ARTICLE - Platelet Biology & its Disorders

CSF1R modulates megakaryopoiesis by targeting RUNX1 in immune thrombocytopenia

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Supplementary Methods

Patient Samples

Newly diagnosed ITP was defined as patients within 3 months of initial diagnosis who had recurrent thrombocytopenia confirmed on two or more occasions. Blood films were examined to exclude pseudothrombocytopenia and to assess cell morphology. Splenomegaly is uncommon, and bone marrow examination in ITP typically shows normal or increased megakaryocytes with impaired maturation. Patients with secondary thrombocytopenia, including autoimmune, hematological, congenital, gestational, drug-induced, infection-associated, or other bone marrow disorders, were excluded[1, 2].

ScRNA-seq

BM samples were obtained via iliac crest aspiration. Erythrocytes were lysed using a hemolytic solution (Solarbio, China), and the remaining cells were washed and resuspended in phosphate-buffered saline (PBS; Solarbio, China) supplemented with 0.04% bovine serum albumin (BSA; Sigma-Aldrich, Japan) to maintain cell viability. Cell viability and concentration were assessed by acridine orange/propidium iodide (AO/PI) staining, and the final cell concentration was adjusted to 1×10^6 cells/mL. Single-cell suspensions were then processed for library preparation following the manufacturer's protocol using the Chromium Next GEM Chip G Single Cell Kit (10× Genomics, Cat# 1000120) and the Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v3.1 (10× Genomics, Cat# 1000121). The process included single-cell capture and library preparation, followed by a quality control assessment of library concentration and fragment size. Qualified libraries were sequenced on the Illumina NovaSeq 6000 platform using a PE150 sequencing strategy to achieve the desired sequencing depth and resolution.

The computational pipeline included demultiplexing, alignment (reference genome and software versions), stringent cell/gene quality-control thresholds (nFeature/nCount and mitochondrial cut-offs), doublet detection, ambient RNA removal, normalization (LogNormalize with log1p or SCTransform), variable-feature selection, batch correction (if applicable), dimensionality reduction, clustering, and marker detection with multiple-testing correction. To facilitate manual cluster annotation, Table S1 provides a comprehensive signature-gene reference listing marker genes for HSC, MEP, MKP, MK, and other clusters, together with their normalized

expression values (cluster-average log-normalized or SCT-normalized) and the percentage of cells expressing each gene in each cluster.

Flow Cytometry

Flow cytometry were conducted using a NovoCyte flow cytometer (ACEA Biosciences, USA). For the detection of CSF1R on human BM-derived MKs, BM cells were labeled with a BV510-conjugated lineage antibody cocktail targeting CD3, CD14, CD16, CD19, CD20, CD56 (BioLegend, USA), an APC-conjugated CD41a antibody (BD Biosciences, USA), and a FITC-conjugated CSF1R antibody (BioLegend, USA). MKs were defined as $Lineage^{-}CD41^{+}$ cells, with the gating strategy shown in Figure S1A.

To assess MK differentiation in vitro, cultured cells were harvested on days 7 and 10 and stained with APC-conjugated CD41a antibody, FITC-conjugated CD42b antibody, and PE-conjugated CD61 antibodies (all from BD Biosciences, USA).

For ploidy analysis, cells were harvested on day 12, stained with APC-conjugated CD41a antibody (BD Biosciences, USA), fixed in cold 70% ethanol, and permeabilized overnight at 4°C. Cells were then incubated with RNase buffer (Abcam, USA) and stained with propidium iodide (BioLegend, USA) for DNA content analysis. The gating strategy is shown in Figure S1B.

Platelets derived from PBMC-induced MKs were collected on day 12 and were labeled with APC-conjugated CD41a antibody (BD Biosciences, USA) to evaluate platelet production.

ELISA

To quantify cytokine levels, the concentrations of M-CSF and IL-34 in BM and PB serum samples were measured using ELISA kits specific for M-CSF and IL-34 (Aimeng Youning, China), following the manufacturer's instructions.

Cytokine dynamics after ITP plasma addition

$CD34^{+}$ HSCs were cultured with ITP plasma for the first 4 days, and the cell culture supernatants were collected at 1, 24, 48, and 72 hours after plasma addition. Cytokine levels were measured using legendplex™ Multi-Analyte Flow Assay Kit (BioLegend, USA), following the manufacturer's instructions.

CSF1R inhibitor treatment

To optimize in vitro dosing of the CSF1R inhibitor, dose-response testing was performed at 0.5, 2, and 8 μM. A concentration of 2 μM was selected for subsequent experiments, as it effectively enhanced megakaryopoiesis while maintaining cell viability. To evaluate the inhibitor's effect on CSF1R signaling, cultured cells from ITP and ITP + CSF1R inhibitor groups were collected on day 4 for Western blot analysis. Additionally, RAW264.7 mouse macrophage cells were treated with the inhibitor and analyzed by Western blot to determine whether downstream signaling pathways were suppressed or activated.

