ARTICLE - Hematopoiesis

Elevated 5-HTR7 deteriorates dysregulated megakaryocytopoiesis in immune thrombocytopenic purpura via upregulating the PKA/Orai1/ERK1/2 pathway

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Supplement materials

Methods

Animals

C57BL/6N mice were obtained from GemPharmatech company. $CD61^{-/-}$ mice were kindly gifted by Professor Yi Wu of the Soochow University. All animal protocols were approved by the Soochow University Animal Care and Use Committee.

Culture of primary MKs from human $CD34^+$ cells in vitro

Human $CD34^+$ cells were obtained from mobilized peripheral blood using an immunomagnetic bead separation system (Easy-Sep, Human CD34 Positive Selection kit, Stem Cell, Canada), with a purity between 90% and 95%. $CD34^+$ cells were cultured in serum-free medium (StemSpan SFEM, Stem Cell) supplemented with, human recombinant(rh) TPO (100 ng/mL, Peprotech, USA), rhSCF (100 ng/mL, Peprotech), IL-3 (10 ng/mL, Peprotech). Cells were seeded at a density of 1×10^5 per well in 24-well plates and cultured for 10-12 days, allowing for differentiation towards MKs. $CD34^+$ cells were cultured in the presence of 10 μL bone marrow supernatant from ITP patients or HCs and 990 μL serum-free medium supplemented with cell factors above.

Flow cytometry analysis and cell sorting

Phenotypic markers of megakaryocytes (MKs) like CD41, CD42 and CD61 were detected by flow cytometry with monoclonal antibody (R&D systems, USA) at indicated time points of culture. For resting platelet detection, pellets containing platelets collected on day 12 of culture were stained with APC-mouse anti-human CD41 and propidium iodide (PI) (R&D systems, USA), resting platelet formation was

determined by the percentage of $CD41^{+}PI^{-}$ particles. For polyploidy investigation, MKs cultured for 10 days were gathered and examined by flow cytometry after being stained with anti-CD41 antibody and PI.

5-HTR7 was detected by flow cytometry as follows: Suspended cells were incubated with mouse anti-human 5-HTR7 primary antibody (R&D, USA) for 30 min，then they were washed and incubated with FITC-conjugated goat anti-mouse secondary antibody (Beyotime, China) for another 30min. The mean fluorescence intensity was measured.

For the cell sorting, cells were incubated with PE anti-CD41 (Biolegend, USA) for 30 min and washed twice with phosphate-buffered saline (PBS) to select viable cells by a fluorescence-activated cell sorter Aria II flow cytometer (Beckman coulter, USA).

Adhesion and migration Assay

Primary MKs were purified using immunomagnetic sorting with an anti-CD61 antibody and subsequently pre-stained with DilC18(3) (Beyotime, China), a red fluorescent cell membrane probe, for 10 min in dark. The cells were then seeded into a 24-well culture plate pre-coated with 100 μg/mL fibrinogen (Sigma-Aldrich, USA) at a density of 1×10^5 cells/well in serum-free medium. After incubation at 37°C for 3 h, the medium was removed, and the cells were washed with PBS. Adherent cells were visualized using an inverted fluorescence microscope (Olympus, Japan).

To assess the migratory capacity of MKs, 24-transwell chambers equipped with 8 μm pore size polycarbonate membranes (Corning, USA) were coated with 20 μg/mL fibrinogen overnight at 4°C. Purified primary MKs (2×10^5) from different groups were

placed into the upper chamber in serum-free medium. The lower chamber contained serum-free medium supplemented with 100 ng/mL stromal cell-derived factor 1α (PeproTech, Canada). After 5h, non-migrated cells were removed and the membrane was fixed with 4% PFA. The migrated cells were then stained with 1% crystal violet (Beyotime, China) for counting.

