

## ORIGINAL ARTICLE

# Increased Levels of Circulating Monocytic- and Early-Stage Myeloid-Derived Suppressor Cells (MDSC) in Acute Myeloid Leukemia

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### SUMMARY

**Background:** To investigate the levels of circulating myeloid-derived suppressor cells (MDSC) in patients with primary acute myeloid leukemia (AML), and to explore the relationship between the number of MDSC and AML.

**Methods:** Peripheral blood samples from 29 patients with primary AML and 30 healthy controls were collected. CD33, CD11b, HLA-DR, CD14, and CD15 were used to label cells, and flow cytometry was used to analyze the numbers of total MDSC and subgroups eMDSC (early-stage MDSC), M-MDSC (monocytic MDSCs), PMN-MDSC (polymorphonuclear-MDSCs) or G-MDSC (granulocytic-MDSC) via two gating strategies. Presence of MDSC in AML was determined after assessment of clinical data.

**Results:** Phenotypic analysis of MDSC by the two gating strategies was consistent. Compared with healthy controls, the numbers of total MDSC (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup>) and G-MDSC (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup>CD14<sup>-</sup>CD15<sup>+</sup> or CD14<sup>-</sup>CD15<sup>+</sup>CD11b<sup>+</sup>) in peripheral blood of AML patients were lower ( $p < 0.05$ ), while numbers of M-MDSC (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup>CD14<sup>+</sup>CD15<sup>-</sup> or HLA-DR<sup>-LOW</sup>CD14<sup>+</sup>) and eMDSC (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-LOW</sup>CD14<sup>-</sup>CD15<sup>-</sup>) were higher ( $p < 0.05$ ). The levels of G-MDSC in peripheral blood of AML-M2 patients were higher than those in other subtypes, along with total MDSC, while the levels of eMDSC and M-MDSC in AML-M3 patients were higher than those in other subtypes.

**Conclusions:** The high frequency of HLA-DR<sup>-LOW</sup>CD14<sup>+</sup>M-MDSC and CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-LOW</sup>CD14<sup>-</sup>CD15<sup>-</sup>eMDSC in peripheral blood of AML patients indicates potential for MDSC as a diagnostic index in AML.

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#### KEY WORDS

myeloid-derived suppressor cell (MDSC), monocytic MDSC (M-MDSC), early-stage MDSC (eMDSC), acute myeloid leukemia (AML), flow cytometry

## INTRODUCTION

Tumorigenesis and tumor progression represent a complex, long-term battle between tumor and host immune system. On the one hand, the immune defense network, composed of the innate and acquired immune responses, antagonizes the formation and development of a tumor. On the other hand, the tumor microenvironment can induce immune tolerance through a variety of mechanisms, which promote tumor occurrence and development [1,2]. Myeloid-derived suppressor cells (MDSC) are a group of heterogeneous cells with negative immunomodulatory function, which form the immune defense against tumor cells together with regulatory T cells (Treg) and other cell subsets. They induce tumor immune escape and play a key role in the pathogenesis of cancer, chronic inflammation, infection, autoimmune diseases, trauma, and graft-versus-host disease [1,3-6]. A large number of studies have shown that in patients with cancer, the levels of circulating MDSC are closely associated with the development and prognosis of tumors. MDSC cannot only inhibit immune responses, but also promote tumor growth, constituting an important part of the tumor microenvironment [7,8].

At present, due to the biochemical and functional heterogeneity of MDSC, there is no consensus on MDSC immunophenotypic markers for different tumors, and their heterogeneity confuses the functional characterization of MDSC in different disease conditions. MDSC sub-classification is therefore essential clinically, as the subsets are functionally different and mediate different mechanisms of activation and immunosuppression [2]. Markers of MDSC are summarized as follows and reflect immunology, morphology, and cell function. The phenotype of human total MDSC is mainly defined as CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>LOW</sup>, which can be further classified into PMN-MDSC (polymorphonuclear-MDSCs) or G-MDSC (granulocytic-MDSC; CD11b<sup>+</sup>HLA-DR<sup>+</sup>CD14<sup>+</sup>CD33<sup>+</sup>CD15<sup>+</sup>), M-MDSC (monocytic MDSCs; CD11b<sup>+</sup>HLA-DR<sup>+</sup>CD14<sup>+</sup>CD33<sup>+</sup>CD15<sup>+</sup>), and eMDSC (early-stage MDSC) or i-MDSC (immature MDSCs; Lin<sup>-</sup>HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup>), according to expression of markers such as Lin (including CD3, CD14, CD15, CD19 and CD56) [3,4,9-13]. Among these, G-MDSC contains PMN-MDSC, with the morphology of PMN-MDSC being more mature according to phenotypic analysis [14].