Immunofluorescence Assay

To evaluate proplatelet formation, 24-well plates (Corning, USA) were pre-coated with fibrinogen (100 μg/mL, Sigma-Aldrich, Japan) and incubated overnight at 4°C. Cultured MKs were plated and incubated for 24 hours at 37°C. Cells were then fixed and stained with anti-human CD41 antibody (Abcam, China), anti-human β-tubulin antibody(Abcam, China), phalloidin (Abcam, China), and DAPI (Beyotime, China). Cellular morphology and proplatelet structures were observed by confocal microscopy (Leica BC SP8, Germany).

Meg01 cell culture

Meg01 cells (Bena Biotech, China) were cultured in RPMI 1640 medium (Gibco, China) supplemented with 10% fetal bovine serum (FBS; SenBeiJia, China). To investigate the role of CSF1R in megakaryopoiesis, Meg01 cells were transduced with lentiviruses (Genechem, China) for CSF1R knockdown or overexpression. Transduced cells were selected based on fluorescence tagging and puromycin resistance. Successful gene modification was confirmed by qPCR analysis.

qPCR

Total RNA was extracted from cultured MKs using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA (1000 ng) was reverse-transcribed into cDNA using the 5× All-In-One RT MasterMix (Abmgood, USA). qPCR was performed using 2× SYBR Green qPCR Master Mix (Low ROX) (Bimake, China). Primer sequences for target genes are listed in Table S7 and all primers were sourced from Genewiz (China). Gene expression levels were quantified using comparative Ct method and normalized to the housekeeping gene GAPDH.

Western Blot

Cells were lysed on ice using RIPA lysis buffer containing protease and phosphatase inhibitors (Beyotime, China). Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Equal amounts of protein were subjected to SDS-PAGE and transferred to 0.2 μm PVDF membranes (Merck Millipore, USA). Membranes were blocked with 5% BSA or nonfat milk for 1 hour at room temperature, followed by incubation with primary antibodies overnight at 4°C. The following primary antibodies were used: CSF1R (Abcam, Canada), p-AKT (CST, USA), AKT (CST, USA), RUNX1/AML1 (Abcam, Canada), GAPDH (CST, USA), β-Actin (CST, USA). After washing, membranes were incubated with secondary antibodies, and signals were detected using Enhanced Chemiluminescence kit (Beyotime, China).

Optimization of CSF1R inhibitor dosing in vivo

In vivo dose optimization of the CSF1R inhibitor was performed using a passive ITP mouse model[3]. C57BL/6N WT mice received tail vein injections of anti-CD42 antibody (R300, 0.1 $\mu g \cdot g^{-1}$, Emfret, Germany) twice, 24 h apart (day 0-1) to induce thrombocytopenia, while the control group received PBS. Following model establishment, mice were randomized to receive oral CSF1R inhibitor (0.1, 0.5, or 1 $mg \cdot kg^{-1}$) or CMC vehicle once daily for 3 consecutive days (day 1-3). Peripheral blood counts were monitored to assess treatment effects. The 0.5 $mg \cdot kg^{-1}$ dose was selected as the minimal effective and well-tolerated dose, whereas 1 $mg \cdot kg^{-1}$ caused mortality in two mice within 24 h, indicating potential toxicity at higher doses.

CSF1R Inhibition on platelet clearance

To determine whether CSF1R inhibition influences macrophage-mediated platelet phagocytosis, platelets from C57BL/6N WT mice were labeled via tail vein injection with DyLight488-conjugated anti-GPIbβ IgG (1 $\mu g \cdot g^{-1}$, emfret, Germany). Mice were then randomized to receive oral CSF1R inhibitor (0.1, 0.5, or 1 $mg \cdot kg^{-1}$) or CMC vehicle once daily for 3 consecutive days. Complete blood counts were measured at 0, 24, 48, and 72 hours after the first administration to monitor platelet survival.

Supplementary Tables

Table S1 Specifically expressed genes in hematopoietic cell clusters (details in Excel file)

Table S2 Clinical characteristics of 26 newly diagnosed ITP patients

ID	Age (year)	Sex (F/M)	Source	PLT ($\times 10^9$/L)
1	56	M	BM	8
2	60	M	BM	32
3	20	M	BM	9
4	50	M	BM	9
5	71	F	BM	10
6	56	F	BM	11
7	58	M	BM	21
8	47	F	BM	37
9	35	F	BM	2
10	34	F	BM	17
11	37	F	BM	37
12	58	F	BM	36
13	29	F	BM	40
14	14	M	BM	8
15	45	F	BM	1
16	62	F	BM	24
17	64	F	BM	11
18	60	M	BM	12
19	34	M	BM	6
20	70	F	BM	18
21	33	F	BM	53
22	74	M	BM	28
23	46	F	BM	10
24	21	F	BM	13
25	45	M	BM	2
26	28	F	BM	35

M: male; F: female; PB: peripheral blood; BM, bone marrow; PLT: platelet.

