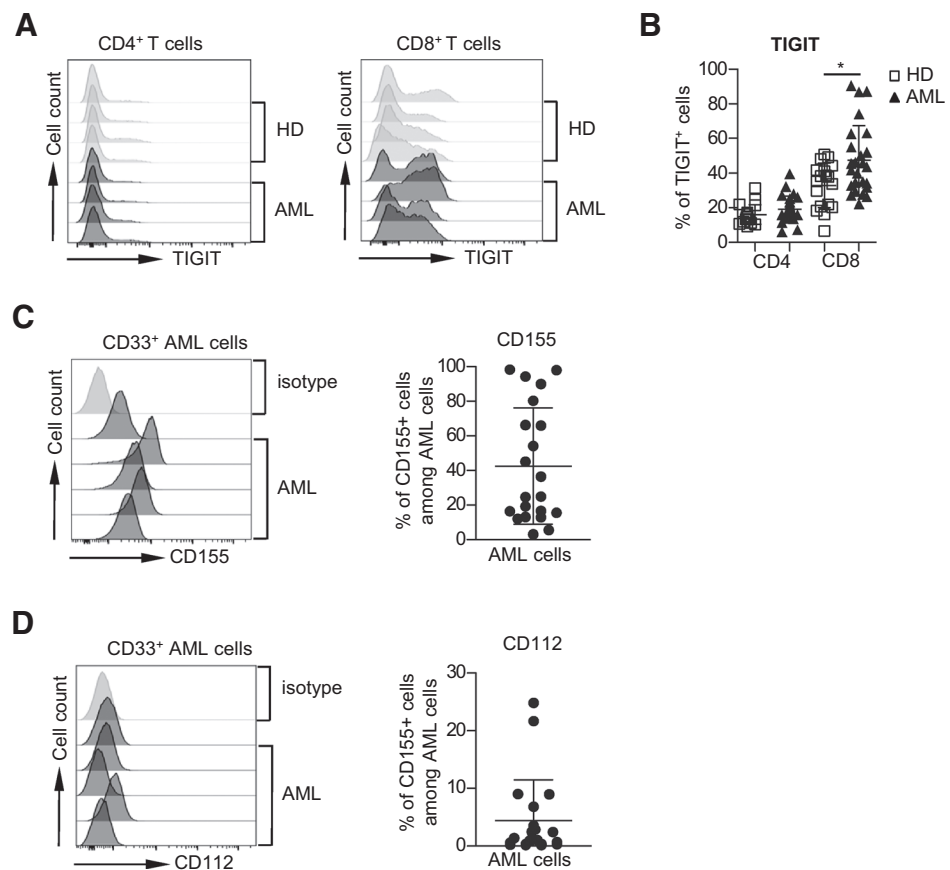
To determine whether TIGIT is involved in the pathogenesis of AML, we assessed PBMCs from 25 AML patients at initial diagnosis for TIGIT expression on both CD4⁺ and CD8⁺ T cells. PBMCs from 18 healthy donors (HD) were tested as controls. As shown in Fig. 1A and B, the frequency of TIGIT⁺ cells among CD8⁺ T cells from AML patients was significantly higher than those from HD (mean frequency 47.4%±19.9% vs. 32.9%±12.9%, *P* = 0.0101). In contrast, expression of TIGIT on CD4⁺ T cells was comparable between AML patients and HD. We further evaluated expression of TIGIT ligands, CD155 and CD112, on leukemia cells (gated on CD45⁺ CD33⁺) from AML patients. Although the levels are variable, the majority of patients express CD155 (high-affinity ligand) on their leukemia cells (mean frequency 42.5%±33.7%, Fig. 1C and D). In contrast, the expression of CD112 (low-affinity ligand) is minimal in most AML patients (mean frequency 4.4%±7.0%, Fig. 1C and D). Taken together, these data suggest a potential role for TIGIT/CD155 signaling in AML.

High expression of TIGIT on CD8⁺ T cells at initial diagnosis associates with primary refractory disease in AML patients

On the basis of the level of TIGIT expression on CD8⁺ T cells, we defined high-TIGIT (TIGIT>47.4%) versus low-TIGIT

Figure 1.

TIGIT is elevated on CD8⁺ T cells in patients with AML. Flow-cytometry analysis of surface expression of TIGIT was performed on PBMCs collected from AML patients at initial diagnosis as well as HDs. A, histograms display the expression of TIGIT gated on CD4⁺ (left) and CD8⁺ T cells (right). Representative data from 4 HDs and 4 AML patients are shown. B, the plot of percentages of TIGIT⁺ cells on CD4⁺ and CD8⁺ T cells from HDs (*n* = 18) versus AML patients (*n* = 25). Each spot represents an individual patient or HD. *P* values were obtained by unpaired *t* test. C and D, representative flow data (left) and plots (right) showing expression of CD155 (C) and CD112 (D) on CD33⁺ leukemia cells from AML patients (*n* = 21); *, *P* < 0.05.



