All-trans retinoic acid protects mesenchymal stem cells from immune thrombocytopenia by regulating the complement–interleukin-1β loop

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Supplementary Methods

Inclusion and exclusion criteria for patients

Patients with other causes of thrombocytopenia, such as systemic lupus erythematosus (SLE), APS, thrombotic thrombocytopenic purpura (TTP), disseminated intravascular coagulation (DIC), drug induced thrombocytopenia, liver disease (hepatitis B or C virus infection and cirrhosis), bone marrow failure, cancer and pregnancy were excluded. The diagnosis of SLE and APS complied with the Systemic Lupus International Collaborating Clinics (SLICC) criteria for SLE (1, 2) and the guidelines for APS (3, 4). Antiphospholipid antibodies, including lupus anticoagulant (LA), anti-cardiolipin antibody (aCL) and anti-β2-GPI antibody, were determined at the time of ITP diagnosis and at least 12 weeks apart. The presence of any antibody on any occasions was excluded from the study.

We excluded patients who had congestive heart failure, severe arrhythmia, aspartate aminotransferase and alanine aminotransferase concentrations three times or more the upper limit of the normal threshold criteria, creatinine or serum bilirubin concentrations each 1.5 times or more than the normal range, and active or previous malignancy. We considered patients who remain unresponsive to the rescue treatment to be withdrawn from the study. Rescue treatment was defined as platelet or plasma transfusion and hemostatic therapy, and started in patients who were bleeding or had a platelet count less than 10×10^9/L. For patients with platelet counts between 10×10^9/L and 30×10^9/L, use of rescue treatment was dependent on physician’s assessment. Patients were not withdrawn unless severe adverse events or drop-out occurred.
MSCs isolation

MSCs isolation was described in our previous publication (5, 6). The BM mononuclear cells were plated at a density of 10^7 cells/mL in media supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic-antimycotic and incubated at 37 °C in a 5% (vol/vol) humidified CO2 chamber. The medium was exchanged after 48 h and every 3 to 4 days thereafter. When cultures reached 80% confluence, the cells were passaged and frozen for the next study. To confirm the human MSC phenotype, plastic adherent cells were analyzed for the expression of surface-specific antigens using flow cytometry. The cells were stained with CD14 (555387), CD19 (557697), CD34 (550761), CD45 (563791), CD105 (563803), CD90 (563070), CD73 (560847) and HLA-DR (555559). Fluorescein isothiocyanate (FITC)-conjugated, allophycocyanin (APC)-conjugated, peridinin chlorophyll protein (PerCP)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies (All purchased from BD Biosciences, San José, CA). The FITC-, PE-, APC- and PerCP-conjugated isotypes were used as negative controls. The analysis was performed using a flow cytometer (Beckman Coulter, Co, USA). Once the third passage was reached, the MSCs were plated into 96-well tissue culture dishes in culture medium. Proliferation was assessed during each day of culture by measuring the 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye absorbance of the cells at 570 nm.

To explore the effect of ATRA on MSCs in vitro, the cells were detached using 0.25% trypsin-EDTA at 37 °C for 1 minute after removing the supernatant from the ITP-MSC cultures. Based on our previous publication (6), the cells were seeded in flasks at 1×10^6 cells/ml and treated with ATRA (50 μg/ml, Sigma-Aldrich, MO, USA) for 48 hours. Cell proliferation assay, apoptosis
assay and expression of signaling proteins MyD88, NF-κB p38 MAPK and ERK1/2 were then evaluated.

The phenotypes of mice bone marrow MSCs were analyzed using the following antibodies: PE conjugated-CD29 (14-0291-82), CD45 (14-0451-82), CD105 (14-1051-82), CD34 (11-0341-82) and CD90 (48-0902-82). All antibodies were purchased from eBioscience, San Diego, USA).