Table S3 Clinical characteristics of 26 newly diagnosed ITP patients

ID	Age (year)	Sex (F/M)	Source	PLT ($\times 10^9$/L)
1	56	M	BM	8
2	60	M	BM	32
3	20	M	BM	9
4	50	M	BM	9
5	71	F	BM	10
6	56	F	BM	11
7	58	M	BM	21
8	47	F	BM	37
9	53	F	BM	39
10	56	M	BM	2
11	50	F	BM	18
12	55	M	BM	25
13	18	F	BM	21
14	63	F	BM	4
15	42	M	BM	34
16	72	F	BM	32
17	43	F	BM	4
18	58	F	BM	36
19	14	M	BM	8
20	62	F	BM	24
21	45	F	BM	1
22	34	M	BM	26
23	60	F	BM	5
24	60	M	BM	12
25	64	F	BM	11
26	56	M	BM	2

M: male; F: female; PB: peripheral blood; BM, bone marrow; PLT: platelet.

Table S4. Clinical characteristics of 30 newly diagnosed ITP patients

ID	Age (year)	Sex (F/M)	Source	PLT ($\times 10^9$/L)
1	52	M	PB	45
2	27	F	PB	45
3	49	M	PB	11
4	48	F	PB	10
5	12	F	PB	29
6	34	F	PB	44
7	18	F	PB	48
8	32	F	PB	36
9	71	M	PB	3
10	25	F	PB	33
11	17	M	PB	12
12	17	M	PB	6
13	15	M	PB	27
14	71	F	PB	12
15	50	F	PB	48
16	10	M	PB	23
17	50	F	PB	34
18	34	F	PB	31
19	72	F	PB	33
20	54	F	PB	2
21	82	M	PB	48
22	69	M	PB	26
23	77	F	PB	50
24	53	F	PB	5
25	41	F	PB	30
26	38	M	PB	6
27	52	M	PB	7
28	48	F	PB	16

29	60	M	PB	32
30	49	F	PB	3

M: male; F: female; PB: peripheral blood; BM, bone marrow; PLT: platelet.

Table S5. Clinical characteristics of the newly diagnosed ITP patients

ID	Age (year)	Sex (F/M)	PLT ($\times 10^9$/L)	platelet-associated antibody (%)	Anti-GP antibodies				Application
					anti-GPIb/IX	anti-GPIb	anti-GPIIb	anti-GPIIIa	
1	45	F	1	56.3	-	-	-	-	scRNA-seq
2	50	F	18	21.6	-	-	-	-	validation
3	60	F	5	18.6	-	+	-	+	validation
4	62	F	24	46.4	-	-	-	+	validation
5	14	M	8	26.5	-	-	-	-	validation
6	42	M	34	38.0	-	-	-	-	validation

M: male; F: female; PLT: platelet; anti-GP, anti-glycoprotein.

Table S6. Clinical characteristics of the newly diagnosed ITP patients

ID	Age (year)	Sex (F/M)	PLT ($\times 10^9$/L)	platelet-associated antibody (%)	Anti-GP antibodies			
					anti-GPIb/IX	anti-GPIb	anti-GPIIb	anti-GPIIIa
1	15	M	15	45.1	+	+	-	+
2	43	F	4	26.3	-	-	+	-
3	48	F	8	35.4	-	+	+	+
4	18	F	21	41.8	-	+	-	+
5	64	F	11	28.7	-	-	-	-
6	60	M	12	46.7	-	-	-	-
7	34	M	26	41.7	-	-	-	-
8	63	F	4	35.8	-	-	-	-

M: male; F: female; PLT: platelet; anti-GP, anti-glycoprotein.

Table S7. The primer sequences for each gene

Gene	Forward primer	Reverse primer
CD41a	TGGAACGTCCTAGAAAAGACTGA	CTTCACAGTAACGCTTGTCCC
CD42b	CTGTGAGGTCTCCAAAGTGGC	GTGAGGCGAGTGTAAGGCATC
CD61	AGTAACCTGCGGATTGGCTTC	GTCACCTGGTCAGTTAGCGT
CSF1R	TCC AAA ACA CGG GGA CCT ATC	CGG GCA GGG TCT TTG ACA TA
RUNX1	CCA CCT ACC ACA GAG CCA TCA A	TTC ACT GAG CCT CGG AAA AG
GAPDH	GTC TCC TGA CTT CAA CAG CG	ACC ACC CTG TTG CTA GCC AA

Supplementary Figure Legends

Figure S1. Gating strategy for MKs. (A) Gating strategy used to identify Lineage$^-$CD41$^+$ MKs from BM cells. (B) Gating strategy for ploidy analysis of MKs. MK, megakaryocyte; BM, bone marrow.

Figure S2. Levels of M-CSF and IL-34 were in PB supernatants measured by ELISA in ITP patients (n = 30) and HDs (n = 20). Data are shown as mean ± SD. ns, not significant. M-CSF, macrophage colony-stimulating factor; IL-34, interleukin-34, PB, peripheral blood; ITP, immune thrombocytopenia; HDs, healthy donors.

Figure S3. In vitro MK differentiation and cytokine dynamics after ITP plasma treatment. (A) Schematic representation of the in vitro MK differentiation assay. (B) Cytokine dynamics in CD34$^+$ HSC cultures treated with ITP plasma during the first 4 days of differentiation. Cytokine levels were measured at 1, 24, 48, and 72 hours after plasma addition (n = 3). Data are shown as mean ± SD. MK, megakaryocyte; ITP, immune thrombocytopenia.