(TIGIT<47.4%) subgroups in AML patients. The mean value (47.4%) of TIGIT expression on CD8⁺ T cells of the 25 AML patients evaluated in our study was used as the cutoff here. Nine patients are classified in the high-TIGIT group and 16 in the low-TIGIT group. We analyzed clinical characteristics and found no significant difference between the two groups in terms of age, gender, WBC, Hemoglobin, platelet, and cytogenetic features. Blast percentage and absolute counts in peripheral blood tended to be higher in patients who express high TIGIT, but did not reach statistical significance (Table 1). No difference was detected in blast percentage of bone marrow. We next investigated whether the level of TIGIT is associated with failure to achieve complete remission (CR) after induction chemotherapy, which is defined as primary refractory disease in AML (37). Three patients died of induction-related complications before we were able to assess CR and one patient went to hospice without induction treatment due to multiple co-morbidities and poor performance. Among the 21 AML patients of whom the induction response was able to be evaluated, we found a significantly higher rate of primary refractory disease in the high-TIGIT group compared with the low-TIGIT

Table 1. High-TIGIT associates with primary refractory disease in AML patients

Total (n = 25)	High-TIGIT (n = 9)	Low-TIGIT (n = 16)	P
Age, y			
Median	64	58	0.136
Range	24-77	24-72	
Gender			
Male	4	6	1.000
Female	5	10	
WBC, × 10⁹/L			
Median	42.2	31.4	0.677
Range	12.4-205.1	5.0-364.6	
Hemoglobin, g/dL			
Median	8.3	8.4	0.803
Range	7-9.2	7-10.6	
Platelet, × 10⁹/L			
Median	53	39	0.861
Range	14-105	14-108	
ANC, × 10⁹/L			
Median	0.9	4.2	0.251
Range	0-34.6	0-47.1	
PB blast (%)			
Median	84	56.7	0.803
Range	22.4-97	0.9-98	
Absolute blast counts, × 10⁹/L			
Median	32	17.5	0.559
Range	10-174	0-342	
BM blast, %			
Median	66	59	0.413
Range	34.5-88	1.5-97	
Cytogenetics			
Poor	1	2	0.910
Intermediate	7	13	
Good	1	1	
Flt3 ITD mutation^a			
Positive	3	6	1.000
Negative	6	9	
Primary refractory disease^b	5/8 (62.5%)	1/13 (7.7%)	0.014

Abbreviations: ANC, absolute neutrophil counts; BM, bone marrow; ITD, internal tandem duplication; PB, peripheral blood; WBC, white blood cell.

^aFlt3 ITD mutation data were not available for one patient; thus, data from 24 patients (9 of high-TIGIT, 15 of low-TIGIT) are shown.

^bThree patients died before assessment of remission; one patient went to hospice without induction therapy due to co-morbidities and poor performance status; therefore, 21 patients (8 of high-TIGIT and 13 of low-TIGIT) were able to be assessed for primary refractory disease.

group (5/8 (62.5%) versus 1/13 (7.7%), $P = 0.014$, Table 1). These data demonstrate an association of TIGIT expression on CD8⁺ T cells with primary refractory disease in AML.

Elevated TIGIT expression on CD8⁺ T cells post-alloSCT associates with AML relapse

Leukemia relapse post-alloSCT is another challenging clinical scenario in AML management. To determine whether TIGIT associates with AML relapse posttransplantation, we examined PBMCs from cohorts of patients who had received alloSCT. Samples from 5 patients who suffered leukemia relapse and from 6 patients who remained in remission were selected for study. Patients' clinical characteristics were described previously (22). Age and gender were evenly distributed in relapse versus remission patients. Time point (posttransplant) of sample collection, conditioning regimen, and donor resource are largely comparable between the two cohorts. Flow-cytometry analysis of TIGIT expression on CD4⁺ and CD8⁺ T cells was performed. We observed a significantly higher percentage of TIGIT⁺ cells among CD8⁺ T cells in patients who had leukemia relapse compared with that of patients who remained in remission ($P = 0.0352$; Fig. 2A). CD4⁺ T cells showed a similar trend but did not achieve statistical significance ($P = 0.1660$; Fig. 2B). Thus, TIGIT expression on CD8⁺ T cells associates with leukemia relapse post-alloSCT.

