Expression of PD-L1, PD-L2, PD-1 and CTLA4 in myelodysplastic syndromes is enhanced by treatment with hypomethylating agents

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Abstract

Blockade of immune checkpoints is emerging as new form of anticancer therapy. We studied the expression of PD-L1, PD-L2, PD-1 and CTLA4 mRNA expression in CD34+ cells from MDS, CMML and AML patients (N=124). Aberrant up-regulation (≥2 fold) was observed in 34%, 14%, 15% and 8% of the patients respectively. Increased expression of these 4 genes was also observed in PBMNC (N=61). The relative expression of PD-L1 from PBMNC was significantly higher in MDS (p=0.018) and CMML (p=0.0128) compared to AML. By immunohistochemical (IHC) analysis, PD-L1 protein expression was observed in MDS CD34+ cells, whereas stroma/non-blast cellular compartment was positive for PD-1. In a cohort of patients treated with epigenetic therapy, PD-L1, PD-L2, PD-1 and CTLA4 expression was upregulated. Patients resistant to therapy had relative higher increments in gene expression compared to patients that achieved response. Treatment of leukemia cells with decitabine resulted in a dose dependent up-regulation of above genes. Exposure to decitabine resulted in partial demethylation of PD-1 in leukemia cell lines and human samples. This study suggests PD-1 signaling may be involved in MDS pathogenesis and resistance mechanisms to HMAs. Blockade of this pathway can be a potential therapy in MDS and AML.
Keywords
programmed death-1; myelodysplastic syndromes; DNA methylation

INTRODUCTION

Myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell disorders characterized by bone marrow failure, dysplasia of myeloid blood cell lineages, and increased risk of developing acute myeloid leukemia (AML). The pathogenesis of MDS is multifactorial including both cell intrinsic alterations, such as mutations, and cell extrinsic stimuli such as immune deregulation. Across different categories of MDS, alterations of natural killer (NK) cells, T cells, FOXP3+ regulatory T cells (Tregs), as well as myeloid derived suppressor cells have been reported.

Leukemia cells harbor both genetically and epigenetically dysregulated genes which can be recognized as foreign antigens by the immune system. However spontaneous leukemia remissions are a rare occurrence, suggesting an endogenous immune dysfunction preventing the control of leukemia growth. Negative immune regulatory factors have been proposed to contribute to a suppressive microenvironment that protects cancer cells from immune destruction. This has led to clinical efforts aimed at overcoming immune tolerance in cancer such as the monoclonal antibody (mAb) ipilimumab, a blocking mAb directly against the co-inhibitory T cell receptor CTLA4 (cytotoxic T lymphocyte-associated antigen 4). Other co-inhibitory molecules include programmed death-1 (PD-1) and its ligand, programmed death ligand 1 (PD-L1; B7-H1). Anti-PD-1 monoclonal antibodies have produced responses in patients with solid tumors.

The use of the hypomethylating agents (HMAs) 5-azacytidine and 5-aza-2′ deoxycytidine are a standard in patients with higher risk MDS and commonly used in older patients with AML. However resistance to HMAs is an almost universal phenomenon. While the mechanisms of resistance to HMAs in myeloid malignancies are not understood, it is known that MDS therapy with the above agents can induce immune reaction alterations. Importantly, recent studies have identified that demethylation of the locus encoding PD-1 leads to exhaustion of CD8+ T cells after chronic viral infection. To further evaluate the role of immune checkpoints in patients with myeloid malignancies, we performed an expression analysis of PD-L1, PD-L2, PD-1, CTLA4 in MDS, CMML, AML and in a cohort of patients treated with epigenetic therapy. This study provides important information for the understanding of the role of the PD-1/PD-L1 axis and its ligands in myeloid leukemia pathogenesis and in mechanism of resistance to HMAs.

MATERIALS AND METHODS

Cell culture and treatment

Human leukemia cell lines HL-60, NB4, THP1, U937, ML1, OCI-AML3 and HEL were cultured in RPMI 1640 medium. KG1 cell line was maintained in IMDM medium. All cells were obtained from the American Type Culture Collection. THP1 and KG1 cells were
plated at low density before drug treatment and subsequently treated with 5-aza-2′-deoxycytidine (DAC) (Eisai Pharmaceuticals, Woodcliff Lake, NJ) or cytarabine (Ara-C) (Sigma-Aldrich, MO).