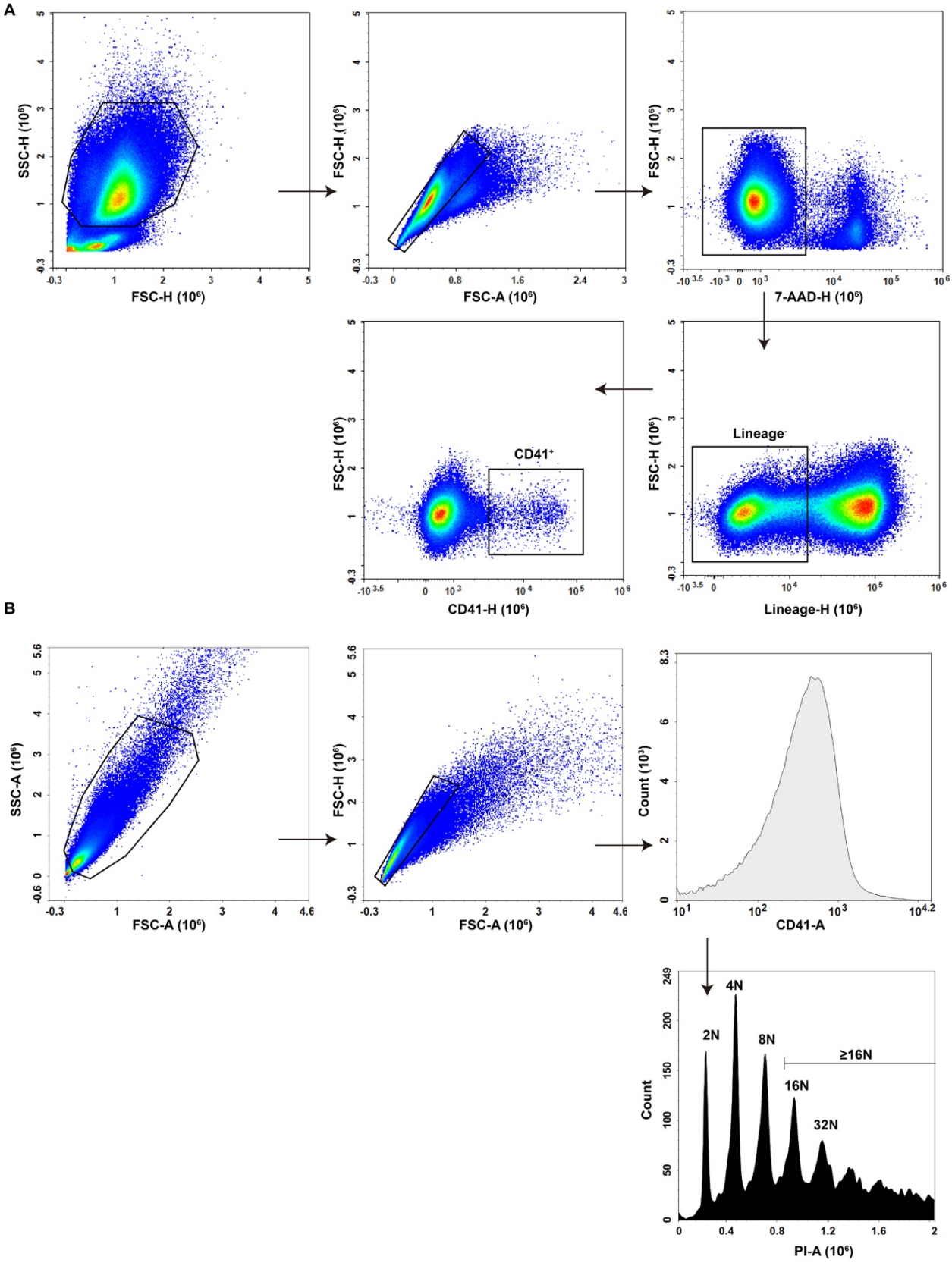
Figure S4. In vitro CSF1R inhibitor dosing and signaling assessment. (A) Dose-response testing of the CSF1R inhibitor (0.5, 2, and 8 μM) to determine the optimal concentration (n = 3). (B) Western blot analysis of CSF1R in ITP and ITP + CSF1R inhibitor-treated cells on day 4 (n = 3). (C) Western blot of RAW264.7 cells showing suppression of downstream signaling (P-AKT) without activation after CSF1R inhibitor treatment (n = 3). (D) qPCR analysis of mRNA levels of CD41a, CD42b, and CD61 in cultured MKs (n = 3). Data are shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ITP, immune thrombocytopenia; MK, megakaryocyte.

Figure S5. CSF1R inhibition enhances megakaryocyte differentiation in vitro. (A-B) The proportion of MKs expressing CD41a, CD42b, or CD61, derived from CD34$^+$ HSCs cultured with pooled plasma from anti-glycoprotein-positive or anti-glycoprotein-negative ITP patients for 7 or 10 days, with or without CSF1R inhibitor (n = 3). Data are shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns, not significant. HSC, hematopoietic stem cell; MK, megakaryocyte; ITP, immune thrombocytopenia.