Collectively, these results demonstrate a strong correlation of TIGIT expression on CD8⁺ T cells in patients with refractory AML and post-alloSCT progression, suggesting that TIGIT may play a critical role in the pathogenesis of AML.

TIGIT⁺ CD8⁺ T cells in AML exhibit phenotypes that are consistent with exhaustion

T cells are generally divided into four subsets: naïve T cells (T_N , CCR7⁺CD45RA⁺), central memory T cells (T_{CM} , CCR7⁺CD45RA⁻), effector memory T cells (T_{EM} , CCR7⁻CD45RA⁻), and terminally differentiated effector cells (T_{EMRA} , CCR7⁻CD45RA⁺; ref. 38). Consistent with a previous report showing that the majority of T cells in AML patients fall within the activated subsets (39), we observed a significantly increased proportion of T_{EMRA} cells in AML patients compared with that of healthy controls ($38.5\% \pm 20.3\%$ vs. $26.3\% \pm 9.3\%$, $P = 0.0342$, Fig. 3A and B). We then assessed the expression of TIGIT on each subset of CD8⁺ T cells and observed significantly higher expression of TIGIT on T_{CM} , T_{EM} , and T_{EMRA} compared with that of T_N . This occurred in both AML patients as well as HD (Fig. 3C and D). Notably, TIGIT was expressed highest on T_{EMRA} , which are considered terminal effector cells with limited functional capacity and high susceptibility to apoptosis, thus more consistent with an exhaustion phenotype. There was a trend of increased TIGIT expression on T_{EMRA} CD8⁺ T cells in AML patients compared with that of HD, although no statistical significance was reached ($P = 0.0588$).

To further characterize the phenotype of CD8⁺ T cells that express TIGIT in patients with AML, we tested the expression of a number of inhibitory receptors on TIGIT⁺CD8⁺ T cells. We found significantly higher expression of PD-1, CD160, and 2B4 on TIGIT⁺ CD8⁺ T cells, compared with that of TIGIT⁻ CD8⁺ T cells (Supplementary Fig. S1A-S1C). In addition, expression of TIGIT was tightly correlated with expression of these receptors (Supplementary Fig. S1G-S1I). In contrast, expression of BTLA, TIM-3, and LAG-3 on TIGIT⁺ CD8⁺ T cells was comparable with that of TIGIT⁻ CD8⁺ T cells

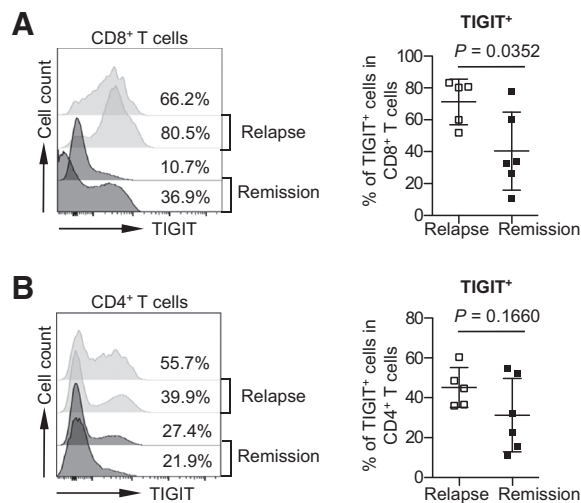


Figure 2. Expression of TIGIT is enhanced on CD8⁺ T cells from patients with leukemia relapse post-transplant. Flow-cytometry analyses of TIGIT were performed on PBMCs collected from AML patients post alloSCT. A and B, representative histograms and plots display the expression of TIGIT gated on CD8⁺ (A) and CD4⁺ T cells (B) from patients with relapse ($n = 5$) versus remission ($n = 6$). Each square represents data from one patient. P values were obtained by the unpaired t test; *, $P < 0.05$

(Supplementary Fig. S1D–S1F). No correlation was observed between expression of TIGIT and BTLA, TIM-3 or LAG-3 (Supplementary Fig. S1J–S1L).

Taken together, our study of CD8⁺ T cells from AML patients demonstrates a phenotypic association of TIGIT with the T_{EMRA} subset and upregulation of multiple negative regulators that are crucial in T-cell exhaustion, suggesting a role for TIGIT in T-cell exhaustion and AML pathogenesis.

TIGIT⁺ CD8⁺ T cells from AML patients show functional defects, which can be reversed by TIGIT knockdown.

