

# Induction of M-MDSCs with IL6/GM-CSF from adherence monocytes and inhibition by WP1066

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**Abstract.** Peripheral blood monocytes acquire the phenotype of myeloid-derived suppressor cells (MDSCs) by induction of cytokine or co-culture with cancer cells and are widely used to model MDSCs for *in vitro* studies. However, the simplest method of plastic adhesive sorting is poorly described as the purity of monocyte resulting from this method is the lowest compared with flow cytometry cell-sorting and magnetic beads sorting. Therefore, the present study aimed at investigating the effect of the plastic adhesive monocyte isolation techniques on the resulting MDSCs phenotype. Monocytes were allowed to adhere for 1 h and cultured with IL6 and granulocyte-macrophage colony-stimulating factors (GM-CSF) for 7 days. Plastic adhesion sorting resulted in early low monocyte yield and purity, but high purity of MDSCs was obtained by refreshing the induction medium. The resulting MDSCs were the major subpopulation of CD33<sup>+</sup>CD11b<sup>+</sup>CD14<sup>+</sup>CD15<sup>-</sup>human leukocyte antigen (HLA)<sup>-low</sup> cells and provided the potent capacity to suppress T cell proliferation and cytokine IFN- $\gamma$  production. Moreover, the induced MDSCs were inhibited by STAT3 inhibitor WP1066, resulting in downregulation of phosphorylated-STAT3 and PD-L1 expression and upregulation of apoptosis respectively. In conclusion, the present study described the generation of monocytic MDSCs from adherence monocytes and the inhibition of STAT3 inhibitor WP1066 on

the induced MDSCs. The present study contributed to the development of a new clinical drug, WP1066 targeting MDSC.

## Introduction

Myeloid-derived suppressor cells (MDSCs) are heterogeneous, immature cell populations of myeloid origin including monocyte/macrophage, granulocyte and dendritic cells, which are one of the major components of the tumor microenvironment protecting cancer cells from the host immune system attack (1-3). MDSCs promote tumor growth by producing immunosuppressive molecules in the TME such as interleukin-10 (4), transforming growth factor- $\beta$  (5) and reactive oxygen species, as well as expressing cell surface receptors that inhibit T cell proliferation and activation and producing molecules that directly promote tumor growth and invasions such as vascular endothelial growth factor (6) and matrix metalloproteinases (7). There are two major subsets of MDSCs, identified in both mice and humans: Monocytic MDSCs (M-MDSCs) are morphologically and phenotypically similar to monocytes whereas polymorphonuclear MDSCs (PMN-MDSCs) are similar to neutrophils. In mice, M-MDSCs and PMN-MDSCs are defined as CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>high</sup> cells and CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>low</sup> cells, respectively (8). In humans, M-MDSCs and PMN-MDSCs are defined as CD11b<sup>+</sup>CD14<sup>+</sup>CD15<sup>-</sup>CD33<sup>+</sup>HLA-DR<sup>-/low</sup> cells and CD11b<sup>+</sup>CD14<sup>-</sup>CD15<sup>+</sup>CD33<sup>+</sup> cells, respectively (9,10). It is reported that MDSCs accumulate in patients with renal cell carcinoma (9,11), breast cancer (12,13), ovarian cancer (14,15), melanoma (16) and head and neck cancer (17,18). In some clinical studies, MDSCs are therapeutic targets alone (19-21) or combined with other target drugs such as PD-1/PD-L1 and CTLA-4 immune checkpoint inhibitors (22-24).

In *in vitro* MDSCs studies, there are some different methods to generate MDSCs in mice and humans. One of the major methods is that mouse primary bone marrow cells or human peripheral blood mononuclear cells are co-cultured with mouse or human tumor cell lines or cytokines and then purified by magnetic beads or flow sorting with the use of myeloid cell surface markers such as CD33, CD11b or CD14 (25-29). Another major method is to first obtain high purity myeloid cells, such as monocytes, by magnetic beads

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or flow sorting and then co-culturing with tumor cell lines or cytokines. Taking a reductionist approach, the combination of GM-CSF and IL6 could induce normal peripheral blood mononuclear cells (PBMCs) to become CD33<sup>+</sup> HLA-DR<sup>low</sup> MDSCs more consistent and potency than combinations of tumor cell-secreted cytokines (30). The present study hypothesized that an obvious limitation of the studies isolating induced MDSCs or freshly monocytes by magnetic beads or flow sorting is the high cost of magnetic bead sorting kits or flow cytometers with sorting function. To overcome this problem, the present study showed that MDSCs were induced from monocytes by plastic adhesive sorting with the cytokine combination of IL6 and GM-CSF. This new model could significantly simplify the manufacturing process of MDSCs and promote drug discovery with MDSCs.

## Materials and methods

**Isolation of PBMCs.** Peripheral blood mononuclear cells (PBMCs) were isolated from anonymous healthy donors. Briefly, the buffy coats were diluted 1:1 in 0.9% NaCl, then centrifuged at 400 × g for 30 min at room temperature, according to the protocol of density gradient centrifugation (Ficoll-Paque). Following centrifugation, PBMCs on the second layer were harvested and washed twice with 30 ml of cold PBS. All human blood samples (150-200 ml) were collected between March 2018 and September 2020 and were performed under the guidelines of the Medical Ethics Committee of the Affiliated Tianyou Hospital, College of Life and Health Sciences, Wuhan University of Science and Technology. (institutional review board approval: WUSTLL-20180009). Each patient involved in the study was asked to sign a written informed consent form and agreed to the use of their samples in scientific research. All the specimens were anonymized and handled according to accepted ethical and legal standards.