Figure S6. Effect of CSF1R ligands (M-CSF and IL-34) on megakaryopoiesis in ITP (n = 3). Data are shown as mean ± SD. CSF1R, colony-stimulating factor 1 receptor; ITP, immune thrombocytopenia; M-CSF, macrophage colony-stimulating factor; IL-34, interleukin-34.

Figure S7. In vivo CSF1R inhibitor dosing and its effect on platelet clearance. (A) Dose-response testing of the CSF1R inhibitor (0.1, 0.5, 1 $mg \cdot kg^{-1}$) in a passive ITP mouse model to determine the optimal concentration. Administration of 1 $mg \cdot kg^{-1}$ caused mortality in two mice within 24 hours (data not quantified) (n = 3). (B) Platelet clearance assessment using DyLight488-conjugated anti-GPIbβ IgG labeled platelets (n = 3). Data are shown as mean ± SD. $*P < 0.05$.

Figure S1.


A
SSC-H (10⁶)
FSC-H (10⁶)
FSC-H (10⁶)
FSC-A (10⁶)
FSC-H (10⁶)
7-AAD-H (10⁶)
FSC-H (10⁶)
CD41⁺
CD41-H (10⁶)
FSC-H (10⁶)
Lineage⁻
Lineage-H (10⁶)
B
SSC-A (10⁶)
FSC-A (10⁶)
FSC-H (10⁶)
FSC-A (10⁶)
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Count
2N
4N
8N
16N
32N
≥16N
PI-A (10⁶)

Figure S2.

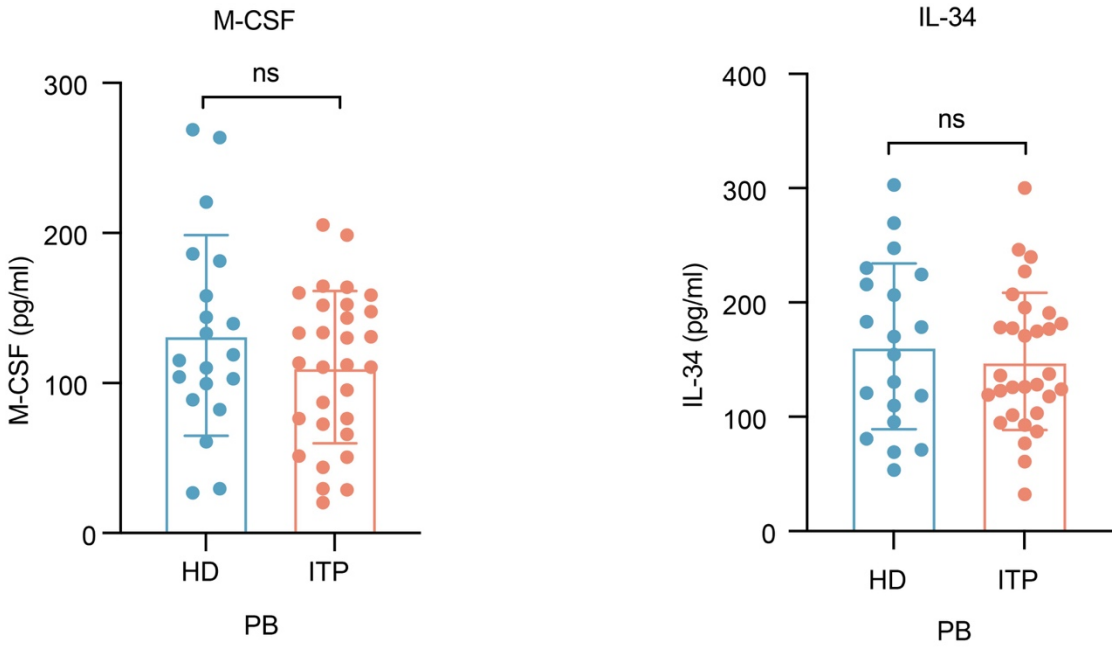

M-CSF
ns
300
200
100
0
M-CSF (pg/ml)
HD
ITP
PB
IL-34
ns
400
300
200
100
0
IL-34 (pg/ml)
HD
ITP
PB

Figure S3.