Immunofluorescence staining

Mouse femurs were fixed with 4% paraformaldehyde, decalcified, embedded in paraffin, and sectioned. Then the tissue sections were subjected to immunofluorescence staining using the tyramide signal amplification (TSA) technique. These sections were incubated overnight at 4°C with antibodies to CD105 (Abcam, UK) and CD41 (Abcam, UK). Subsequently, they were incubated with appropriate secondary antibodies, washed, and then stained with DAPI.

Western Blot

Primary MKs from various groups were collected and lysed on ice using RIPA Lysis Buffer containing protease and phosphatase inhibitors (1×) (Beyotime, China). The BCA Protein Assay Kit (Beyotime, China) was utilized to determine protein concentrations. Proteins were separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking, the membrane was incubated overnight at 4 ℃ with primary antibodies to ERK1/2, Akt, MEK (Cell Signaling Technology, USA), Orai1 (Abcam, UK), and Stim1 (Proteintech, USA). The membranes were then thoroughly washed with Tris-buffered saline containing 0.1% Tween 20. Next, the blots

were incubated with species-specific horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The reactive bands were then detected and captured. Western blot band intensities were quantified using ImageJ software, with normalization to β-Actin, MEK, ERK1/2 or AKT (Cell Signaling Technology, USA) for each group.

Quantitative real-time PCR

Total RNA from murine MKs was extracted with TRIzol reagent (Invitrogen, USA). The RNA was then converted into cDNA using the 5× All-In-One RT Master Mix (Abcam, UK). Quantitative real-time PCR (qRT-PCR) was performed for 5-HTR7 using a real-time PCR instrument (Applied Biosystems, USA). Each reaction was replicated three times, and the mouse Actin mRNA was used to normalize the results. Real-time PCR data was analyzed using the $2^{-\triangle\triangle CT}$ method. The primers were: 5-HTR7 (mouse): Forward (5' to 3') CTATGGCAGAGTCGAGAAA; reverse (5' to 3') CAATCAGGTAGTTGGAGGG; Actin (mouse): Forward (5' to 3') GCTCCTAGCACCATGAAGAT; reverse (5' to 3'): GTGTAAAACGCAGCTCAGTA.

For qRT-PCR of bone marrow mononuclear cells (BMMNCs) from healthy controls (HCs) or ITP patients, the protocol was the same as the above. The primers were: 5-HTR7 (human): Forward (5' to 3') CGAAGATGATTCTCTCCGTCTG; reverse (5' to 3') GCGGTAGAGTAAATCGTATAGCC. GAPDH (human) Forward (5' to 3') CTCTGCTCCTCCTGTTCGAC; reverse TTAAAAGCAGCCCTGGTGAC.

Intracellular calcium measurement of MKs

Prepared cells were firstly labeled with anti-human CD41 for 30 minutes and washed as mentioned above. Then the cells were incubated with Fluo-4/AM at 5 μM (Beyotime, China) for 30 min at 37 °C and washed twice. Flow cytometry was performed using a 488 nm excitation laser, and fluorescence emission was collected through a 530/30 nm bandpass filter. The mean fluorescence intensity (MFI) of Fluo-4 AM-loaded $CD41^+$ cells was recorded as F_{sample}, while the MFI of unstained $CD41^+$ cells (processed identically without Fluo-4 AM) served as the background signal (F_0). The normalized fluorescence intensity ($\triangle F=F_{sample} - F_0$) was calculated to quantify the relative resting calcium concentration in MKs.

Store-operated calcium entry (SOCE) measurement

100 μg/mL poly-L-lysine was coated in Nuck lab-Tek chamber slide system (Thermo Fisher Scientific, USA) at 4℃ for 24 h. Human purified $CD34^+$ cells cultured to day 8 were seeded at 1×10^6/mL, treated with DMSO or SB269970A (4 μM). After 24 h, the medium was discarded and cells were stained with Rhod-2 (3-5 μM) for 45 min at 37℃ in calcium free HBSS. After washing, the cells were re-suspended with 200 μL calcium-free HBSS under a confocal microscope (x-y-t mode, one record per second). When the baseline was stabilized, the basal fluorescence was recorded as F_b, then thapsigargin was added to final concentration at 2 μM. 60 seconds later, $CaCl_2$ was added (2 μM), and fluorescence was recorded as F_t. The images were recorded and fluorescence variation (F_t/F_b) was quantified with the software of Leica Application Suite X 3.4.2 [1].