**RNA extraction and Microarray**

MSCs were placed in Trizol (Invitrogen, Carlsbad, CA, USA) and processed for RNA extraction using the RNeasy kit according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA). The integrity of the RNA was assessed using denaturing RNA agarose gel electrophoresis. The quantity of the RNA samples was assessed by absorbance spectrometry using a NanoDrop 2000 (Thermo, Waltham, MA, USA). For mRNA microarray experiments, total RNA was purified and subjected to first-strand cDNA and second-strand cDNA synthesis. cDNA was generated and labeled with biotin, and then fragmented to a suitable size. Hybridization was performed with Affymetrix mRNA microarray (CapitalBio Corp, Beijing, China) according to the manufacturer’s instructions. After washing and staining, arrays were scanned and the imaging data were extracted with Affymetrix GeneChip Command Console Software (CapitalBio Corp). The significantly changed genes were selected based on $p$ value $<$0.05 and $>$2-fold as criteria.

Normalized RNA-seq expression data were preranked based on the fold change among the three groups. The Hallmark curated Gene sets in MSigDB database 5.0 were used for GSEA
analysis. GSEA 3.0 was used to perform the analysis. Gene sets were tested for enrichment in rank ordered lists via GSEA using a classic statistics and compared to enrichment results from 1000 random permutations of the gene set to obtain \( p \) values. A corrected \( p \)-value was obtained from the analysis using the FDR q-value correction. On the basis of this correction, the cutoff for significance was established at a \( p \) value <0.05.

To assess biological relationships among genes, we used the Ingenuity Pathway Analysis software (IPA, Ingenuity System, Redwood City, CA, USA; http://www.ingenuity.com). The pathway, upstream and network analysis was performed using IPA. The canonical pathways generated by IPA are the most significant for the uploaded data set. Fischer’s exact-tests with FDR option was used to calculate the significance of the canonical pathway. IPA computes a score for each network according to the fit of the set of supplied focus genes. These scores indicate the likelihood of focus genes to belong to a network versus those obtained by chance. A score > 2 indicates a ≤ 99% confidence that a focus gene network was not generated by chance alone.

*Immunofluorescence assay (IFA)*

Cell monolayers were fixed in 4% paraformaldehyde for 10 min and washed with PBS. The fixed cells were permeabilized with 0.25% Triton X-100 in PBS for 15 min. After blocking in PBS containing 3% BSA for 30 min at 4°C, samples were incubated overnight at 4°C with primary antibody at 1: 200 in PBS containing 3% BSA. After washing with PBS containing 0.05% Tween (PBS-T), samples were incubated for 15 min with secondary antibody in PBS containing 3% BSA, washed again and incubated with 0.5 \( \mu \)g/ml 4-,6-diamidino-2-phenylindole (DAPI) in PBS for 1
min. Samples were mounted in FluorSave Reagent (Calbiochem, San Diego, CA, USA). Pictures were obtained using an IX71 Olympus Fluorescence Microscope equipped with a digital camera or a Nikon ECLIPSE Ti Confocal Laser-Scanning Microscope. Nikon NIS Elements Ar Microscope Imaging Software was used for image analysis and 3-D rendering. Mouse monoclonal antibodies to C1q (ab71940, Abcam, Cambridge, MA, USA), C3b (ab11871, Abcam), rabbit polyclonal antibodies to C4d (ab36075, Abcam), C5b-9 (ab55811, Abcam), rabbit monoclonal antibodies to CD90 (ab133350, Abcam), CXC L12 (ab155090, Abcam) and IL-1β (12703, Cell Signaling Technology, CST, Danvers, MA, USA) were used as primary antibodies. FITC labeled goat anti-rabbit IgG (ab7064, Abcam) and FITC labeled goat anti-mouse IgG (ab6785, Abcam) were used as secondary antibodies.