To assess the functional status of TIGIT⁺ CD8⁺ T cells from AML patients, we performed functional assays to test cytokine release upon *in vitro* stimulation with anti-CD3 and anti-CD28. TIGIT⁺ CD8⁺ T cells had significantly lower intracellular TNF α , IFN γ , and IL2 compared with TIGIT⁻ CD8⁺ T cells (Fig. 4A). We next assessed TIGIT⁺ CD8⁺ T cells for their susceptibility to apoptosis, which is also an important feature of exhaustion. Expression of both Annexin V and CD95 are significantly elevated on TIGIT⁺ CD8⁺ T cells (Fig. 4B), suggesting that they are highly susceptible to apoptosis. We further evaluated the proliferation ability of TIGIT⁺ CD8⁺ T cells from AML patients by assessing ki-67 expression and performing CFSE-based proliferation assays. Strikingly, TIGIT⁺ CD8⁺ T cells exhibited significantly higher levels of ki67 expression compared with TIGIT⁻ CD8⁺ T cells (Supplementary Fig. S2A). Consistently, TIGIT⁺ CD8⁺ T cells showed an increased proliferation index in the CFSE assay (Supplementary Fig. S2B). In addition, perforin staining was elevated on TIGIT⁺ CD8⁺ T cells (Supplementary Fig. S2C), indicating a stronger killing potential. Thus TIGIT⁺ CD8⁺ T cells are functionally impaired to some degree by displaying low capacity of cytokine production and high susceptibility to apoptosis, but retaining their capacity for proliferation and potential to kill.

To better understand the contribution of TIGIT in inhibition of CD8⁺ T-cell function in AML patients, we used a specific siRNA to knockdown TIGIT expression in CD8⁺ T cells from AML patients (Fig. 4C). Intracellular cytokine-staining assays were performed on CD8⁺ T cells that had TIGIT knocked down. We observed a significant increase of TNF α and IFN γ production after TIGIT knockdown (Fig. 4D). Consistently, CD8⁺ T cells were less susceptible to apoptosis upon TIGIT knockdown manifested by decreased expression of Annexin V after siRNA transfection (Fig. 4E). These data demonstrate a pivotal role for TIGIT in inhibition of cytokine release and induction of apoptosis, thus suppressing T-cell function in AML patients.

CD226 is downregulated on CD8⁺ T cells in AML patients

Recent studies suggest that TIGIT can exert inhibitory regulation by competing with its costimulatory counterpart, CD226, for their common ligand CD155 (28, 29, 40). To determine whether CD226 is involved in TIGIT-mediated immune suppression in AML patients, we evaluated the expression of CD226 on CD8⁺ T cells from HD and patients with AML. In contrast with the upregulation of TIGIT, CD226 was significantly decreased in AML patients compared with that of HD ($68.2\% \pm 8.2\%$ vs. $77.5\% \pm 13.6\%$, $P = 0.0492$, Fig. 5A and B). In addition, the proportion of CD226⁺ cells was significantly lower among TIGIT⁺ CD8⁺ T cells compared with TIGIT⁻ CD8⁺ T cells ($54.8\% \pm 17.8\%$ vs. $83.1\% \pm 12.6\%$, $P < 0.0001$; Fig. 5C and D). Thus, TIGIT expression is inversely associated with expression of the CD226 costimulatory receptor.

Discussion

TIGIT was first identified as an inhibitory receptor expressed by activated CD4⁺ T cells, Tregs, and NK cells (28–31). Recent studies demonstrate its immunosuppressive effect on CD8⁺ T cells and antitumor activity (34, 35). In a mouse model of colon cancer and breast cancer, expression of TIGIT was elevated on CD8⁺ tumor-infiltrating lymphocytes (TIL). Blockade of TIGIT and PD-L1 synergistically enhanced CD8⁺ TIL function and resulted in tumor rejection (34). The increased expression of TIGIT was also reported in several human solid tumors. Most recently an elegant study from Chauvin and colleagues (35) demonstrated that in patients with advanced melanoma, TIGIT is upregulated on tumor antigen-specific CD8⁺ T cells of PBMCs and CD8⁺ TILs. Importantly, these TIGIT-expressing CD8⁺ T cells co-express the inhibitory receptor PD-1, and combined blockade of TIGIT and PD-1 increased their proliferation and cytokine production. These observations suggest that TIGIT is an important negative modulator for antitumor CD8⁺ T-cell responses, and thus may represent a target for cancer immunotherapy. However, direct evidence supporting a clinical role for TIGIT in AML patients has not been provided. Our study on a large cohort of patients with AML demonstrated a significant elevation of TIGIT expression on CD8⁺ T cells. TIGIT⁺ CD8⁺ T cells displayed features of exhaustion and compromised functional status. Importantly, TIGIT knockdown by siRNA restored their function manifested by increased cytokine release and reduced apoptosis. To our knowledge, this is the first evidence for a role of TIGIT in AML pathogenesis, providing a strong rationale for novel leukemia therapeutics that target TIGIT to restore CD8⁺ T-cell function and antitumor immunity.