**Monocytes isolation with plastic adhesion.** For monocytes isolation, PBMCs were resuspended in RPMI-1640 medium (Dalian Meilun Biotech Co., Ltd.) supplemented with 10% Fetal Bovine Serum (ExCell Bio. ExCell Biotech Co., Ltd.), seeded in a surface-treated 6-well plate at 2 × 10<sup>7</sup> cells/ml in 2 ml. After 1 h of adherence in a 5% CO<sub>2</sub> container at 37°C, non-adherent cells were removed by washing twice with RPMI-1640 medium and the remaining adherent cells were mostly monocytes.

**T cell isolation and activation.** For suppression assay, PBMCs isolated from volunteer blood in the above steps were used to sort T cells. According to the instructions (Pan T Cell Isolation kit, human, Miltenyi Biotec GmbH), the PBMCs cell pellet was first resuspended in 40 μl of buffer per 10<sup>7</sup> total cells; second, 10 μl of Pan T Cell Biotin-Antibody Cocktail, 30 μl of buffer and 20 μl of Pan T Cell MicroBead Cocktail was added per 10<sup>7</sup> total cells successively. Then, the LS Column was placed in the magnetic field of a suitable MACS Separator (MidiMACS; Miltenyi Biotec GmbH), the cell suspension added into the column and the flow-through containing unlabeled cells, representing the enriched T cells, was collected. Finally, an appropriate amount of anti-CD3/CD28 magnetic

beads was added to activate the T cells (the ratio of magnetic beads to T cells was 2:1).

**IL6/GM-CSF induce monocytes to M-MDSCs.** For the induction of M-MDSCs, adherent cells were kept cultured in a surface-treated 6-well plate in the complete induction medium [RPMI-1640 with 10% FBS, 10 ng/ml IL6 (PeproTech, Inc.) and 10 ng/ml GM-CSF (PeproTech, Inc.)] for 7 days. The medium was refreshed on day 3 and day 5.

**Flow cytometry.** Briefly, adherent cells were harvested with 10 min of dissociation with a gentle non-enzymatic cell dissociation reagent Versene (Gibco; Thermo Fisher Scientific, Inc.) followed by gently scraping. Antibody staining was performed in PBS supplemented with 2% FBS for 30 min at 4°C in the dark. For intracellular antibody staining, cells were treated with the True-Nuclear Transcription Factor Buffer Set. The antibodies used were: CD3 (UCHT1, cat. no. 300458), CD22 (HIB22, cat. no. 302524), CD14 (63D3, cat. no. 367104), CD15 (HI98, cat. no. 301908), CD33 (HIM3-4, cat. no. 303304), CD11b (ICRF44, cat. no. 301324), HLA-DR (TU39, cat. no. 361707), phosphorylated (p)-STAT3 (A16089B, cat. no. 698905), PD-L1 (29E.2A3, cat. no. 329705) (all from BioLegend, Inc.) and two SinoBiological antibodies of Bcl-2 (cat. no. 100126-R204-F), Caspase3 (cat. no. 10050-MM02) and corresponding isotype control. All the antibodies were diluted 1:2.

**Suppression assay.** The inhibitory function of the induced M-MDSCs were evaluated by their ability to inhibit the proliferation of allogeneic T cells in the following Suppression Assay: T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; 5 μM; MilliporeSigma) and seeded at 2 × 10<sup>5</sup> cells per well in 96-well plates with 100 μl RPMI-1640 medium at 37°C for 4 days. M-MDSCs from the above cytokine induction cultures were added to T cells at ratios of 1:4. T cell proliferation was provided by anti-CD3/CD28 stimulation beads (Gibco; Thermo Fisher Scientific, Inc.) and IL2 (Beijing T&L Biotechnology Co., Ltd.). Suppression Assay wells were measured by flow cytometry for T cell proliferation after four days. T cells with or without stimulation and activated T cells co-cultured with monocytes were used as controls.

**Reverse transcription-quantitative (RT-q) PCR for suppression gene expression.** For the suppression gene expression study, monocytes and M-MDSCs were harvested, then total cellular RNA was extracted from 5 × 10<sup>6</sup> cells using the RNeasy Mini kits (Qiagen GmbH). RNA (1 μg) was reverse transcribed to cDNA with random hexamer primers using the Transcriptor First Strand cDNA Synthesis kit (Roche Molecular Diagnostics). Afterward, relative cDNA was amplified with gene specific-primers using Hieff qPCR SYBR Green Master Mix (Shanghai Yeasen Biotechnology Co., Ltd.) and run on a Bio-Rad CFX Maestro with PCR cycling conditions: 95°C 5 min; 95°C 15 sec; 55°C 15 sec; and 72°C 30 sec for 30 cycles. (Bio-Rad Laboratories, Inc.). Data were acquired and analyzed using Bio-Rad CFX Maestro 1.1 software (Bio-Rad Laboratories, Inc.). Gene expression was normalized to the house-keeping gene (GAPDH) and fold change relative to monocytes was determined. Gene specific primers for reverse transcription-quantitative PCR are shown

Table I. Gene specific primers for reverse transcription-quantitative PCR.