Single-cell RNA capture, sequencing, and processing

The single-cell lysates of thymuses from primary cells and bone marrow mononuclear cells (BMMNCs) were loaded per channel onto a ChromiumTM controller (10x Genomics) after lysis of red blood cells, to generate gel-bead-in-emulsions (GEMs). High-throughput sequencing of the library was performed using the paired-end sequencing model of the Illumina sequencing platform at LC Bio (Zhejiang, China). Data were obtained from CellRanger Version 4.0.0 against Mus_musculus. GRCm38.96 served as the reference genome and then normalized using the Seurat "LogNormalize" function. Principal component analysis (PCA) dimensionality reduction was performed using "RunPCA" after quality control. A resolution parameter of 0.4 was used for the samples. Differential gene expression (DEG) was filtered using a maximum adjusted P-value of 0.05 for Gene Ontology analysis. 68 scRNA-seq plots were generated using ggplot2 (v3.3.5), and volcano maps were generated using the EnhancedVolcano (v 1.10.0) package in R 4.0.0. Violin maps and heat maps were generated using the "Vlnplot" and "Doheatmap" functions in Seurat [2].

Reference

1. Luo R, Gourrierec PL, Antigny F, Bedouet K, Domenichini S, Gomez AM, et al. STIM2 variants regulate Orai1/TRPC1/TRPC4-mediated store-operated Ca(2+) entry and mitochondrial Ca (2+) homeostasis in cardiomyocytes. Cell Calcium. 2024; 119: 102871

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Legends

Supplemental Figure 1. Transcriptional profiling of megakaryocyte bone marrow mononuclear cells (BMMNCs) from the healthy control (HC) and ITP patient. Gene transcription in megakaryocyte precursor progenitors (MKPs) from HCs and ITP patients was profiled with single-cell RNA sequencing (scRNA-seq). (A) Quality control and (B) batch effect correction of the samples. (C) Cell cluster based on differential genes. (D) Dimensional reduction using uniform manifold approximation and projection (UMAP) and (E) t-distributed stochastic neighbor embedding (t-SNE). (F) Differential genes between the HC and the ITP patient (n=1); (G) Relative expression of 5-HTR7 mRNA in BMMNCs between HCs and ITP patients.

BMMNCs derived from a HC and a newly diagnosed ITP patient were analyzed by scRNA-seq. After quality control and batch effect correction (Figure S1A-B), single-cell profiles were included in the downstream analyses (ITP, n=12211; HC, n=12778). Dimensional reduction using uniform manifold approximation and projection (UMAP) and t-distributed stochastic neighbor embedding (t-SNE) showed the effective integration of the datasets from different samples (Figure S1D-E). We manually annotated the cell clusters into 14 different cell types with distinct gene expression patterns (Figure S1C). These populations included HSCs, granulocyte-macrophage progenitors (GMP), granulocytes, dendritic cells (DC), B cells, $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells (NK), megakaryocyte-erythroid progenitors (MEP), megakaryocyte progenitors (MKP), erythrocytes, MKs and two unknown clusters (could not be identified). As for differential gene expression, we found that the expression of 5-HTR7 was higher in MKPs from ITP than in cells from HC (Figure S1F). The relative expression of 5-HTR7 of BMMNCs was significantly higher in ITP patients than that in HCs (Figure S1G)

