CSF1R modulates megakaryopoiesis by targeting RUNX1 in immune thrombocytopenia

Haohao Han,[1-3*] Meng Zhou,[1-3*] Jiaqian Qi,[1-3*] Xiaofei Song,[1-3] Xueqian Li,[1-3] Ziyan Zhang,[1-3] Tingting Pan,[1-3] Xiaoyan Xu,[1-3] Mengting Guo,[1-3] Depei Wu[1-3] and Yue Han[1-4]

[1]National Clinical Research Center for Hematologic Diseases, Jiangsu Institute of Hematology, The First Affiliated Hospital of Soochow University, Suzhou; [2]Institute of Blood and Marrow Transplantation, Collaborative Innovation Center of Hematology, Soochow University, Suzhou; [3]Key Laboratory of Thrombosis and Hemostasis of Ministry of Health and [4]State Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou, China

**HH, MZ and JQ contributed equally as first authors.*

Correspondence: Y. Han
hanyue@suda.edu.cn

D. Wu
drwudepei@163.com

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Abstract

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by platelet destruction and defective megakaryopoiesis. However, the mechanisms underlying megakaryocyte (MK) dysfunction in ITP remain unclear. To address this, we performed single-cell RNA sequencing (scRNA-seq) on bone marrow cells from a newly diagnosed ITP patient. ScRNA-seq analysis revealed a marked upregulation of *colony-stimulating factor 1 receptor (CSF1R)* in MK compared with healthy control. This finding was independently validated by flow cytometry in additional clinical samples. *In vitro*, MK differentiation and maturation were significantly impaired in ITP, and these defects were rescued by inhibition of CSF1R. In an active murine model of ITP, CSF1R inhibition accelerated platelet recovery. Mechanistically, elevated CSF1R expression suppressed the transcription factor RUNX1, a key regulator of megakaryopoiesis. In conclusion, our findings identify CSF1R as a previously unrecognized regulator of megakaryopoiesis and suggest it represents a promising therapeutic target in ITP.

Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by isolated thrombocytopenia, yet its precise pathogenesis remains elusive. Traditionally, ITP has been attributed to antibody-coated platelets that are cleared primarily by macrophages in the spleen via interactions with Fcγ receptors and complement receptors.[1-3] Desialylated platelets mediated by antibody can also be cleared in the liver.[4,5] Additionally, cytotoxic T lymphocytes ($CD8^+$) not only destroy autologous platelets via apoptosis and perforin/granzyme-mediated cytotoxicity,[6] but also induce platelet desialylation, thereby promoting hepatic clearance.[7]

However, there is growing evidence to indicate that, beyond the excessive immune-mediated platelet destruction, defective maturation of megakaryocytes (MK) also plays a pivotal role in the pathogenesis of ITP.[2,8] Megakaryopoiesis is a highly regulated and complex process within the bone marrow (BM), beginning with the lineage commitment of hematopoietic stem cells (HSC) toward megakaryocytic progenitors (MKP). These progenitors undergo multiple rounds of endomitosis to form MK, which subsequently extend proplatelets and release platelets into the bloodstream.[9,10] Several factors contribute to the defective MK differentiation and platelet production in ITP. For instance, MK express platelet-specific glycoproteins such as GPIIb/IIIa and GPIb/IX,[11-13] which serve as targets for autoantibodies in ITP patients. These antibodies not only promote platelet clearance but also impair MK maturation, leading to reduced MK numbers and defective platelet production.[14,15] In addition, impaired MK apoptosis mediated by cytotoxic T lymphocytes, as well as abnormalities in the BM microenvironment, including mesenchymal stem cell dysfunction, dysregulated cytokine profiles, and reduced thrombopoietin (TPO) levels, have also been associated with decreased megakaryopoiesis.[10,16] Therefore, therapeutic stimulation of MK has become a key strategy to elevate platelet counts in ITP patients. TPO receptor agonists (TPO-RA), such as eltrombopag and romiplostim, significantly enhance platelet production and achieve sustained responses in a substantial proportion of patients.[8,17,18] Nevertheless, in some patients, resistance or intolerance to TPO-RA occurs due to adverse events, including thrombosis, hepatotoxicity, or progressive BM fibrosis, manifested by increased reticulin levels.[19-21] These limitations highlight the urgency to further

explore alternative molecular pathways involved in MK dysfunction in ITP.

The colony-stimulating factor 1 receptor (CSF1R, CD115) is an essential receptor tyrosine kinase predominantly expressed on various myeloid cells. It plays a central role in regulating cell survival and function via interaction with its ligands, macrophage colony-stimulating factor (M-CSF) and interleukin-34 (IL-34).[22] Beyond its established function in monocytes and macrophages, CSF1R is also moderately expressed in HSC, common myeloid progenitors, and common lymphoid progenitors, indicating its broader involvement in early hematopoietic development.[23,24] Importantly, CSF1R expression has also been identified in MK.[25,26] Grabert *et al.*[25] used CRISPR-Cas9 gene editing to create a CSF1R-T2A-FRed knock-in mouse model, in which a fluorescent reporter was fused to the C-terminus of CSF1R; they found that CSF1R-FRed is expressed not only in monocytes and macrophages but also in MK and $CD41^+$ progenitors within mouse BM. Consistently, Cortegano *et al.*[26] reported that $CD45^{++}CD11b^+CD115^+$ ($CSF1R^+$) embryonic progenitors can give rise not only to myeloid/monocytic cells but also to a small population of immature adult-type $CD45^+$ MK. In adult C57BL/6 mice, approximately 26% of BM MK ($Ter119^-CD45^+CD9^{++}CD41^{++}CD42c^+$) express CSF1R, reflecting the biological heterogeneity of MK developmental pathways. However, the functional role of CSF1R in MK remains unclear. Whether CSF1R directly regulates MK differentiation and maturation, and the underlying mechanism, is still unknown. In this study, we identified aberrantly increased CSF1R expression in the BM of ITP patients. We further demonstrate that CSF1R impairs MK maturation by downregulating the transcription factor RUNX1, thereby contributing to the defective thrombopoiesis in ITP.

Methods

Patient samples

Single-cell RNA sequencing (scRNA-seq) was performed on BM cells from a newly diagnosed ITP patient and a matched healthy donor (HD) at the First Affiliated Hospital of Soochow University to identify subset-specific gene expression changes (*Online Supplementary Methods*). Cell clusters were annotated using specific marker genes reported previously (*Online Supplementary Table S1*). For validation, BM samples from 26 ITP patients and 18 HD (clinical characteristics summarized in *Online Supplementary Table S2*) were used. Levels of CSF1R ligands (M-CSF and IL-34) were measured by enzyme-linked immunosorbent assay (ELISA) in BM (26 ITP, 18 HD) and peripheral blood (30 ITP, 20 HD) samples, with patient characteristics summarized in *Online Supplementary Tables S3* and *S4*. Detailed inclusion and exclusion criteria for ITP are provided in the *Online Supplementary Methods*. All research programs used in the study are in line with the guidelines of the Ethics Committee of Soochow University and are in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all participants.

Cell culture and treatment *in vitro*

$CD34^+$ cells were extracted from the HD peripheral blood mononuclear cells using the EasySep Human CD34 Positive Selection Kit (Stem Cell Technologies, Canada) via immunomagnetic separation. Purified cells were seeded at 1×10^5 cells/well in Stem Span SFEM II medium (Stem Cell Technologies, Canada) supplemented with 100 ng/mL TPO, 100 ng/mL stem cell factor (SCF), and 10 ng/mL interleukin-3 (IL-3) (Pepro Tech, USA) to induce MK differentiation.[27] Cultures were maintained for 10-12 days, with 50% medium replaced on days 4, 7, and 10, and 1% fresh plasma from ITP patients or HD was supplemented at each change to maintain plasma-derived factors. Cytokine dynamics were monitored during the first four days after plasma addition (*Online Supplementary Methods*). The CSF1R inhibitor (cFMS Receptor Inhibitor II, 2 μM; MCE, USA) was added on days 0, 4, and 7 and maintained throughout differentiation. Details of inhibitor validation and flow cytometric gating strategies for MK and ploidy analysis are provided in the *Online Supplementary Methods* and shown in *Online Supplementary Figure S1A* and *B*, respectively.