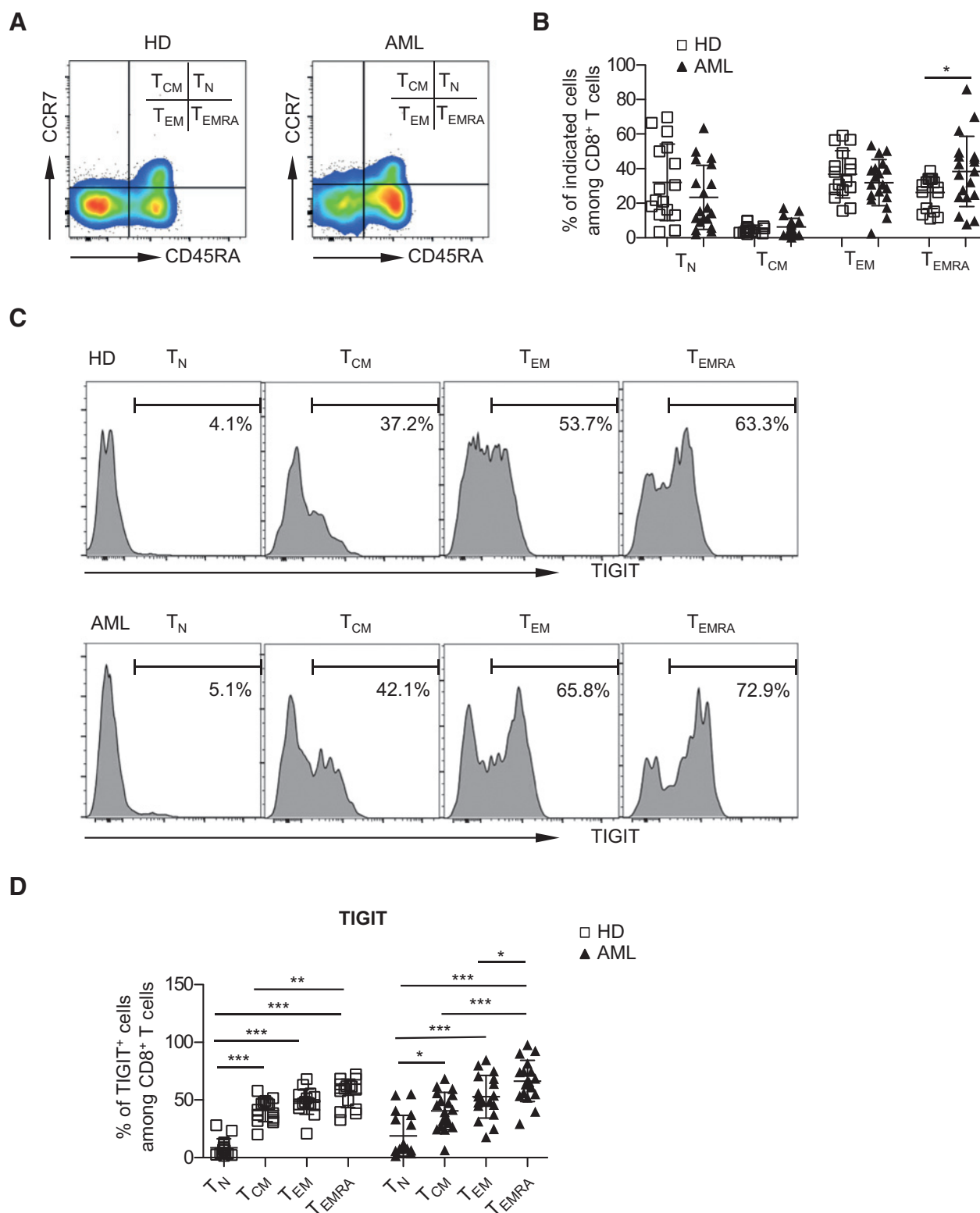


Figure 3. TIGIT is preferentially expressed on T_{EMRA} cells, which are increased in AML. A and B, distribution of T_N , T_{CM} , T_{EM} , and T_{EMRA} in CD8⁺ T cells from HDs versus AML patients. Representative flow data gated on CD8 (A) and plots (B) of the percentage of each subset in HDs ($n = 16$) or AML patients ($n = 19$) are shown. P values were obtained by the unpaired t test. C and D, expression of TIGIT among each subset (T_N , T_{CM} , T_{EM} , and T_{EMRA}) of CD8⁺ T cells was analyzed. Representative histograms (C) and plots (D) of percentage of TIGIT expression among each subset of CD8⁺ T cells from HDs ($n = 15$) or AML patients ($n = 18$) are shown. P values were obtained by the Mann-Whitney U test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