**Human specimens**

Bone marrow specimens from 124 patients with MDS (n=69), CMML (n= 46), and AML (n=9) were obtained from the leukemia department tissue bank at MD Anderson Cancer Center (MDACC) following institutional guidelines. Patient characteristics are shown in Table 1. CD34+ cells were sorted from bone marrow specimens using CD34+ MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer’s protocol. Bone marrow samples from 4 healthy donors were obtained from Alcells (Emeryville, CA). Peripheral blood (PB) samples from 61 patients with MDS, CMML or AML treated with epigenetic therapy and 6 healthy volunteers were analyzed (Supplemental table 1 and 2). PB mononuclear cells were isolated from patient samples by Ficoll density-gradient centrifugation. Finally, 45 bone marrow biopsy samples and 11 CD34+ cell cytopsins from MDS, CMML and AML patients were obtained from the hematopathology department at MDACC. Four normal bone marrow biopsy samples were used as controls.

**Real time PCR**

Real time PCR was performed using total cellular RNA as previously described (Supplemental Information).

**Immunohistochemical analysis**

Tissue sections from bone marrow biopsies were incubated with specific monoclonal antibodies against human PD1 (Cell Marque, CA) or human PD-L1 (BioLegend, CA). Detailed protocol is described in Supplemental Information. Using a cytospin preparation of CD34+ cells from MDS patients, immunohistochemical analysis was performed with the above human PD-L1 and PD-1 specific antibody.

**Multicolor flow cytometry**

APC mouse anti-human PD-1 and PE mouse anti-human PD-L1 antibodies were obtained from BD Biosciences (San Jose, CA). KG-1 cells with and without DAC treatment were incubated with antibodies for 20 minutes. Multicolor flow cytometry analysis was performed with a BD FACSCalibur instrument (San Jose, CA). Data were analyzed with the BD Cell Quest Pro software (San Jose, CA).

**DNA bisulfite treatment and methylation analysis**

For PD-1 DNA methylation analysis, two methods were employed. First, pyrosequencing after bisulfite treatment of genomic DNA was performed as previously described. Primers are shown in Supplemental table 3. To confirm the primary methylation analysis, methylation of PD1 CpG island was subsequently evaluated using a bisulfite-sequencing assay. A 340-bp amplicon that contains 10 CpG sites was analyzed (Supplemental table 3). Detailed information is described in Supplemental Information.
Statistical analysis

Associations between patient characteristics, gene expression levels and overall survival were analyzed. Categorical variables were compared using $\chi^2$ or Fisher’s exact test, and continuous variables using the Wilcoxon-rank sum test. Associations between gene expression and continuous variables were calculated using linear regression analysis. Relative gene expression was evaluated both as a continuous and as a ternary categorical variable, divided into three cohorts of samples without expression, relative expression between 0 and 2 fold, and ≥2 fold relative expression. Overall survival (OS) and overall response rate (ORR) was analyzed using the Kaplan-Meier method, with differences evaluated by the log-rank test. Analyses were performed using STATA software, version 12.0 (College Station, TX) and SAS 9.0.

RESULTS

Expression of PD-L1, PD-L2, PD-1 and CTLA4 in CD34+ cells from MDS, CMML and AML patients

We first evaluated the mRNA expression levels of PD-L1, PD-L2, PD-1 and CTLA4 by Q-PCR in bone marrow CD34+ cells from 124 patients. This included 69 with MDS, 46 with CMML, and 9 with AML. Seventy two (58%) patients were previously untreated. Patient characteristics are shown in Table 1. Relative mRNA expression was quantified using normal CD34+ cells as controls. Expression levels are shown in Figure 1A and Supplemental Table 4. No significant differences in gene expression were observed when comparing patients with or without prior therapy (Table 2). In summary, the mean expression of PD-L1 in the 69 MDS patients was 28.13 (range 0–843), PD-L2 was 1.2 (range 0–22.5), PD-1 was 0.82 (range 0–36), and CTLA4 was 0.77 (range 0–25). Up-regulation (≥2 fold) of PD-L1 was observed in 36% of MDS CD34+ samples, PD-L2 in 12%, 8% for PD-1 and 6% for CTLA4, respectively. In the 46 CMML, the mean expression of PD-L1 was 8.8 (range 0–199), PD-L2 was 1.1 (range 0–15.7), PD-1 was 5.5 (range 0–77), and CTLA4 was 0.85 (range 0–6). Up-regulation (≥2 fold) of PD-L1 was observed in 32% of CMML CD34+ samples, 14% for PD-L2, 26% for PD-1 and 14% for CTLA4, respectively. In the 9 AML patients compared to normal CD34+ cells, the mean expression of PD-L1 was 2.5 (range 0–16), PD-L2 was 1.3 (range 0–4.1), PD-1 was 1.5 (range 0–11.7), and CTLA4 was 0.16 (range 0–0.77). Up-regulation (≥2 fold) of PD-L1 was observed in 25% of AML CD34+ samples, 33% for PD-L2, 22% for PD-1 and 0 for CTLA4 respectively.