Studies have found that MDSC are associated with the occurrence and development of solid tumors including melanoma, lung cancer, breast cancer, hepatocellular carcinoma, colorectal carcinoma, pancreatic carcinoma, renal cell carcinoma, and bladder cancer [7,13,15-22]. In addition, it has been reported that in patients with circulatory system diseases, such as non-Hodgkin's lymphoma (NHL), chronic myeloid leukemia (CML), multiple myeloma (MM), and myelodysplastic syndrome (MDS), or recipients of hematopoietic cell transplantation, MDSC have immunosuppressive function or promote disease progression [7,23-26]. The immune sys-

tem plays a critical role in the occurrence and development of circulatory system diseases. Leukemia describes a group of highly heterogeneous hematopoietic malignant tumors, which mainly involves hematopoietic stem cells and hematopoietic progenitor cells, resulting in a large number of immature myeloid or lymphoid cells in the bone marrow (BM) and peripheral blood [27]. Acute myeloid leukemia (AML) is the most common type of leukemia in adults and is a malignant neoplasm of immature, BM-derived myeloid cells exhibiting variable differentiation [28]. There are few studies of MDSC in human AML. Currently, studies have shown that the levels of total MDSC, M-MDSCs, and G-MDSCs are increased in BM and peripheral blood of patients with AML [29,30]. However, these have some limitations in their further sub-typing of MDSC and their sample sizes. Therefore, focusing on MDSC, we combined morphological analysis, expanded sample size, and detection of circulating MDSC subsets in patients with primary AML, providing a theoretical basis for use of MDSC as a potential diagnostic and therapeutic monitoring index.

## MATERIALS AND METHODS

### Patients and samples

The diagnosis and classification of AML was based on "Standard of diagnosis and curative effect of blood disease" [31]. From December 2016 to September 2017, peripheral blood from 29 cases diagnosed with AML for the first time in a tertiary care hospital were collected using EDTA-K2 as anticoagulant. They included 14 males and 15 females, aged from 26 to 85 years with an average age of 54. Among them, the percentages of blast cells in the blood of 26 cases were  $\geq 20\%$  and  $< 20\%$  in the other 3 cases; however, acute leukemia was diagnosed according to BM morphology. Thirty healthy cases were included in the healthy control group, including 15 males and 15 females, aged 16 to 71 years with an average age of 42. This study was approved by the ethics committee, and informed consent was obtained from all donors.

### Reagents and instruments

Fluorescent labeled mouse anti-human antibodies were: HLA-DR-FITC (BD, Lot: 555811), CD11b-APC (BD, Lot: 550019), CD33-PE (BD, Lot: 555450), CD15-PerCP-Cy<sup>TM</sup>5.5 (BD, Lot: 560828) or CD15-PE-Cy<sup>TM</sup>7 (BD, Lot: 560827), CD14-APC-Cy<sup>TM</sup>7 (BD, Lot: 557831) or CD14-PerCP-Cy<sup>TM</sup>5.5 (BD, Lot: 550787). Other reagents used were: RBC Lysing Buffer 10 x (BD, Lot: 5358501); Flow Cytometry Staining Buffer Solution (eBioscience, Lot: E00015-1642). Instruments were: Centrifuge B320 (BAIYANG); vortex mixer; FACS CantoII (Beckton Dickinson).

## Methods

Following the recommended dosage of fluorescent-labeled antibodies, anti-HLA-DR, CD11b, CD33, CD14, and CD15 were added to flow cytometry tubes and mixed with 100  $\mu$ L peripheral blood, then incubated at room temperature in the dark for 20 minutes. 10 x red blood cell lysis buffer was diluted with distilled water to 1 x working solution, then 2 mL of 1 x working solution was added to each tube and incubated for 10 minutes in the dark. After centrifugation at 300 g for 5 minutes, the supernatant was discarded and cells re-suspended in 2 mL PBS buffer, centrifuged at 300 g for 5 minutes, and then supernatant was discarded and cells re-suspended in 500  $\mu$ L PBS prior to flow cytometry.