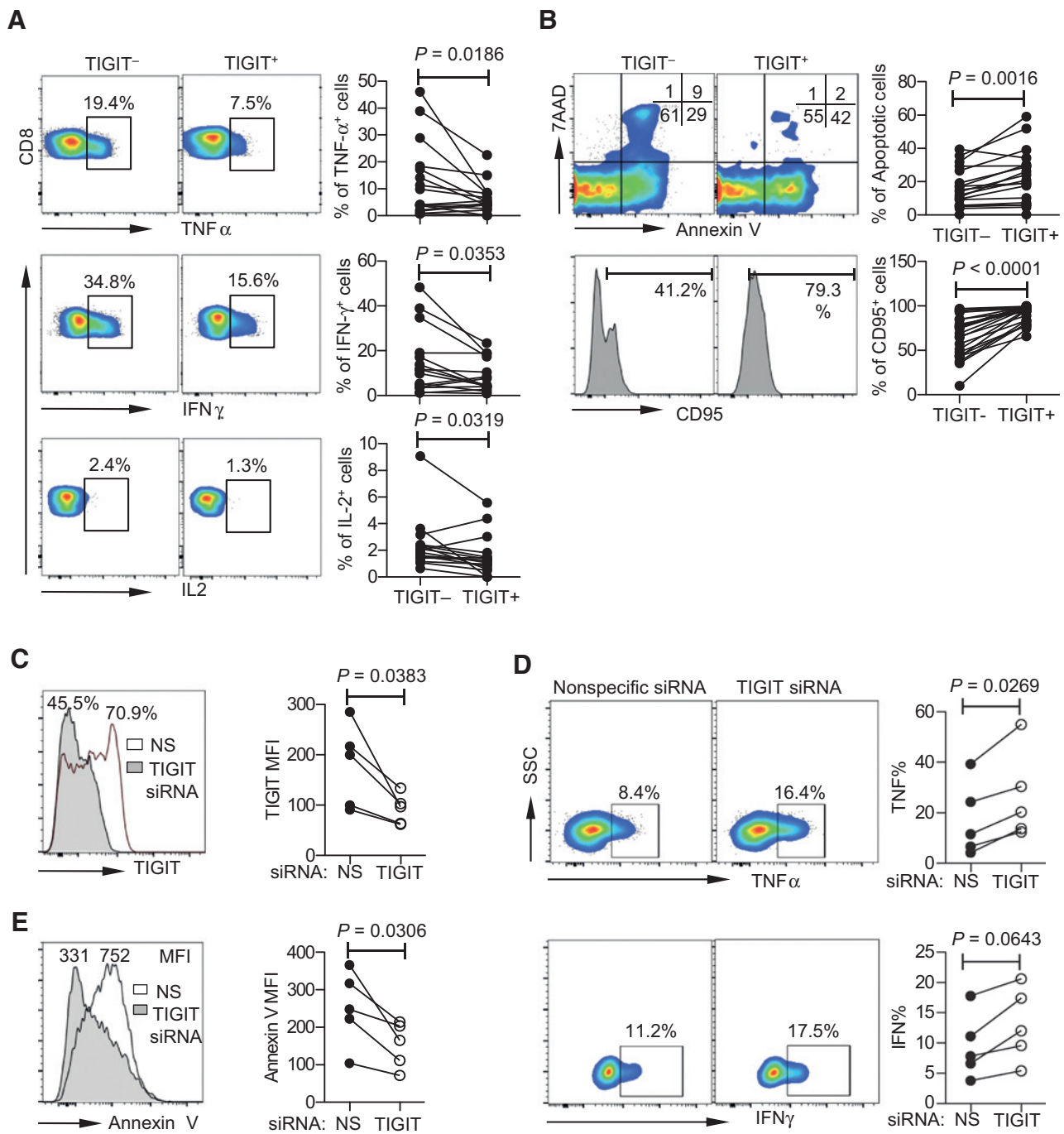


Figure 4. TIGIT⁺ CD8⁺ T cells from AML patients show functional defects in cytokine production and high susceptibility to apoptosis, which can be reversed by TIGIT knockdown. A, intracellular cytokine production of TNF α , IFN γ , and IL2 by TIGIT⁻ and TIGIT⁺ CD8⁺ T cells from AML patients ($n = 16$) upon *in vitro* anti-CD3/anti-CD28 stimulation. Shown are representative flow data (left) and summary data (right) for TNF α , IFN γ , and IL2, respectively. P values were obtained by the Wilcoxon matched-pairs signed rank test. B, the percentage of apoptotic cells (Annexin V⁺ 7AAD⁻) and expression of CD95 on TIGIT⁻ and TIGIT⁺ CD8⁺ T cells from AML patients ($n = 19$). Representative flow data (left) and plot of percentage of apoptotic cells (Annexin V⁺ 7AAD⁻, right) are shown. P values were obtained by the paired *t* test (Annexin V) or Wilcoxon matched-pairs signed rank test (CD95). C to E, purified CD8⁺ T cells from AML patients ($n = 5$) were transfected with indicated siRNA and cultured *in vitro* for 3 days. Intracellular cytokine production in response to anti-CD3 and anti-CD28 stimulation was evaluated by flow cytometry. The susceptibility of apoptosis was analyzed by depicting expression of Annexin V. C, TIGIT knockdown significantly decreased TIGIT expression on CD8⁺ T cells. Representative flow data (left) and a plot of TIGIT MFI (right) on CD8⁺ T cells transfected with nonspecific (NS) siRNA vs. TIGIT siRNA are shown. D, TIGIT knockdown increases cytokine production. Representative flow data (left) and plots (right) of the percentage of TNF α and IFN γ production are shown. E, TIGIT knockdown reduces apoptosis. Representative histogram (left) and plot (right) of Annexin V expression in 7AAD⁻ CD8⁺ T cells are shown. P values were obtained by the paired *t* test.

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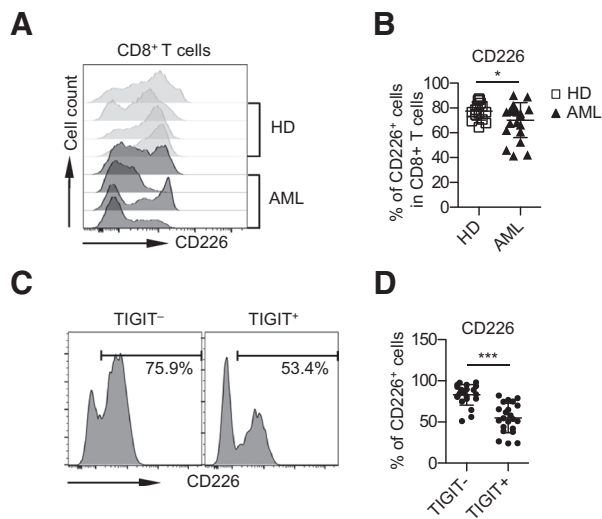


Figure 5. CD226 is downregulated on CD8⁺ T cells in AML patients. A and B, flow-cytometry analysis of PBMCs from HDs or AML patients for CD226 expression on CD8⁺ T cells. Representative flow data from 4 HDs and 4 AML patients are shown in A. A plot of the percentage of CD226⁺ cells among CD8⁺ T cells from HDs ($n = 15$) and AML patients ($n = 