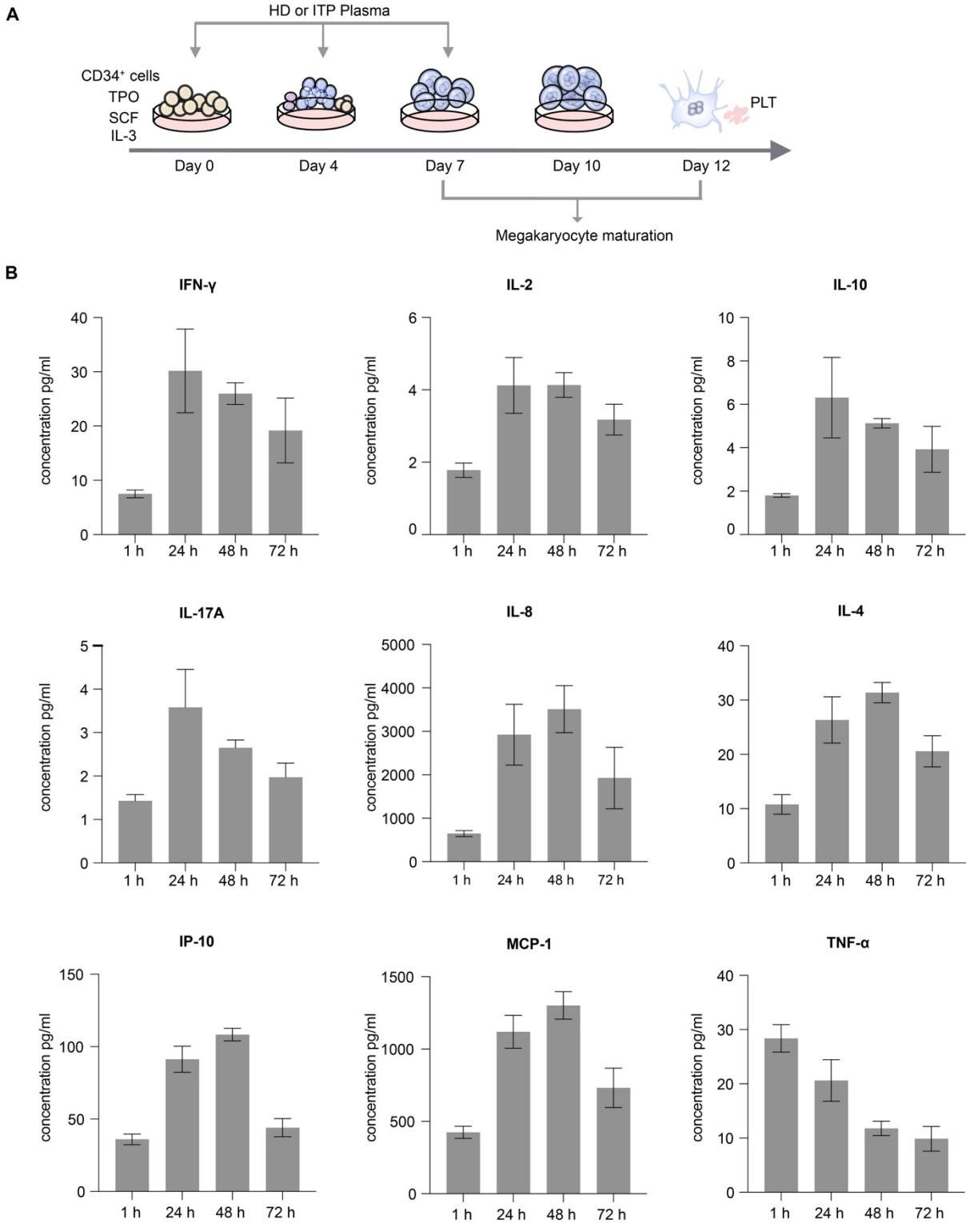

A
HD or ITP Plasma
CD34⁺ cells
TPO
SCF
IL-3
PLT
Day 0
Day 4
Day 7
Day 10
Day 12
Megakaryocyte maturation
B
IFN-γ
IL-2
IL-10
IL-17A
IL-8
IL-4
IP-10
MCP-1
TNF-α
concentration pg/ml
1 h
24 h
48 h
72 h

Figure S4.