We analyzed PD-L1 membranous protein expression in 4 cytospins from MDS CD34+ cells corresponding to 2 patients with high Q-PCR PD-L1 levels and 2 negative controls by immunohistochemistry (IHC) analysis using monoclonal antibody directed against PD-L1. A perfect correlation was observed between protein and mRNA expression. We then expanded the IHC analysis for both PD-L1 and PD-1 to bone marrow biopsies from 45 patients and CD34+ cytospins from 7 patients with MDS, CMML and AML. Positive was considered when more than 5% of cells carried membrane marker. In the 45 bone marrow biopsies, the leukemic blasts of 9 patients (20%) were positive for PD-L1 (Figure 2A), 3 (7%) were positive for PD-1 (Figure 2B). All 4 normal controls were negative for both PD-
L1 and PD-1. Interestingly, positive PD-1 expression in stroma/non-blast cellular compartment was also observed (Figure 2B). In CD34+ cytospins from 7 patients: 6 were positive and 1 negative for PD-L1 (Figure 2C). All, except one that was negative, were rarely positive for PD-1 (Figure 2D).

Clinical implications of expression patterns

Correlations between gene expression and clinicopathologic characteristics were then evaluated and are detailed in Supplemental Table 5 for the n=124 bone marrow specimens from patients with MDS, CMML or AML. Statistically significant relationships were identified between elevated PD-1 expression and increased age (p=0.008), while elevated PD-L2 expression correlated significantly with female gender (p=0.005). Both elevated PD-L1 and CTLA4 expression correlated with MDS subtype according to WHO criteria, (p=0.034) and (p=0.012), respectively. Additionally, elevated CTLA4 expression correlated with higher white blood cell count (p=0.021), lack of prior therapy (p=0.022) and lower MDS IPSS score (p=0.027). We then performed an analysis of the impact on survival of the 4 gene expression in patients that had not received prior treatment. Patients with lower expression of PD-L1 had a none significant trend towards better survival (31.5 months versus 16.2, p=0.24).

Expression of PD-L1, PD-L2, PD-1 and CTLA4 in PBMNC cells from MDS, CMML and AML patients

We then analyzed mRNA expression levels of PD-L1, PD-L2, PD-1 and CTLA4 in peripheral blood mononuclear cells (PBMNC) of a cohort of 61 patients treated with epigenetic therapy, including 24 patients with MDS, 5 with CMML and 32 with AML (Figure 1B and Table 3). Relative mRNA expression was quantified using normal PBMNC cells as controls (N=6). Increased gene expression of these four genes in the PBMNCs of patients with myeloid malignancies was also observed. In summary, in the 24 MDS patients, the mean expression of PD-L1 was 4.4 (range 0–22.1), PD-L2 was 2.7 (range 0–19.5), PD-1 was 5.2 (range 0–26.5), and CTLA4 was 1.0 (range 0–9.5). Up-regulation (≥2 fold) of PD-L1 was observed in 50% of MDS samples, 9% for PD-L2, 61% for PD-1 and 8% for CTLA4 respectively. In the 5 CMML patients, the mean expression of PD-L1 was 12.4 (range 0.12–55.3), PD-L2 was 2.2 (range 0.03–6.5), PD-1 was 4.4 (range 0.03–17.2), and CTLA4 was 1.0 (range 0.001–3.9). Up-regulation (≥2 fold) of PD-L1 was observed in 40% of CMML samples, 40% for PD-L2, 40% for PD-1 and 3% for CTLA4 respectively. In the 32 AML patients, the mean expression of PD-L1 was 1.7 (range 0–8.3), PD-L2 was 0.9 (range 0–7.4), PD-1 was 3.9 (range 0–32.4), and CTLA4 was 0.4 (range 0–2.3). Up-regulation (≥2 fold) of PD-L1 was observed in 32% of AML samples, 12.9% for PD-L2, 39% for PD-1 and 3% for CTLA4 respectively. The relative expression of PD-L1 was significantly higher in MDS (p=0.018) and CMML (p=0.0128) compared to AML. Of interest, mRNA expression of these 4 genes was significantly higher in PBMNC than in CD34+ cells except PD-L1 (Figure 1C).
Up-regulation of PD-L1, PD-L2, PD-1 and CTLA4 expression in MDS, CMML and AML patients treated with hypomethylating agents

