# DMAG, a Novel Countermeasure for the Treatment of Thrombocytopenia

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# Abstract

**Background:** Thrombocytopenia is one of the most common hematological disease that can be life-threatening caused by bleeding complications. However, the treatment options for thrombocytopenia remain limited.

**Methods:** In this study, giemsa staining, phalloidin staining and flow cytometry were firstly used to identify the effects of 3,3'-Di-O-methylellagic acid 4'-glucoside (DMAG), a natural ellagic acid derived from *Sanguisorba officinalis* L. (SOL) on megakaryocyte differentiation in HEL cells. Then, thrombocytopenia mice model was constructed by X-ray irradiation to evaluate the therapeutic action of DMAG on thrombocytopenia. Next, network pharmacology approachs were carried out to identify the targets of DMAG. Moreover, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrich-ment analyses were performed to elucidate the underling mechanism of DMAG against thrombocytopenia. Finally, Molecular docking simulation, molecular dynamics simulation and western blot analysis were used to explore the relationship between DAMG with its targets.

**Results:** DMAG significantly promoted megakaryocyte differentiation and maturation of HEL cells. DMAG administration accelerated platelet recovery and megakaryopoiesis in thrombocytopenia mice. Network pharmacology revealed that ITGA2B, ITGB3, VWF, PLEK, TLR2, BCL2, BCL2L1 and TNF were the core targets of DMAG. GO and KEGG pathway enrichment analyses suggested that the core targets of DMAG were enriched in PI3K-Akt signaling pathway, hematopoietic cell lineage, ECM-receptor interaction and platelet activation. Molecular docking simulation and molecular dynamics simulation further indicated that ITGA2B, ITGB3, PLEK and TLR2 displayed strong binding ability with DMAG. Finally, western blot analysis evidenced that DMAG up-regulated the expression of ITGA2B, ITGB3, VWF and PLEK.

**Conclusion:** DMAG plays a critical role in promoting megakaryocytes differentiation and platelets production and might be a promising medicine for the treatment of thrombocytopenia.


# Introduction

Platelets, a major type of blood cells, play a critical role in hemostasis, inflammation, thrombosis and immunity (Eto and Kunishima 2016). These small anucleated cells are the final products of megakaryocytes differentiation and maturation. Megakaryocytes (MK) derive from hematopoietic stem cells (HSCs) and undergo a continuous and complex maturation program (Nishikii et al. 2015). Megakaryocytes increase their size and become polyploid through repeating cycles of DNA replication without cell division, a process called endomitosis (Mattia et al. 2002). At the end, megakaryocytes extend the voluminous protrusions into the lumen of sinusoids, and under the shearing force of flowing blood, the protrusions are cut off and platelets shed into the blood (Patel et al. 2005). Various cytokines, chemokines, signaling pathways and transcription factors regulate MK differentiation through transcription and microenvironmental mechanisms at multiple levels. Thrombopoietin (TPO) is the most important mediators of megakaryopoiesis. It binds its receptor, c-MPL, activating a number of

downstream signaling pathways, including JAK2/STATs, MAPK/ERK, and PI3K/Akt signaling pathway (Bianchi et al. 2016; Eto and Kunishima 2016). Since megakaryocytes are located in marrow bone (BM), the BM microenvironment plays a vital role in both megakaryocyte differentiation and maturation, and platelet production (Leiva et al. 2018). For instance, the BM extracellular matrix (ECM) is an acellular component that provides physical support for hematopoiesis. The ECM matrix proteins, such as VWF, fibrinogen, fibronectin, collagen type IV and laminin bind to corresponding receptors of megakaryocyte, and regulate proplatelet reorganization and platelet formation (Guo et al. 2015).

Any abnormity in the process of megakaryocyte differentiation and maturation, as well as platelet release can lead to platelets disorders (Krishnegowda and Rajashekaraiah 2015). Particularly, thrombocytopenia, an important clinical problem that may cause by either impaired megakaryocyte maturation or insufficient platelets production (Greenberg 2017). The severe thrombocytopenia can cause intra-cerebral and intra-abdominal bleeding, which can be fatal. Currently, platelet transfusion is the most direct and effective method for treating thrombocytopenia (Stroncek and Rebulla 2007). However, due to the platelets shortage and the common occurrence of allergic transfusion reactions (ATRs), the clinical application of platelet transfusion is largely limited (Wang et al. 2017). Promoting the body's own platelets production has become the effective strategy for clinical intervention of thrombocytopenia. The most widely used drugs for increasing the level of patients' platelets is thrombopoietin receptor agonists (TPO-RA), such as Eltrombopag and Romiplostim. But the drugs are not only expensive but also can increase the risk of thromboembolic complications (Garnock-Jones 2011; Yuan et al. 2019). Therefore, it is urgent and necessary to discovery novel therapeutic drugs with high efficacy and safety for the treatment of thrombocytopenia.

