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Decitabine improves platelet recovery by down-regulating IL-8 level in MDS/AML patients with thrombocytopenia

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1. Introduction

Myelodysplastic syndromes (MDS) represent a heterogeneous group of clonal disorders characterized by ineffective hematopoiesis, leading to cytopenia and a predisposition to acute myeloid leukemia (AML) [1,2]. Thrombocytopenia (platelet [PLT] count $< 100 \times 10^9$/L) occurs in 40%–65% of MDS patients and is associated with bleeding complications [3]. PLT transfusion can protect these patients from serious bleeding, but platelet alloimmunization may occur when patients receive multiple transfusions, leading to ineffective infusion [4]. In order to identify potential novel strategies to increase PLT counts, decrease the need for PLT transfusions and reduce the number of bleeding episodes with a reasonable tolerance, it is of high importance to better understand the molecular mechanisms underlying thrombocytopenia.

Deregulation of various cytokines has been found in patients with MDS/AML and may contribute to thrombocytopenia by inhibiting the proliferation and differentiation of hemopoietic stem cells/progenitor cells (HSCs/HPCs). Compared with healthy donors, these patients have an imbalance of multiple cytokines such as thrombopoietin (TPO), interleukin-6 (IL-6) and interleukin-8 (IL-8) [5–7], which may inhibit the proliferation of HSCs/HPCs. Broxmeyer et al. found that the human macrophage inflammatory protein family of cytokines, including macrophage inflammatory protein (MIP)-2α, macrophage chemotactic and activating factor (MCAF), platelet factor 4 (PF4) and IL-8, synergistically suppresses the proliferation of human myeloid progenitor cells [6]. In addition, it was proposed that the cytopenia in AML patients might result from impaired differentiation at the HSC–progenitor transition but not depletion of normal HSCs [8]. Furthermore, several inflammation-associated cytokines, such as serum insulin-like growth factor binding protein 1 (IGFBP1), regulated upon activation normal T cell expressed and secreted (RANTES) and IL-8, were found to be significantly elevated in patients with prolonged isolated thrombocytopenia after allogeneic HSC transplantation (allo-HSCT), which might inhibit both the cell expansion and megakaryocytic differentiation of HSPCs [9]. Therefore, the abnormal expression of thrombopoietic and inflammatory cytokines may play an important role in the thrombocytopenia of MDS/AML patients.

Abnormal cytokine expression could be derived from the bone marrow microenvironment and $CD34^+$ cells. Mesenchymal stem cells (MSCs) are important stromal cells of the bone marrow microenvironment that secrete a large number of cytokines to regulate different functions of various cells [10–12]. For example, MSCs regulate the survival, proliferation and differentiation of HSCs by secreting some hematopoiesis-supporting cytokines [13]. Moreover, it was reported that the MSCs of MDS patients cannot support hematopoiesis and even can lead to the occurrence of MDS [14,15]. Moreover, the levels of several cytokines in stem and progenitor cells of MDS/AML patients were found to be abnormal [16]. However, it remains to be explored whether the abnormal cytokine levels contribute to the diminished PLT counts in MDS/AML patients.

Decitabine, a hypomethylating agent that inhibits DNA methyltransferase, has been approved for the treatment of MDS/AML patients with thrombocytopenia [17,18]. Decitabine effectively increases PLT counts and reduces the need for PLT transfusions [19]. Several studies have shown that the effects of decitabine in thrombocytopenia patients may be due to enhanced megakaryocyte differentiation and maturation,

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which accelerates the production of PLTs [20–22]. However, no significant difference in megakaryocyte number in bone marrow smears was found in these patients [20]. Therefore, the precise mechanism underlying the improvement of PLT counts in MDS/AML patients with decitabine treatment warrants further investigation.

In this study, we found that the IL-8 concentration in peripheral blood (PB) was markedly elevated in MDS/AML patients with thrombocytopenia and significantly inversely correlated with PLT count in MDS/AML patients. Importantly, the levels of IL-8 were significantly decreased after decitabine treatment both *in vivo* and *in vitro*. These findings revealed a potential mechanism by which decitabine improves PLT recovery in MDS/AML patients.