To study the effect of epigenetic therapies on the expression of these 4 genes, we performed a sequential analysis of mRNA expression of PD-L1, PD-L2, PD-1 and CTLA4 in 61 patients treated with epigenetic therapy at MDACC (Supplemental Table 1). PBMNC were analyzed by Q-PCR on days 0 and sequential by depending on specific protocol during first cycle of therapy. During first course of epigenetic therapy, PD-L1, PD-L2, PD-1 and CTLA4 gene expression was upregulated (≥2 fold) in 57%, 57%, 58% and 66% of the patients respectively at any time during the first cycle of therapy. A summary of gene expression patterns during therapy is shown in Figure 3A. All 61 patients received combination therapy except for 8 patients treated with single-agent decitabine. In those eight patients, we also observed PD-L1, PD-L2, PD-1, and CTLA4 up-regulation (data not shown). We then analyzed the relationship between expression and response to hypomethylating agents. We focused on study 2007-0685 (NCT00948064) (azacitidine + vorinostat) because the study is completed and only allowed patients with previously untreated disease. In this group of patients, there was a trend towards increased expression of these 4 genes in resistant patients compared to sensitive patients. No difference in baseline levels was observed between sensitive and resistant patients (Figure 3B). We then performed a survival analysis for these patients. Patients without up-regulation of PD-L1, PD-L2, PD-1 or CTLA4 expression after treatment were grouped as 0, patients with up-regulation were grouped as 1. The median survival was 11.7 and 6.6 months (p=0.122) for PD-L1 in group 0 and group 1, and 12.5 and 4.7 months (p=0.029) for PD-L2 (Figure 3C) respectively. No significant survival difference was observed related to PD-1 and CTLA4 expression level for this group of patients.

mRNA Expression of PD-L1, PD-L2, PD-1 and CTLA4 in leukemia cell lines

To investigate the suitability of using leukemia cell lines to study the effect of hypomethylating agents in AML and MDS, we analyzed the mRNA expression level of PD-L1, PD-L2, PD-1 and CTLA4 in the following acute myeloid leukemia cell lines: HL-60, NB4, THP1, U937, ML1, OCI-AML3, HEL and KG1. The general mRNA expression levels in cell lines were very low but detectable. PD-L1 was detectable in all cell lines examined except OCI-AML3. HEL expressed the highest levels. PD-L2 was detectable in THP-1, U937, OCI-AML3 and HEL, whereas CTLA4 and PD-1 were only detected in KG1 cells (Supplemental Figure 1).

5-aza-2′ deoxycytidine enhances PD-L1, PD-L2, PD-1 and CTLA4 expression in acute myeloid leukemia cell lines