**Enzyme-linked immunosorbent assay (ELISA)**

For detecting complement components deposition on the surface of MSCs, samples from the patients were obtained after diagnosis and prior to medicine intervention, which was accompanied by collection of samples from healthy volunteers simultaneously. All bone marrow samples were obtained from 8 a.m. to 11 a.m.. The deposition of complement components was quantified using commercially available monoclonal mouse anti-human C1q (ab71940, Abcam), C3b (MA1-70053, eBioscience), C4d (MA1-83082, eBioscience) and C5b-9 (ab66768, Abcam). Reference intervals were determined for the deposition of C1q, C4d, C3b and C5b-9 on bone marrow derived MSCs from 20 healthy volunteers. The complement components deposition of enrolled 58 consecutive newly diagnosed ITP patients and 42 healthy controls were expressed as a ratio to the reference intervals to facilitate inter-assay comparison.
TNF-α (DTA00C, R&D Systems, Minneapolis, MN, USA), IL-1β (DLB50, R&D Systems), CXCL12 (DSA00, R&D Systems), C3a (ab133037, Abcam) and C5a (DY2037, R&D Systems) levels were assessed in bone marrow supernatant or MSCs cell lysates. All of the tests were processed with ELISA kits according to the manufacturer’s instructions. MSCs were centrifuged at 500g to completely separate them from probable remained microvesicles in supernatant, and then, they were washed by PBS. Viable cells were counted in a hemocytometer chamber using Trypan Blue staining. All results of cytokine levels by ELISA were given for per 10⁶ cells.

**Cell Proliferation Assay**

The MSC proliferation assay was performed using the Cell Counting Kit-8 (CCK-8) assay kit (CK04-3000T, Dojindo Molecular Technologies, Rockville, MD), according to the manufacturer’s protocols. Generally, cells were seeded onto 96-well cell culture cluster plates at a concentration of 2×10⁴ cells per well in volumes of 100 ml and grown overnight. Cell Counting Kit-8 reagents were added to a subset of wells and incubated for 2 hours at 37 °C, and the absorbance of the samples was measured using an enzyme-linked immunosorbent assay plate reader at a wavelength of 450 nm.

**Apoptosis assays**

Cell apoptosis were analyzed by Annexin V-PE/7-AAD stained flow cytometry (559763, BD Biosciences). MSCs were harvested through trypsinization and washed with PBS and then centrifuged to collect the pellet. The pellet was resuspended in 1 × binding buffer at a density of 5.0×10⁵ cells per ml. One hundred microliters of the binding buffer was incubated with 5 μl of
PE-conjugated Annexin V and 5 μl of 7-AAD and added to samples for 15 min at room temperature (25 °C) in the dark. Samples were analyzed by flow cytometer using NovoExpress. Early apoptotic (PE positive, 7-AAD negative), late apoptotic, and dead cells (PE positive, 7-AAD positive) can be discriminated on the basis of a double-labeling for Annexin V-PE and 7-AAD and analyzed by flow cytometry.

**Western blotting**

The cells were washed twice with ice-cold PBS and extracted with lysis buffer (Sigma-Aldrich) for 45 minutes on ice. Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were then blocked with Tris-buffered saline with Tween (TBST) containing 5% non-fat dry milk for 1 h. The membranes were then incubated overnight at 4°C with monoclonal antibodies (1:500-1000 dilution) against ERK1/2 (4370, CST), p38 MAPK (8690, CST), MyD88 (4283, CST), p65 (8242, CST) and β-actin (4970, CST). The membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody for 1 h. The blots were developed using an enhanced chemiluminescence kit. A quantitative analysis was performed by scanning the blots and calculating the relative intensities in relation to the corresponding actin signal using Quantity One (Bio-Rad, Laboratories, Hercules, CA, USA). The analysis was normalized against a housekeeping protein β-actin.

**Determination of CXCR4 expression on megakaryocyte surface**
Supplementary 12 consecutive newly diagnosed ITP patients and 8 healthy controls at Institute of Hematology, Peking University People’s Hospital were enrolled in this study. Megakaryocytes were isolated and stained with anti-CXCR4-PE and subsequently analyzed using a flow cytometer. Platelets were acquired using light FSC and SSC thresholds, gated by characteristic FSC-SSC dot plots. FL1-FSC dot plots and FL1-histograms were analyzed (7).

Analysis of CD34+ cells

The isolated bone marrow mononuclear cells were plated at 1×10^7 cells/100 mm in a culture dish for 90 min. The non-attached cells were then incubated with microbead-conjugated anti-CD34 monoclonal antibody at 4°C for 20 min and processed through a MiniMACS magnetic separation column (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) to obtain the CD34+ cell fraction. CD34+ cell preparations were analyzed by flow cytometry using the single platform CD34 assay kit (Stem-Kit™, Beckman Coulter Co, USA) to measure the direct counts of CD34+ cells (Beckman Coulter, Co, USA) (8).