2. Materials and methods

2.1. Retrospective study

A retrospective study was designed to evaluate a cohort of 52 MDS/AML patients from January 2015 to June 2018 without chemotherapy or radiotherapy at the hematopoietic stem cell transplantation center of Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences. Clinical and laboratory data were extracted from the computerized database and the patients' charts. We collected the clinical data of 37 MDS (including 10 with RCMD, 12 with RAEB1, 13 with RAEB2, 1 with MDS−U, and 1 with MDS and myelofibrosis) and 15 AML (including 3 with M2, 3 with M4, and 9 with M5) patients. Ten healthy donors were enrolled as controls. Peripheral blood samples were collected using a vacuum blood collection tube with EDTA and immediately sent to the laboratory for the detection of plasma cytokines by professional technicians. We excluded two patients whose PLT count increased beyond the normal range (PLT $> 300 \times 10^9$/L) when exploring the relationship between cytokines and thrombocytopenia. All patients involved in this study were treated in the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences. This study was approved by the ethics committee of Blood Diseases Hospital, Chinese Academy of Medical Sciences, and written consent was obtained from all patients.

2.2. Preparation of BM-MNCs and $CD34^+$ cells

Fresh bone marrow samples were collected in a vacuum blood collection tube with EDTA and processed within 4 h. Bone marrow mononuclear cells (BM-MNCs) were separated using Ficoll-Hypaque density centrifugation medium according to the manufacturer's protocols (Union Stem Cell & Gene Engineering Company, Tianjin, China). Then, the BM-MNCs were enriched with 2 cycles of immunomagnetic bead separation using a MiniMACS $CD34^+$ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The rates of $CD34^+$ cells were > 95% as verified by flow cytometry (FACS Canto II, BD Biosciences, San Jose, CA).

2.3. Cell culture

BM-MNCs were seeded at a density of 5×10^6 cells/mL in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Gibco), 1% L-glutamine (Gibco) and 1% Minimal Essential Media (MEM) Non-Essential Amino Acids (NEAA, Gibco). The MSC medium was exchanged with fresh medium every 3 days. Cells were grown to 80%–90% confluency and passaged using a 0.25% trypsin solution. $CD34^+$ cells were seeded at a density of 5×10^5 cells/mL in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) containing 20% BIT 9500 (STEMCELL Technologies), 1% penicillin/streptomycin (Gibco), 50 ng/mL human thrombopoietin (TPO, PeproTech, Rocky Hill, NJ), 10 ng/mL human IL-3 (PeproTech), 100 ng/mL human stem cell factor (SCF, PeproTech), and 100 ng/mL human FMS-like trysine kinase 3 ligand (Flt3L, PeproTech). MSCs and $CD34^+$ cells were treated with 0.5 μM decitabine (SIGMA, 3050 Spruce Street Saint Louis Mo 63103 USA) for 72 h at 37 °C in a humidified atmosphere in 5% CO_2.

2.4. Quantitative real-time polymerase chain reaction (PCR)

Total mRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity and quality of mRNA were measured with a Nanodrop 2000 instrument (Thermo Scientific). cDNA was reverse transcribed with oligo-dT as primer (Promega, Madison, WI, http://www.promega.com). Real-time PCR was performed using the SYBR Green PCR kit (Qiagen) following the manufacturer's protocol with an ABI 7900HT system (Thermo Scientific). Actin was used as the reference gene. The results were analyzed using the comparison Ct (-ΔΔCt) method and are presented as fold changes relative to the respective controls. The primer sequences used were as follows: *ACTIN*: forward, 5′-CTCTTCCAGCCT TCCTTCCT-3′; reverse, 5′-AGCACTGTGTGTTGGCGTACAG-3′; *IL-8*: forward, 5′-AGTGCTAAAGAACTTAGATG-3′; reverse, 5′-TATGAATTCT CAGCCCTCTT-3′.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Bone marrow samples were collected in a vacuum blood collection tube with EDTA. Bone marrow plasma was separated from patients and cryopreserved in −80 °C. All samples were thawed at the time of detection. Cytokines levels were measured using ELISA kits (Neobioscience, China) according to the manufacturer's instructions. The values were read at an optical density (OD) of 450 nm, and protein levels were determined using a standard curve. The limit of sensitivity of the assays was determined to be 4 pg/mL.