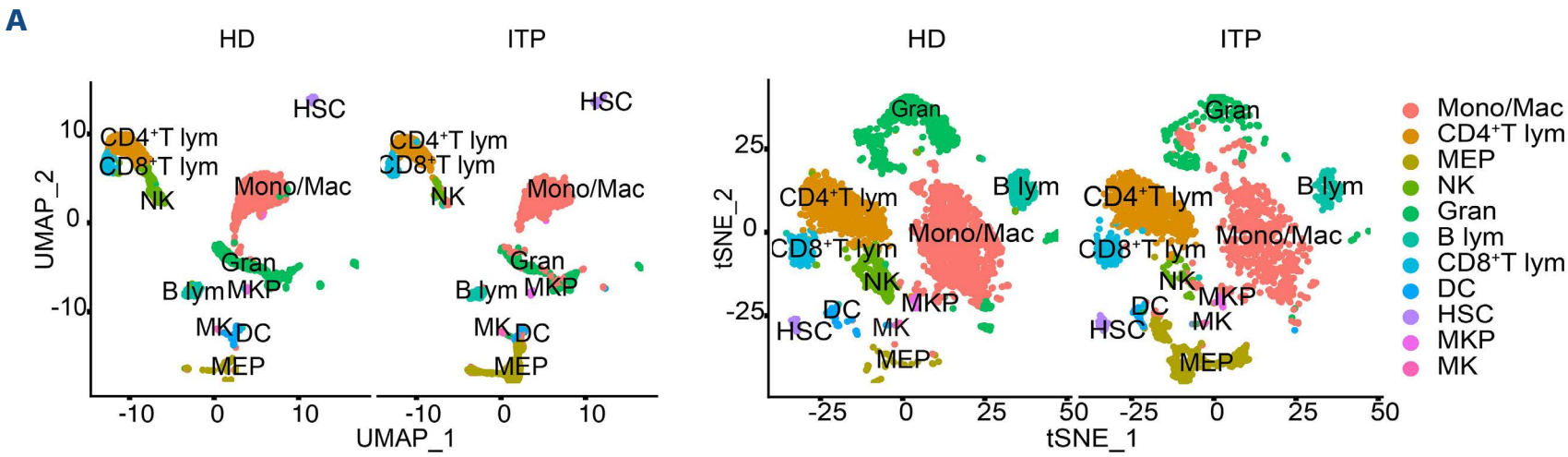
Active mouse immune thrombocytopenia model and colony-stimulating factor 1 receptor inhibition

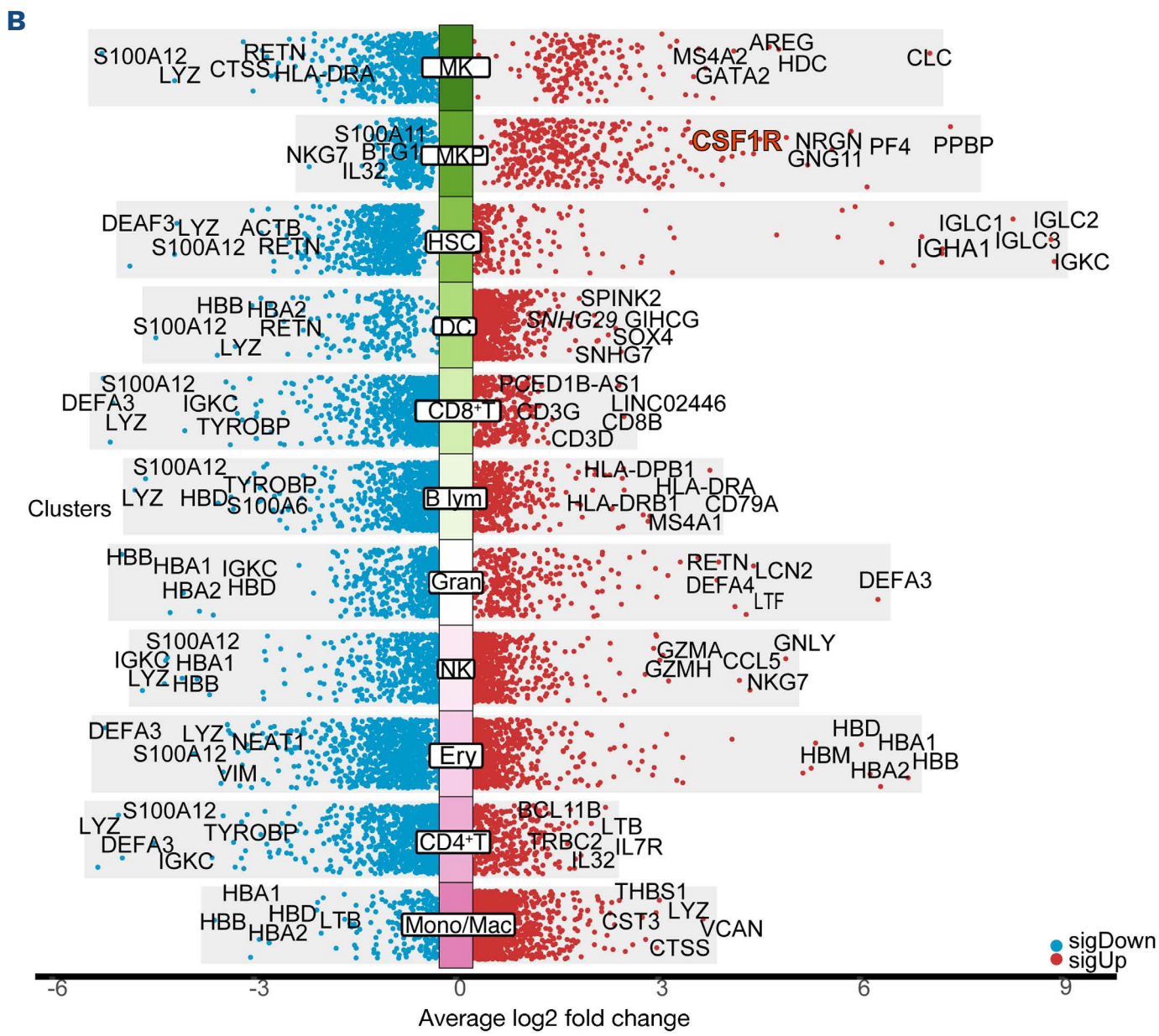
Wild-type (WT) C57BL/6N mice were obtained from the Laboratory Animal Center of Soochow University and heterozygous CD61 knockout ($CD61^{+/-}$) mice on the same background were kindly provided by Dr. Yi Wu (Soochow University, China). Animal experiments were approved by the Animal Ethics Committee of Soochow University.