Lymphocytes were gated out based on forward scatter/side scatter and served as internal negative controls for each sample. Flow cytometry was performed using a FACS CantoII (Beckton Dickinson) and data were analyzed using BD FACS Diva 8.0.1 software.

## Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). The data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). Differences in means were analyzed using parametric tests or non-parametric test (Mann-Whitney for unpaired samples), based on their distribution patterns. Comparisons between groups were performed using one-way ANOVA (Newman-Keuls).  $p < 0.05$  was considered statistically significant.

## RESULTS

In this study, two flow cytometry gating strategies were selected based on previous reports on total MDSC and their subgroups. CD33, CD11b, HLA-DR, CD14, and CD15 antibodies were used as markers to analyze the levels of circulating MDSC in the healthy control group (HD) and primary acute myeloid leukemia (AML) group.

### MDSC flow cytometry gating strategy I and associated data in peripheral blood

As shown in Figure 1, gating strategy I [13,21,30] first selects all cells except lymphocytes into the P1 gate, then selects HLA-DR<sup>-LOW</sup> cells into the P2 gate. CD33<sup>+</sup>CD11b<sup>+</sup> cells in the P2 gate are then selected, with cells in the P3 gate being CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-LOW</sup> (total MDSC; Figure 1A). P3 cells are then classified, according to expression of CD14 and CD15, into four sub-types: CD14<sup>+</sup>CD15<sup>+</sup> (G-MDSC), CD14<sup>+</sup>CD15<sup>-</sup>, CD14<sup>-</sup>CD15<sup>+</sup> (M-MDSC) and CD14<sup>-</sup>CD15<sup>-</sup> (eMDSC) (Q1 - Q4; Figure 1B).

Flow cytometry results for circulating MDSC in the healthy donor (HD) and AML groups are shown in Figure 1. HLA-DR-negative cells in the AML group were significantly lower than those in the healthy controls (data not shown; Figure 1A). Circulating CD33<sup>+</sup> and

CD11b<sup>+</sup> cells in patients with AML were further analyzed, and the results showed that levels of total MDSC (CD33<sup>+</sup>CD11b<sup>+</sup> HLA-DR<sup>-</sup>) in patients with AML were lower than those of healthy controls (AML: 33,155  $\pm$  26,094,  $n = 28$ ; HD: 77,086  $\pm$  20,999,  $n = 27$ ; Figure 1A). Both comparisons showed statistical significance ( $p < 0.05$ ).

By analyzing expression of CD14 and CD15 in CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-LOW</sup> cells, we found that the number of G-MDSC (CD14<sup>+</sup>CD15<sup>+</sup>) in patients with primary AML was lower than in healthy controls (AML: 28,740  $\pm$  27,295,  $n = 27$ ; HD: 75,551  $\pm$  20,751,  $n = 27$ ; Figure 1B). The number of eMDSC (CD14<sup>-</sup>CD15<sup>-</sup>) in patients with primary AML was significantly higher than in the healthy group (AML: 3,211  $\pm$  5,532,  $n = 27$ ; HD: 638.7  $\pm$  740.2,  $n = 27$ ; Figure 1B); the level of M-MDSC (CD14<sup>+</sup>CD15<sup>-</sup>) in patients with AML was higher than in healthy controls (AML: 1,437  $\pm$  3,951,  $n = 28$ ; HD: 46.43  $\pm$  121.9,  $n = 27$ ; Figure 1B); differences in both groups were statistically significant ( $p < 0.05$ ).

### MDSC flow cytometry gating strategy II and associated data in peripheral blood

As shown in Figure 2, gating strategy II focuses on analysis of MDSC subgroups [10]. Firstly, all cells except doublets, debris, and lymphocytes were assigned to the P1 gate (Figure 1A), then CD14<sup>+</sup>CD15<sup>+</sup> cells were selected as the P6 gate. CD11b<sup>+</sup> cells were named PMN (PMN-MDSCs) and are equivalent to G-MDSC in gating strategy I. We found that the percentage of PMN-MDSCs (CD14<sup>+</sup>CD15<sup>+</sup> CD11b<sup>+</sup>) in patients with primary AML was lower than in healthy controls (AML: 12,629  $\pm$  13,454,  $n = 28$ ; HD: 47,332  $\pm$  16,525,  $n = 29$ ; Figure 2A).