Gene	Forward primer (5' à 3')	Reverse primer (5' à 3')
GAPDH	TTAAAAGCAGCCCTGGTGAC	CTCTGCTCCTCCTGTTTCGAC
VEGF	CACACAGGATGGCTTGAAG	AGGGCAGAATCATCACGAAG
NOX2	TGCCAGTCTGTGCGAAATCTGC	ACTCGGGCATTACACACC
TGF-β	GCAGAAGTTGGCATGGTAGC	CCCTGGACACCAACTATTGC
PDL1	TATGGTGGTGCCGACTACAA	TGCTTGTCCAGATGACTTCG
ARG1	GTTTCTCAAGCAGACCAGCC	GCTCAAGTGCAGCAAAGAGA
PDL2	ACCGTGAAAGAGCCACTTTG	GCGACCCCATAGATGATTATGC

in Table I. All experiments were performed in at least three independent experiments. RNA extraction, cDNA synthesis, and qPCR were performed according to the manufacturer's protocols.

**IFN-γ ELISA assay.** According to the protocol (Human IFN-γ ELISA kit, U-CyTech biosciences), 50 μl of diluted coating antibody solution and 100 μl PBS was added to each well of the ELISA plate, then incubated overnight at 4°C. Then, 100 μl of diluted standard/blank/samples was added to the wells and the plate sealed and incubated for 2 h at 37°C. Diluted detection antibody solution (100 μl) was added and incubated for 1 h at 37°C. Finally 100 μl of diluted SPP conjugate, 100 μl of TMB substrate solution and 100 μl of stop solution was added into each well (resulting in a yellow color) and the plate read at 450 nm within 30 min.

**WP1066 treatment on induced M-MDSCs.** After 7 days of IL6/GM-CSF induction, M-MDSCs induced *in vitro* were treated with different concentrations of STAT3 inhibitor WP1066 (0, 5 and 10 μM for 24 h and DMSO without WP1066 as a control. Flow cytometry of p-STAT3 expression levels and suppression protein PD-L1 expression was determined. In addition, the toxicity analysis of WP1066 on induced M-MDSCs was also determined by detecting the level of cell apoptosis and the expression of apoptosis-relative protein Bcl-2 and Caspase3 by flow cytometry.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc.). The results were shown as means ± SEM from at least three independent experiments. Single comparison between two groups was analyzed by Student's t test. Comparisons between multiple groups were determined using ANOVA followed by the Newman-Keuls multiple-comparison test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**The purity of monocytes before and after plastic adhesion.** Isolating monocytes from PBMCs and co-culturing with tumor cells or inducing with cytokines is a common way to obtain MDSCs for *in vitro* studies. The present study wanted to explore the effect of monocytes on cell yield, purity and monocyte phenotype using the plastic adhesion isolation method.

Peripheral blood mononuclear cells were isolated from healthy donors by density gradient centrifugation with a median PBMCs count of 720.5x10<sup>6</sup> cells with a range (540.0x10<sup>6</sup> to 790.0x10<sup>6</sup>) and median viability of 96.5% (90.0-97.8). The median monocytes percentage and cell count in PBMC were 11% (9.8-21.6) and 77.6x10<sup>6</sup> (50.6x10<sup>6</sup>-176.8x10<sup>6</sup>) cells respectively. After 1 h of plastic adhesion enrichment, the total number of cells reduced by 76%, with an average of 165.0x10<sup>6</sup> cells and the median viability was 98.4%. The median percentage of monocytes increased to 21.8% as the number of 34.6x10<sup>6</sup> on average (Fig. 1A and B). Compare with monocytes separated from flow cytometry, monocytes isolated by plastic adhesion were mixed with a proportion of lymphocytes. However, after 7 days of induction with IL6 and GM-CSF, the mean of the total cells was 23.1x10<sup>6</sup> and the median percentage of monocytes increased to 94% (82-98%).

**The presence of non-monocytes during MDSCs induction.** Unlike the high purity of monocytes produced by isolation methods such as flow cytometry cell sorting or magnetic bead sorting, there were numerous non-monocytes such as CD3<sup>+</sup> T cells or CD22<sup>+</sup> B cells during the MDSCs induction process of the present study. As shown in Fig. 1A and B, the median percentage of CD3<sup>+</sup> T cells in 1 h of adherent cells was 52.6% (34.8-68.0%). Meanwhile, median percentage of CD22<sup>+</sup> B cells in 1 h of adherent cells was 16.6% (9.7-23.5%); median percentage of CD3<sup>+</sup>CD22<sup>-</sup>CD14<sup>-</sup> cells was 10% (6.8-15.9%). However, after 3 PBS washes and medium changes in the 7 days of the culture process, non-monocytes all decreased to the lowest level ~2-5% (Fig. 1C and D).

**The phenotype of MDSCs.** Similar to the common definition of human MDSCs (3), the phenotype of *in vitro*-generated MDSCs was evaluated for CD33, CD11b, HLA-DR, CD14 and CD15 expression by flow cytometry in the present study. As described above, human PMN-MDSCs were defined as CD11b<sup>+</sup>CD14<sup>-</sup>CD15<sup>+</sup>CD33<sup>+</sup> cells and M-MDSCs were defined as CD11b<sup>+</sup>CD14<sup>+</sup>CD15<sup>-</sup>CD33<sup>+</sup>HLA-DR<sup>low</sup> cells. As shown in Fig. 2A, MDSCs show high expression of CD33, CD11b and CD14 and low expression of HLA-DR. However, MDSCs did not show CD15 expression. The results indicated that the method generated only a single subset of CD11b<sup>+</sup>CD14<sup>+</sup>CD15<sup>-</sup>CD33<sup>+</sup>HLA-DR<sup>low</sup> M-MDSCs but no CD11b<sup>+</sup>CD14<sup>-</sup>CD15<sup>+</sup>CD33<sup>+</sup> PMN-MDSCs. At the same time, the present study also revealed the changes of above surface marker expression compared with 1 h adherent monocytes (data not shown).