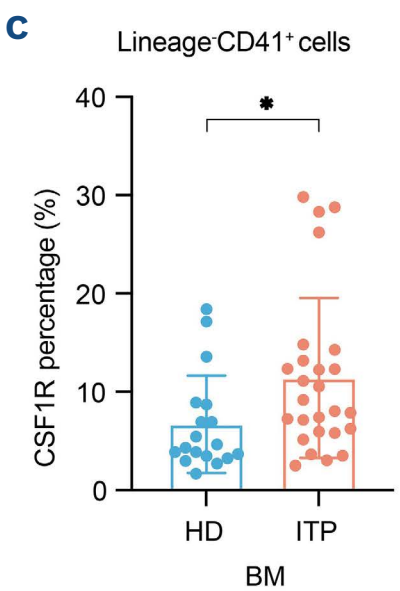
An active ITP model was established by transfusing $CD61^{-/-}$ mice weekly with 1.5×10^8 WT platelets for six weeks, as previously described[28] with minor modifications. Splenocytes (1×10^7 cells) from immunized $CD61^{-/-}$ mice were transferred into WT recipients to induce ITP.[29,30] To assess the effect of CSF1R inhibition, mice received oral administration of the CSF1R inhibitor (cFMS Receptor Inhibitor II, 0.5 $mg{\cdot}kg^{-1}$; MCE, USA) or vehicle (carboxymethyl cellulose [CMC]) for three consecutive days, commencing approximately one week after splenocyte transfer, coinciding with the onset of severe thrombocytopenia. Platelet counts were monitored regularly, and BM samples collected on day 16 were processed for hematoxylin-eosin staining.

Statistical analysis

Data were analyzed using GraphPad Prism 9.0. Student *t* test was used for comparisons between two groups and one-way ANOVA for multiple group comparisons. All data are presented as mean ± standard deviation (SD). $P<0.05$ was considered statistically significant.







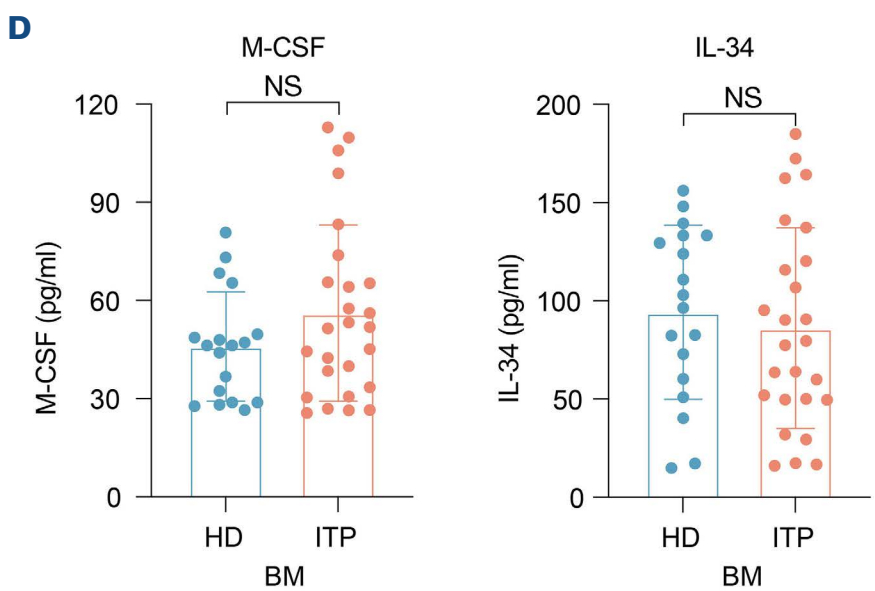


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Figure 1. CSF1R expression is increased in megakaryocytes from patients with immune thrombocytopenia. (A) Single-cell RNA sequencing (scRNA-seq) was performed on bone marrow (BM) cells from a newly diagnosed immune thrombocytopenia (ITP) patient and a matched healthy donor (HD), followed by dimensionality reduction and clustering analyses using UMAP and t-SNE (N=1). (B) Comparative analysis of transcriptional profiles revealed differential gene expression between the ITP and HD samples, with sigUp (red) indicating genes with log2 fold change ≥0 and sigDown (blue) indicating genes with log2 fold change ≤0 (N=1). (C) Colony-stimulating factor 1 receptor (CSF1R) expression in BM megakaryocytes (MK) was evaluated by flow cytometry in ITP patients (N=26) and HD (N=18). (D) Concentrations of CSF1R ligands (macrophage colony-stimulating factor [M-CSF] and interleukin-34 [IL-34]) were quantified in BM supernatants from ITP patients (N=26) and HD (N=18) using ELISA. Data are presented as mean ± standard deviation. *P<0.05; NS: not significant.

Results

Colony-stimulating factor 1 receptor is upregulated in megakaryocytes of immune thrombocytopenia patients

To investigate the alterations in hematopoiesis associated with ITP, we performed scRNA-seq of BM cells from a newly diagnosed ITP patient and an HD. The ITP patient was a 65-year-old female presenting with a platelet count of 10×10^9/L, and normal white blood cell and hemoglobin levels. Following quality control, dimensionality reduction and clustering analysis were conducted using UMAP and t-SNE methods (Figure 1A). Cell populations were manually annotated based on distinct gene expression patterns, including HSC, megakaryocyte-erythroid progenitors (MEP), MKP, MK, granulocytes, monocyte/macrophages, B lymphocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, natural killer cells, and dendritic cells. Both the ITP patient and HD exhibited relatively low proportions of MK and MKP. Nevertheless, a marked expansion of MEP was observed in ITP (Figure 1A), suggesting a skewing of HSC differentiation toward the erythroid lineage. Notably, *CSF1R* expression was upregulated in MKP from the ITP patient (Figure 1B). To validate this observation, we assessed CSF1R expression in BM-derived MK from 26 ITP patients and 18 HD by flow cytometry. The ITP cohort included 10 males and 16 females with a median age of 46.5 years (range: 14-74 years) and a median platelet count of 12.5×10^9/L (range: 1-53×10^9/L). MK from ITP patients exhibited significantly higher CSF1R expression than those from HD (Figure 1C). To determine whether altered ligand availability contributed to CSF1R upregulation, we measured concentrations of its ligands, M-CSF and IL-34, in both BM and PB supernatants. ELISA revealed no significant differences between ITP patients and HD (Figure 1D, *Online Supplementary Figure S2*), suggesting that CSF1R overexpression may be largely independent of ligand-mediated regulation. These findings implicate CSF1R as a critical contributor to impaired MK maturation in ITP and warrant further investigation into its mechanistic role in disease pathogenesis.

Immune thrombocytopenia plasma induces colony-stimulating factor 1 receptor upregulation in cultured megakaryocytes

To further examine the role of CSF1R in megakaryopoiesis in ITP, we employed an *in vitro* culture system in which $CD34^+$ HSC were cultured with plasma derived from ITP patients or HD (*Online Supplementary Figure S3A*). Clinical characteristics of the ITP patients are summarized in *Online Supplementary Table S5*. These plasma samples were used for scRNA-seq analysis and subsequent validation experiments. Cytokine profiling after ITP plasma addition revealed transient peaks of TNF-α, IFN-γ, IL-2, IL-4, IL-8, IL-10, IL-17A, IP-10, and MCP-1 within 24-48 hours, followed by a gradual decline, indicating that these factors remain biologically active between medium changes (*Online Supplementary Figure S3B*). On day 4 of culture, cells exposed to plasma from a single ITP patient or a matched HD were harvested for scRNA-seq analysis. Dimensionality reduction and clustering analyses revealed a marked reduction in the proportion of MKP and MK in ITP compared to HD (Figure 2A). Conversely, the ITP group exhibited an increased number of MEP, suggesting a lineage bias toward erythropoiesis in response to ITP plasma (Figure 2B), consistent with our prior observations in patient samples. Differential gene expression analysis revealed upregulation of CSF1R in MK cultured with ITP plasma in the discovery pair (Figure 2C). To validate this result, we evaluated the CSF1R expression on cultured MK at days 4, 7, and 10. On day 4, CSF1R expression was significantly elevated in ITP compared to HD. However, its expression declined progressively on days 7 and 10, with no significant differences observed between groups at later time points (Figure 2D). These findings indicate that *CSF1R* expression is transiently induced during early MK differentiation in response to ITP plasma, supporting a potential regulatory role in the impaired megakaryopoiesis observed in ITP.