*Sanguisorba officinalis* L. (SOL) has been used as herbal medicine for treating hemorrhage syndrome for a long time in Asia and Europe (Gawron-Gzella et al. 2016; Bai et al. 2019). In recent decades, SOL was used to treat myelosuppression induced by chemotherapy or radiotherapy in clinic (Ma et al. 2015). Our previous study demonstrated that the ethanol extract of SOL exhibited remarkable therapeutical effect against leukopenia in mice (Wang et al. 2020). We also found that two ellagic acids, 3,3',4-tri-O-methylellagic acid-4'-O-β-D-xyloside and 3,3',4-tri-O-methylellagic acid isolated from SOL significantly stimulated hematopoietic progenitor cell proliferation and megakaryocyte differentiation (Chen et al. 2017). However, the active compounds of SOL in promoting hematopoietic recovery and their underling molecular mechanism remain largely unknown. In the present study, we aim to investigate the effects of DMAG on megakaryocyte differentiation and maturation *in vitro*, and evaluate the therapeutic action on thrombocytopenia *in vivo*. We further seek to elucidate the molecular mechanism of DMAG in the treatment of thrombocytopenia using network pharmacology method and experimental verification.

# Materials And Methods

## Chemicals

DMAG, 3,3'-Di-O-methylellagic acid 4'-glucoside (CAS:51803-68-0, purity ≥ 98%) was obtained from Chengdu Push Bio-technology Co., Ltd (Chengdu, China).

### Cell culture

HEL cells were purchased from American Type Culture Collection (Rockville, MA, USA). The cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% $CO_2$.

### Giemsa staining

$4.0 \times 10^4$ HEL cells were seeded in 6-well plates and were treated with DMAG (10, 20 and 40 μM) for 6 days. The cells were harvested and washed with PBS for twice. Then cells were fixed with fixing solution (methanol: glacial acetic acid = 3:1 (v/v)), and stained with Giemsa solution (Solarbio, Beijing, China) for 5 min. The stained cells were finally photographed under electron microscope (10Í).

### Phalloidin staining

After treatment for 6 days, cells were harvested for F-actin staining by using phalloidin (Solarbio, Beijing, China) according to the manufacturer's instructions. In brief, the cells were fixed with 4% paraformaldehyde for 15min and transfused with 0.05% Triton X-100 for 10min at the room temperature. After that, cells were washed twice with PBS and TRITC Phalloidin (Solarbio, Beijing, China) was performed for 30min in dark at room temperature, then add DAPI to counter-stain the nucleus for 5 minutes. Finally, the representative images were captured using the inverted fluorescence microscope (Nikon Ts2R/FL, Japan).

### Flow cytometry analysis for megakaryocyte differentiation

After treating with DMAG (10, 20 and 40 μM) for 6 days, HEL cells were harvested and washed with PBS for twice, then labeled with FITC-anti-CD41 (4A Biotech, Beijing, China) and PE-anti-CD61 (BioLegend, USA) antibody on ice in the dark for 30 min. The samples were resuspended in 400 μL PBS for analysis by flow cytometry (BD Biosciences, San Jose, CA, USA).

### Megakaryocytes ploidy assay

HEL cells were treated with DMAG (10, 20 and 40 μM) for 6 days and then harvested for DNA ploidy analysis using CycleTEST™ PLUS DNA Reagent Kit (Cycletest Plus DNA Reagent, BD) according to the manufacturer's instructions. Then, we used the cell cycle analysis module of high-content screening (HCS) to detect the megakaryocytes ploidy again. Briefly, HEL cells were collected and washed twice with PBS, then cells were transferred to a 96-well plate at a density of $2 \times 10^5$cells/well. DAPI (100 nM, Solarbio, Beijing, China) was added and incubated at room temperature in the dark for 10 minutes. Lastly, the sample was detected by the ImageXpress Micro4 (Molecular Devices, USA).

### Establishment the thrombocytopenia mice model and treatment with DMAG

Specifc-pathogen-free Kunming mice (KM), 8- to 10-week-old, were purchased from Da-shuo Biotechnology Limited (Chengdu, Sichuan, China). The mice were maintained under standard condition (22 ± 2°C, 55 ± 5 % humidity and 12 hours light / dark cycle). All experimental procedures were approved by the laboratory animal ethics committee of the Southwest Medical University (Luzhou, China). Except for the control group, the other mice were irradiated by X-ray (4 Gy) to establish thrombocytopenia mice model. According to the level of peripheral blood, the mice were randomly divided into 4 groups (6 male mice and 6 female mice in each group): control group, model group, TPO positive group, DMAG group. The mice in control group and model group were intraperitoneally administered with normal saline per day. The mice in TPO positive group and DMAG group were intraperitoneally administered with TPO (3000 U/kg) or DMAG (5 mg/kg) per day. On day 0, 3, 7, 10, and 14, the blood were collected from eyes' venous plexus for hematologic parameters analysis by Hematology Analyzer (SYSMEX XT-1800Iv, Kobe, Japan). On day 10, the femurs were collected and fixed in 10% formaldehyde for 24 hours. After decalcification for a month, the femurs were embedded in paraffin and cut into 5μm thick sections. Then the samples were stained with hematoxylin and eosin (H&E). Images were captured using Olympus BX51microscope (Olympus Optical).