HLA-DR<sup>-LOW</sup> cells in the P1 gate were attributed a P4 gate, and CD14<sup>+</sup>CD15<sup>-</sup> cells further assigned to the P5 gate. On this basis, CD33<sup>+</sup>CD11b<sup>+</sup> cells were named e (eMDSC). The percentage of eMDSC (HLA-DR<sup>-LOW</sup>CD14<sup>+</sup>CD15<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup>) in patients with primary AML was significantly higher than in the HD group (AML: 849.9  $\pm$  2,280,  $n = 29$ ; HD: 512.5  $\pm$  2,137,  $n = 30$ ; Figure 2B).

HLA-DR<sup>-LOW</sup> and CD14<sup>+</sup> cells were selected from the P1 gate and named M (M-MDSCs). The level of M-MDSC (HLA-DR<sup>-LOW</sup>CD14<sup>+</sup>) in patients with AML was higher than in healthy controls (AML: 4474  $\pm$  13,950,  $n = 28$ ; HD: 715.8  $\pm$  2,300,  $n = 26$ ; Figure 2C); this difference was statistically significant ( $p < 0.05$ ).

### Levels of circulating total MDSC and their subtypes in patients with AML M2-M5

According to verified clinical data, patients with AML included in this study were classified into four types, AML M2 - M5, according to FAB classification. As shown in Figure 3 A&B, levels of circulating total MDSC and G-MDSC in patients with AML-M2 were higher than those of other subtypes, followed by those in AML-M4. As shown in Figure 3 C&D, levels of circulating eMDSC and M-MDSC in patients with AML-