Colony-stimulating factor 1 receptor inhibition enhances megakaryopoiesis *in vitro*

To determine whether CSF1R upregulation functionally impairs megakaryopoiesis, $CD34^+$ HSC were cultured with plasma from ITP patients or HD, with or without 2 μM CSF1R inhibitor. This concentration, selected from dose-optimization experiments, efficiently promoted megakaryopoiesis (*Online Supplementary Figure S4A*), suppressed CSF1R between medium changes (*Online Supplementary Figure S4B*), and blocked downstream signaling without inducing activation (*Online Supplementary Figure S4C*). Exposure to ITP plasma markedly reduced

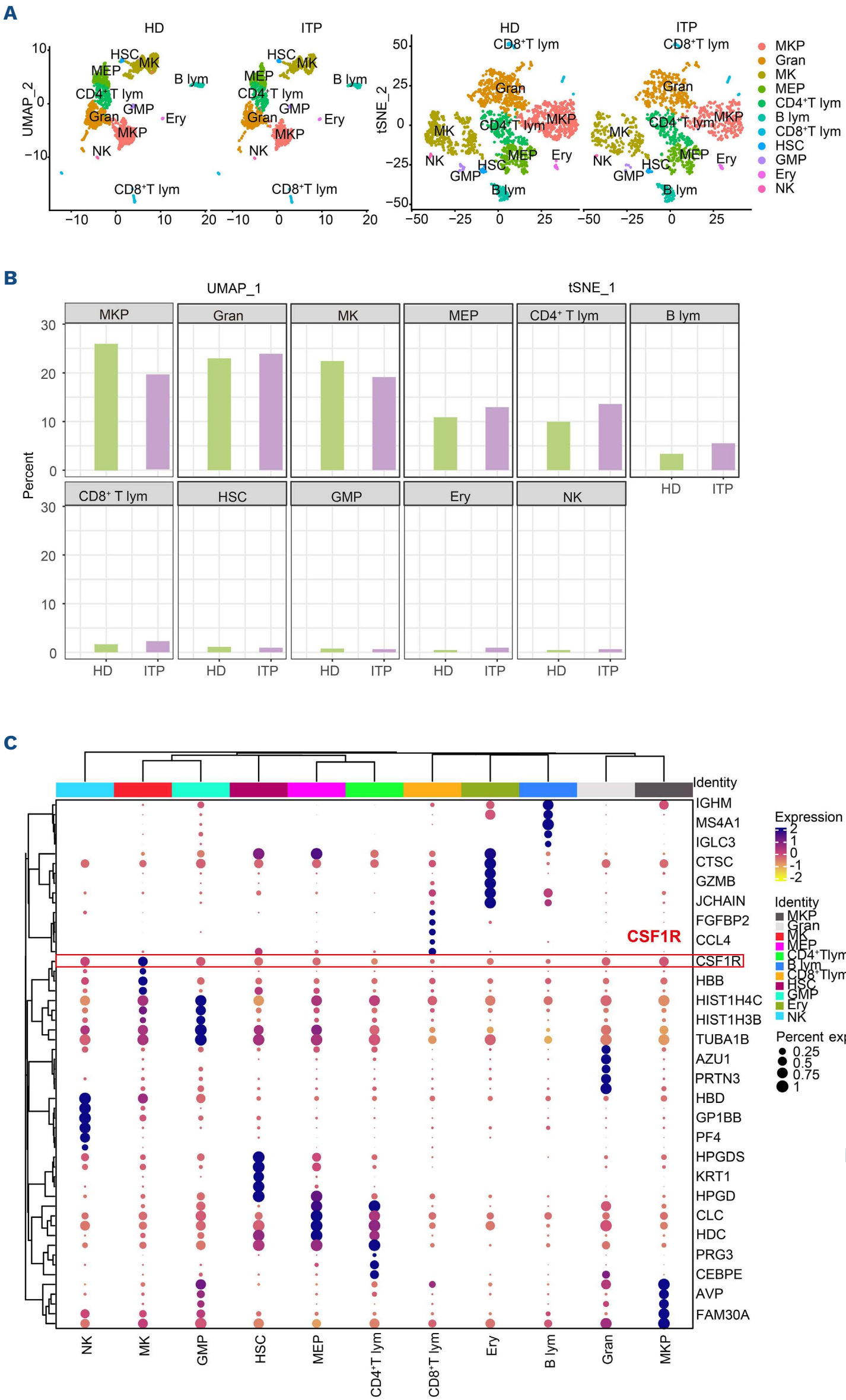

Figure 2. CSF1R expression is upregulated in megakaryocytes cultured with immune thrombocytopenia plasma. (A) UMAP and t-SNE plots of single-cell RNA sequencing (scRNA-seq) data showing cellular clusters derived from CD34⁺ hematopoietic stem cells (HSC) cultured for four days with plasma from an immune thrombocytopenia (ITP) patient and a matched healthy donor (HD) (N=1). (B) Quantification of distinct cell populations identified by scRNA-seq (N=1). (C) Illustrative scRNA-seq visualization of relative colony-stimulating factor 1 receptor (CSF1R) expression in megakaryocytes (MK) cultured with ITP or HD plasma at day (D) 4 in the discovery pair (N=1). (D) Flow cytometric analysis of CSF1R expression in cultured MK at D4, D7, and D10. CSF1R was significantly elevated in ITP-derived MK at D4, but differences were not observed at later time points (N=3). Data are presented as mean ± standard deviation. *P<0.05; NS: not significant.