### Acquisition of candidate targets of DMAG against thrombocytopenia

PharmMapper (https://lilab.ecust.edu.cn/pharmmapper/index.php) and Swiss database (http://www.swisstargetprediction.ch/index.php) were used to identify targets of DMAG. The GeneCards database (https://www.genecards.org/) and DisGeNET database (http://www.disgenet.org/) were used to retrieve targets related to thrombocytopenia. The common targets of DMAG and thrombocytopenia were considered as potential targets of DAMG against thrombocytopenia. Venn diagram was drawn on Jvenn website (http://jvenn.toulouse.inra.fr/app/example.html) to obtain the overlapped targets of DMAG and thrombocytopenia. The component-target-disease network was constructed by cytoscape_v3.7.1 software.

### Construction of protein-protein interaction (PPI) network and identification of core targets of DMAG against thrombocytopenia

PPI network was constructed by STRING database (http://string-db.org) and visualized by Cytoscape_v3.7.1 software. The screening condition of core targets of DMAG was as follows: Degree was greater than or equal to twice the median, Betweenness Centrality (BC) and Closeness Centrality (CC) were greater than or equal to the median.

### Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of core targets

Database for Annotation, Visualization and Integrated Discovery database (DAVID, https://david.ncifcrf.gov/) was used to obtain Gene Ontology (GO) and Kyoto Encyclopedia of

Genes and Genomes (KEGG) pathway analyses. Visualization of GO and KEGG pathway analyses by using GraphPad Prism v9.1.0.221 software and OmicShare website (https://www.omicshare.com/tools/).

### Molecular docking simulation and molecular dynamics simulation

Molecular docking simulation was used to explore the binding ability between DMAG and its core targets. The crystal structures of core targets were obtained from the RCSB Protein Data Bank (https://bivi.co/visualisation/rcsb-protein-data-bank). Sybyl-X 2.0 software was used for structural modification of these structures, including residue modification and repair, hydrogenation and charging. The 3D structure of DMAG was constructed based on the PubChem database (https://pubchem.ncbi.nlm.nih.gov), its partial atomic charges were calculated by the Gasteiger Hückel method, energy minimizations were performed using the Tripos force field and the Powell conjugate gradient algorithm convergence criterion of 0.01 kcal/mol Å. After binding pocket was generated using the Protomol generation technique of SYBYL, the molecular docking between DMAG and core proteins were simulated by Sybyl-X 2.0(Ragunathan et al. 2018). Molecular docking simulation was visualized utilizing Pymol and Ligplus software. Molecular dynamics simulation is a widely used tool to explore the dynamic binding of compounds to proteins. We use the DMAG-protein complex obtained by molecular docking to establish a molecular dynamics model using AMBER18 software. Then, the biological macromolecule system was optimized, and the conditions were set as follows: the DMAG-protein complex was dissolved in the TIP3P water model containing $H_2O$, $Na^+$ ions and $Cl^+$ ions, and the temperature was heated to 300 K. After all optimizations are completed, a continuous simulation of 25ns will be performed.

### Western blotting

HEL cells were collected after treated with DMAG (10, 20 and 40 μM) for 4 days. Total protein was extracted by RIPA lysis buffer (CST, MA, UAS) supplemented with protease inhibitors (Sigma, St Louis, MO). Protein was quantified with the Quick Start™ Bradford 1 × Dye Protein Assay Reagent (Bio-Rad, CA, USA). An equal amount of protein (30 μg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skim milk powder in phosphate-buffered saline (PBS) for 60 min, the membrane was incubated with primary antibodies overnight at 4 °C followed by the HRP-bound secondary antibody for 60 min at 37 °C. The protein bands were visualized with ECL Western Blotting detection reagent (4A Biotech Co., Ltd., Beijing, China) and detected by the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). The proteins were quantified with ImageJ software. Primary antibodies were as follows: β-actin (CST, MA, USA), ITGB3 (Proteintech, USA), ITGA2B (Proteintech, USA), PLEK (Proteintech, USA), VWF (Proteintech, USA), BCL2 (CST, MA, UAS), BAX (CST, MA, UAS) and TNFα (CST, MA, UAS). Secondary antibodies were as follows: Mouse Anti-rabbit IgG (CST, MA, USA) and Anti-mouse IgG (CST, MA, USA). A β-actin antibody was used as a control.

# Result

## DMAG induces the differentiation and maturation of HEL cells

Given that ellagic acids derived from SOL displayed high activity in promoting megakaryocyte differentiation in our previous study, the effect of DMAG on the differentiation of HEL cells was assess in the present study. We found another ellagic acids, DMAG, derived from SOL (Supplemental S1), that a large number of big cells appeared in DMAG (20 and 40 μM)-treated groups, while little in control group after 6 days of culture (Figure 1a). Giemsa staining and phalloidin staining showed that cells in DMAG (20 and 40 μM)-treated groups had larger size and more number of nucleus than that of control group (Figure 1b and 1c). Then the expression of megakaryocytes-specific marker CD41 and CD61 was detected by flow cytometry. The results showed that the expression of CD41 and CD61 was significantly increased in DMAG-treated groups in a concentration-dependent manner compared with control group (Figure 2a). Moreover, ploidy assay revealed that DMAG treatment remarkably increased the DNA ploidy in a concentration-dependent manner (Figure 2b). Correspondingly, high-content assay showed the similar results with ploidy assay detected by flow cytometry (Figure 2c). These data suggest that DMAG has a strong ability to induce differentiation and maturation of HEL cells. Taken together, above data suggest that DMAG has a strong ability to induce differentiation and maturation of HEL cells.