2.6. Flow cytometry

MSCs were labeled with phycoerythrin (PE)-CD31, allophycocyanin (APC)-CD34, PE-CD45, PE-CD73, PE-90, and APC-CD105 antibodies (BD Biosciences) for 30 min at room temperature in darkness. $CD34^+$ cells were labeled with APC-CD34. The cells were subjected to flow cytometric analyses on FACSCanto II instruments (BD Biosciences).

2.7. Statistical analyses

Differences in cytokine levels were analyzed by the Mann–Whitney test. Paired *t*-test was used to compared the changes of IL-8 before and after decitabine treatment. Nominal variables were compared between patient groups by χ^2 tests. For continuous variables, data were analyzed using the independent samples t-test. Statistical analyses were performed using SPSS 18.0 software or GraphPad 5.0. P values < 0.05 represented a significant difference.

3. Results

3.1. IL-8 is elevated and negatively correlated with PLT counts in MDS/AML patients

We previously found that several inflammation-associated cytokines, including IL-8, were notably up-regulated in allo-HSCT patients with prolonged thrombocytopenia and inhibited the generation of megakaryocytes and PLTs [9]. To explore the potential relationship between cytokines and PLT counts in MDS/AML patients, we collected and analyzed the clinical data for cytokine levels in PB plasma of MDS (n = 37) and AML (n = 15) patients without chemotherapy or radiotherapy as well as healthy donors (n = 10). The median age of 10 healthy donors is 40 (14–56) and the median age of 52 patients is 47 (19–63). There was no significant difference in age between patients and healthy donors (P = 0.536). The tested cytokines included IL-1β