Supplemental Figure 2. Single-cell RNA sequencing of human primary MKs cultured

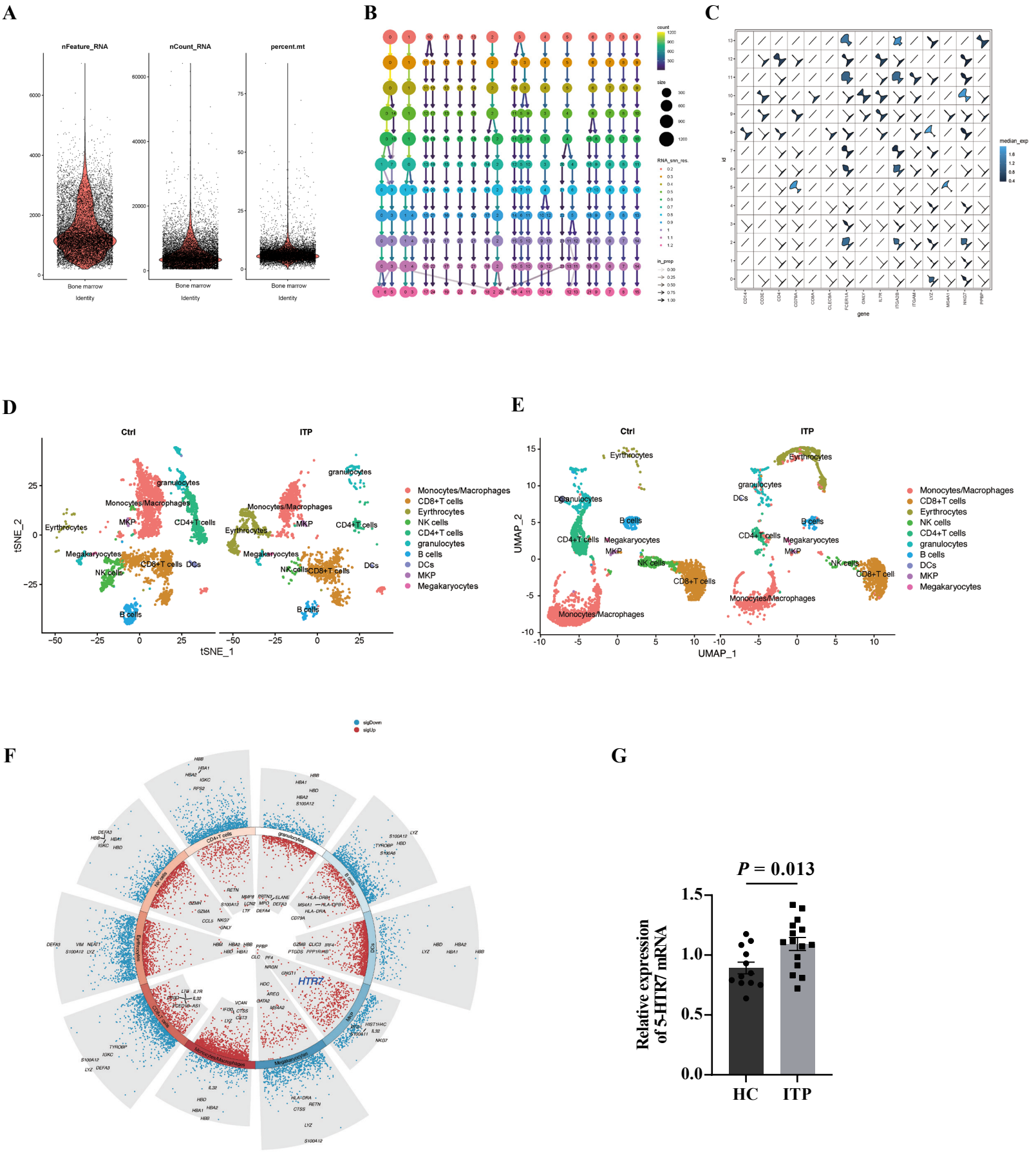
with ITP bone marrow supernatant (BMS), treated with DMSO or SB269970A. (A) Quality control and batch effect correction of the samples. (B) Cell cluster based on differential genes. Dimensional reduction using (C) t-SNE and (D) UMAP.