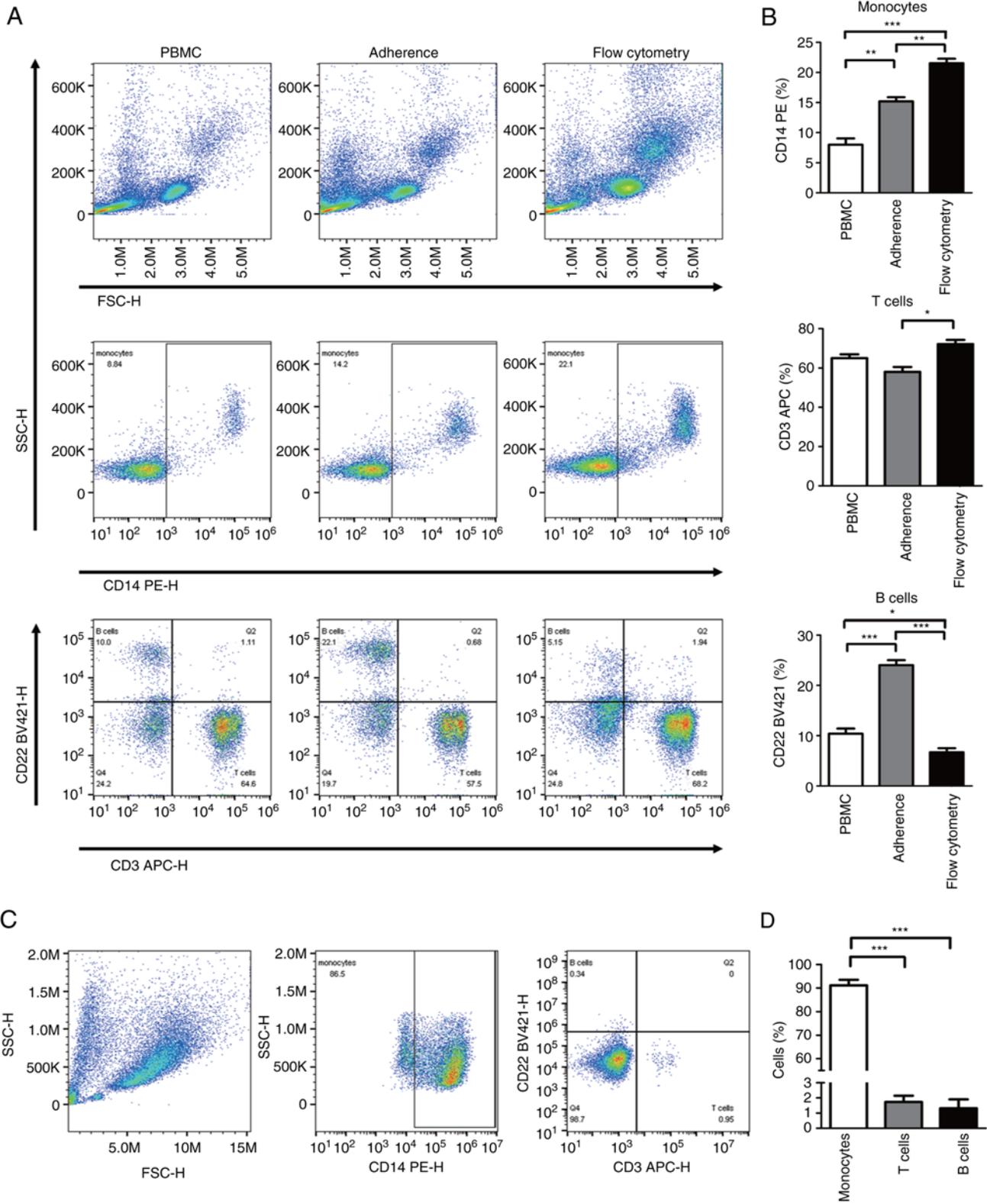


Figure 1. Purity of monocytes before and after plastic adhesion of PBMCs. (A) Streaming representation graph and (B) quantitative analysis of T cells, B cells and monocytes on PBMCs, adherent cells and flow cytometry sorted cells before IL6 and GM-CSF induction. (C) Streaming representation graph and (D) quantitative analysis of T cells, B cells and monocytes after 7 days of IL6 and GM-CSF induction. \*P<0.05, \*\*P<0.001, \*\*\*P<0.0001. PBMCs, peripheral blood mononuclear cells; GM-CSF, granulocyte-macrophage colony-stimulating factors.