To study the effect of hypomethylating agent decitabine (DAC) on PD-L1, PD-L2, PD-1 and CTLA4 expression in AML cell lines, KG-1 and THP1 cells were treated with DAC at different concentrations for three days. Medium was changed every 24 hours and fresh drug was added daily. mRNA expression levels were analyzed by Q-PCR (Figure 4A, 4B). Treatment with DAC resulted in a significant increase in gene expression in both cell lines at concentration of 1μM and above. For instance, in KG-1 cells, PD-L1 upregulation could be observed at concentrations of 1μM (6.2 fold), and continued to increase in a concentration
dependent manner (fold 11.8 at 10 uM). At a concentration of 1uM of treatment, PD-1 was induced 38.4 fold compared to control, and continued to increase to 59.3 at 10 uM. CTLA4 expression was induced by 12.5 fold at 1 uM and 49.4 fold at 10 uM respectively. In THP-1, PD-L1 and PD-L2 both were up-regulated following a concentration dependent manner. At a concentration 1uM, PD-L1 and PD-L2 were induced by 3.6 and 5.3 fold, and continued to increase to 14.8 and 26.8 with 10 uM of treatment separately. To study whether this effect on leukemia cell lines was specific to DAC or could be observed with other agents, we treated KG-1 and THP1 with cytarabine (Ara-C) at different concentrations (0, 0.5, 1, 3, 5, 10uM) for 24 hours in KG-1 cells and 24 hours, 48 hours in THP1 cells. Cell viability by trypan blue staining was used to determine the effect of Ara-C on the two cell lines (Supplemental figure 2). mRNA expression levels were analyzed by Q-PCR (Figure 4A, 4B). In KG-1 cells, induction of PD-L1, PD-1 and CTLA4 expression was not observed except for PD-1 at concentrations of 10uM (3-fold), CTLA4 at 5uM (2-fold) and 10uM (5-fold). In THP1 cells, no effect on gene expression of PD-L1 was observed in 24 and 48 hours of Ara-C treatment, PD-L2 was slightly induced (4 fold) begin at 5uM with 24 hours of treatment and 3 fold begin at 1 uM with 48 hours, no concentration dependent effect was observed.

To detect antigen expression levels of PD-L1 and PD-1 in DAC treated myeloid leukemia cell line KG-1, we performed multicolor flow cytometry analysis. In KG-1 cells (Figure 4C), we observed an increase of PD-L1 and PD-1 antigen expression at concentrations as low as 0.1uM of DAC treatment. Both PD-L1 and PD-1 expression were increased in a concentration dependent manner.

**DNA demethylation of PD-1 in decitabine treated leukemia cell line and patients treated with hypomethylating agents**

The role of DNA methylation causing repression of PD-1 expression after T cell receptor (TCR) stimulation was recently reported in an *in vivo* model of acute infection. Consistent with these results, we detected PD-1 methylation in the same CpG island loci reported above in DNA from normal PBMNC, leukemia cell lines, and MDS and AML patient PBMNC. We observed PD-1 methylation in all of these samples. There was no difference in PD-1 methylation levels between normal controls and MDS, AML patients, whereas higher methylation level observed in leukemia cell lines (Figure 5A). Treatment of KG-1 cell line with DAC resulted in demethylation of PD-1. Hypomethylation could be observed at concentrations of 1uM and above (Figure 5B). We confirmed the pyrosequencing results in DAC treated KG-1 cells using bisulfite sequencing (Figure 5C). We also studied the dynamics of PD-1 demethylation in the group of patients treated with vorinostat in combination with azacitidine (Figure 5D). DNA hypomethylation could be observed in both resistant and sensitive cases. That said, baseline methylation levels were higher in resistant patients compared to sensitive (P<0.05). No PD-L1 methylation was observed in normal controls and AML patients (data not shown).
**Discussion**

PD-1 is a negative costimulatory receptor on activated T lymphocytes which counters the activation signal provided by T cell receptor ligation.\(^{28}\) PD-1 can also be induced in NK cells, B cells and monocytes.\(^{28}\) The two ligands of PD-1 are PD-L1 and PD-L2. They have distinct cellular expression patterns. Expression of PD-L2 is largely restricted to antigen presenting cells (APCs) while PD-L1 is broadly expressed in tissues and can be further induced by exposure to interferon IFN-\(\gamma\).\(^{28}\) PD-L1 is the major ligand for PD-1 mediated immune-suppression. Increased evidence suggests that PD-L1 expression on solid tumor cells is capable of dampening antitumor immune responses, and blockade of PD-L1 inhibits tumor growth and delays progression in murine models.\(^{28}\) However, evidence supporting a functional role for this pathway in myeloid leukemia is lacking.

In this study, we first demonstrated that PD-1 and its two ligands, PD-L1 and PD-L2, as well as CTLA4, are aberrantly upregulated in 8 to 34% of bone marrow CD34+ cells from patients with myeloid leukemias. There was a trend towards increased expression in MDS. Recent studies suggest that the role of the immunologic compartment may change over time from autoimmune into immune-suppressive mechanisms as MDS progresses from early into more advanced stages.\(^{3,29-31}\) PD-L1 and PD-L2 have been found to be expressed in solid tumors,\(^{32,33}\) correlation between PD-1 ligands expression on tumors cells and poor prognosis has been reported.\(^{34}\) In the CD34+ cells from a group of patients without prior treatment, we observed that lower expression of PD-L1 was associated with a tendency to longer survival. A larger cohort analysis will be needed to expand these results.