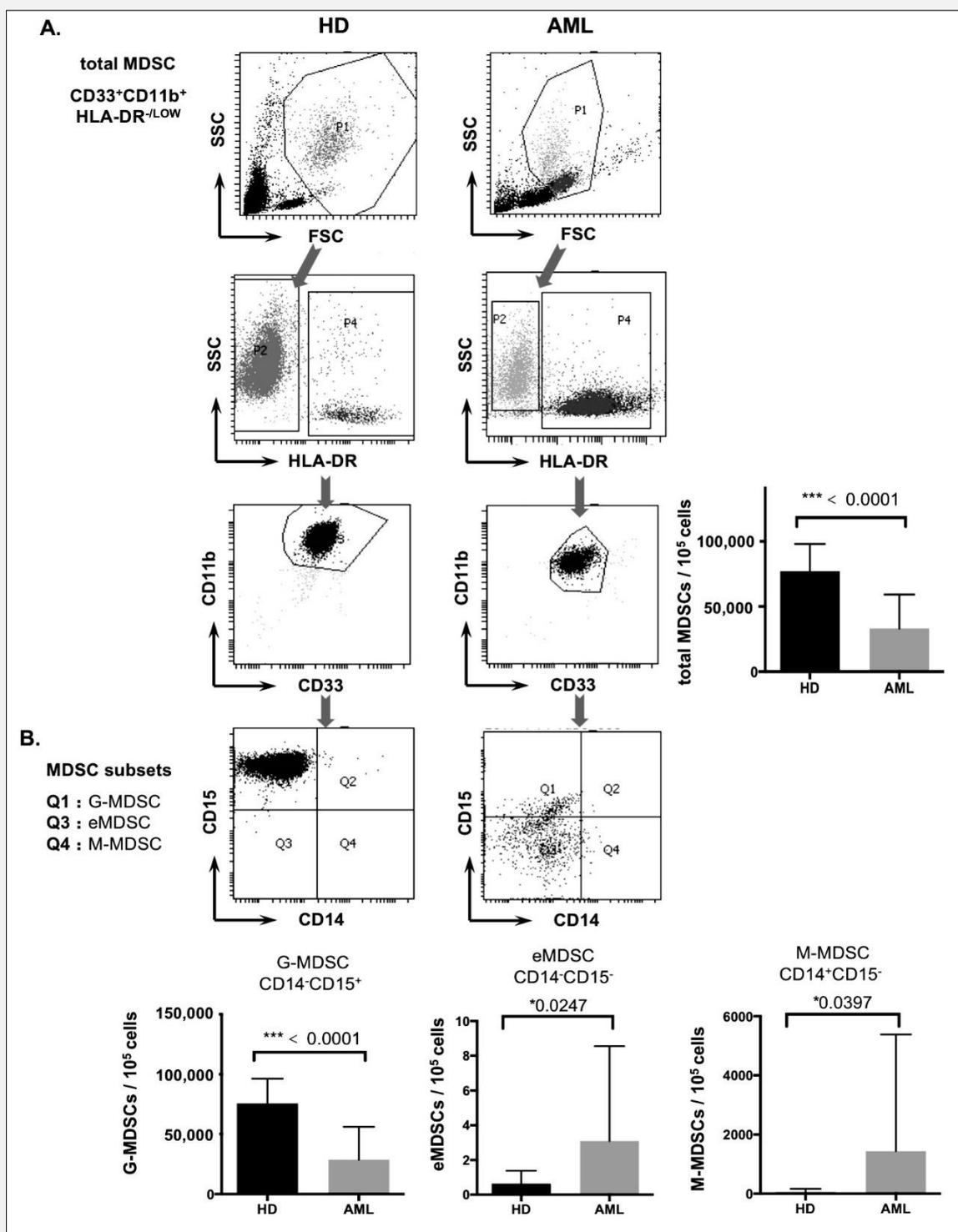


Figure 1. MDSC flow cytometry gating strategy I and associated data in peripheral blood of HD and AML groups.

A. Gating to exclude doublets, debris, and lymphocytes based on SSC and FSC; representative flow cytometry plots with gating strategy based on selection of the HLA-DR negative population, followed by gating out CD11b/CD33-double positive cells, giving total MDSC. B. Total MDSC were sub-classified into four groups: CD14<sup>-</sup>CD15<sup>+</sup> (G-MDSC), CD14<sup>+</sup>CD15<sup>+</sup>, CD14<sup>+</sup>CD15<sup>-</sup> (M-MDSC) and CD14<sup>-</sup>CD15<sup>-</sup> (eMDSC), according to expression of CD14 and CD15 (Q1 - Q4).