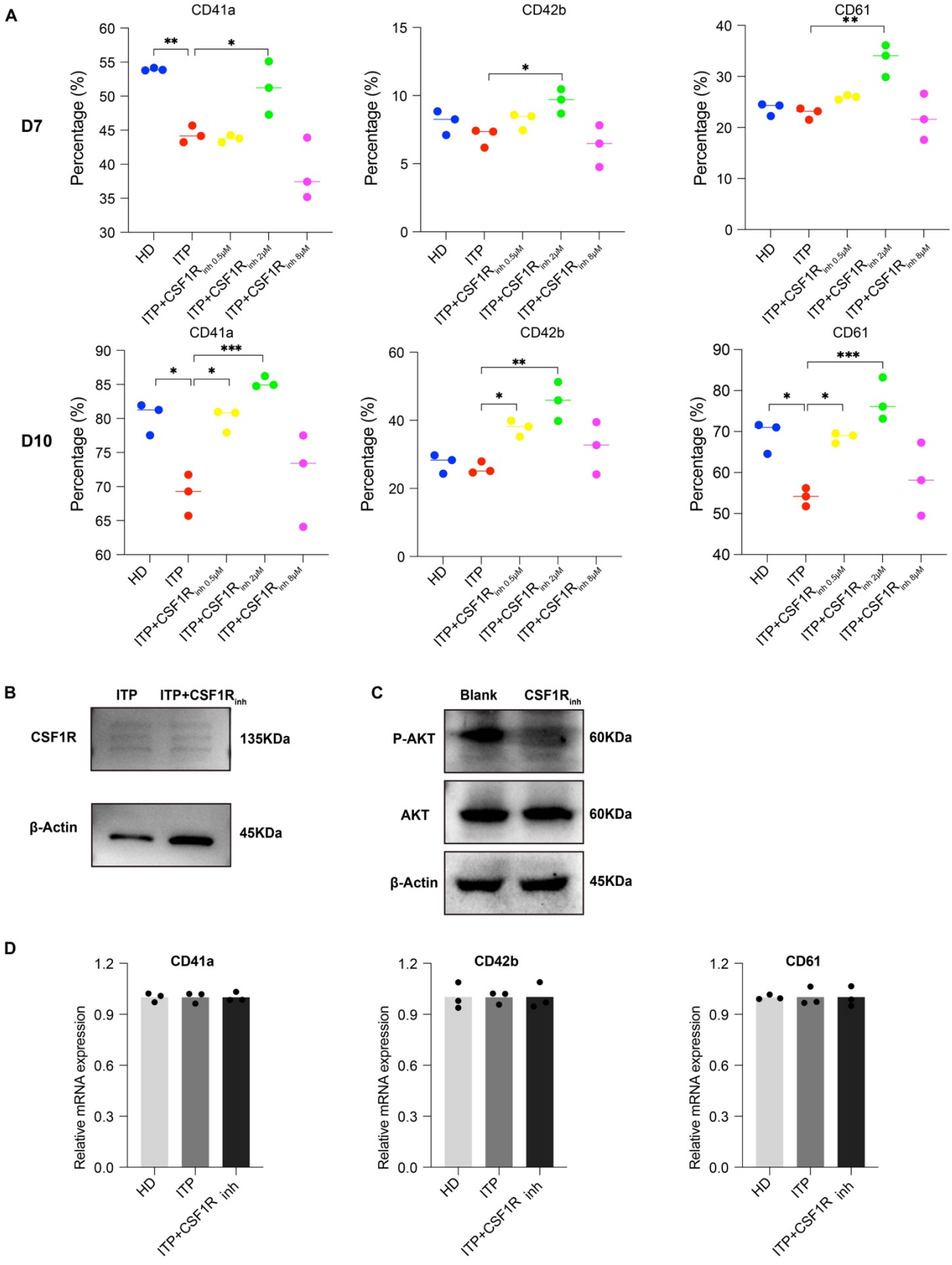
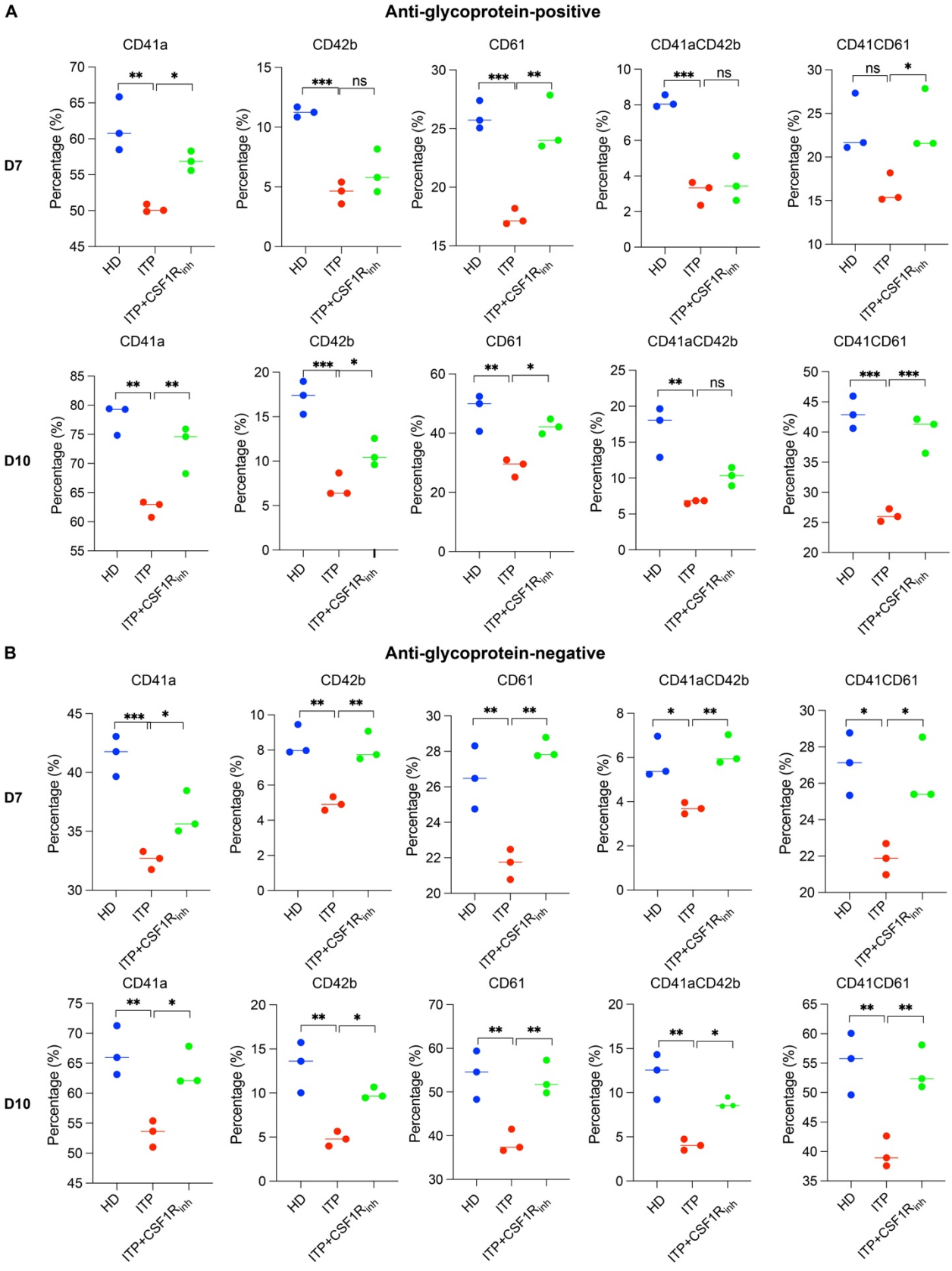