Analysis for colony-forming unit-megakaryocyte (CFU-MKs)

Megakaryocytes were cultured on semisolid serum-free medium (04961, StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. After dehydration, fixation and sequential staining according to the manufacturer’s instructions (04962, StemCell Technologies), the colonies were quantified and each cluster of three or more megakaryocytes was scored as a colony under a fluorescence microscope. CFU-MKs were scored according to the manufacturer’s protocol based on their size, which reflects the maturation stage of the progenitor
giving rise to each colony. The colonies were rated as large (50 cells, arising from more primitive megakaryocyte progenitors), medium (21-49 cells) and small (3-20 cells, derived from more mature megakaryocyte progenitors) colonies (6).

The modified monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay

The assay was carried out as previously described in detail by Hou et al (Eur J Haematol. 2003). In brief, washed platelets were incubated with patient platelet eluate and solubilized in Tris-buffered saline (TBS), containing 1% Triton X-100. After 30 min incubation at 4°C, insoluble material was removed by centrifugation and the supernatant was diluted 1:4 with TBS/0.5% Triton X-100/0.05% Tween-20. Platelet glycoprotein-specific monoclonal antibodies for GPIIb/IIIa and GPIb/IX (Immunotech S.A., Marseille, France) were incubated with patient serum for investigation. The absorbance was recorded at 405 nm. An absorbance higher than the mean absorbance + 3 SD recorded for the controls was considered as positive (9).

Analysis of DNA methylation

Bisulfite treatment was performed for the genomic DNA (500 ng) of each sample using the EZ DNA Methylation-Gold™ Kit (D5005 & D5006, Zymo Research Corporation, CA, USA) according to the manufacturer's instructions. After bisulfite treatment, a 40 μl PCR was carried out in 3.2 μl bisulfite treated DNA (30 ng), 36 μl Platinum® PCR Supermix High Fidelity (12532016, Invitrogen, Waltham, MA, USA), and 200 nM of each primer. Thermal cycler conditions consisted of an initial activation step at 95°C for 5 min, followed by a 3-step PCR program of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s for 50 cycles. Following PCR, gel electrophoresis to determine
purity was performed using 10 µl of each PCR product. Percent DNA methylation in the IL-1β promoter was quantified using PyroMark™ MD (Qiagen) according to the manufacturer's instructions. The primers used for pyrosequencing (Supplementary Table 4) were designed with Pyrosequencing™ Assay Design Software Ver 2.0 (Qiagen). Two primer sets were designed for IL-1β promoter. The -299 and -256 CpG sites of IL-1β promoter were covered by IL-1β_1 primer set and the -20 and +13 CpG sites were covered by IL-1β_2 primer set (10).

**Real-time PCR**

Relative quantification of gene expression was performed with an ABI Prism 7500 detection system (Applied Biosystems, Warring-ton, UK). Reactions were performed in triplicate, with 18srRNA as the internal control. Primer Express 3.0 software (Applied Biosystems) was used to design primers across exon-exon boundaries. Messenger RNA expression was quantified according to the $2^{-\Delta\Delta Ct}$ method. The 20 µl reaction mixture was used contained 1µl of complementary DNA, 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), and 250 nM of each primer. Thermal cycler condition consisted of an initial activation step at 95°C for 10 min, followed by a 2-step PCR program of 95°C for 15s and 60°C for 60 s for 40 cycles. A dissociation curve was obtained for each quantitative PCR run. The primers used for RT-PCR are shown in Supplementary Table 5.

**Gene silencing**

IL-1β-siRNA expression vectors were constructed using standard cloning procedures. The viral vector GV115 (http://www.genechem.com.cn/Zaiti.aspx?zt=GV115) carrying IL-1β siRNA
sequences have been published previously and were purchased from Genechem (GeneChem Inc., Shanghai, China). The IL1β-RNAi sequences targeted the following sequences (5’ to 3’): (1) GCCAGGATATAACTGACTT; (2) GATTTGTCTTCAACAAGAT and (3) TGCGTGTTGAAAGATGATA. MSC transduction was performed at a multiplicity of infection of four to achieve 50% infection and ten to achieve 100% infection.