*The suppressive ability of MDSCs and suppress gene expression.* The inhibitory effect of MDSCs generated from adherent monocytes was evaluated by their ability to inhibit allogeneic T cell proliferation and IFN- $\gamma$  secretion. As only M-MDSCs were

produced, high-purity MDSCs were separated with Versene for 10 min and carefully scraped off the surface of the treated 6-well plate. MDSCs were then mixed with CFSE-labeled allogeneic T cells at MDSCs: T cells ratios of 1:4, followed by stimulation

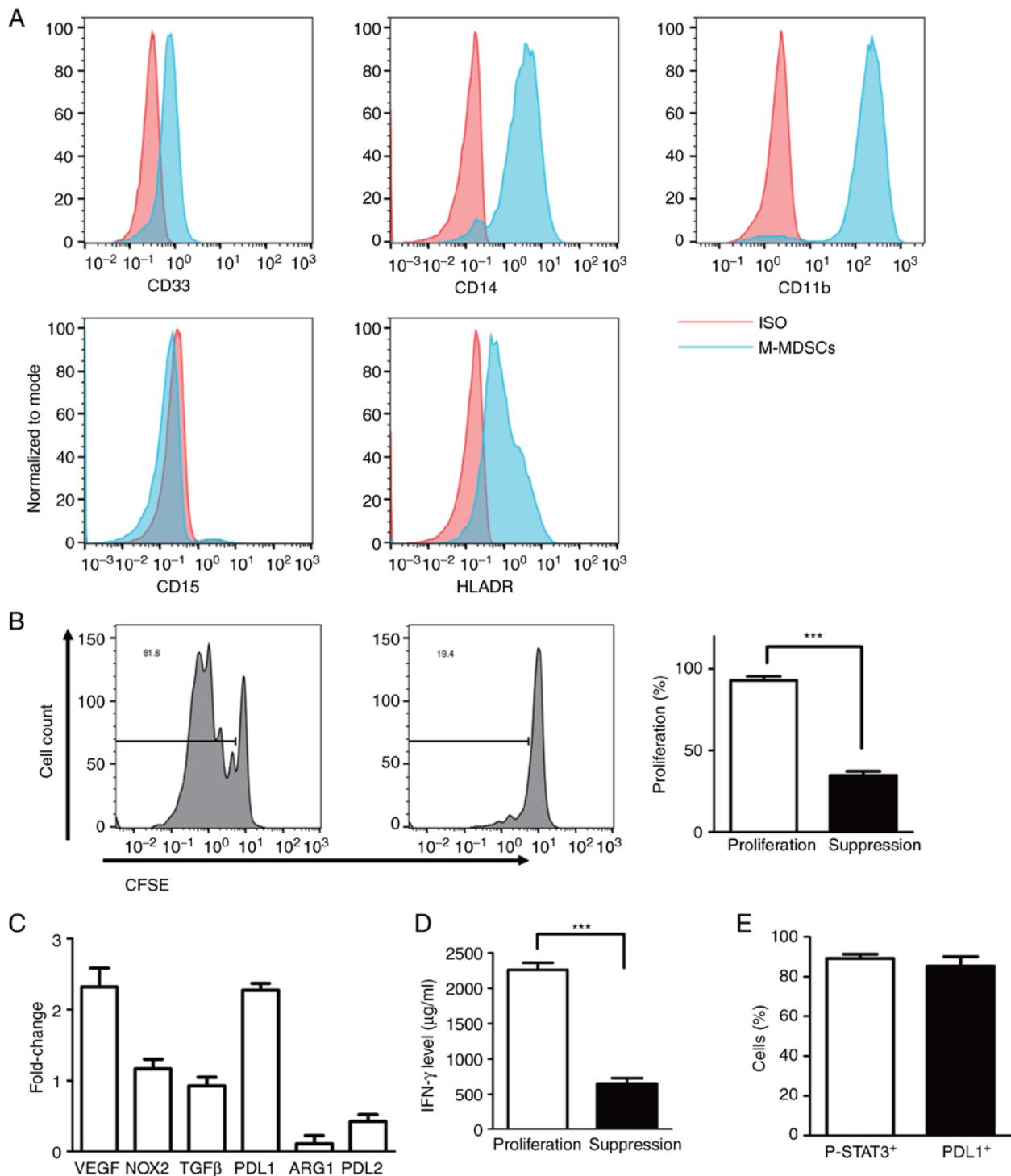


Figure 2. Phenotype of induced MDSCs and suppressing ability. (A) Flow analysis of the cell-surface expression of CD33, CD11b, CD14, CD15 and HLA-DR on *in vitro* induced MDSCs with IL6/GM-CSF for 7 days. (B) and (D) The ability of suppressing T cells proliferation in M-MDSCs at ratio of 1:4 as determined by CFSE label assay and secretion of IFN- $\gamma$  by ELISA. (C) Gene expression of relative suppressive mechanisms in MDSCs such as VEGF, NOX2, TGF- $\beta$ , PD-L1, ARG1, PD-L2 in induced M-MDSCs as determined by reverse transcription-quantitative PCR. (E) Percentage of positive p-STAT3 and PDL1 cells in induced M-MDSCs. \*\*\* $P < 0.0001$ . MDSCs, myeloid-derived suppressor cells; HLA, human leukocyte antigen; GM-CSF, granulocyte-macrophage colony-stimulating factors; M-MDSCs, monocytic MDSCs; CFSE, carboxyfluorescein succinimidyl ester; NOX2, NADPH oxidase 2; PD-L, programmed death ligand; ARG1, arginase 1; p-, phosphorylated.

with anti-CD3/CD28 beads. T cell proliferation was evaluated by flow cytometry after 4 days. Notably, the proliferation of T cells was significantly inhibited with 98% of proliferation T cells with control compared with 36% of proliferation T cells with MDSCs co-culture (Fig. 2B). To reveal the molecular

mechanism of suppressing T cell proliferation, some putative suppressor genes including VEGF, ARG1, PD-L1, PD-L2, NOX2 and TGF $\beta$  were tested by RT-qPCR. As shown in Fig. 2C, the suppressor genes of PD-L1 and VEGF were shown to be upregulated, which may affect the inhibitory ability of MDSCs.