Overexpression of these genes was also observed in PBMC. Except for PD-L1, the baseline expression of the other three genes was significantly higher in PBMC than in bone marrow CD34+ cells. We observed a correlation between protein and mRNA expression for PD-L1. Engagement of PD-1 by PD-L1 leads to the inhibition of T cell receptor-mediated lymphocyte proliferation and cytokine secretion.\(^{35}\) Tumor cells may suppress the function of tumor infiltration T cells by modulating PD-1. PD-1 has been reported to be up-regulated on tumor infiltration T cells in melanoma and lung cancer.\(^{17}\) In AML and MDS bone marrow biopsies, we observed that blasts were positive for PD-L1 whereas stroma/non-blast cellular compartment were positive for PD-1. Thus, our results suggest that PD-1 ligands expressed on tumor cells may act through PD-1 positive stroma within the tumor microenvironment of AML and MDS patients.

PD-1 methylation plays a role in memory CD8+ T cell differentiation.\(^{24}\) DNA methylation is involved in repression of PD-1 expression after T cell receptor (TCR) stimulation in an *in vivo* model of acute infection. Demethylation and site specific remethylation of PD-1 locus was coupled with development of fully functional memory CD8+ T cell.\(^{24}\) T cell exhaustion during chronic viral infection is associated with maintained demethylation of PD-1 locus.\(^{24}\) DNA-methyltransferase inhibitors such as 5-azacitidine and decitabine are widely used in the treatment of MDS.\(^{18-20}\) A significant portion of patients do not response to the hypomethylating treatment or lose response to therapy. The prognosis of patients that lose response to HMAs is very poor and the mechanisms of resistance are unknown.\(^{21,36}\) It has been postulated that hypomethylating agents exert their effects in part via regulation of expression of cell surface antigens,\(^{37}\) expression that could in turn be involved in resistance...
to HMA.\textsuperscript{38} We performed an analysis of sequential samples from patients treated in different clinical trials of epigenetic therapy. Induction of PD-L1, PD-L2, PD-1 and CTLA4 expression was demonstrated in these patients. This data suggests that HMAs can modulate PD-1/PD-L1 axis in MDS and AML patients. And that increased expression may be associated with resistance to therapy.

We modeled the data from the primary clinical samples in leukemia cell lines. PD-L1, PD-L2, PD-1 and CTLA4 were induced by DAC treatment in a concentration dependent manner. This was not observed with Ara-C. This has important implications for the development of combination strategies of HMAs with PD-1 and PD-L1 inhibitors in leukemia. We also showed here that DAC induced PD-1 reactivation in KG-1 cells was related to PD-1 demethylation in the same loci as reported.\textsuperscript{24} In the group of patients treated with vorinostat in combination with azacitidine, though pretreatment methylation level was significantly higher (p<0.05) in patients resistant to therapy than sensitive, PD-1 dynamic demethylation range was more sensitive also. Gene reactivation may depend more on demethylation level other than baseline methylation level.

In conclusion, our results suggest that deregulation of PD-1/PD-L1 axis may contribute the MDS pathogenesis and that induction of PD-1 and PD-1 ligands may be involved in HMAs resistance. We predicted that blockade of this pathway alone or in combination with HMAs can be a potential therapy in MDS patients.

\section*{Supplementary Material}

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Increased mRNA expression of PD-L1, PD-L2, PD-1 and CTLA4 in MDS, CMML and AML patients
(A) Increased mRNA expression of PD-L1, PD-L2, PD-1 and CTLA4 genes in CD34+ cells from MDS, CMML and AML patients by real-time PCR analysis, normal CD34+ cells were used as normal control. N = number of patients. MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia. (B) Increased mRNA expression of PD-L1, PD-L2, PD-1 and CTLA4 in PBMNCs from MDS, CMML and AML patients, normal PBMNCs were used as normal controls. PBMNC, peripheral blood mononuclear cells. (C) Comparisons of mRNA expression levels of PD-L1, PD-L2, PD-1 and CTLA4 between CD34+ cells and PBMNC from MDS, CMML and AML patients.
Figure 2. Immunohistochemical analysis of PD-L1 and PD-1 membranous expression in CD34+ cell cytospin and bone marrow biopsy samples from MDS, CMML and AML patients