## DMAG administration increased platelet count in mice with thrombocytopenia

In order to evaluate the therapeutical effect of DMAG on thrombocytopenia, the thrombocytopenia mice model was constructed by 4 Gy X-ray total body irradiation. The results showed that the platelet count in all irradiation groups reached the lowest point on day 7 (Figure 3a). However, the platelet count in DMAG-treated group and TPO group was significant higher compared with the control group (Figure 3a), indicating that DMAG administration decelerated the rate of descent of platelet after irradiation damage. The platelet counts gradually recover after reaching the lowest point and the platelet count in DMAG-treated group and TPO group was higher than that of the control group (Figure 3a), indicating that DMAG administration enhanced platelet recovery when the mice encountered irradiation. The therapeutical effect of DMAG *in vivo* was further proved by H&E staining, which demonstrated that DMAG treatment significantly increased the number of megakaryocytes in bone marrow (Figure 3b). These results suggest that DMAG administration has potential for the treatment of thrombocytopenia induce by irradiation

## Identification of core targets of DMAG against thrombocytopenia

The targets of DMAG against thrombocytopenia were predicted by network pharmacology. Through databases screening, a total of 206 targets were identified as targets of DMAG and 295 targets as targets related to thrombocytopenia. 16 common targets were considered as potential targets of DMAG for the treatment of thrombocytopenia (Figure 4a and 4b). The core targets were screened out through two screenings: using Cytoscape_v3.7.1 software, first remove the two unrelated targets, and then set the screening conditions of Degree>2, BC>0.0042735, CC>0.43333333. The top 8 target proteins of ITGA2B, ITGB3, VWF, PLEK, TNF, TLR2, BCL2, BCL2L1 were regarded as core proteins (Figure 4c).

## Enrichment analysis of the core targets of DMAG acting on thrombocytopenia

GO and KEGG enrichment analyses were performed to elucidate the underling mechanism of DMAG against thrombocytopenia. GO enrichment analysis showed that the core targets were mainly enriched in platelet degranulation, platelet aggregation, extracellular matrix organization and integrin-mediated signaling pathway (BP), cell surface, extracellular space, membrane and platelet alpha granule membrane (CC), identical protein binding, protease binding, protein homodimerization activity and protein binding (MF) (Figure 5a), which were important for development and function of megakaryocyte and platelet. KEGG enrichment analysis revealed that the core targets were significantly enriched in PI3K-Akt signaling pathway, hematopoietic cell lineage, ECM-receptor interaction and platelet activation (Figure 5b), which played a vital role in megakaryocytes differentiation and platelet production.

### Molecular docking stimulation and molecular dynamics simulation verification

To further explore the direct relationship between DMAG and its core targets, molecular docking stimulation and molecular dynamics simulation were used to predict their binding possibility. Docking score > 5 kcal·mol$^{-1}$ was regarded as a high binding strength. The information of compound-protein docking was listed in Table 1, and visualization for compound-protein combination was shown in Figure 6a. According to the docking results, the binding scores of ITGB3, ITGA2B, PLEK, TLR2 with DMAG were all greater than 6, indicating that DMAG had a good affinity with the crystal structure of these four core proteins. In the results of molecular dynamics simulation, the binding of ITGB3, PLEK and TLR2 with DMAG was all reached equilibrium state at about 15ns, 20ns and 20ns respectively (Figure 6b, supplemental GIF). BCL2 and BCL1 have low binding scores with DMAG, but they have performed well in molecular dynamics simulation (Supplemental S2, supplemental GIF). These results suggested that DMAG might directly bind to ITGB3, ITGA2B, TLR2 and PLEK.

**Table 1.** Docking score of DMAG with the core proteins.

| proteins | Docking score (kcal·mol$^{-1}$) |
|---|---|
| ITGA2B | 6.3757 |
| ITGB3 | 6.2011 |
| PLEK | 6.6829 |
| TLR2 | 6.8015 |
| BCL2L1 | 4.7380 |
| BCL2 | 3.9747 |

### Validation of the expression of core targets by western blot

According to network pharmacology analysis, the expression of core targets of DMAG against thrombocytopenia was validated by western blot. The results showed that the expressions of ITGA2B, ITGB3, VWF and PLEK, which were related to megakaryocytes differentiation and platelet production were obviously up-regulated induced by DMAG (Figure 7). The results indicated that DMAG might stimulate

megakaryocyte differentiation and platelet production via activation of PI3K-Akt, hematopoietic cell lineage, ECM-receptor interaction and platelet activation signaling pathway.