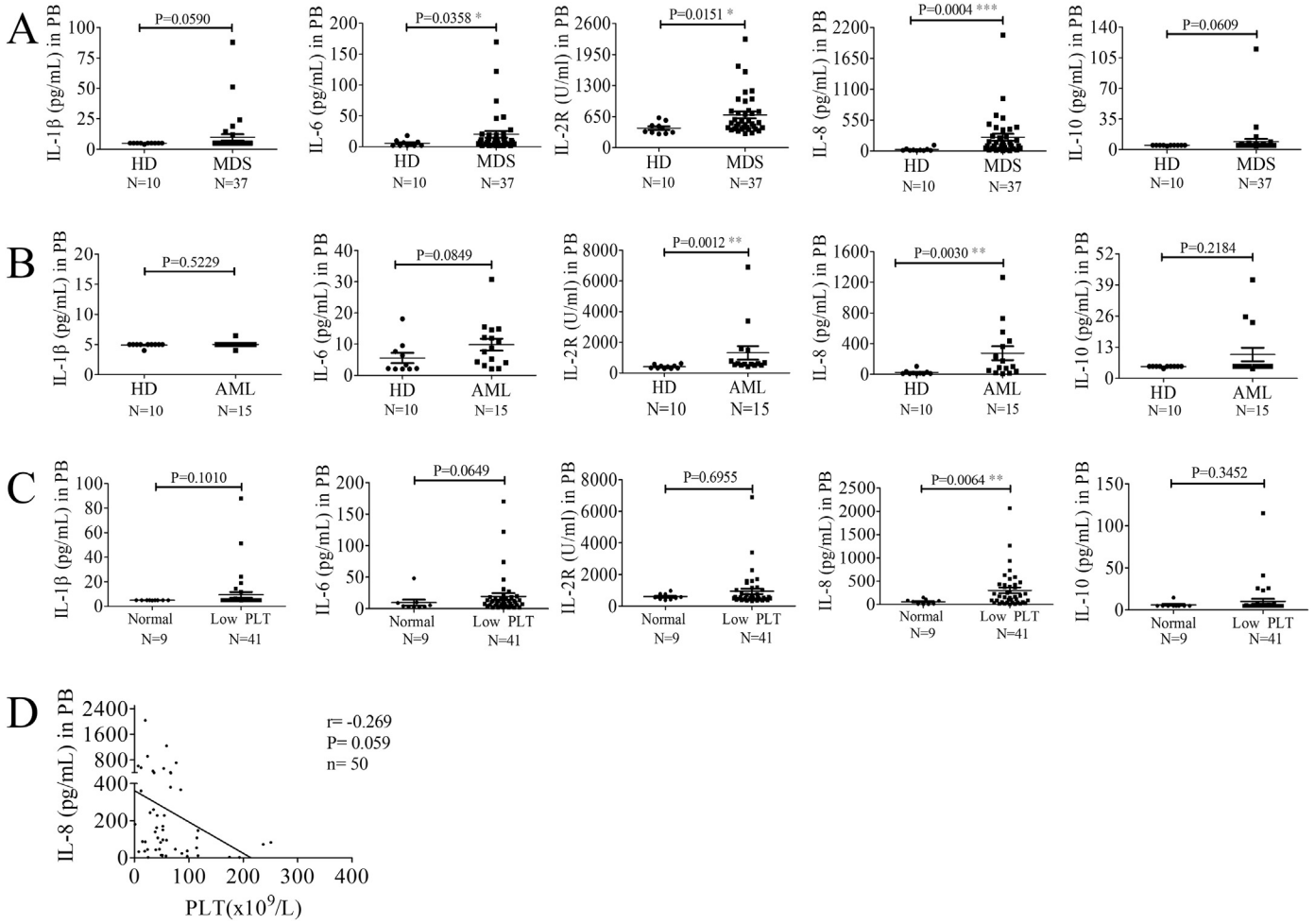

Fig. 1. IL-8 levels negatively correlated with PLT counts.
A. Clinical data of the concentrations of cytokines in PB plasma of MDS patients who were not received chemotherapy or radiotherapy and HDs.
B. Clinical data of the concentrations of cytokines in PB plasma of AML patients who were not received chemotherapy or radiotherapy and HDs.
C. The relationship between cytokines and thrombocytopenia in MDS/AML patients.
D. Correlation analysis of PLT counts and IL-8 in all MDS/AML patients.
The error bars represent standard error of mean (SEM). "n" represents the number of samples. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

(pg/mL), IL-6 (pg/mL), IL-2R (U/mL), IL-8 (pg/mL) and IL-10 (pg/mL). Compared with healthy donors ($n = 10$), the concentrations of IL-8 and IL-2R were significantly higher in both MDS and AML patients. IL-6 expression was also increased in MDS patients but not in AML, whereas there was no significant alteration of the levels of IL-1β and IL-10 in either MDS or AML patients (Fig. 1A, B).

We divided these patients into two groups according to PLT counts, one group with a normal PLT number (PLT = 100–300 × 10^9/L, $n = 9$) and the other with thrombocytopenia (PLT < 100 × 10^9/L, $n = 41$). The clinical characteristics of the two groups are summarized in Table 1. Between the MDS/AML patients with or without thrombocytopenia, no statistically significant differences were found in gender, median age, median white blood cell (WBC) count, median hemoglobin, median blasts, underlying disease, or karyotype classification ($P > 0.05$, Table 1). There also were no significant differences in the IL-1β, IL-6, IL-2R and IL-10 levels between the two groups. However, the levels of IL-8 were dramatically higher in MDS/AML patients with thrombocytopenia than in those without thrombocytopenia (Fig. 1C, Table 1). Furthermore, the IL-8 concentration had a significantly inverse correlation with the PLT count (Fig. 1D). These results suggest that elevated IL-8 may contribute to thrombocytopenia in MDS/AML patients.