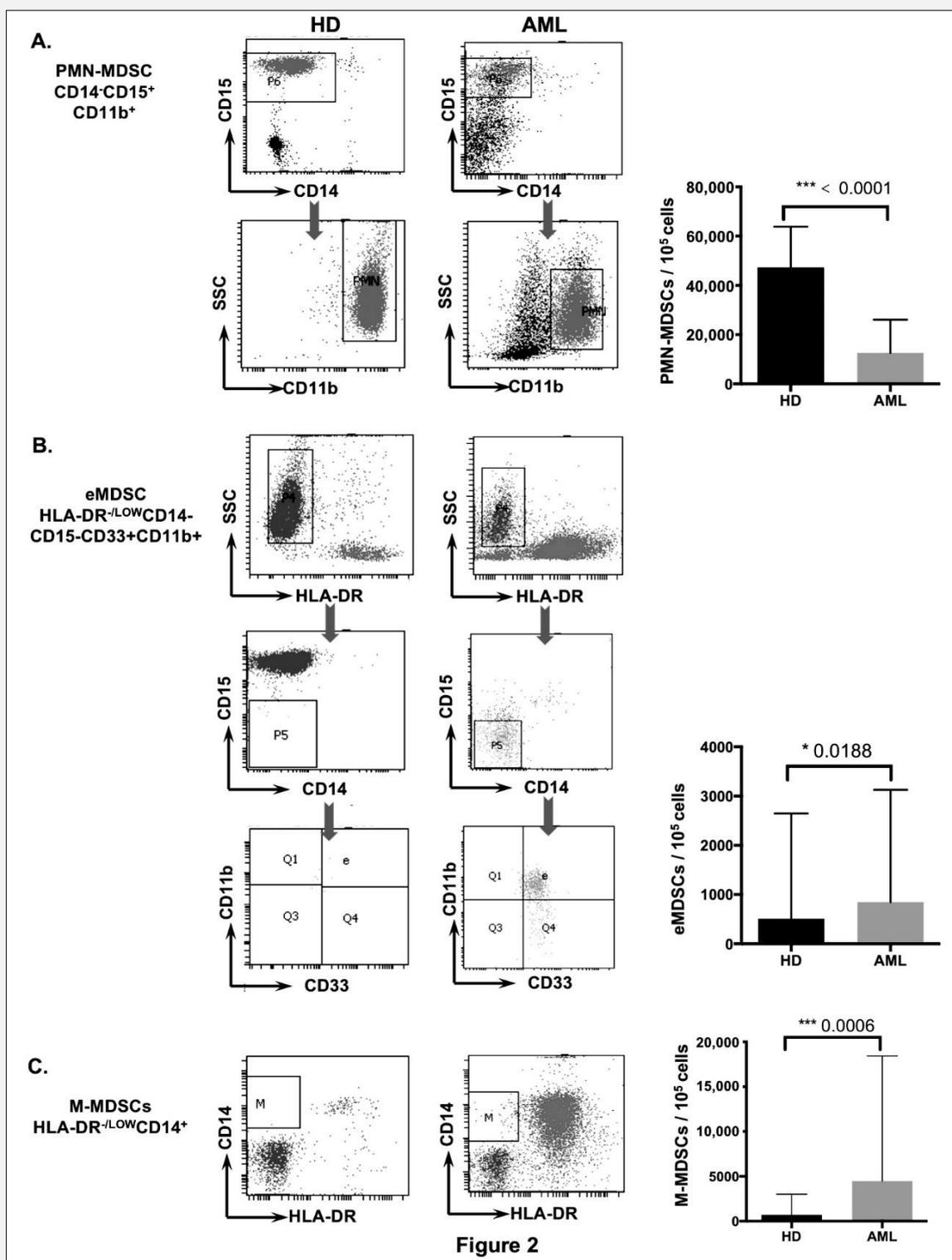
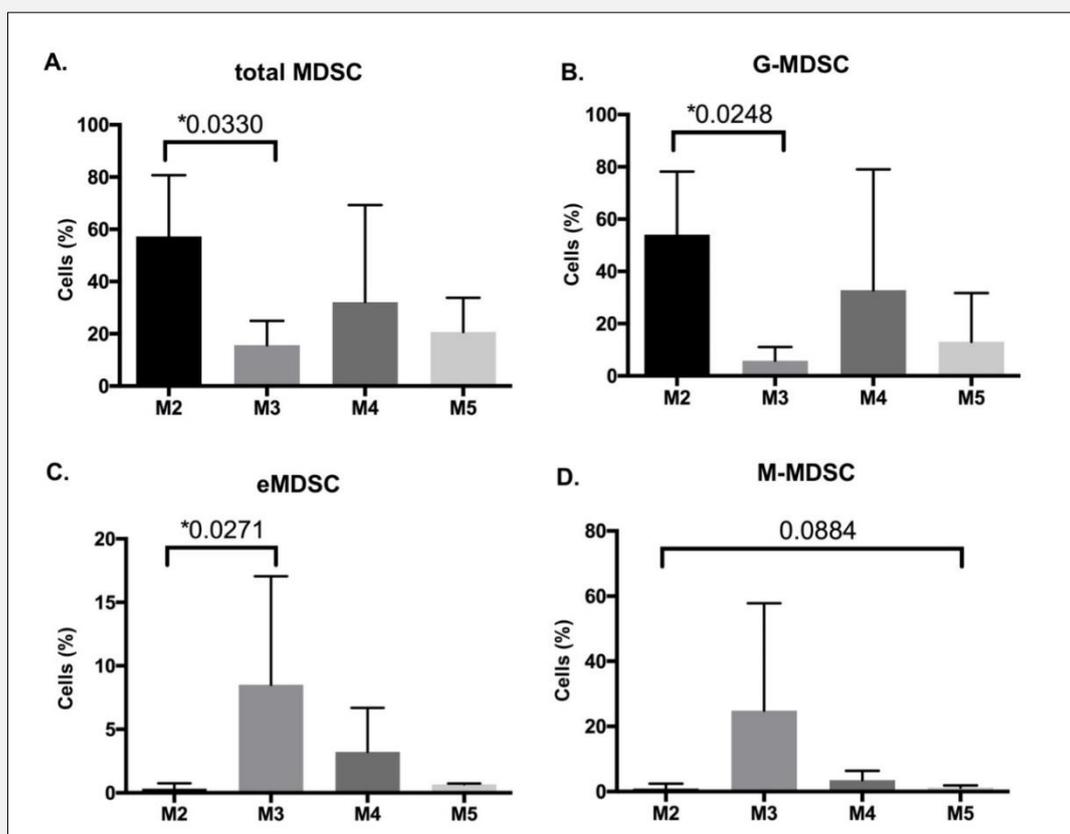


Figure 2. MDSC flow cytometry gating strategy II and associated data in peripheral blood of HD and AML groups.