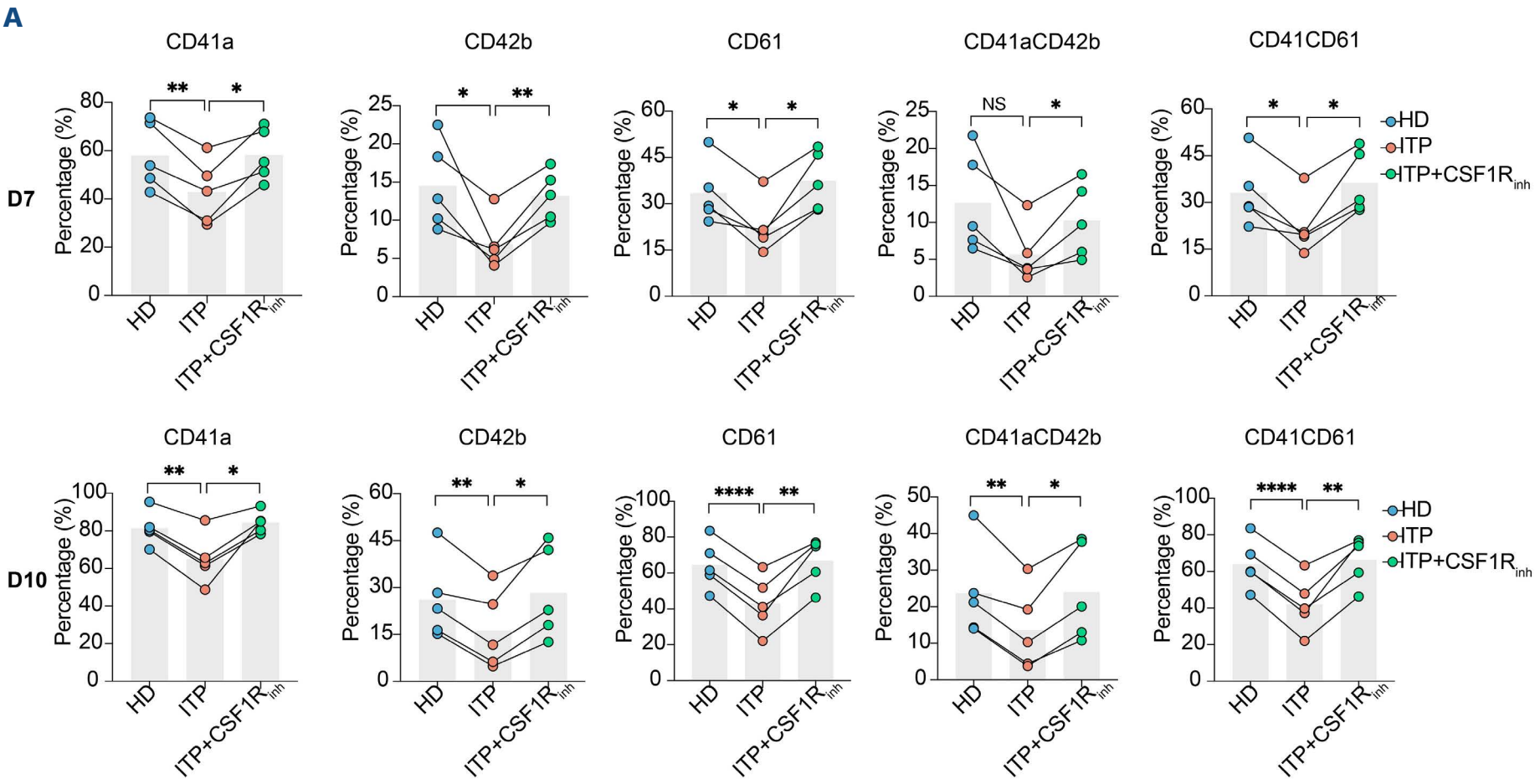

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Figure 3. CSF1R inhibition restores megakaryocyte differentiation and maturation in immune thrombocytopenia *in vitro*. (A) Flow cytometric analysis of the proportion of megakaryocytes (MK) expressing CD41a, CD42b, or CD61, derived from $CD34^+$ hematopoietic stem cells (HSC) cultured with plasma from immune thrombocytopenia (ITP) patients or healthy donors (HD) for seven or ten days, with or without colony-stimulating factor 1 receptor (CSF1R) inhibitor treatment (N=5). (B) Polyploid distribution of MK cultured under the indicated conditions, assessed by flow cytometry on day (D) 10 (N=3). (C) Quantification of platelet production from cultured MK on D12 (N=3). (D) Confocal microscopy of $CD41^+$ MK cultured with ITP plasma, with or without CSF1R inhibitor (N=3). Scale bar is 50 μm (CD41, red; α-tubulin, green; phalloidin, purple; DAPI, blue). Data are presented as mean ± standard deviation. **P*<0.05, ***P*<0.01, *****P*<0.0001; NS: not significant.

the proportion of MK expressing CD41a, CD42b, or CD61 at various stages of differentiation. This inhibitory effect was fully reversed by CSF1R inhibition (Figure 3A). No significant differences were observed in the mRNA levels of CD41a, CD42b, and CD61 among the groups (*Online Supplementary Figure S4D*). To further assess whether the effect of CSF1R on megakaryopoiesis is influenced by anti-glycoprotein autoantibodies, we performed additional experiments using pooled plasma from anti-glycoprotein-positive or anti-glycoprotein-negative ITP patients (*Online Supplementary Table S6*). Under both conditions, CSF1R inhibition consistently promoted megakaryopoiesis (*Online Supplementary Figure S5A, B*), suggesting that its effect may be independent of anti-glycoprotein antibody status.

We next assessed MK ploidy distribution and platelet production. MK cultured with ITP plasma exhibited a higher proportion of low-ploidy cells (4N: 33.10% ± 2.30% *vs*. 25.97% ± 2.16%, *P*=0.032) and a significant reduction in high-ploidy MK (≥ 16N: 27.43% ± 1.04% *vs*. 38.49% ± 1.02%, *P*=0.002) (Figure 3B), accompanied by reduced platelet release (Figure 3C). In contrast, CSF1R inhibition restored normal ploidy distribution (≥16N: 38.81% ± 0.95% *vs*. 27.43% ± 1.04%, *P*=0.003) and significantly rescued platelet production (126,296 ± 10,035 *vs*. 63,122 ± 4,277, *P*=0.015) (Figure 3B, C).

Confocal fluorescence microscopy further revealed a reduced number of $CD41^+$ MK in ITP cultures, characterized by smaller cell size and absence of cytoplasmic extensions. In contrast, CSF1R inhibition markedly increased the number of $CD41^+$ MK, which displayed enlarged cell bodies, multinucleated nuclei, irregular membrane contours, and pseudopod-like cytoplasmic protrusions, all indicative of proplatelet formation (Figure 3D).

Given the restorative effects of CSF1R inhibition on MK function, we next assessed whether CSF1R ligands (M-CSF and IL-34) influenced megakaryopoiesis in ITP. Neither cytokine significantly influenced MK differentiation, suggesting that the regulatory effects of CSF1R in ITP are likely independent of its ligand levels (*Online Supplementary Figure S6*). These results are consistent with our earlier observations (Figure 1D, *Online Supplementary Figure S2*).

To further validate the functional role of CSF1R in MK, we established stable Meg01 cell lines with lentiviral-mediated CSF1R knockdown (lv-shCSF1R) or overexpression (lv-oeCSF1R). Efficient knockdown and overexpression of CSF1R were confirmed by quantitative real-time PCR (qPCR) (Figure 4A, B). CSF1R knockdown significantly increased the proportions of $CD41^+$ (50.41% ± 0.15% *vs*. 34.47% ± 1.41%, *P*<0.001), $CD61^+$ (62.43% ± 1.83% *vs*. 48.11% ± 2.35%, *P*=0.009), and $CD41^+CD61^+$ (43.28% ± 0.82% *vs*. 26.94% ± 1.01%, *P*<0.001) MK compared with controls (Figure 4C). Conversely, CSF1R overexpression significantly reduced these populations ($CD41^+$ MK, 46.10% ± 3.85% *vs*. 63.02% ± 2.28%, *P*=0.019; $CD61^+$ MK, 36.82% ± 2.72% *vs*. 59.94% ± 3.37%, *P*=0.006; $CD41^+CD61^+$ MK, 26.38% ± 2.75% *vs*. 48.35% ± 4.63%, *P*=0.015) (Figure 4D). Polyploidy analysis further revealed that CSF1R knockdown decreased low-ploidy MK (2N: 7.29% ± 0.59% *vs*. 12.45% ± 0.88%, *P*=0.008; 4N: 19.42% ± 1.16% *vs*. 23.52% ± 0.59%, *P*=0.034) and increased high-ploidy MK (≥16N: 54.74% ± 1.43% *vs*. 44.98% ± 0.33%, *P*=0.003), indicating enhanced maturation (Figure 4E). Conversely, CSF1R overexpression shifted the ploidy distribution toward less mature MK, increasing 4N MK (33.86% ± 0.93% *vs*. 28.68% ± 0.61%, *P*=0.010) and decreasing ≥16N MK (30.52% ± 1.19% *vs*. 38.78% ± 1.43%, *P*=0.011) (Figure 4F).

Collectively, these findings underscore the pivotal role of CSF1R in MK differentiation and maturation and highlight its potential contribution to the pathophysiology of ITP.