# Discussion

Thrombocytopenia is a very common blood disorder that is caused by multiple reasons, such as radiotherapy and chemotherapy treatments. Severe thrombocytopenia can lead to bleeding that is fatal. Whoever, there are still no effective and safe drugs for the rapid treatment of thrombocytopenia. SOL, a well known traditional herbal medicine, has long been used for the treatment of various wounds, particularly burns, internal haemorrhage, inflammatory, cancers and metabolic diseases (Zhao et al. 2017). In our previous study, we found that SOL and its ellagic acids had remarkably activities against leukopenia and in promoting megakaryocyte differentiation, respectively (Chen et al. 2017; Wang et al. 2020). In the present study, we demonstrated that another ellagic acid derived from SOL, DMAG, significantly promoted megakaryocyte differentiation *in vitro* and stimulated platelet formation *in vivo*. Combined with network pharmacology analysis and experimental verification, we explored the targets and elucidated the underling molecular mechanism of DMAG against thrombocytopenia.

We first evaluated the pro-differentiation activity of DMAG *in vitro*. After the HEL cells were treated with DMAG, the cell size, the expression of megakaryocytes-specific marker CD41 and CD61, number of nucleus and DNA ploidy were both remarkably increased, indicating DMAG could promote megakaryocyte differentiation and maturation. Since megakaryocytes are the precursors of platelets, the acceleration of megakaryocyte differentiation induced by DMAG may be conducive to platelet formation. We thereby identified the therapeutic effects of DMAG on thrombocytopenia. As expected, DMAG administration significantly accelerated platelet recovery in mice with thrombocytopenia induced by X-ray irradiation. The increased number of platelets either caused by enhanced megakaryopoiesis and platelets production, or decreased platelets destruction or clearance. H&E staining results revealed that DMAG elevated the number of BM megakaryocytes in mice with thrombocytopenia. The results of *in vitro* and *in vivo* experiments proved that DMAG ameliorated radiation-induced thrombocytopenia in mice at least partly through promotion of megakaryopoiesis and megakaryocyte differentiation. This therapeutic effect of DMAG was similar to nanocurcumin, human growth hormone (hGH) and insulin-like growth factor-1 (IGF-1), which promoted megakaryocyte differentiation *in vitro* and platelet recovery in irradiated mice (Xu et al. 2014; Chen et al. 2018; Mortazavi Farsani et al. 2020).

Network pharmacology was carried out to identify the targets of DMAG against thrombocytopenia. We found 8 proteins (ITGA2B, ITGB3, VWF, PLEK, TNF, TLR2, BCL2 and BCL2L1) were the core targets of DMAG for the treatment of thrombocytopenia. Integrins are cell surface receptors that play a crucial role in both platelet activation, adhesion and aggregation (Guidetti et al. 2015). The ITGA2B gene, also called CD41, encodes for the αIIb which exclusive express in megakaryocytes, platelets and some hematopoietic progenitor cells. The ITGB3 gene, also known as CD61, encodes for β3. These two proteins can form a fibrinogen receptor, αIIbβ3, an integrin that is crucial for platelet aggregation through binding of soluble fibrinogen (Nurden et al. 2011). The homozygous mutations in ITGA2B or ITGB3 locus could cause Glanzmann thrombasthenia, a bleeding disorder (Nurden et al. 2013). The heterozygous activating

mutations in the membrane-proximal region of the αIIb and β3 subunit could lead to congenital macrothrombocytopenia (Ghevaert et al. 2008; Kunishima et al. 2011; Nurden et al. 2011). Now, CD41 and CD61 were regard as early markers of megakaryocyte differentiation (Psaila et al. 2016). Von Willebrand factor (VWF), a large multimeric adhesive glycoprotein, is a well-known mediator of platelet-vessel wall interaction and platelet-platelet interactions under high shear-stress conditions. Reduced or dysfunctional levels of VWF can lead to inherited von Willebrand disease (VWD), an inherited bleeding disorder (Ruggeri 2007). In type 2B VWD, gain-of-function mutations in VWF cause enhanced binding of mutated VWF multimers to platelets through a direct interaction with its receptor GPIba (Bury et al. 2019). Emerging studies had demonstrated that VWF and its receptor played a critical role in megakaryocytopoiesis and platelet production. Abnormalities of GPIb-IX-V expression or an abnormal interaction between newly synthesized VWF with GPIb-IX-V in the megakaryocytes of a family with VWD2B caused by VWF R1308P lead to impaired megakaryocytopoiesis and thrombocytopenia (Nurden et al. 2006; Nurden et al. 2010). VWF promoted proplatelet formation (PPF) and platelet production when the human megakaryocytes exposure to high shear rates (Poirault-Chassac et al. 2013). The expression of VWF has been considered as a sensitive and distinct marker for megakaryocytes (Tomer 2004). Pleckstrin (PLEK), a prominent substrate of protein kinase C (PKC) in platelets and leukocytes, has long been considered as a marker of platelet activation (Lian et al. 2009). Previous study had demonstrated that inhibition of PKC by bisindolylmaleimide, GF109203X, a highly selective inhibitor of PKC, suppressed the expression of CD61, phosphorylation of pleckstrin, and megakaryocyte differentiation induced by phorbol 12-myristate 13-acetate (PMA) (Hong et al. 1996). Pleckstrin-null platelets from a pleckstrin null knockout mouse exhibited a marked defect in granule secretion, aggregation, actin polymerization and mild thrombocytopenia (Lian et al. 2009). Toll-like receptors (TLRs) are essential components of the innate immune response and are activated upon interaction with different pathogen-associated molecular patterns (PAMPs). There are 13 TLRs in humans and mice. Among them, TLR1, TLR2, TLR4 and TLR6 are both expressed on megakaryocytes and platelets. TLR2, which forms functional heterodimers with either TLR1 or TLR6, recognizes a wide spectrum of microbial pathogenassociated molecules, such as virus, fungal, bacterial pathogens and their ligands (LP and Schattner 2017). It has been reported that stimulation of Meg-01 cells by Pam3CSK4, a specific synthetic TLR2 ligand, activating NF-kappaB, ERK-MAPK, and PI3K/Akt pathways, which leads to up-regulation of transcription factors associated with megakaryocyte maturation, thereby increasing megakaryocyte ploidy. In addition, after the mice are treated with Pam3CSK4, the platelet level initially drops and then returns to normal level, accompanied by an increase in megakaryocyte maturation (Beaulieu et al. 2011). Recent studies have shown that stimulation of Dami cells by heat killed lacto bacillus (HKL), another TLR2 ligand, leading to up-regulation of TLR2 and cytokine production, mainly IL-6, which is essential for megakaryocyte generation and CD41 expression. Additionally, TLR2 induction activates wnt b-catenin signalling pathway components, indicating a cross talk between wnt and TLR pathway leading to megakaryocyte maturation (Undi et al. 2016). BCl-$X_L$ is the key pro-survival protein that is essential for survival of megakaryocyte and platelet (Josefsson et al. 2020). Megakaryocyte-specific deletion of BCl-$X_L$ in mice triggers megakaryocyte apoptosis and a failure of platelet shedding, which leading to severe thrombocytopenia (Josefsson et al. 2011). The megakaryocytic lineage-specific deletion of both MCL1 and BCL-XL causes