3.2. MSCs and CD34$^+$ cells are the potential sources of elevated IL-8 levels

To verify the increase of IL-8 in the BM plasma of MDS/AML patients, we collected the bone marrow (BM) plasma of additional sets of MDS/AML patients ($n = 14$) and healthy donors (n = 14) and tested the IL-8 levels by ELISA. Consistent with the results in PB plasma, the concentration of IL-8 was significantly increased in the BM plasma of MDS/AML patients compared to that in healthy donors (Fig. 2A). Both CD34$^+$ cells and BM niche cells may contribute to the increased IL-8 production in MDS/AML patients. To investigate the potential sources of the aberrantly increased IL-8 levels, we first isolated CD34$^+$ cells from the BM of MDS/AML patients and healthy donors and cultured these cells *in vitro*. Detection of IL-8 expression by real-time PCR showed that IL-8 was over-expressed by the CD34$^+$ cells from MDS/AML patients compared to its expression in the normal CD34$^+$ cells from healthy donors (Fig. 2B). We further isolated and cultured the MSCs to evaluate IL-8 levels. Interestingly, IL-8 was also up-regulated in MSCs derived from MDS/AML patients (Fig. 2C). Collectively, these results indicate both CD34$^+$ cells and MSCs are potential sources of the increased IL-8 production that leads to reduced PLT generation in MDS/AML patients.

Table 1
Characteristics of patients.

	PLT < 100 × 10^9/L	PLT = 100–300 × 10^9/L	*P* value
Patients (n)	41	9	
Gender (M/F)	27/14	7/2	0.4870
Median age (year)	43 (19–63)	39 (14–56)	0.5010
Median WBC, X10^9/L (range)	5.02 (0.49–23.72)	2.84 (0.62–8.93)	0.0520
Median hemoglobin, g/L (range)	78.15 (44–150)	91.22 (40–139)	0.1260
Median blasts, %(range)	18.65 (0–93.3)	13.87 (1.14–50.8)	0.8800
Cytokines			
IL-1β (pg/ml)	4.99 (4.90–5.00)	9.39 (4.90–87.90)	0.1010
IL-6 (pg/ml)	9.55 (2.00–48.10)	19.33 (2.00–170.00)	0.0649
IL-2R (U/ml)	600.11 (382–972)	943.37 (310–6897)	0.6955
IL-8 (pg/ml)	**302.92 (7.77–2066)**	**59.23 (6.56–152)**	**0.0064**
IL-10 (pg/ml)	6.08 (5.00–14.70)	10.28 (5.00–115)	0.5006
Underlying disease			
MDS/AML	6/3	29/12	0.8100
Karyotype classification			0.8140
Normal karyotype	20	4	
Abnormal karyotype	21	5	

Data are presented as n, median (range). F, female; M, male; WBC, white blood cells; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia.

3.3. Decitabine down-regulates IL-8 expression

It has been reported that decitabine improves PLT counts in MDS/AML patients with thrombocytopenia [19,20], but the underlying mechanisms remain largely unknown. To assess whether decitabine enhances PLT generation by inhibiting the abnormal increase in IL-8, we collected four cases of MDS/AML patients who had elevated IL-8 levels and accepted decitabine (Xian Janssen Pharmaceutical Ltd, 34 Wanshou north road, Xincheng district, Xi'an city, Shanxi province) chemotherapy (20 mg/m^2/day, 5 days for 1 cycle). The levels of IL-8 in PB plasma before and after decitabine chemotherapy were detected by ELISA. We found that the concentration of IL-8 in PB plasma was dramatically reduced after decitabine chemotherapy (Fig. 3A). To extend our observation, MSCs and $CD34^+$ cells isolated from the MDS/AML patients were cultured *in vitro* and treated with 0.5 μM decitabine for 72 h. The relative IL-8 expression in $CD34^+$ cells obviously decreased after decitabine treatment (Fig. 3B). Similarly, decitabine treatment significantly reduced IL-8 expression in MSCs from the BM of MDS/AML patients (Fig. 3C). Together, these *in vitro* and *in vivo* data demonstrate that decitabine may reduce IL-8 expression, thereby enhancing PLT recovery in MDS/AML patients.

4. Discussion

The results of this study revealed that expression of the inflammation-associated cytokine IL-8 is increased both in MDS and AML patients with thrombocytopenia, and the abnormal high amounts of IL-8 were derived from $CD34^+$ cells and MSCs in the BM of MDS/AML patients. Importantly, for the first time, we demonstrated that decitabine chemotherapy decreased IL-8 expression in MDS/AML patients with thrombocytopenia. These findings may reveal a novel mechanism by which decitabine promotes PLT recovery in MDS/AML patients.