(A) Illustrations of PD-L1 membranous expression in bone marrow biopsy samples from MDS, CMML and AML patients (X1000): a, Representative case show strong PD-L1 membranous expression in blasts; b, Representative case show negative PD-L1 membranous expression in blasts. (B) Illustrations of PD-1 membranous expression in bone marrow biopsy samples from MDS, CMML and AML patients (X1000): a, Representative case show PD-1 membranous expression in blasts; b, Representative case show negative PD-1 membranous expression in blasts, but positive PD-1 expression in stroma/non-blast compartment. (C) Illustrations of PD-L1 membranous expression in CD34+ cell cytospin from MDS, CMML and AML patients (X1000): a, Representative case show strong PD-L1 membranous expression; b, Representative case show negative PD-L1 membranous expression. (D) Illustrations of PD-1 membranous expression in CD34+ cell cytospin from MDS, CMML and AML patients (X1000): a, Representative case show partial PD-1 membranous expression; b, Representative case show negative PD-1 membranous expression.
Figure 3. Induction of PD-L1, PD-L2, PD-1 and CTLA4 expression in patients treated with epigenetic therapy
(A) Summary of dynamics of PD-L1, PD-L2, PD-1 and CTLA4 mRNA expression in 61 patients treated with different forms of epigenetic therapy, expression on day 0 was baseline expression before treatment, most patients received 5 days of hypomethylating agent treatment. (B) Induction of PD-L1, PD-L2, PD-1 and CTLA4 expression in patients treated in a phase 2 trial of vorinostat in combination with azacitidine. C=course, D=days on therapy. Resistance: patients had no response to treatment; Responing: patients acquired complete remission after treatment. (C) Overall survival by comparison of PD-L2 upregulation after treatment in group of patients from a phase 2 trial of vorinostat in combination with azacitidine. Group 0: group of patients without PD-L2 expression induction (≥2 fold) after treatment; Group 1: group of patients acquired PD-L2 expression induction (≥2 fold) after treatment.
Figure 4. Induction of PD-L1, PD-L2, PD-1 and CTLA4 expression in leukemia cell lines KG-1 and THP1 treated with hypomethylating agent

(A) Induction of PD-L1, PD-1 and CTLA4 mRNA expression in KG-1 treated with different concentrations of decitabine and cytarabine. (B) Induction of PD-L1 and PD-L2 mRNA expression in THP1 treated with different concentrations of decitabine and cytarabine. (C) Flow cytometry analysis of PD-1 and PD-L1 protein expression levels in KG-1 treated with different concentrations of decitabine.
Figure 5. PD-1 methylation in leukemia cell lines, MDS and AML patients with and without treatment of hypomethylating agents
(A) PD-1 methylation in leukemia cell lines, AML patients and normal controls. (B) Dynamics of PD-1 methylation in KG-1 leukemia cell line treated with decitabine by pyrosequencing analysis, N=Number. (C) Bisulfite sequencing analysis of dynamics of PD-1 methylation with decitabine treatment of KG-1. (D) Dynamics of PD-1 methylation in AML and MDS patients from group of patients treated with vorinostat in combination with azacitidine, C=Course; D=days on therapy.
### Table 1