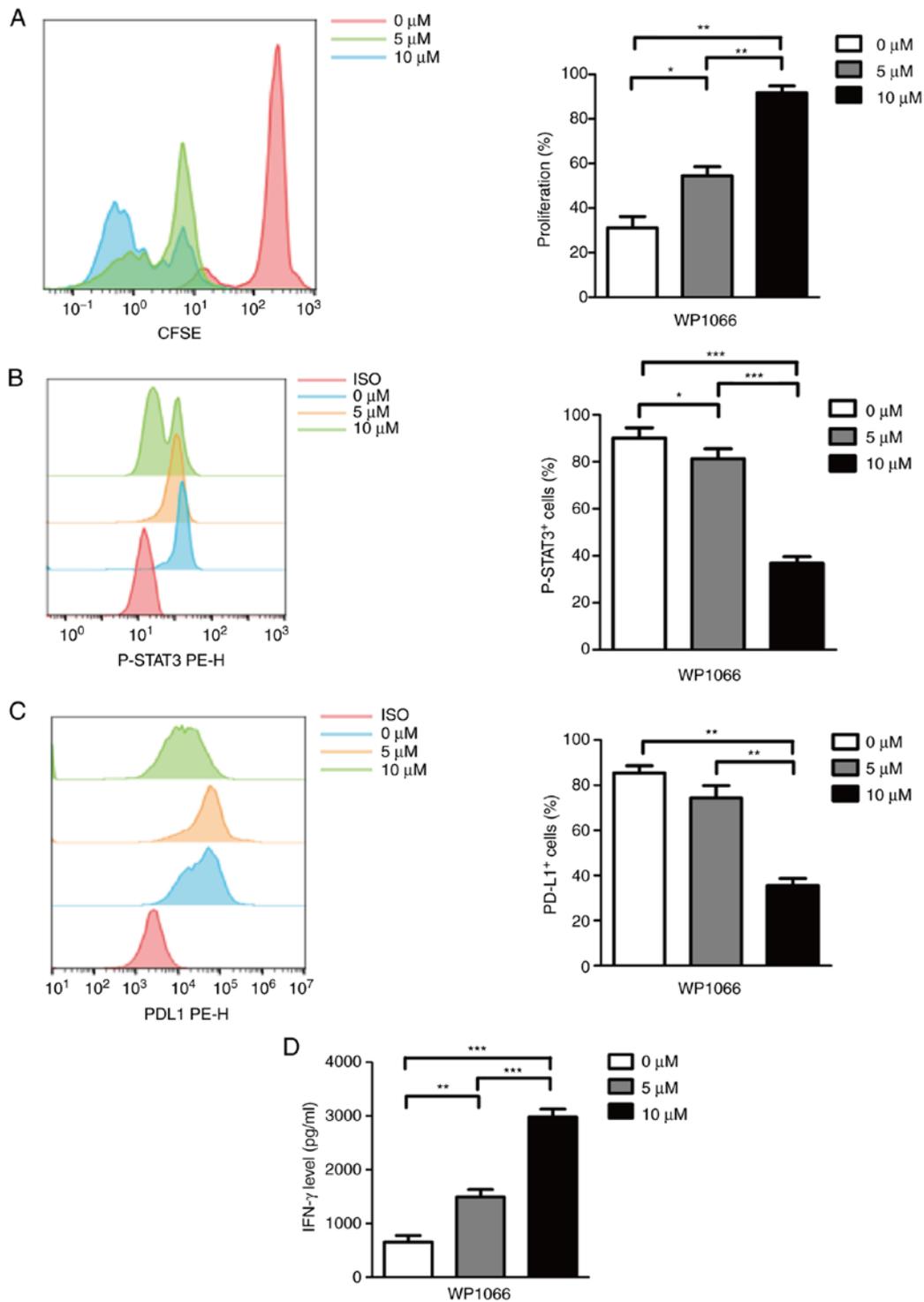


Figure 3. WP1066 treatment leads to downregulation of p-STAT3 and PD-L1. (A) Flow analysis and quantitative analysis of the ability to suppress T cells proliferation when M-MDSCs were treated with 0, 5 and 10  $\mu\text{M}$  STAT3 inhibitor WP1066 for 24 h. (B) p-SATA3 positive cells and (C) PD-L1 positive cells were determined by flow cytometry and quantitative analysis. (D) Secretion of IFN- $\gamma$  on treated M-MDSCs by ELISA. \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ . p-, phosphorylated; PD-L, programmed death ligand; M-MDSCs monocytic myeloid-derived suppressor cells.

Last, the percentage of p-STAT3 and PD-L1 positive cells both reached ~90% (Fig. 2E). As shown in Fig. 2D, the level of IFN- $\gamma$  was significantly decreased in suppressed T cells.