Colony-stimulating factor 1 receptor inhibition facilitates platelet recovery in an active murine model of immune thrombocytopenia

Based on preliminary experiments, a CSF1R inhibitor dose of 0.5 $mg{\cdot}kg^{-1}$ was selected as the minimal effective and well-tolerated dose (*Online Supplementary Figure S7A*), whereas a higher dose of 1 $mg{\cdot}kg^{-1}$ caused mortality in 2 mice within 24 hours, indicating potential toxicity. Therefore, a dose of 0.5 $mg{\cdot}kg^{-1}$ was used in all subsequent experiments. To investigate the *in vivo* role of CSF1R in ITP, an active ITP mouse model was established (Figure 5A). Platelet counts in ITP mice were significantly reduced compared with healthy controls at eight days after the transfer of immunized splenocytes, confirming successful disease induction. Subsequently, ITP mice received the CSF1R inhibitor or vehicle control (CMC) for three consecutive days. Notably, CSF1R inhibitor-treated mice exhibited increased MK numbers in the BM (Figure 5B), along with a significant elevation in platelet counts on day 16 (891 ± 64×10^9/L *vs*. 547 ± 47×10^9/L, *P*=0.005) and day 20 (1,007 ± 40×10^9/L *vs*. 590 ± 113×10^9/L, *P*=0.025), compared with the CMC group (Figure 5C). Analysis of macrophage-mediated platelet clearance revealed no significant difference between CSF1R inhibitor-treated and control groups (*Online Supplementary Figure S7B*). Together, these results demonstrate that CSF1R inhibition promotes megakaryopoiesis and enhances

platelet recovery in ITP without affecting platelet clearance.

RUNX1 as a target of colony-stimulating factor 1 receptor

Our findings thus far underscore the crucial role of CSF1R in MK differentiation and maturation. To explore the downstream targets mediating this effect, we conducted RNA-seq on CD61$^+$ MK cultured with ITP plasma (N=3) or ITP plasma supplemented with a CSF1R inhibitor (N=3). Differential expression analysis identified 166 genes that were significantly altered between the two groups, with 108 genes downregulated and

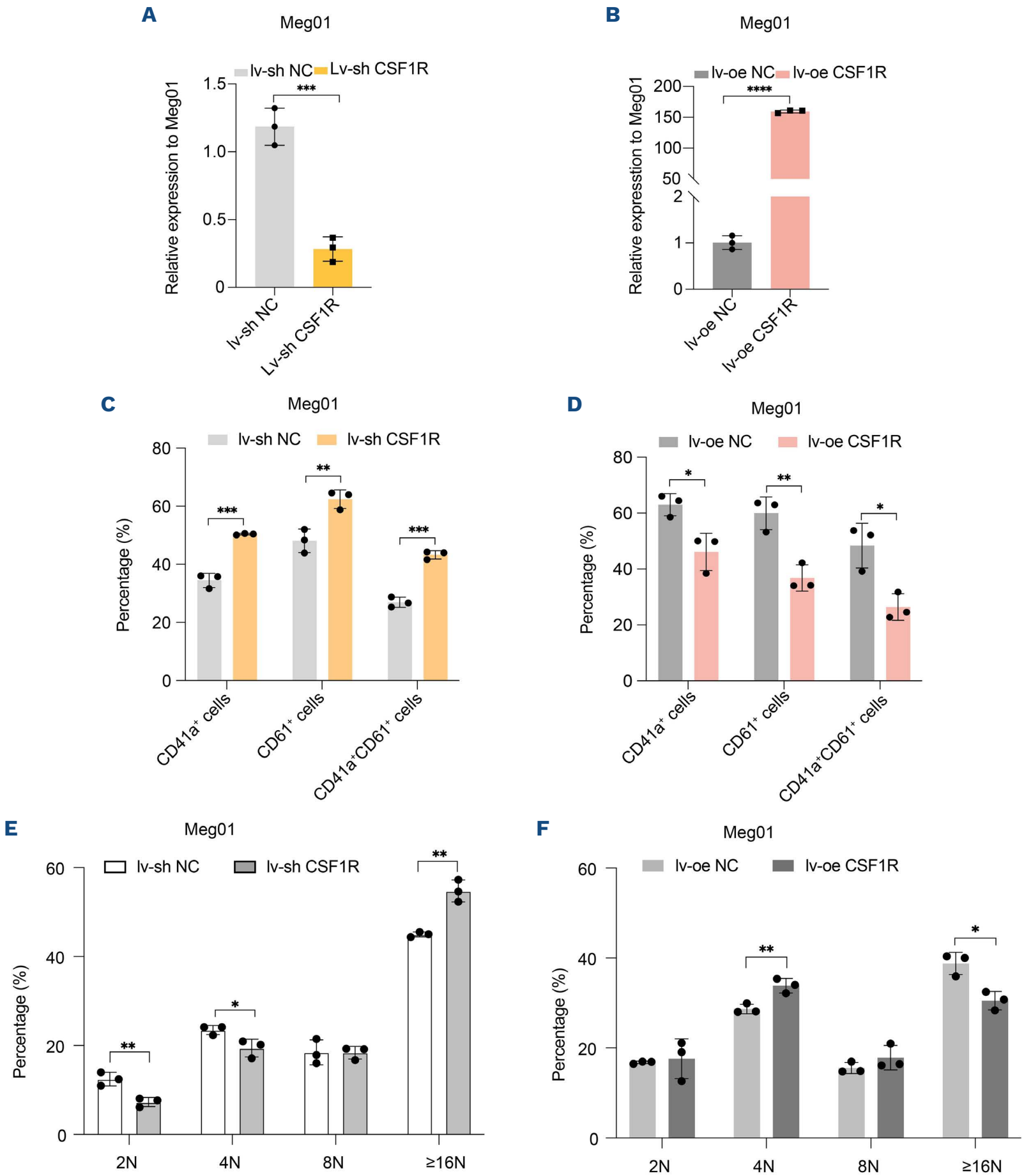

Figure 4. Altered CSF1R Expression modulates megakaryopoiesis in Meg01 cells. (A and B) Quantitative real-time polymerase chain reaction (qPCR) analysis confirming colony-stimulating factor 1 receptor (CSF1R) knockdown (lv-sh CSF1R) or overexpression of CSF1R (lv-oe CSF1R) in the Meg01 cells compared with respective negative controls (lv-sh NC or lv-oe NC) (N=3). (C and D) Flow cytometric analysis of the proportion of megakaryocytes (MK) following CSF1R knockdown or overexpression (N=3). (E and F) Polyploid distribution of Meg01 cells after modulation of CSF1R expression (N=3). Data are shown as mean ± standard deviation. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

58 upregulated upon CSF1R inhibition. Among the upregulated transcripts, we noted a marked increase in RUNX1, a key transcription factor essential for megakaryopoiesis (Figure 6A, B). qPCR confirmed that *RUNX1* mRNA expression was significantly elevated in MK treated with the CSF1R inhibitor (Figure 6C). At the protein level, RUNX1 was similarly upregulated in Meg01 cells following CSF1R knockdown (Figure 6D) and conversely downregulated upon CSF1R overexpression (Figure 6E). Collectively, these findings identify RUNX1 as a downstream target of CSF1R signaling and suggest that modulation of RUNX1 may underlie the regulatory role of CSF1R in MK development under ITP conditions.

Discussion

Recent advancements in the understanding of ITP pathophysiology and its clinical management have markedly improved patient outcomes.[1,8,13,31-33] Yet treatment resistance and relapse remain significant challenges, leading to refractory or chronic ITP that negatively impacts patients' quality of life. In this study, we report for the first time a substantial upregulation of CSF1R in MK derived from ITP patients, as confirmed by scRNA-seq and validated in clinical BM samples. Our findings further demonstrate that CSF1R modulates megakaryopoiesis by regulating the expression of RUNX1, a key regulator of MK differentiation and maturation. These insights suggest that targeting CSF1R and its downstream signaling pathways may offer new therapeutic strategies for patients with ITP.