20$) are shown in B. P values were obtained by the Mann-Whitney U test. C and D, depicting the CD226 expression on TIGIT⁻ versus TIGIT⁺ CD8⁺ T cells. Representative flow data (C) and a plot (D) of CD226⁺ percentage on TIGIT⁻ versus TIGIT⁺ CD8⁺ T cells from AML patients ($n = 20$) are shown. P values were obtained by unpaired t test; *, $P < 0.05$; ***, $P < 0.001$.

Our study shows that TIGIT⁺ CD8⁺ T cells exhibit features of T-cell exhaustion. TIGIT expression is associated with upregulation of several inhibitory regulators that play key roles in T-cell exhaustion. Interestingly, although enhanced PD-1, CD160, and 2B4 were observed on TIGIT⁺ CD8⁺ T cells, no correlation was found between TIGIT expression and other markers known to be involved in T-cell exhaustion such as TIM-3, BTLA, and LAG-3. Consistent with exhaustion, TIGIT⁺ CD8⁺ T cells showed decreased production of cytokines and high susceptibility to apoptosis, which were partially reverted by TIGIT knockdown. In contrast, TIGIT⁺ CD8⁺ T cells retained and even displayed higher proliferative and cytotoxic capacity. Several possibilities are considered to explain the discrepancy. First, different tumor types may use different negative regulatory mechanisms to evade immune attack. Consistent with a previous study in melanoma (35), we observed a tight association of TIGIT with PD-1 expression on CD8⁺ T cells of AML patients, supporting a therapeutic strategy of dual TIGIT and PD-1 blockade in AML. In addition, our finding that other inhibitors such as CD160 and 2B4 are also likely involved in TIGIT mediated AML progression, provides a new therapeutic avenue to explore for AML control in which combining blockade of multiple inhibitory receptors might be the most effective measure. Second, extensive studies of T-cell exhaustion in chronic viral infections suggest that loss of function during exhaustion follows a reproducible hierarchical program in which exhausted T-cells lose some of their functions while retaining others at an early stage (13, 41–43). In fact, it has been reported that T cells from chronic lymphocytic leukemia patients exhibit an exhausted defect in proliferation and cytotoxicity, but retain the capacity for cytokine production (27). In our study, TIGIT⁺ CD8⁺ T cells from AML