Gene overexpression

The lentivirus covered the signal sequence and coding region cDNA sequence of IL-1β was purchased from Genechem. Briefly, IL-1β (NM_000576.2 (117-269aa)) with the signal sequence was amplified using the 5’-GAGGATCCCGGGTACCGGTCGCCACCATGGAAATCTGCAGAGGCCTCCGCAGTCA CCTAATCACTCTC-3’ and 5’-TCCTTTGTAGTCCATACCGGAAGCACAAATTTGCATGGTG-3’ primer and cloned into the GV365 vector (http://www.genechem.com.cn/Zaiti.aspx?zt=GV365).

Immunohistochemical

Bone marrow biopsy tissues were obtained from the posterior superior iliac spine. BM tissues were fixed in 4% formaldehyde for 48 hours before decalcification (4% hydrochloric acid for 7 hours). Bone marrow was then embedded in paraffin and sliced into 3-mm-thick sections. Bone marrow sections (3 mm) underwent heat-mediated antigen retrieval (Dako, Carpinteria, CA) to uncover the surface antigens, were blocked with 5% goat serum, and were then incubated in 3% H2O2 to quench the endogenous peroxidase activity. The Tyramide Signal Amplification™ (TSA™,
PerkinElmer, Waltham, MA) method was applied to enhance the antigen signals according to the manufacturer’s instructions. Briefly, the sections were incubated with rabbit polyclonal antibody to CD41 (1: 200, 18308-1-AP, Proteintech, Chicago, IL, USA), CD31 (1:800, 11265-1-AP, Proteintech) and IL-1β (1:100, ab9722, Abcam) respectively in antibody dilution solution overnight at 4°C. Subsequently, the sections were incubated with HRP-conjugated secondary antibodies (anti-rabbit, MP-7601, Vector, Burlingame, CA, USA) in PBS for 2 h at room temperature. After washout, the sections were visualized by an incubation with 100-300μl of the Biotin Amplification Working Solution onto each slide for 3-10 min at room temperature. Sections were rinsed with PBS three times and mounted with DAPI-containing Fluoroshield (ab104139, Abcam). Primary and/or secondary antibody omission controls under identical staining conditions resulted in no fluorescent signal. Evaluation of MSCs apoptosis in niche was performed with TUNEL and CD90 staining. TUNEL staining (K403, Biovision, Milpitas, CA, USA) was used concomitantly with rabbit monoclonal antibody to CD90/Thy1 (1:100, ab133350, Abcam).

The bone-associated region was defined as between 0-100 μm from the endosteal surface within the diaphysis. The immediately adjacent region of the same size was defined as between 100-200 μm from the endosteal surface. The definition of CD41+ megakaryocytes physically associated with CD31+ vessels was that between 0-10 μm from the sinusoidal endothelium within the diaphysis (11). Total CD41+ megakaryocytes were manually enumerated for 8 to 12 images per section.

*In situ hybridization*
Analysis of CXCL12 transcripts was performed on BM biopsy sections. CXCL12 probes (No.422991, Advanced Cell Diagnostics, Hayward, CA, USA) was used according to the manufacturer’s instructions. Briefly, the BM biopsy sections were deparaffinized and pretreated according to the manufacturer’s protocol. The target probes were hybridized for 2 h in a hybridization oven at 40 °C before performing amplification steps according to the manufacturer. Staining for CXCL12 was categorized into six grades: 0, (+), 1+, 2+, 3+ and 4+ in accordance with the manufacturer’s guidelines, with the addition of (+) grade (for manufacturer’s guidelines, see www.acdbio.com). The (+) grade was introduced to include samples with distinct but scattered staining for CXCL12. Slides were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the cells were incubated with 1:2,000-diluted 4′,6′-diamidino-2-phenylindole (DAPI) for 5 min (to stain the nucleus and kinetoplast). The bone-associated region was defined as between 0-100 μm from the endosteal surface within the diaphysis. The immediately adjacent region of the same size was defined as between 100-200 μm from the endosteal surface (11).