embryonic lethality in association with failure of megakaryopoiesis and systemic haemorrhage (Kodama et al. 2012). Tumour Necrosis Factor-a (TNF-a), a pro-inflammatory cytokine, plays an important role in inflammation, anti-tumor responses and homeostasis. It is well known that TNF-a can regulate a wide spectrum of biological processes, such as cell proliferation, differentiation and apoptosis (Tian et al. 2014). These studies demonstrate the critical role of above core targets of DMAG in regulating megakaryocyte differentiation and platelet production. In order to explore the direct relationship between DMAG and its core targets, molecular docking stimulation and molecular dynamics simulation were performed. The results showed that DMAG had a strong binding ability with ITGB3, ITGA2B, PLEK and TLR2, indicating that DMAG might regulate megakaryocyte differentiation and platelet production by directly binding to ITGB3, ITGA2B, PLEK and TLR2.

Through GO enrichment analysis, we found that the above targets were mainly associated with platelet degranulation, platelet aggregation, extracellular matrix organization and integrin-mediated signaling pathway (BP), cell surface, extracellular space, membrane and platelet alpha granule membrane (CC), identical protein binding, protease binding, protein homodimerization activity and protein binding (MF), which were all involved in development and function of megakaryocyte and platelet (Bianchi et al. 2016; Eto and Kunishima 2016; Leiva et al. 2018). According to KEGG enrichment analysis results, the core targets of were mainly enriched in PI3K-Akt signaling pathway, hematopoietic cell lineage, ECM-receptor interaction and platelet activation. PI3K-Akt pathway is one of the most crucial signaling pathways that is activated in response to various types of stimulations, including cytokines, growth factors, hormones, integrin and extracellular matrix (ECM) proteins. This pathway regulates a wide spectrum of cellular processes, such as cell cycle, proliferation, survival, differentiation and death (Guidetti et al. 2015; Moroi and Watson 2015). It is known that PI3K-Akt pathway is located downstream of TPO/MPL signaling pathway, activating several transcription factors to derive megakaryocyte differentiation and maturation, as well as platelet production (Bianchi et al. 2016). Emerging evidences have demonstrated that PI3K-Akt pathway is also activated by several platelet receptors, including GPIb-IX-V, ITAM-bearing receptors, G-protein-coupled receptors, and integrins, that regulates platelet activation and haemopoiesis (Guidetti et al. 2015). The ECM is the non-cellular structure that provide tissue cohesion and rigidity. The BM ECM is crucial for normal hematopoiesis. It contains variety of proteins, including fibrinogen, collagens, fibronectin and laminin, as well as multiple soluble proteins, such as chemokines, cytokines and secreted enzymes. Different ECM components possess diverse function in regulating megakaryocyte development and platelet formation (Leiva et al. 2018). For instance, type III and IV collagens stimulate megakaryocyte maturation and platelet production via the PI3K/Akt signaling pathway (Abbonante et al. 2017). Fibrinogen binds to αIIbβ3 on the surface of megakaryocyte to stimulate proplatelet formation and platelet release (Larson and Watson 2006). In the present study, through western blot validation of the expression of core targets, we found that these signaling pathways were activated by DMAG.