Supplemental Figure 3. Maturation and resting platelet formation of primary MKs treated with agonist and antagonist of 5-HTR7. (A) Expression of CD41 and CD42 of MKs cultured ex vivo for 10 days. (B) Statistical analysis of resting platelet formation. Data were mean ± SEM. ns, not significant. *, $P<0.05$. ***, $P<0.001$. ****, $P<0.0001$.

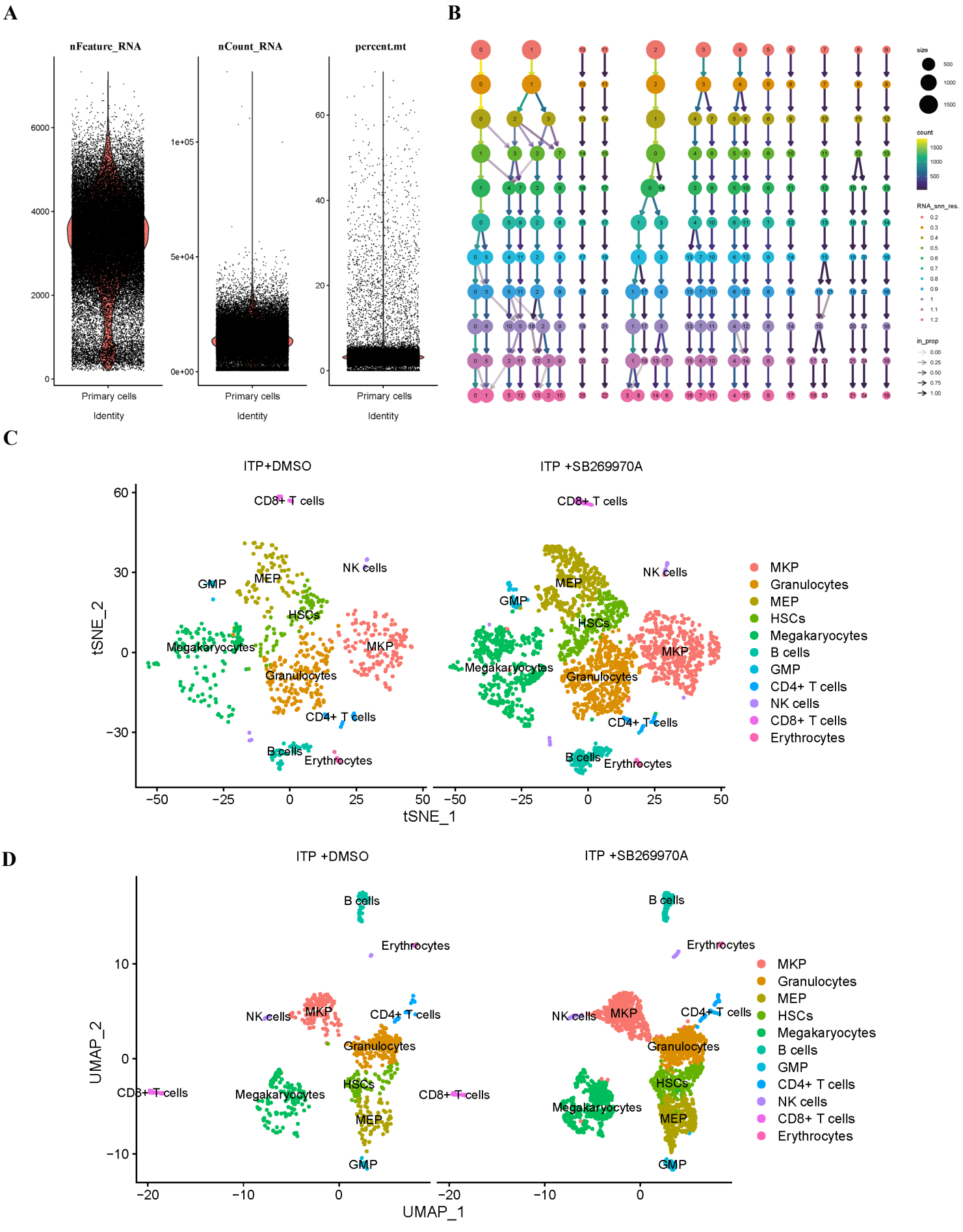
Supplemental Figure 4. Maturation and resting platelet formation of primary MKs cultured with ITP BMS that were pretreated with agonist or antagonist of 5-HTR2. (A) Expression of CD41 and CD42 of MKs cultured ex vivo for 10 days. (B) Quantification of resting platelet formation. Data represent mean ± SEM, ns, not significant.