Animal model and treatment

Wild-type (WT) C57BL/6 mice (6–8 weeks) were purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). C57BL/6 CD61 KO mice were kindly provided by Professor Junling Liu (Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory of Tumor Microenvironment and Inflammation, Shanghai Jiao Tong University School of Medicine, Shanghai, China) and bred in the Animal Centre of Peking University People’s Hospital. The immunization of CD61 KO mice with WT platelets were conducted as previously (12). Severe combined immunodeficient (SCID) mice of C57BL/6 background (J001913, 6-8 weeks of age) as spleen cell transfer recipients were purchased from Jackson Laboratory (Bar
Harbor, ME, USA). All animal experiments were approved by the Ethics Committee of Peking University People’s Hospital and undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

ITP mice were established as described previously (12). On the day before splenocyte transfer, screened SCID mice were injected intraperitoneally with 50 μL of rabbit anti-asialo GM1 (014-09801, Wako Pure Chemical Industries Ltd) to primarily remove natural killer cells and on the day of splenocyte transfer, the SCID mice were subjected to 200 cGy total body irradiation to inhibit recipient innate immune responses and enhance engraftment. Within 3 hours of irradiation, certain mice were injected intraperitoneally with 100 μL of the indicated splenocyte preparations (at 5×10⁴/mL final). Blood was drawn by retro-orbital bleeding. Platelet counts were recorded weekly. The SCID mice were killed 4 weeks after irradiation.

ITP mice received ATRA (Sigma-Aldrich) of 5 mg/kg (n = 15) and 20 mg/kg (n = 15) dissolved in DMSO for 10d by intraperitoneal injection, respectively. WT mice and control ITP mice received equivalent dosage of DMSO. 4 of ITP mice died in the treatment group of 5 mg/kg ATRA intraperitoneal injection within 4 weeks after irradiation. 2 of ITP mice died in the treatment group of 20 mg/kg ATRA intraperitoneal injection within 4 weeks after irradiation.

Six mice of every group were used for isolation of bone marrow MSCs. Bone marrow cells were collected by flushing femurs and tibias with 2 mL needles. The cells were seeded in a flask at a density of 10⁶/cm². The basic culture medium for the isolation of MSC was the MesenCult™
Proliferation Kit (Stemcell Technologies, Vancouver, Canada) or Dulbecco’s modified Eagle’s medium containing 10% FBS. Three days later, non-adherent cells were removed from the culture. The cells were cultured at 37°C in an atmosphere maintaining 5% CO₂ and passed at 80% confluence. Cells of passage 7 to passage 12 were used.

Femurs and tibias from remained mice of every group were fixed in 10% formaldehyde for 24 hours. The prepared samples were embedded in paraffin and sliced. Sections were partly staining with hematoxylin and eosin (H&E) by standard methods. Some sections were incubated with rabbit polyclonal antibody to C5b-9 (1 µg/ml, ab55811, Abcam), CXCL12 (1:1000, ab9797, Abcam), CD31 (1:200, ab28364, Abcam) and IL-1β (1:100, ab9722, Abcam) for immunohistochemical. Evaluation of MSCs apoptosis in niche was performed with TUNEL and CD90 staining. TUNEL staining (K403, Biovision, Milpitas, CA, USA) was used concomitantly with rat monoclonal antibody to CD90/Thy1 (1:300, ab3105, Abcam).

Statistics

Data are presented as the means ± SD. unless otherwise indicated. Tukey’s multiple comparison tests, paired t-tests, χ² tests and unpaired Student’s t-tests were used to determine the statistical significance as appropriate using SPSS 22.0 (IBM, Armonk, NY, U.S.A.). p values < 0.05 were considered statistically significant. * p <0.05, ** p <0.01, *** p <0.001.
Supplementary Reference


Supplementary Table 1. Comparison of the complement deposition on MSCs of patients with ITP and healthy controls.

<table>
<thead>
<tr>
<th>Complement components</th>
<th>MSC-ITP-C⁺ (n = 26)</th>
<th>MSC-ITP-C⁻ (n = 32)</th>
<th>MSC-control (n = 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1q</td>
<td>1.1 ± 0.5</td>
<td>0.9 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>C4d</td>
<td>2.1 ± 0.3</td>
<td>1.2 ± 0.5</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>C3b</td>
<td>1.2 ± 0.6</td>
<td>0.8 ± 0.4</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>C5b-9</td>
<td>3.2 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.4</td>
</tr>
</tbody>
</table>

ITP, immune thrombocytopenia; MSCs, mesenchymal stem cells.
Supplementary Table 2. Baseline characteristics of ITP patients.