**Patient characteristics**

|                          | Total N=124 | No Prior Therapy N=72 | Prior Therapy N=52 | p Value |
|--------------------------|-------------|-----------------------|--------------------|---------|
| **Age, years**           |             |                       |                    |         |
| Median                   | 69          | 68                    | 0.982              |         |
| Range                    | 33–89       | 51–87                 |                    |         |
| **Sex**                  |             |                       |                    |         |
| Male                     | 51          | 33                    | 0.384              |         |
| Female                   | 21          | 19                    |                    |         |
| **BM blast, %**          |             |                       |                    |         |
| Median                   | 5           | 7                     | 0.448              |         |
| Range                    | 0–33        | 1–31                  |                    |         |
| **WBC, X10^9/L**         |             |                       |                    |         |
| Median                   | 6           | 4                     | 0.361              |         |
| Range                    | 0.8–73.6    | 1.4–83.5               |                    |         |
| **Hemoglobin, g/dL**     |             |                       |                    |         |
| Median                   | 10          | 10                    | 0.522              |         |
| Range                    | 7.1–14.6    | 7–14.4                |                    |         |
| **Platelet, X10^9/L**    |             |                       |                    |         |
| Median                   | 84          | 65                    | 0.316              |         |
| Range                    | 14–488      | 6–321                 |                    |         |
| **Neutrophil, X10^9/L**  |             |                       |                    |         |
| Median                   | 51          | 47                    | 0.527              |         |
| Range                    | 0–89        | 5–86                  |                    |         |
| **Dx**                   |             |                       |                    |         |
| MDS-U/RA/RARS/RCMD/      | 19          | 14                    | 0.99               |         |
| RCMD-Rs                  |             |                       |                    |         |
| RAEB                     | 20          | 16                    |                    |         |
| CMML                     | 28          | 18                    |                    |         |
| AML/RAEBT                | 1           | 1                     |                    |         |
| AML                      | 4           | 3                     |                    |         |
| **IPSS**                 |             |                       |                    |         |
| H                        | 6           | 6                     | 0.636              |         |
| I-2                      | 17          | 13                    |                    |         |
| I-1                      | 25          | 22                    |                    |         |
| L                        | 19          | 8                     |                    |         |
| NA                       | 5           | 3                     |                    |         |
| Cytogenetics | Total N=124 | No Prior Therapy N=72 | Prior Therapy N=52 | p Value |
|-------------|-------------|-----------------------|--------------------|---------|
| Dip         | 39          | 27                    | 0.801              |         |
| 5/-7-       | 13          | 8                     |                    |         |
| Other       | 16          | 13                    |                    |         |
| IM          | 3           | 4                     |                    |         |
| ND          | 1           | 0                     |                    |         |

BM, bone marrow; WBC, white blood cell; MDS-U, myelodysplastic syndrome unclassified; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RCMD-Rs, refractory cytopenia with multilineage dysplasia and ring sideroblasts; RAEB, refractory anemia with excess blast; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; RAEBT, refractory anemia with excess blasts in transformation; IPSS, international prognostic scoring system; NA, not applicable; IM, insufficient metaphases; ND, not done.
Table 2
PD-L1, PD-L2, PD-1 and CTLA4 expression in bone marrow CD34+ cells

|                  | Total N=124 | No Prior Therapy N=72 | Prior Therapy N=52 | p Value |
|------------------|-------------|-----------------------|--------------------|---------|
| PD-L1            |             |                       |                    |         |
| Mean             | 29.84       | 4.41                  | 0.14               |         |
| Range            | 0–843.3     | 0–36.7                |                    |         |
| PD-L2            |             |                       |                    |         |
| Mean             | 0.73        | 1.78                  | 0.089              |         |
| Range            | 0–15.2      | 0–22.5                |                    |         |
| PD-1             |             |                       |                    |         |
| Mean             | 1.79        | 3.68                  | 0.31               |         |
| Range            | 0–32.6      | 0–76.7                |                    |         |
| CTLA4            |             |                       |                    |         |
| Mean             | 1           | 0.43                  | 0.23               |         |
| Range            | 0–25.1      | 0–6.0                 |                    |         |
Table 3

Summary of the PD-L1, PD-L2, PD-1 and CTLA4 expression in PBMNC

|          | Disease | Mean (Range) | Fold ≥ 2 (%) |
|----------|---------|--------------|--------------|
| **PD-L1**| MDS (24)| 4.4 (0–22.1) | 50           |
|          | CMML (5)| 12.4 (0.12–55.3) | 40           |
|          | AML (32)| 1.7 (0–8.3)   | 32           |
| **PD-L2**| MDS (24)| 2.7 (0–19.5)  | 9            |
|          | CMML (5)| 2.2 (0.03–6.5) | 40           |
|          | AML (32)| 0.9 (0–7.4)   | 12.9         |
| **PD-1** | MDS (24)| 5.2 (0–26.5)  | 61           |
|          | CMML (5)| 4.4 (0.03–17.2) | 40           |
|          | AML (32)| 3.9 (0–32.4)  | 39           |
| **CTLA4**| MDS (24)| 1.0 (0–9.5)   | 8            |
|          | CMML (5)| 1.0 (0.001–3.9) | 3            |
|          | AML (32)| 0.4 (0–2.3)   | 3            |