CSF1R is expressed at low levels in HSC, and activation of the M-CSF/CSF1R signaling pathway promotes myeloid lineage commitment by inducing PU.1, a master regulator of myelopoiesis.[23] CSF1R expression is markedly increased during macrophage and monocyte differentiation, where it regulates cell survival, proliferation, migration, and chemotaxis.[22] Abnormal CSF1R expression has been closely associated with tumor progression through reprogramming macrophages within the tumor microenvironment, contributing to immune evasion, tumor growth, invasion and metastasis.[34,35] In addition, the M-CSF/CSF1R signaling pathway is also involved in the pathogenesis of various inflammatory diseases, including rheumatoid arthritis,[36] lupus nephritis,[37] and chronic graft-*versus*-host disease.[38] CSF1R expression has also been detected in BM MK, indicating a possible involvement in megakaryocytosis.[25,26] It is also essential for the proliferation and viability of the acute megakaryoblastic leukemia cell line MKPL-1.[39] The ROS-CSF1R signaling pathway has been shown to influence the differentiation of granulocyte-monocyte progenitors and MEP at the common myeloid progenitor stage.[40] Our scRNA-seq analysis revealed an increased erythroid lineage and decreased MK population in the BM of ITP patients. These observations were based on a single patient-donor pair. Further investigation is required to determine whether CSF1R directly regulates MEP lineage commitment toward erythroid *versus* megakaryocytic differentiation.

A previous study also reported elevated CSF1R protein expres-

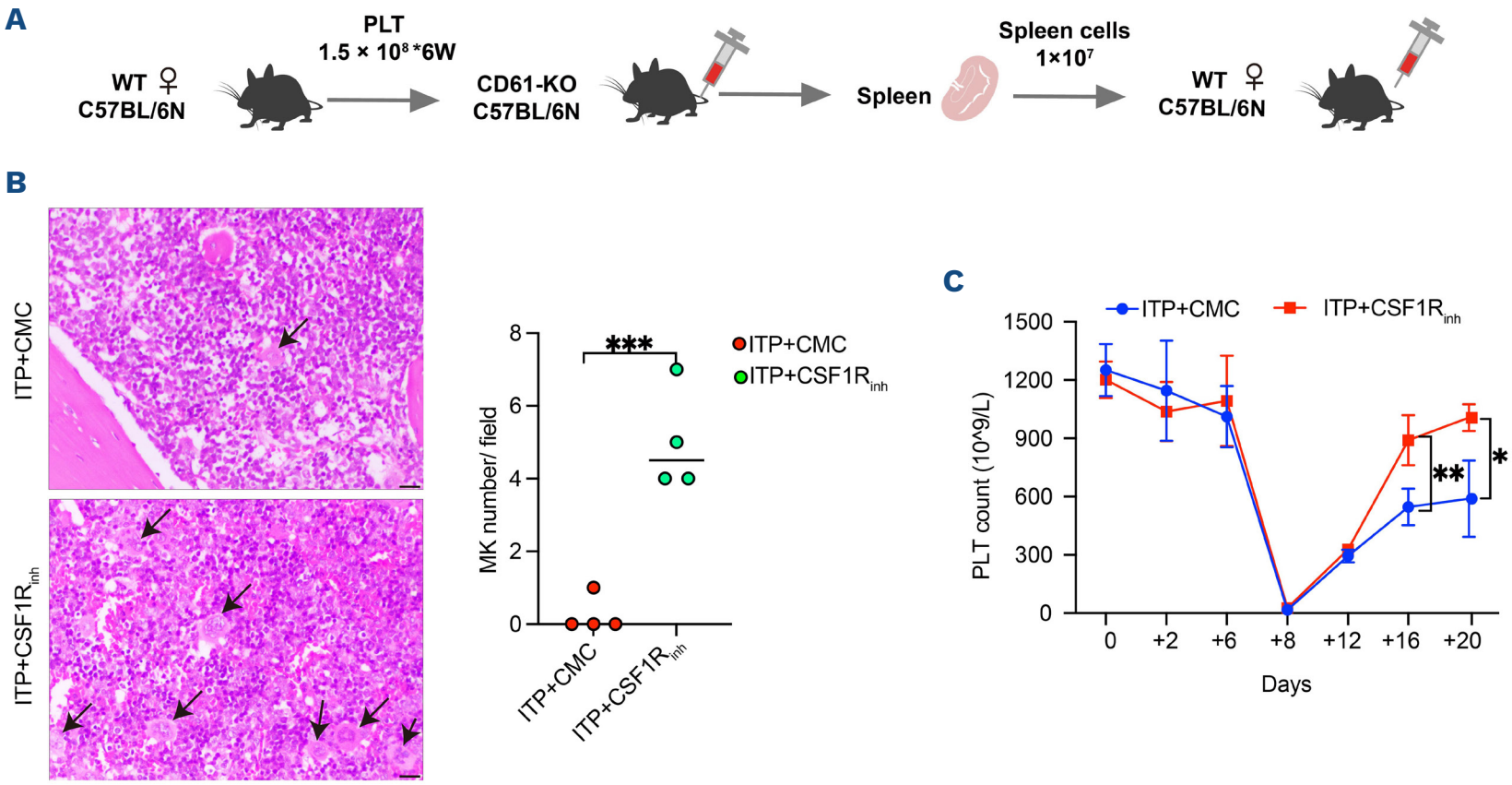

Figure 5. CSF1R inhibitor facilitates platelet recovery in an active murine immune thrombocytopenia model. (A) Schematic representation of the active immune thrombocytopenia (ITP) mouse model. (B) Bone marrow (BM) samples were collected 16 days after splenocyte transfer and stained with hematoxylin & eosin staining to evaluate megakaryocytes (MK) numbers. Scale bar is 25 µm. Black arrows indicate MK (N=4). (C) Platelet counts (PLT) were monitored regularly in colony-stimulating factor 1 receptor (CSF1R) inhibitor-treated *versus* carboxymethyl cellulose (CMC)-treated ITP mice (N=4). Data represent mean ± standard deviation. **P*<0.05, ***P*<0.01, ****P*<0.001. KO: knockout; WT: wild-type.

sion in BM mononuclear cells of ITP patients.[41] The precise role of CSF1R in MK and its contribution to the pathogenesis of ITP remains incompletely understood. Consistent with previous research findings,[14,42] our findings demonstrate that ITP patient plasma impaired MK development, and MK numbers were reduced in an active murine model of ITP. Inhibition or knockdown of CSF1R effectively reversed this impairment, restoring megakaryopoiesis. Moreover, inhibition of CSF1R facilitated platelet recovery in an active murine model of ITP. These findings indicate that aberrant CSF1R expression disrupts MK maturation and platelet production in ITP. Interestingly, the regulatory role of CSF1R in megakaryopoiesis in ITP appears to be independent of its ligands, M-CSF or IL-34. Their plasma levels were unchanged in ITP patients, and exogenous supplementation of these ligands had no effect on MK development in our *in vitro* culture system. These results collectively indicate that aberrant CSF1R expression disrupts MK maturation in ITP likely through a ligand-independent mechanism. Moreover, CSF1R inhibition effectively reversed the suppression of megakaryopoiesis induced by pooled plasma from anti-glycoprotein-positive or anti-glycoprotein-negative ITP patients, suggesting that CSF1R-mediated regulation of megakaryopoiesis may not be dependent on anti-glycoprotein antibodies. At present, the relative contributions of anti-glycoprotein antibodies *versus* other plasma components to CSF1R upregulation remain unclear. Future studies using plasma from additional patients or purified IgG will be necessary to elucidate the underlying mechanisms.