<table>
<thead>
<tr>
<th></th>
<th>MSC-ITP-C⁺ (n = 26)</th>
<th>MSC-ITP-C⁻ (n = 32)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, median (range)</td>
<td>37.65 (25-56)</td>
<td>36.06 (19-58)</td>
<td>0.577</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.346</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>9 (34.62)</td>
<td>15 (46.88)</td>
<td></td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>17 (65.38)</td>
<td>17 (53.12)</td>
<td></td>
</tr>
<tr>
<td>Baseline platelet count, ×10⁹/L, median (range)</td>
<td>18.12 (6-29)</td>
<td>17.06 (5-29)</td>
<td>0.576</td>
</tr>
<tr>
<td>Bleeding score at baseline, n (%)</td>
<td></td>
<td></td>
<td>0.312</td>
</tr>
<tr>
<td>0</td>
<td>10 (38.46)</td>
<td>18 (56.25)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11 (42.31)</td>
<td>9 (28.13)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1 (3.85)</td>
<td>3 (9.37)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4 (15.38)</td>
<td>2 (6.25)</td>
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</tr>
<tr>
<td>4</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

ITP, immune thrombocytopenia; MSCs, mesenchymal stem cells.
Supplementary Table 3. Platelet autoantibodies of patients in the MSC-ITP-C⁺ and MSC-ITP-C⁻ groups.

<table>
<thead>
<tr>
<th></th>
<th>MSC-ITP-C⁺ (n = 26)</th>
<th>MSC-ITP-C⁻ (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP with anti-GPIIb/IIIa (n, %)</td>
<td>7 (26.9)</td>
<td>5 (15.6)</td>
</tr>
<tr>
<td>ITP with anti-GPIb/IX (n, %)</td>
<td>3 (11.5)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>ITP with both antibodies (n, %)</td>
<td>11 (42.3)</td>
<td>9 (28.1)</td>
</tr>
<tr>
<td>ITP without detectable antibodies (n, %)</td>
<td>5 (19.2)</td>
<td>18 (56.3)</td>
</tr>
</tbody>
</table>

ITP, immune thrombocytopenia; MSCs, mesenchymal stem cells.
### Supplementary Table 4. Primer pairs and sequencing primers used for pyrosequencing.

<table>
<thead>
<tr>
<th>Amplicon ID</th>
<th>Primer (sequence 5’ to 3’)</th>
<th>Amplicon length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β_1</td>
<td>F (ATGGAAGGGTAAGGAGTAGTAA)</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>R (CCCACATATACTAAAATTTAAACATTCTT)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S (ATACTAAATTTAAACATTCTTCTA)</td>
<td></td>
</tr>
<tr>
<td>IL-1β_2</td>
<td>F (ATGAAGATTGTTGAAGAGAATTTTAGA)</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>R (ATTTCTCAACCTCCTACTTCTACTTTAA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S (ATTTTAGAGTAGTTTGTTGTG)</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse; S, sequencing.
## Supplementary Table 5. Primer pairs used for RT-PCR.