A. PMN-MDSC (CD14<sup>+</sup>CD15<sup>+</sup>CD11b<sup>+</sup>) gating strategy and data in peripheral blood. B. eMDSC (HLA-DR<sup>-LOW</sup>CD14<sup>-</sup>CD15<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup>) gating strategy and data in peripheral blood. C. M-MDSC (HLA-DR<sup>-LOW</sup>CD14<sup>+</sup>) gating strategy and data in peripheral blood.



**Figure 3. Level of circulating MDSC in patients with AML.**

Data for circulating total MDSC and their subsets G-MDSC, eMDSC, and M-MDSC in patients with M2 - M5 subtypes of AML.

M3 were higher than those in other subtypes.

### DISCUSSION

In the past two decades, MDSC has been a hot area in tumor immunity research. MDSC are a group of heterogeneous cells derived from BM progenitor cells. In healthy individuals, BM progenitor cells rapidly differentiate into mature granulocytes, macrophages, and dendritic cells. Under pathological conditions, such as cancer, septicemia, trauma, and transplantation, their differentiation into mature myeloid cells is inhibited, resulting in production of a large number of MDSC [5]. In this study focusing on MDSC, two flow cytometry gating strategies were selected to detect total MDSC (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-LOW</sup>) and their subgroups (G-MDSC, eMDSC and M-MDSC) in peripheral blood of healthy controls (HD) and patients with acute myeloid leukemia (AML) and used to obtain levels of circulating

MDSC in patients with AML. On this basis, the levels of total MDSC and subsets in the different subtypes of AML, M2 - M5, were further analyzed. Our results provide a potential basis for using MDSC as a diagnostic index in AML and lay a foundation for further functional studies of MDSC.

In humans, in addition to CD33<sup>+</sup>, HLA-DR<sup>-LOW</sup> and CD11b<sup>+</sup>, CD14 and CD15 are also important markers to classify MDSC and its subtypes. CD14 identifies monocytic M-MDSC and CD15 identifies granulocytic G-MDSC. MDSC phenotypes of patients with distinct types of tumors are different [2]. MDSC play an important role in multiple solid tumors, and circulating MDSCs are not only a predictive marker for cancer immunotherapy, but also a leading marker for predicting clinical response to systemic chemotherapy in patients with advanced solid tumors [18]. There are few studies on MDSC in AML, although one showed that total MDSC (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-LOW</sup>) increase significantly in BM of newly diagnosed AML patients - levels

of circulating MDSC and their subgroups were not reported [29]. Another study showed that total MDSC, M-MDSCs (CD11b<sup>+</sup>HLA-DR-CD14<sup>+/+</sup>CD33<sup>+</sup>CD15<sup>-</sup>), and G-MDSCs (CD11b<sup>+</sup>HLA-DR-CD14<sup>-</sup>CD33<sup>+</sup>CD15<sup>+</sup>) are frequent in BM and peripheral blood of AML patients, but the sample size was less than 10 [30]. A further study reported circulating CD14<sup>+</sup>HLA-DR<sup>-low</sup> MDSC proportions which were significantly increased in leukemia patients developing acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT), rather than in primary AML [32].

MDSC are heterogeneous in immunophenotype and function in different tumor types and developmental stages. Thus, the choice of reagents and gating strategies are critical parameters for accurate quantification of MDSC [12]. According to previous reports [10,13, 21,30], the phenotype of MDSC in AML patients relies on two gating strategies, so CD33, CD11b, HLA-DR, CD14, and CD15 were selected as the markers for circulating MDSC. The results showed that the quantification of total MDSC and their subtypes in peripheral blood of the HD and AML groups was consistent between the two gating strategies.