Recent studies have identified various factors involving the suppression of thrombopoiesis, including inhibitory cytokines [23], enhanced apoptosis [24], defective responses to growth factors [25] and increased PLT destruction [26]. Among them, a series of inhibitory cytokines plays a vital role in the process of megakaryocytic differentiation and PLT generation. Many studies have shown that levels of multiple inhibitory cytokines, including transforming growth factor (TGF)-β, interferon-α (INF-α) and IL-8, are increased in MDS/AML patients and that these cytokines function to impair the megakaryocytic differentiation at an early progenitor level [6,27,28]. We previously found that high concentrations of inflammation-associated cytokines IGFBP1, IGFBP2, RANTES and IL-8 inhibit the megakaryocytic potential of HSPCs [9]. In the present study, we found that IL-8 was significantly elevated in MDS/AML patients with thrombocytopenia. Together these findings suggest that the increased levels of IL-8 may inhibit PLT production in MDS/AML.

IL-8, also known as CXCL8, is a proangiogenic/proinflammatory chemokine that promotes neutrophil chemotaxis and degranulation and activates multiple intracellular signaling pathways downstream of CXCR1 and CXCR2 [29]. In addition to acting as an immune reaction mediator, IL-8 plays an important role in the metastatic process of various cancer cells by regulating cell proliferation, angiogenesis, migration and invasion [30]. Moreover, IL-8 is a biomarker for telomerase inhibition-mediated growth attenuation of cancer cells that can potentially be used to predict therapeutic response [31]. IL-8 was found to be increased in patients with MDS/AML and positively correlated with disease progression [16,32]. Overexpression of the IL-8 receptor CXCR2 was also identified in AML/MDS patients, and higher CXCR2 expression correlated with worse clinical outcomes [33]. Consequently, blocking the IL-8/CXCR2 signaling axis was shown to be a potential therapeutic strategy against MDS and AML stem cells [16]. Consistently, we found that IL-8 was significantly upregulated in MDS/AML patients. Interestingly, we observed a significantly negative correlation between the IL-8 level in PB and the PLT count in these patients. Given that IL-8 inhibits the proliferation and megakaryocytic differentiation of HSPCs [9], our findings suggest that IL-8 inhibition may improve PLT recovery in MDS/AML patients.

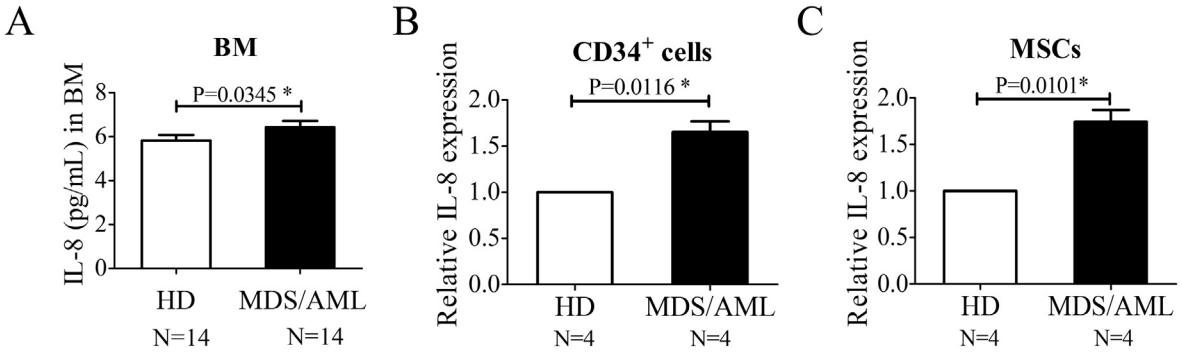

Fig. 2. MSCs and $CD34^+$ cells were potential sources of elevated IL-8.
A. ELISA assay of IL-8 level in BM plasma of MDS/AML patients who were not received chemotherapy or radiotherapy and HDs.
B. The relative IL-8 expression in BM $CD34^+$ cells of MDS/AML patients and HDs.
C. The relative IL-8 expression in BM MSCs of MDS/AML patients and HDs.
The error bars represent standard error of mean (SEM). "n" represents the number of samples. *$P < 0.05$.