Based on previous studies and our findings, we can conclude that DMAG promotes megakaryocyte differentiation and platelet production via directly binding to ITGB3, ITGA2B and TLR2, thereby activation of PI3K-Akt signaling pathway, hematopoietic cell lineage, ECM-receptor interaction and platelet activation.

# Conclusions

In summary, the present study demonstrated that DMAG, a natural ellagic acid derived from SOL could significantly promote megakaryocyte differentiation *in vitro* and enhance platelet recovery in mice with thrombocytopenia. Furthermore, via network pharmacology method and experimental verification, our study also confirmed that the anti-thrombocytopenia activity of DMAG might be mediated by activating PI3K-Akt signaling pathway, hematopoietic cell lineage, ECM-receptor interaction and platelet activation. Altogether, our results suggest that DMAG may be a curative agent for the treatment of thrombocytopenia.

# Declarations

### Supplementary information:

Additional file: **Figure S1.** Identification of DMAG from SOL. (a) Total ion chromatogram of SOL, (b) UV chromatogram at 254nm of DMAG, (c) Fragmentation patterns of DMAG. **Figure S2.** Molecular docking (a) and molecular dynamics simulation (b) of DMAG with its core proteins. **Figure S3.** Origin data of Western blot analysis in Figure 7. **GIF S1.** The GIF of molecular dynamics simulation of DMAG with ITGA2B, **GIF S2.** The GIF of molecular dynamics simulation of DMAG with PLEK, **GIF S3.** The GIF of molecular dynamics simulation of DMAG with TLR2, **GIF S4.** The GIF of molecular dynamics simulation of DMAG with BCL2, **GIF S5.** The GIF of molecular dynamics simulation of DMAG with BCL2L1.

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Not applicable.

### Author contributions

Jianming Wu and Long Wang designed the research. Jing Lin and Xin Shen performed *in vitro* experiments and network pharmacology. Jing Zeng contributed to UHPLC-MS analysis. Long Wang and Sha Liu carried out *in vivo* experiments. Nan Jiang, Yuesong Wu and Hong Li was responsible for molecular docking stimulation and molecular dynamics simulation verification. Jing Lin and Long Wang wrote the original manuscript. Jianming Wu supervised the research.

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### Ethics approval and consent to participate

All the animal care and experimental procedures were approved by laboratory animal ethics committee of the Southwest Medical University (Luzhou, China).

### Consent for publication

All of the authors have consented to publication of this research.

### Competing interests

The authors declare that they have no competing interests.