Our analysis of RNA-seq from ITP plasma-treated MK revealed that CSF1R inhibition upregulated RUNX1 expression, a finding that was corroborated by qPCR and western blot assays. RUNX1 plays a pivotal role in directing the lineage fate of MEP. Its overexpression in primary human MEP enhances megakaryocytic commitment, whereas its inhibition biases differentiation toward the erythroid lineage.[43] RUNX1 haploinsufficiency has been shown to impair megakaryopoiesis by reducing MK-biased hematopoietic progenitor cells, likely

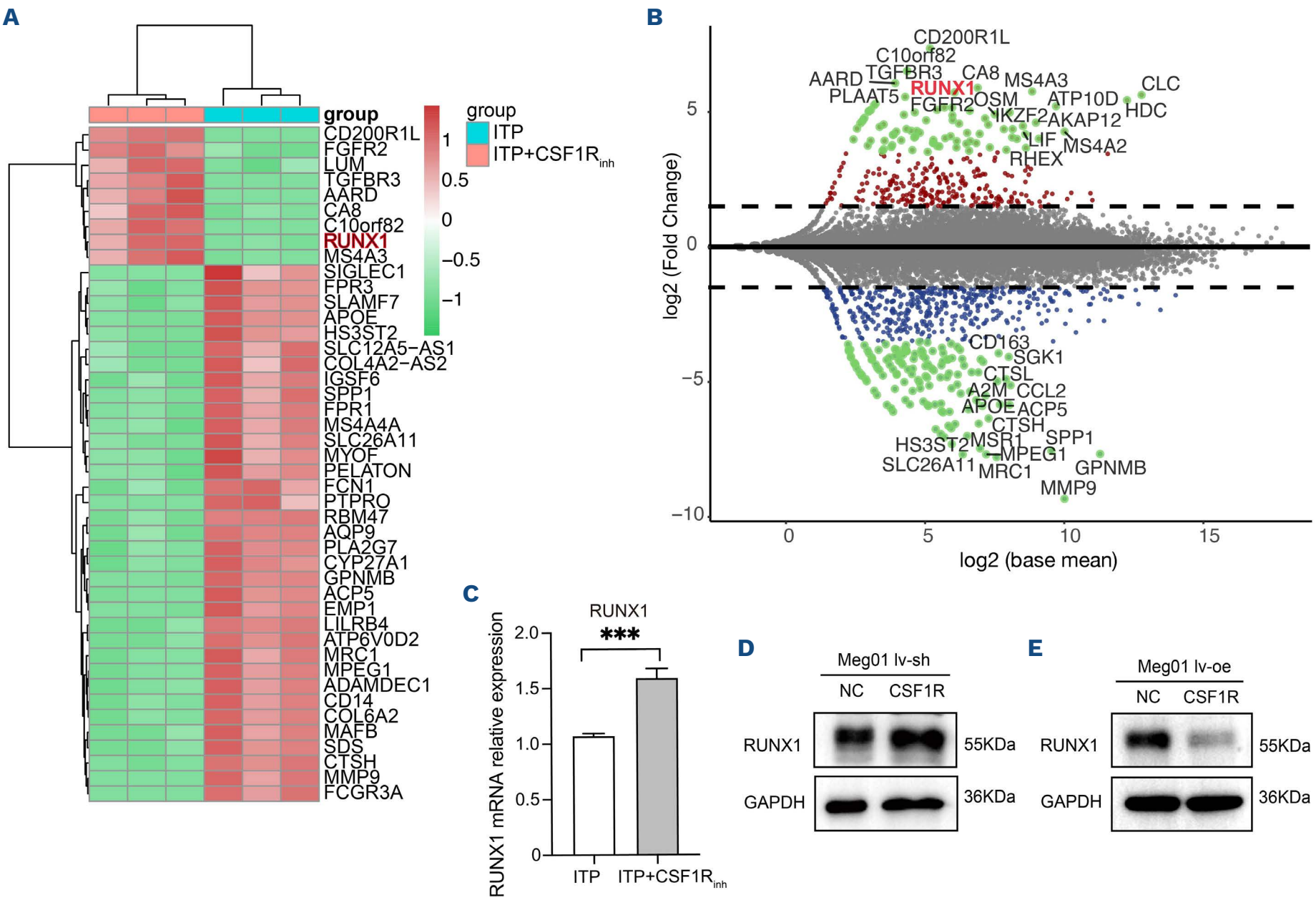

Figure 6. CSF1R regulates megakaryopoiesis via modulation of RUNX1 expression. (A and B) Heatmap and volcano plot illustrating differentially expressed genes between the immune thrombocytopenia (ITP) and ITP+CSF1Rinh groups (N=3). (C) Quantitative real-time polymerase chain reaction (qPCR) validation of RUNX1 mRNA expression in megakaryocytes (MK) cultured with ITP plasma or ITP+CSF1Rinh on day 10 (N=3). (D and E) Western blot analysis of RUNX1 protein levels in Meg01 cells following colony-stimulating factor 1 receptor (CSF1R) knockdown or overexpression (N=3). ***P<0.001.

through TGF-β1, which enhances phosphorylation of c-Jun N-terminal kinase and triggers inflammation-related signaling pathways that affect MK production.[44] Additional research demonstrated that silencing RUNX1 using a lentiviral short hairpin RNA (shRNA) strategy resulted in MK with significantly defective responsiveness to agonists. When infused into immunodeficient NOD scid gamma mice, these MK produced fewer circulating platelets, which exhibited shortened platelet lifespan compared to those transduced with a non-targeting control shRNA.[45] Germline mutations in *RUNX1* are linked to familial platelet disorder with a predisposition to myeloid malignancy, characterized by thrombocytopenia, bleeding tendencies, and increased risk of myeloid malignancy.[46] As a well-established transcription factor, RUNX1 governs MK differentiation, maturation, and platelet production.[47,48]

The interaction between *CSF1R* and RUNX1 appears to be reciprocal and biologically significant in regulating hematopoietic lineage commitment. CSF1R expression in the myeloid lineage largely hinges on prior transcriptional activity of PU.1, and both CSF1R and PU.1 are known direct targets of RUNX1.[49] Intriguingly, in BRAF-mutant melanomas, resistance to BRAF inhibitors has been associated with the upregulation of CSF1R and IL-34, which is also mediated by RUNX1. In this setting, RUNX1 downregulation suppresses CSF1R and IL-34 expression, while CSF1R knockdown or inhibition conversely leads to an increase in RUNX1 levels, suggesting a compensatory feedback loop. This feedback mechanism appears to impede melanoma cell proliferation and invasiveness.[50] Our study supports a similar regulatory mechanism in ITP, where CSF1R inhibition in the context of ITP results in RUNX1 upregulation, thereby enhancing MK maturation and differentiation. RUNX1 encodes multiple isoforms, with RUNX1b and RUNX1c being the major variants, transcribed from the P2 and P1 promoters, respectively. RUNX1b has been shown to be essential for MK differentiation.[48] In this study, we did not distinguish specific RUNX1 isoforms (RUNX1b and RUNX1c) primarily because their gene sequences are highly overlapping. Future studies using rigorously validated primers for isoform-specific qPCR or RNA-seq will be needed to clarify the distinct functions of RUNX1 isoforms in megakaryocyte development.

In conclusion, this study identifies a novel regulatory axis between CSF1R and RUNX1 that governs megakaryopoiesis in ITP. Targeting CSF1R represents a promising therapeutic strategy to restore platelet production and improve outcomes in ITP patients.

Disclosures

No conflicts of interest to disclose.

Contributions

YH and DPW conceived and supervised the study; HHH, MZ and JQQ performed experiments, analyzed data, and drafted the manuscript; XFS, XQL, ZYZ, TTP, XYX and MTG contributed to experiments and data analysis. All authors read and approved the final version of the manuscript for publication.

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Data-sharing statement

All original data can be provided if necessary. The raw single cell sequencing data generated in this study have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE275370.

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