*WP1066 treatment leads to downregulation of p-STAT3 and PD-L1.* As an important component of the immunosuppressive microenvironment of solid tumors, MDSCs have been

extensively studied as therapeutic targets (31). WP1066 is a mature and widely used inhibitor of JAK and STAT3 signaling pathways. However, there are no reports of WP1066 targeting MDSCs. *In vitro* generated MDSCs here were treated with dimethyl sulfoxide (DMSO) plus 0, 5 and 10  $\mu\text{M}$  WP1066 for 24 h to evaluate the inhibitory effect of WP1066 on MDSCs. As shown in Fig. 3A, MDSCs treated with 10  $\mu\text{M}$

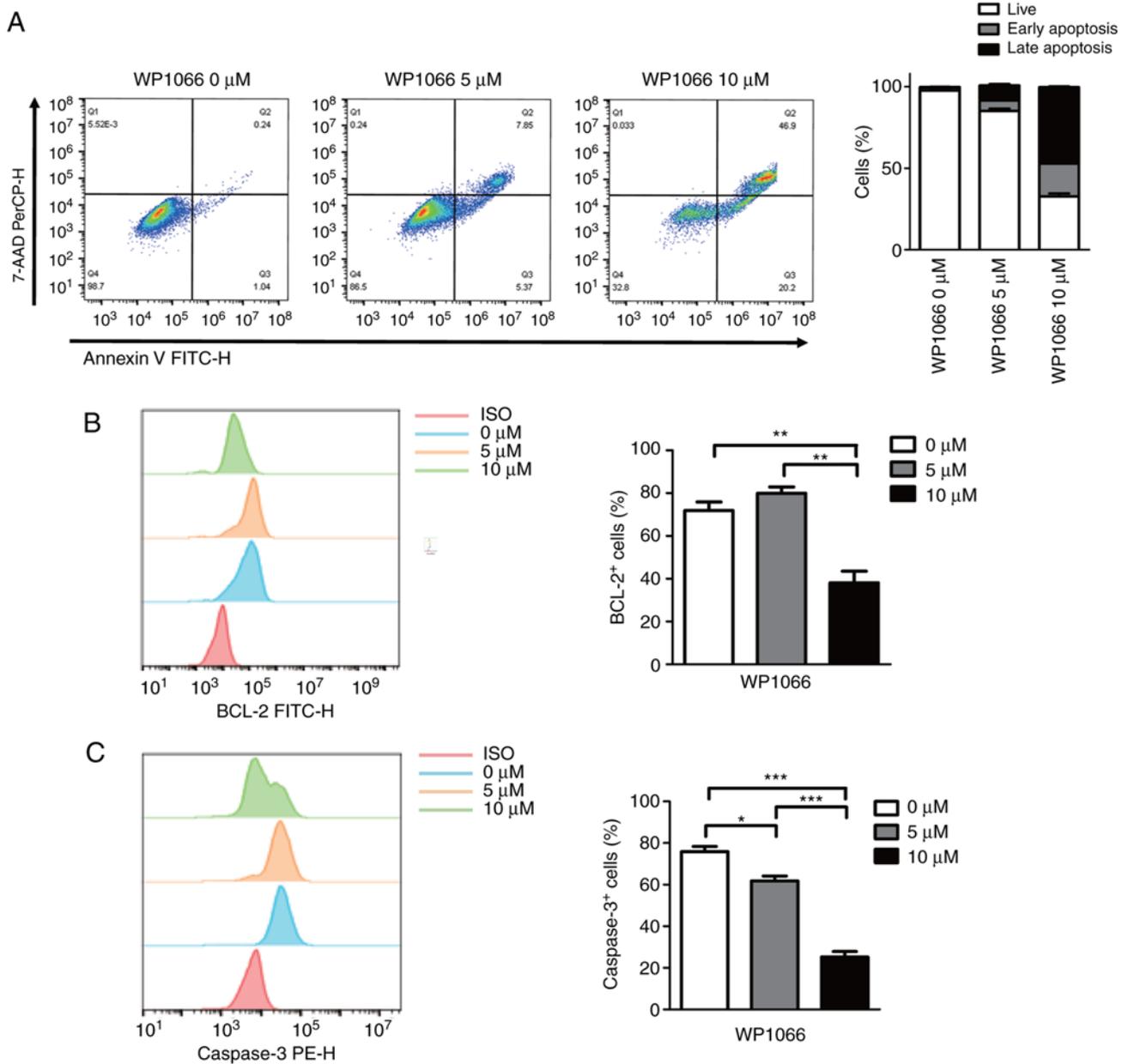


Figure 4. WP1066 treatment leads to upregulation of MDSCs apoptosis and downregulation of apoptosis-related protein. Detection of toxicity of WP1066 on induced M-MDSCs with 0, 5 and 10  $\mu$ M for 24 h determined by (A) apoptosis analysis of FITC/Annexin V and PerCP/7-AAD, (B) apoptosis relative protein Bcl-2 positive cells and (C) apoptosis relative protein Caspase3 positive cells. \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ . MDSCs, myeloid-derived suppressor cells.

WP1066 revealed more effectiveness compared with 0 and 5  $\mu$ M WP1066 in the proliferation of T cells. Similarly, the expression of p-STAT3 (Fig. 3B) and PD-L1 (Fig. 3C) were significantly reduced after 10  $\mu$ M WP1066 treatment. Finally, with the high-intensity inhibitory effect of WP1066 on M-MDSC cells, the ability of T cells to release IFN- $\gamma$  was also effectively restored, as shown in Fig. 3D.