Our data show that the levels of total MDSC (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup>), G-MDSC (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-LOW</sup>CD14<sup>-</sup>CD15<sup>+</sup>) or PMN-MDSC (CD14<sup>-</sup>CD15<sup>+</sup>CD11b<sup>+</sup>) in peripheral blood of patients with primary AML were lower than those of healthy controls, which diverges from previous reports that circulating MDSC in most tumor patients are higher than in healthy controls. A large number of circulating immature myeloid cells appear in AML patients, with major phenotypes CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>+</sup>CD14<sup>-</sup> and AML-M3 (CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup>) [33]. HLA-DR<sup>-</sup> and CD11b<sup>+</sup> gates within MDSC can exclude these leukemia cells. The proportion of neutrophils is highest in normal peripheral blood, and the immunophenotype of neutrophils in normal circulation is CD11b<sup>+</sup>CD15<sup>+</sup>CD14<sup>-</sup>CD33<sup>+</sup>HLA-DR<sup>-</sup>CD66b<sup>+</sup> [14,33], which overlaps with the phenotype of MDSC to some extent. In addition, whole blood, using EDTA-K2 as anticoagulant, was used to analyze MDSC in this study, which differs from most studies using peripheral blood mononuclear cells (PBMC). In the latter case, the pretreatment process will affect the results, with loss of some surface markers resulting in bias; therefore, some researchers recommend use of whole blood to acquire more accurate data [22, 34,35].

Recent studies have suggested that G-MDSC can inhibit the proliferation of T cells by producing reactive oxygen species (ROS), granulocyte colony-stimulating factor (G-CSF) and enzymatic mediators like arginase I (ARG1) after antigen-specific stimulation [14,36]. The morphology of human G-MDSC reflects a heterogeneous population of cells ranging from immature neutrophils to mature polymorphonuclear (PMN) neutrophils [14]. However, some studies have shown that although G-MDSC are frequent in peripheral blood of

cancer patients, they have no immunosuppressive effect on T cells [37]. A small fraction of AML patients in this study could not be classified, due to the lack of case data; however, based on the available classification, levels of circulating total MDSC and G-MDSC in AML-M2 patients were higher than those of other subtypes, followed by AML-M4.

In this study, circulating M-MDSC (CD14<sup>+</sup>CD15<sup>-</sup>HLA-DR<sup>-LOW</sup>) levels in AML patients were higher than in healthy controls, consistent with the literature. M-MDSC have suppressive function, mainly via up-regulation of inducible nitric oxide synthase (iNOS), arginase and immunosuppressive cytokines [36]. M-MDSC represent a potential therapeutic target for cancer therapy, because of their ability to suppress immune responses and their high plasticity and potential for differentiation [11]. The level of peripheral blood M-MDSCs in cancer patients is associated with disease severity [38]. The CD33/CD3-bispecific BiTE<sup>®</sup> antibody construct (AMG 330) may achieve anti-leukemic efficacy through T-cell-mediated cytotoxicity against CD14<sup>+</sup> M-MDSC [39].

The results of this study on MDSC subsets also found that eMDSC levels in AML patients were significantly increased. This subset of MDSCs is also termed promyelocytic MDSC because of morphological resemblance to promyelocytes [35]. eMDSC are present in the blood of patients with breast and colorectal cancer and increased circulating levels of these immunosuppressive myeloid cells correlate with worsened prognosis and radiographic progression [13,40]. Circulating eMDSC and M-MDSC levels in AML-M3 patients were higher than that of other subtypes. The relationship between this subset of cells and acute leukemia therefore needs to be further explored.

For reasons of feasibility, BM samples could not be obtained in this study and analysis of BM was not included. Our results from peripheral blood need to be further verified with larger sample sizes, and we also suggest that further screening and validation of MDSC biomarkers in circulatory system diseases is required, in order to immunologically characterize MDSC in AML more comprehensively. In summary, patients with the circulatory system disease AML were recruited as subjects, and the phenotype and distribution of MDSC in peripheral blood was investigated. Our preliminary data showed that the distribution of total MDSC and their subgroups was different in AML patients compared to healthy controls, indicating that MDSC are closely associated with the onset of AML. This investigation serves to further elucidate the relationship between MDSC, malignant tumors, and antitumor effector cells in AML patients, providing potential new targets for innovation in immunotherapy. This study significantly elucidates the pathogenesis of leukemia and points towards new targeted therapies, as well as providing a theoretical basis for use of MDSC as a therapeutic monitoring index.

**Author Contribution:**

JL designed and performed the research, analyzed data, and wrote the paper; YJZ analyzed data and wrote the paper; HZ, GBM, XZW, and YTZ provided patient samples and analyzed data.

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**Declaration of Interest:**

The authors declare no conflict of interest

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