<table>
<thead>
<tr>
<th>Amplicon ID</th>
<th>Primer (sequence 5’ to 3’)</th>
<th>Amplicon length, bp</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>F (ATGGCCCTAAACAGATGAAGT)</td>
<td>153</td>
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<tr>
<td></td>
<td>R (GCATCTTCTCAGCTTGTC)</td>
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<tr>
<td>CXCL12</td>
<td>F (CTCCTCCTTTCAACCTCAGTGATT)</td>
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<td></td>
<td>R (GAGAAGCAGAAGCAAGATTAAGC)</td>
<td>64</td>
</tr>
<tr>
<td>18srRNA</td>
<td>F (GTAACCCGTGAACCCCAT)</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>R (CCATCCAATCGGTAGTAGCG)</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
Supplementary Figure 1. Complement deposition on the surface of bone marrow MSCs. (A-D) Deposition of complement components C1q, C4d, C3b and C5b-9 on the surface of MSCs from ITP patients and healthy volunteers were detected by indirect immunofluorescence assays.
**Supplementary Figure 2. Intrinsic mRNA alterations in the MSC-ITP-C+ group.** (A-D) Heatmap of the migration-related genes differentially expressed among MSC-ITP-C+, MSC-ITP-C, and MSC-control (MSC-ITP-C+, n = 2; MSC-ITP-C, n = 2; MSC-control, n = 2; FC > 2, p < 0.05, Student t test, Benjamini Hochberg correction). Upregulated genes are shown in red and downregulated genes in green. (E) Canonical pathway analysis of differentially expressed genes of enriched pathways. (F) Disease and functional heat map of the activated or inhibitory relationship between differentially expressed genes and diseases and functions.
Supplementary Figure 3. Flow cytometry analysis of MSC-control, MSC-ITP-C− and MSC-ITP-C+. 
Supplementary Figure 4. IL-1β staining in bone marrow niche. Expression of IL-1β (red) in bone marrow niche assayed by marrow immunohistochemistry of patients from the MSC-ITP-C+ group (A), ITP mice (B), ITP mice treated with 5 mg/kg intraperitoneal injection (C) and ITP mice treated with 20 mg/kg intraperitoneal injection (D). E, endosteum. Scale bar: 100 μm or 50 μm as shown in the figures.
Supplementary Figure 5. CD90 and TUNEL staining in bone marrow niche. Staining of TUNEL on CD90+ MSCs in bone marrow niche assayed by marrow immunohistochemistry of patients from the MSC-ITP-C+ group (A), ITP mice (B), ITP mice treated with 5 mg/kg intraperitoneal injection (C) and ITP mice treated with 20 mg/kg intraperitoneal injection (D). Red: CD90; Green: TUNEL staining; Blue: DAPI. E, endosteum. Scale bar: 100 μm or 50 μm as shown in the figures.
Supplementary Figure 6. No administration effect of ATRA on MSC-ITP-C in vitro. (A) CCK8 proliferative assays of ATRA treated MSC-ITP-C from 3 independent experiments (n = 12). (B) Apoptosis rate of ATRA treated MSC-ITP-C (n = 12). (C, D) Intracellular expression of IL-1β and CXCL12 in untreated and treated MSC-ITP-C by immunofluorescence assays. (E, F) The levels of IL-1β and CXCL12 in MSC-ITP-C cell lysates at day 1 and day 7 with the administration of ATRA from 2 independent experiments (n = 12). (G) Phosphorylation of MyD88, ERK1/2, p38 MAPK and NF-κB in MSC-ITP-C before and after the treatment of ATRA by Western blotting (n = 8).
Supplementary Figure 7. No administration effect of ATRA on MSC-control in vitro. (A) CCK8 proliferative assays of ATRA treated MSC-control from 3 independent experiments (n = 12). (B) Apoptosis rate of ATRA treated MSC-control (n = 12). (C, D) Intracellular expression of IL-1β and CXCL12 in untreated and treated MSC-control by immunofluorescence assays. (E, F) The levels of IL-1β and CXCL12 in MSC-control cell lysates at day 1 and day 7 with the administration of ATRA from 2 independent experiments (n = 12). (G) Phosphorylation of MyD88, ERK1/2, p38 MAPK and NF-κB in MSC-control before and after the treatment of ATRA by Western blotting (n = 8).
Supplementary Figure 8. A murine model of ITP. Splenocytes from CD61 knockout mice immunized with CD61+ platelets were transferred into severe combined immunodeficient (SCID) mouse recipients (C57/B6 background) to induce a murine model of ITP.
Supplementary Figure 9. Numbers of CD34+ cells and CFU-MKS. (A) Numbers of CD34+ cells. (B) CFU-MKS were scored according to their size, which reflects the maturation stage of the progenitors giving rise to each colony. The CFU-MKS were scored as large (50 cells, arising from more primitive MK progenitors), medium (21-49 cells) and small (3-21 cells, derived from more mature MK progenitors) colonies (MSC-ITP-C+, n = 5; MSC-ITP-C-, n = 7; MSC-control, n = 8; One-way ANOVA).
Supplementary Figure 10. Expression of CXCR4 expression on megakaryocyte surface (MSC-ITP-C⁺, n = 5; MSC-ITP-C⁻, n = 7; MSC-control, n = 8; One-way ANOVA).