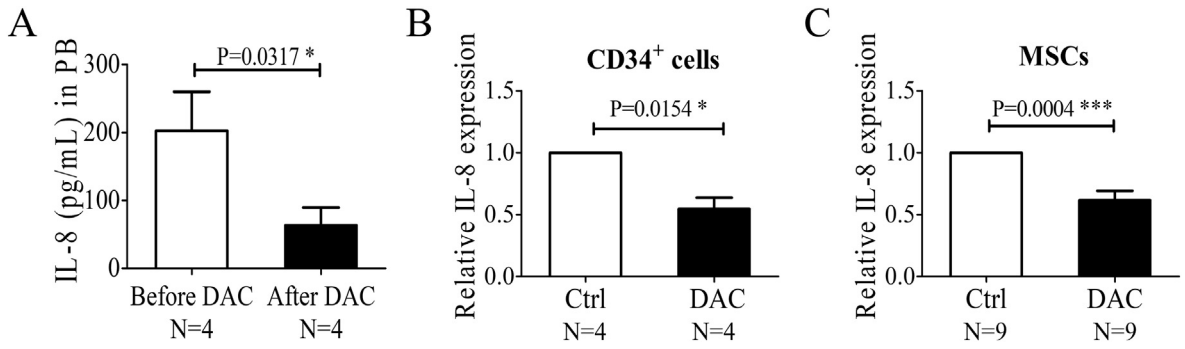

Fig. 3. DAC enhanced PLT production by down-regulating IL-8 expression.
A. Clinical data of IL-8 in PB plasma before and after DAC chemotherapy of 4 cases (2MDS 2AML) with IL-8 rise and accept DAC chemotherapy (20 mg/m^2/day), 5 days for 1 cycle.
B. The relative IL-8 expression in BM CD34$^+$ cells of MDS/AML patients after DAC treatment.
C. The relative IL-8 expression in BM MSCs of MDS/AML patients after DAC treatment.
The error bars represent standard error of mean (SEM). "n" represents the number of samples. *P $<$ 0.05 and ***P $<$ 0.001.

Decitabine is the most widely used inhibitor of DNA methylation, which leads to reactivation of epigenetically silenced tumor suppressor genes. Decitabine has been used to effectively treat MDS/AML by improving red blood cell and PLT counts [34]. Previous studies have shown that decitabine treatment can enhance megakaryocyte differentiation and maturation, thereby accelerating the production of PLTs in MDS/AML patients [20,21]. We previously also demonstrated that decitabine enhanced thrombopoiesis by improving PLT release from mature megakaryocytes derived from CD34$^+$ stem cells [22]. However, the mechanism by which decitabine promotes megakaryocyte differentiation and maturation as well as PLT recovery remains unclear. In this study, we demonstrated, for the first time, that decitabine chemotherapy dramatically reduced the expression of IL-8 both *in vivo* and *in vitro*, and this decrease in IL-8 was accompanied by an increase in PLT production in MDS/AML patients with thrombocytopenia. Therefore, decitabine may enhance PLT production by down-regulating IL-8 expression.

In summary, our findings suggest that the abnormal increase in IL-8 levels in MDS/AML patients may lead to thrombocytopenia, and decitabine chemotherapy might improve PLT recovery by lowering IL-8 levels.

Author contributions

Wenxia Zhang and Cuicui Liu: collection and assembly of data, manuscript writing; Dan Wu, Chen Liang, Leisheng Zhang and Qiuqiu Zhang: clinical sample collection; Yiying Liu, Meijuan Xia, Hongtao Wang, and Pei Su: data collection and analysis; Sizhou Feng, Mingzhe Han and Jiaxi Zhou: provision of study material or patients; FuxuWang and Erlie Jiang: conception and design, final approval of manuscript.

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Declarations of interest

None.

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