*WP1066 treatment leads to upregulation of MDSCs apoptosis.* In addition to reducing the ability of MDSCs to inhibit T cell proliferation, WP1066 treatment also resulted in an upregulation of cell apoptosis in MDSCs. As shown in Fig. 4A, induced MDSCs treated with 5  $\mu$ M WP1066 revealed almost the same level of upregulation of early apoptosis and late apoptosis after 24 h of WP1066 treatment, whereas MDSCs treated with

10  $\mu$ M WP1066 showed more cells with late apoptosis than early apoptosis. Despite the detection of cell viability, classical proteins associated with cell death such as Bcl-2 and Caspase3 were also examined. As shown in Fig. 4B, 5  $\mu$ M WP1066 did not affect Bcl-2 protein expression in the resulting MDSCs, however, 10  $\mu$ M WP1066 showed a significant reduction of Bcl-2<sup>+</sup> cells. Similarly, induced MDSCs treated with 10  $\mu$ M WP1066 showed more decrease in Caspase3<sup>+</sup> cells implying more late apoptosis or dead cells (Fig. 4C).

## Discussion

The present study established a simple *in vitro* model to generate MDSCs from monocytes isolated from fresh PBMCs by plastic adhesion isolation. Plastic adhesion isolation is a simple and

inexpensive widely used technique for the isolation of human monocytes from PBMCs. Evidently, compared with monocytes isolated by magnetic beads or flow sorting, the yield of adherence monocytes was significantly lower and mixed with a high proportion of lymphocytes. However, only M-MDSCs-like cells were successfully generated from adherence monocytes by co-treatment with IL6 and GM-CSF for 7 days similar to CD11b<sup>+</sup>CD14<sup>+</sup>CD15<sup>-</sup>CD33<sup>+</sup>HLA-DR<sup>-low</sup> cells. However, it is not claimed to be the best model to generate MDSCs from human bone marrow or PBMCs. Nevertheless, it is suggested that this model will significantly remove the barriers of research funding and high-tech equipment and allow more researchers to study MDSCs-related problems. In addition, it is hypothesized that the feasibility of this technique in diagnosis aspects is not high because diagnosis requires reliable results and high reproducibility. Although this method is the simplest and cheapest method to obtain monocytes, the reliability and consistency of the conclusions using this method are poor and therefore is not suitable for diagnostic applications.

A limitation of the present study is that it only focused on the most commonly used combination of IL6 and GM-CSF to induce MDSCs with greater inhibitory capacity. Further studies will investigate whether MDSCs can be induced from adherence monocytes with the treatment of other different combinations of cytokines or conditional medium of human cell lines. Based on CD14 and CD16 expression, monocytes could be further divided into three main subsets (32), therefore, another limitation of the present study is that it did not explore the proportion of each subset in adherent monocytes.

The first notable phenomenon is that the presence of no-monocytes in the process of early induction did not affect the successful induction of final MDSCs. It was hypothesized that the powerful function of the cytokine combination of IL6 and GM-CSF masked the influence of other lymphocytes early in the process. As in other studies, the STAT3 signaling pathway was activated in MDSCs induced by IL6/GM-CSF, but the characteristics, phenotype and the mechanisms by which MDSCs inhibited T cell proliferation were different. For example, in Casacuberta-Serra *et al* (33), MDSCs express low levels of CD14, but high PD-L1 expression. Bian *et al* (34), show that arginase-1 is neither inherently expressed in MDSC nor required for MDSC-mediated inhibition. Zhan *et al* (35), reveal that the suppressive function of CD33<sup>+</sup>HLA-DR<sup>low</sup> MDSCs is dependent on the programmed death ligand-1/programmed death ligand-2 pathway. However, the IL6/GM-CSF induced MDSCs in the present study lacked ARG1 and NOX2 expression but upregulated VEGF and PD-L1 expression, suggesting that this model is not a classic MDSCs that suppressed T cells proliferation through L-arginine depletion or RNS production. Furthermore, the expression of PD-L1 was upregulated to suppress T cell proliferation. Therefore, this model may be used for therapeutic targets of PD-L1 on MDSCs, but not for other conventional mechanisms of intracellular regulatory molecules in MDSCs isolated from cancer patients.

STAT3 activation is involved in the generation and function of MDSCs and has been identified as a promising therapeutic target for anticancer drugs (36). The present study demonstrated that the STAT3 inhibitor WP1066 had the potential to reduce the level of immunosuppression and upregulate cell apoptosis in MDSCs. It was observed that

MDSCs treated with 10  $\mu$ M WP1066 for 24 h significantly reduced p-STAT3 levels and PD-L1 expression, suggesting that WP1066 could directly inhibit the function of MDSCs. Furthermore, downregulation of Bcl-2 and Caspase3 in MDSCs indicated that WP1066 could promote MDSCs apoptosis to change the tumor microenvironment.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

XL and TZ participated in research design. HH, YX, QY and TL performed the experiments, analyzed the data and were major contributors in writing the manuscript. LG provided technical guidance and participated in data acquisition and analysis. HH and YX confirm the authenticity of all the raw data. All the authors read and approved the final version of the manuscript.

### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Tianyou Hospital, College of Life and Health Sciences, Wuhan University of Science and Technology. Each patient involved in the study was asked to sign a written informed consent form and agreed to the use of their samples in scientific research.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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