A
D7
D10
CD41a
CD42b
CD61
Percentage (%)
HD
ITP
ITP+CSF1R inh 0.5μM
ITP+CSF1R inh 2μM
ITP+CSF1R inh 8μM
B
ITP
ITP+CSF1R inh
CSF1R
135KDa
β-Actin
45KDa
C
Blank
CSF1R inh
P-AKT
60KDa
AKT
60KDa
β-Actin
45KDa
D
CD41a
CD42b
CD61
Relative mRNA expression
HD
ITP
ITP+CSF1R inh

Figure S5.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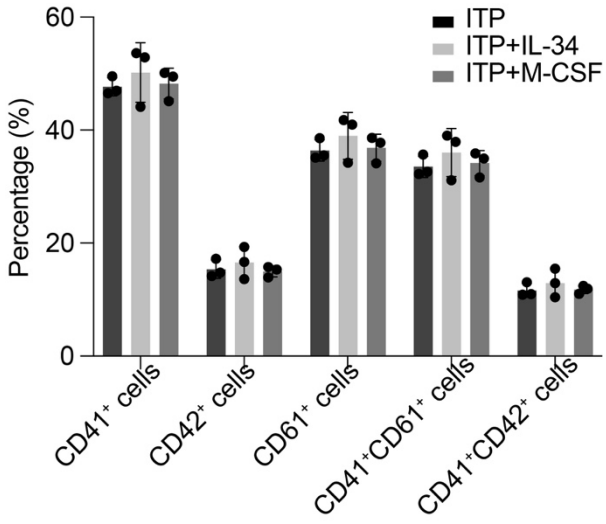
Anti-glycoprotein-positive
B
Anti-glycoprotein-negative
D7
D10
CD41a
CD42b
CD61
CD41aCD42b
CD41CD61
Percentage (%)
HD
ITP
ITP+CSF1R$_{inh}$
ns

Figure S6.


ITP
ITP+IL-34
ITP+M-CSF
Percentage (%)
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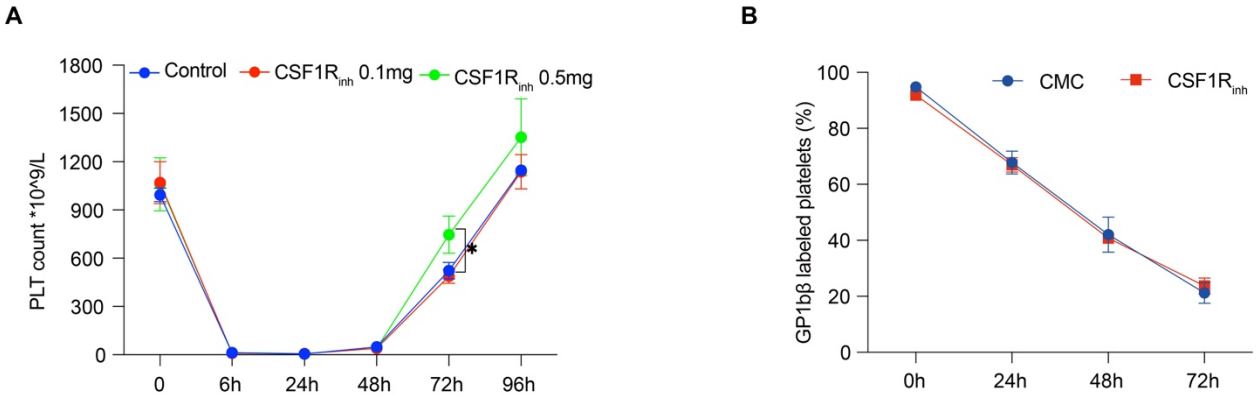
40
20
0
CD41+ cells
CD42+ cells
CD61+ cells
CD41+CD61+ cells
CD41+CD42+ cells

Figure S7.


A
1800
1500
1200
900
600
300
0
PLT count *10^9/L
Control
CSF1R$_{inh}$ 0.1mg
CSF1R$_{inh}$ 0.5mg
*
0
6h
24h
48h
72h
96h
B
100
80
60
40
20
0
GP1bβ labeled platelets (%)
CMC
CSF1R$_{inh}$
0h
24h
48h
72h

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