Supplemental Figure 5. Analysis of the pathways for primary MKs cultured with BMS from HC or ITP. (A) Representative Western blot images of phosphorylated ERK and AKT. Quantification of the phosphorylation of (B) ERK (normalized to ERK) and (C) AKT (normalized to AKT) pathways.

Supplemental Figure 1



Supplemental Figure 2



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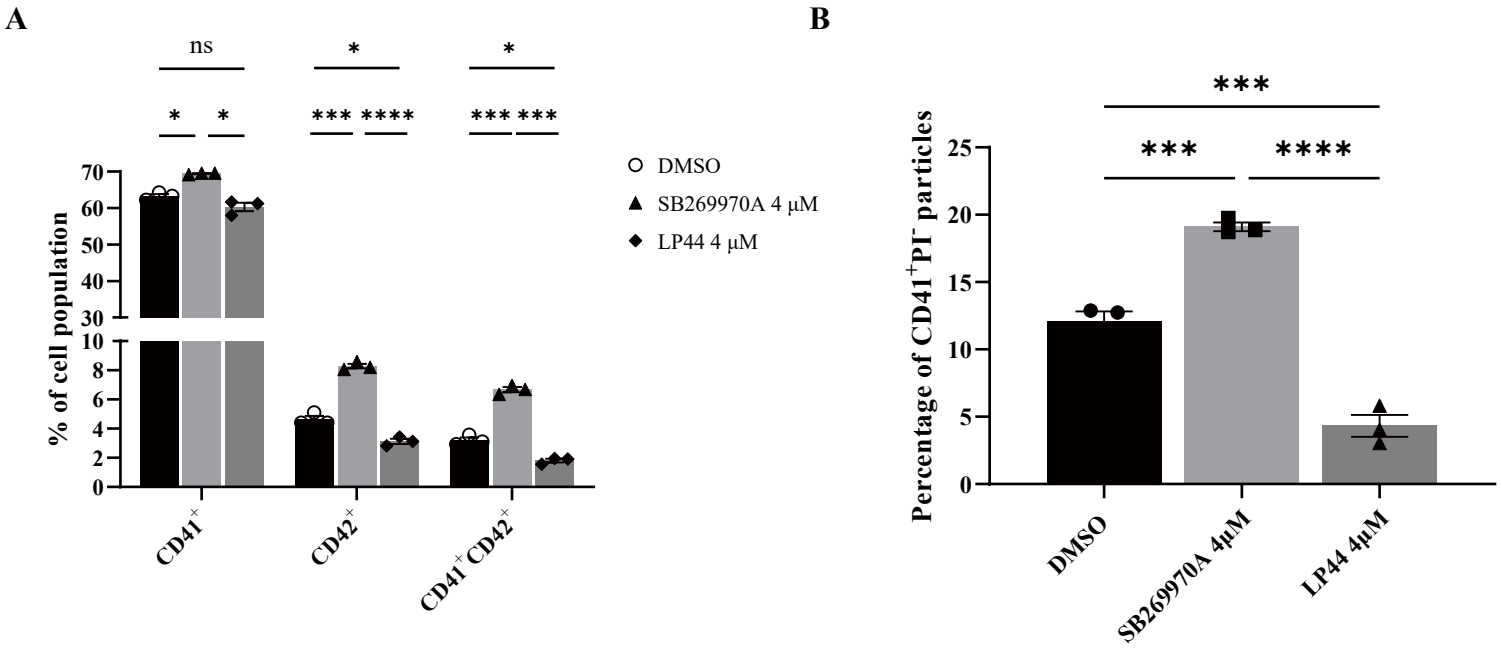
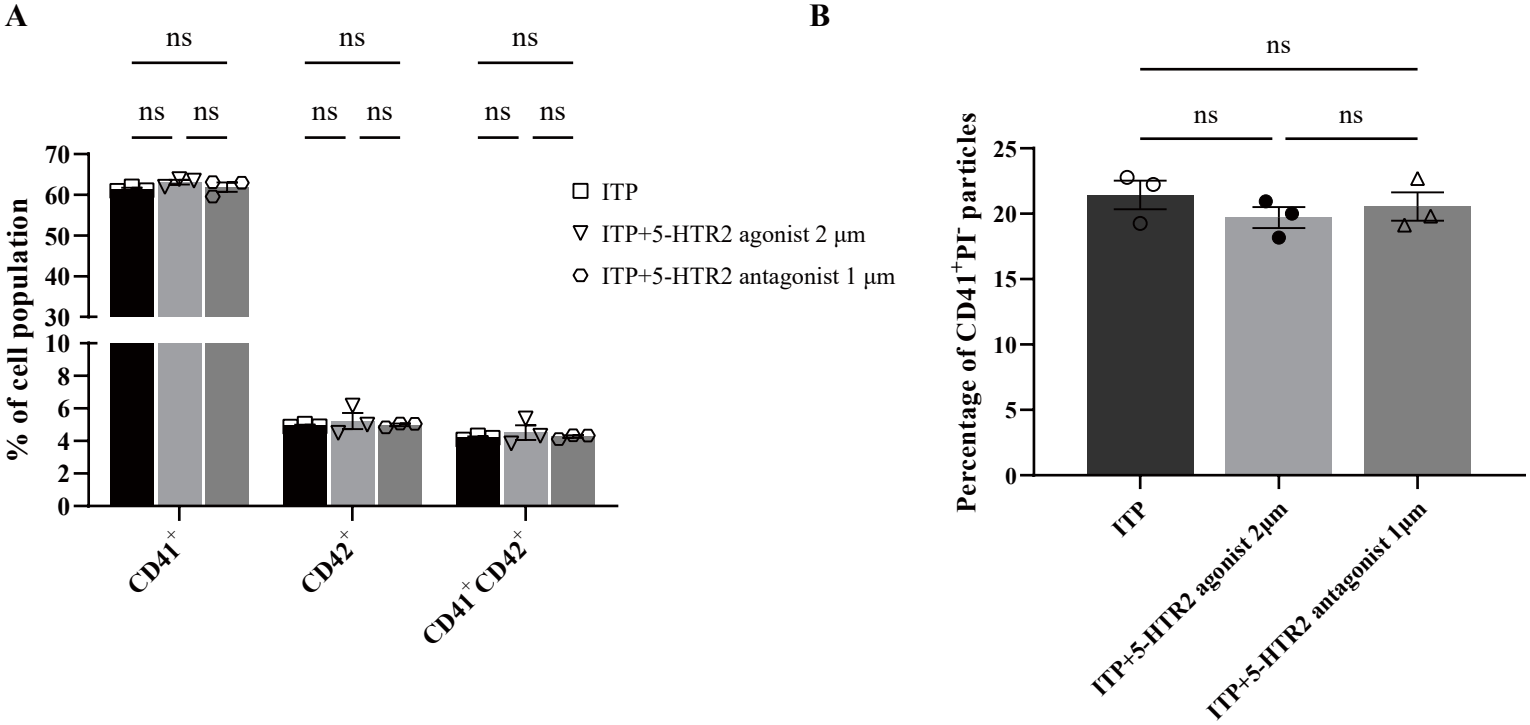


Supplemental Figure 4



Supplemental Figure 5