patients lost the capacity for cytokine production, but retained the capacity for proliferation and cytotoxicity. Third, TIGIT⁺ CD8⁺ T cells tested in our study are polyclonal, which could contribute to the heterogeneity of the results. Further phenotypic and functional dissection of leukemia-specific TIGIT⁺ CD8⁺ T cells would be helpful to clarify the conclusions.

TIGIT could exert negative immunoregulation through multiple mechanisms, including promoting IL10, but dampening IL12 production by dendritic cells (29); direct intrinsic inhibition by recruitment of SHP phosphatases (44, 45); or suppression of the activity of its costimulatory counterpart, CD226 (40). In our study, we observed a significant downregulation of CD226 on CD8⁺ T cells from patients with AML, although these cells have increased expression of TIGIT. Consistently, TIGIT⁺ CD8⁺ T cells expressed lower levels of CD226 compared with that of TIGIT⁻ cells. These results suggest an important role for CD226 in TIGIT-mediated immune suppression in AML patients. Our data are in line with the recent finding that CD226 expression is decreased in CD8⁺ TILs in metastatic melanoma (35). Thus, similar to the relationship between CTLA4 and CD28, TIGIT may suppress CD8⁺ T-cell antitumor responses by inhibiting the costimulatory effect of CD226 in AML.

Our finding that high expression of TIGIT associates with primary refractory disease and leukemia relapse posttransplantation in AML patients has significant clinical implications. Primary refractory disease, defined as failure to achieving CR after 1 to 2 cycles of intense induction therapy, accounts for 10% to 40% of AML patients (37, 46). This represents one of the most challenging clinical scenarios in management of AML and is not typically curable by conventional salvage therapy. AlloSCT offers a higher cure rate. However, this high-risk procedure is often not feasible due to high age, comorbidity, poor performance status, and severe complications of induction treatment. How to optimize the initial therapy and prevent primary refractory disease is currently an active research area. It is crucial to identify the patient population with high risk. Our study reveals an association of high-TIGIT at initial diagnosis with primary refractory disease, suggesting that testing TIGIT might be a feasible approach for early risk stratification. Most importantly, this finding provides a strong rationale for early intervention in that combining induction chemotherapy with agents blocking TIGIT might be the key to improve clinical outcome.

AlloSCT is in many situations the only curative measure for AML. For patients who are fit and able to receive this procedure, alloSCT significantly increases survival. However, leukemia relapse does occur after transplantation. According to the data from CIBMTR, disease relapse is the top cause of death, accounting for approximately 40% of posttransplant deaths (47). Once leukemia relapse occurs, the prognosis is generally poor. Currently, there is no effective treatment available for this patient population. Identification of novel therapeutic targets is critical to develop effective treatments and improve clinic outcomes. Eradication of leukemia in alloSCT largely relies on graft versus leukemia (GVL) mediated by donor T cells (48, 49). Leukemia relapse is considered a failure of GVL. Our finding that high TIGIT expression on CD8⁺ T cells associates with leukemia relapse posttransplantation suggests that negative regulation by TIGIT on donor T cells might contribute to GVL failure and leukemia relapse, making a case for targeting TIGIT for effective treatment of leukemia relapse posttransplantation.

In summary, our study demonstrates that TIGIT is an important negative immune regulator in AML, making it an attractive therapeutic target for effective leukemia control.

Disclosure of Potential Conflicts of Interest

D.F. Claxton is a consultant/advisory board member for Bristol-Meyers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Kong, J. Zhang, D.F. Claxton, W.C. Ehmann, W.B. Rybka, H. Zheng

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Kong, H. Zeng, H. Zheng

Writing, review, and/or revision of the manuscript: Y. Kong, T.D. Schell, D.F. Claxton, W.C. Ehmann, W.B. Rybka, M.R. George, H. Zeng, H. Zheng

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