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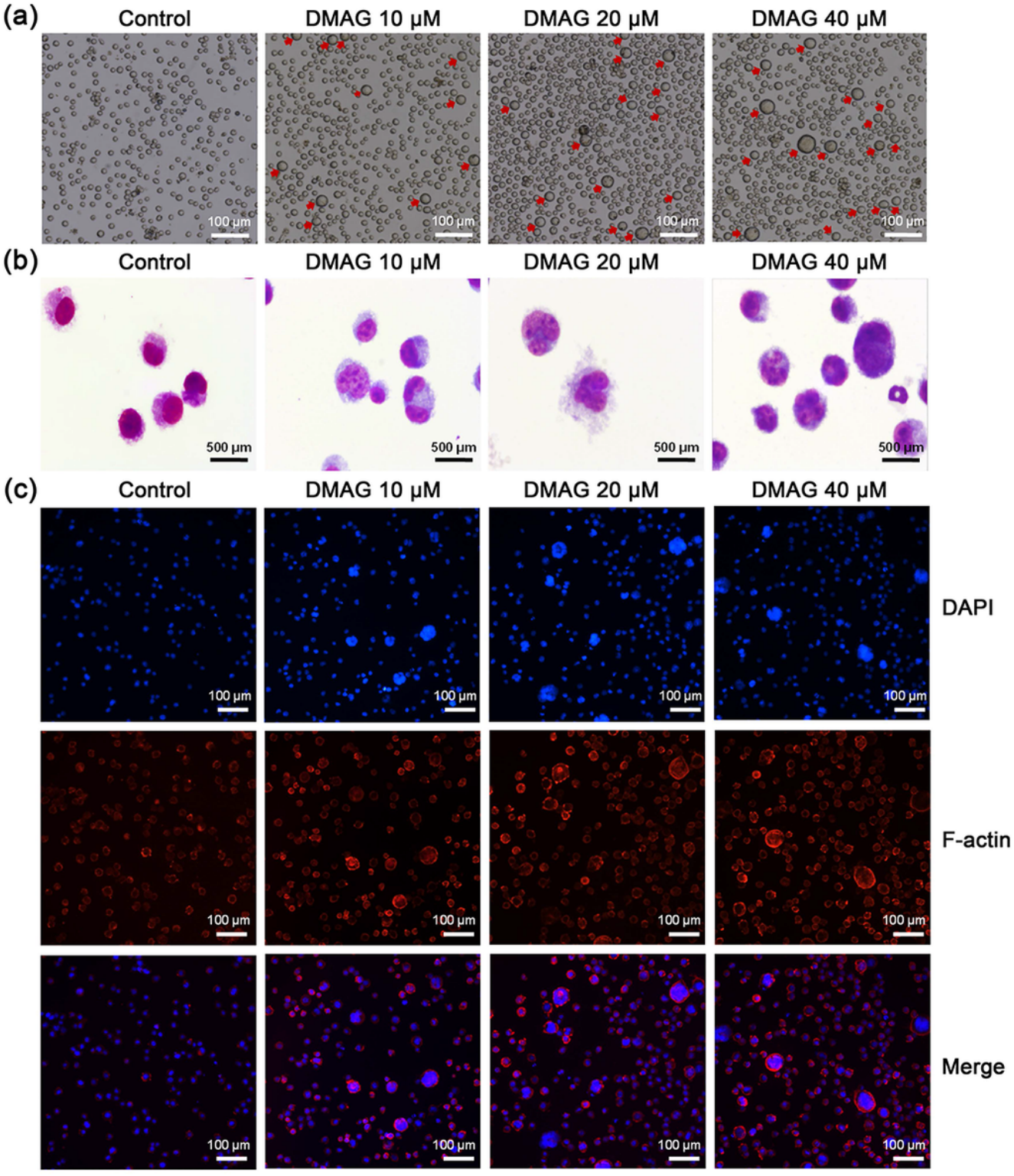
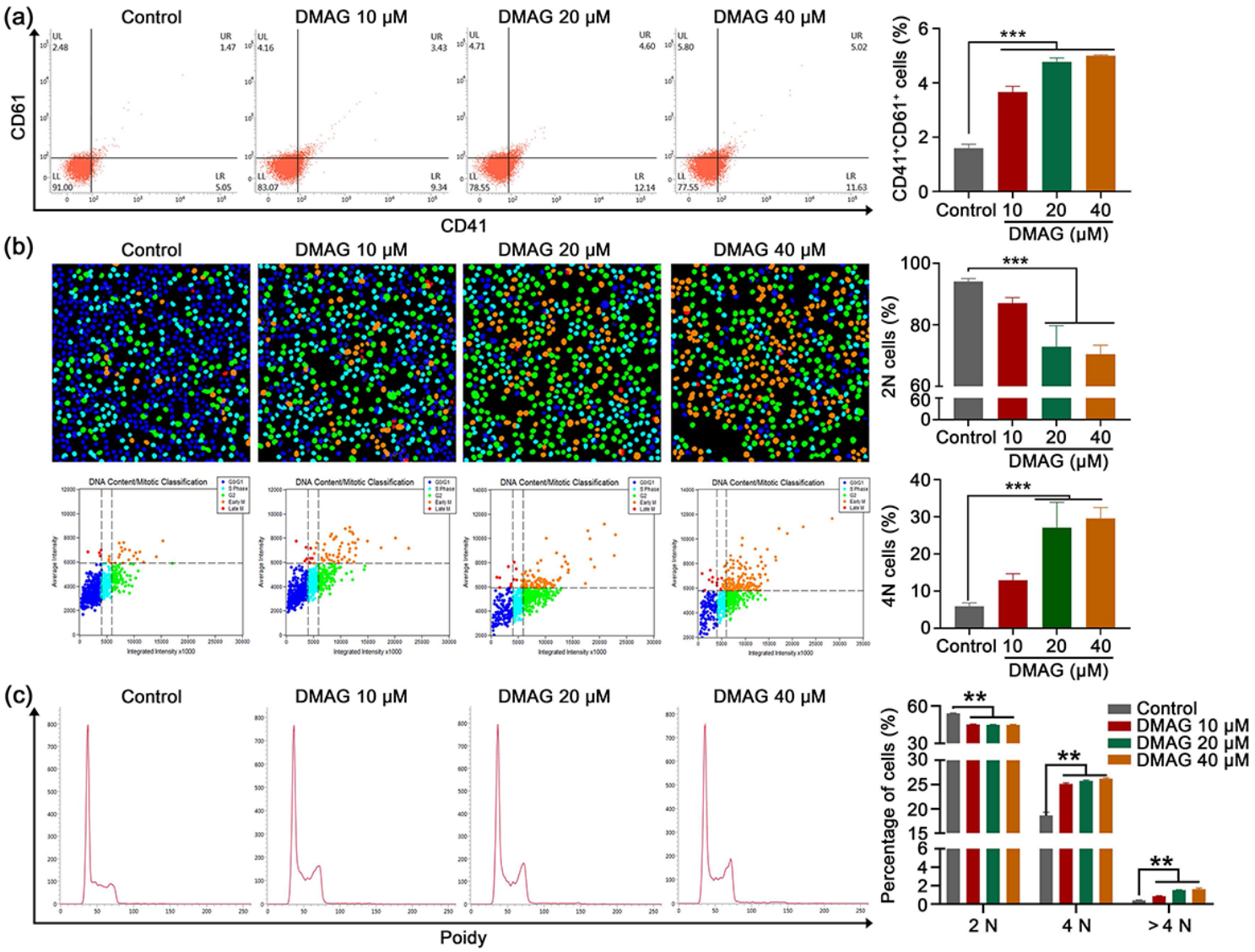
# Figures



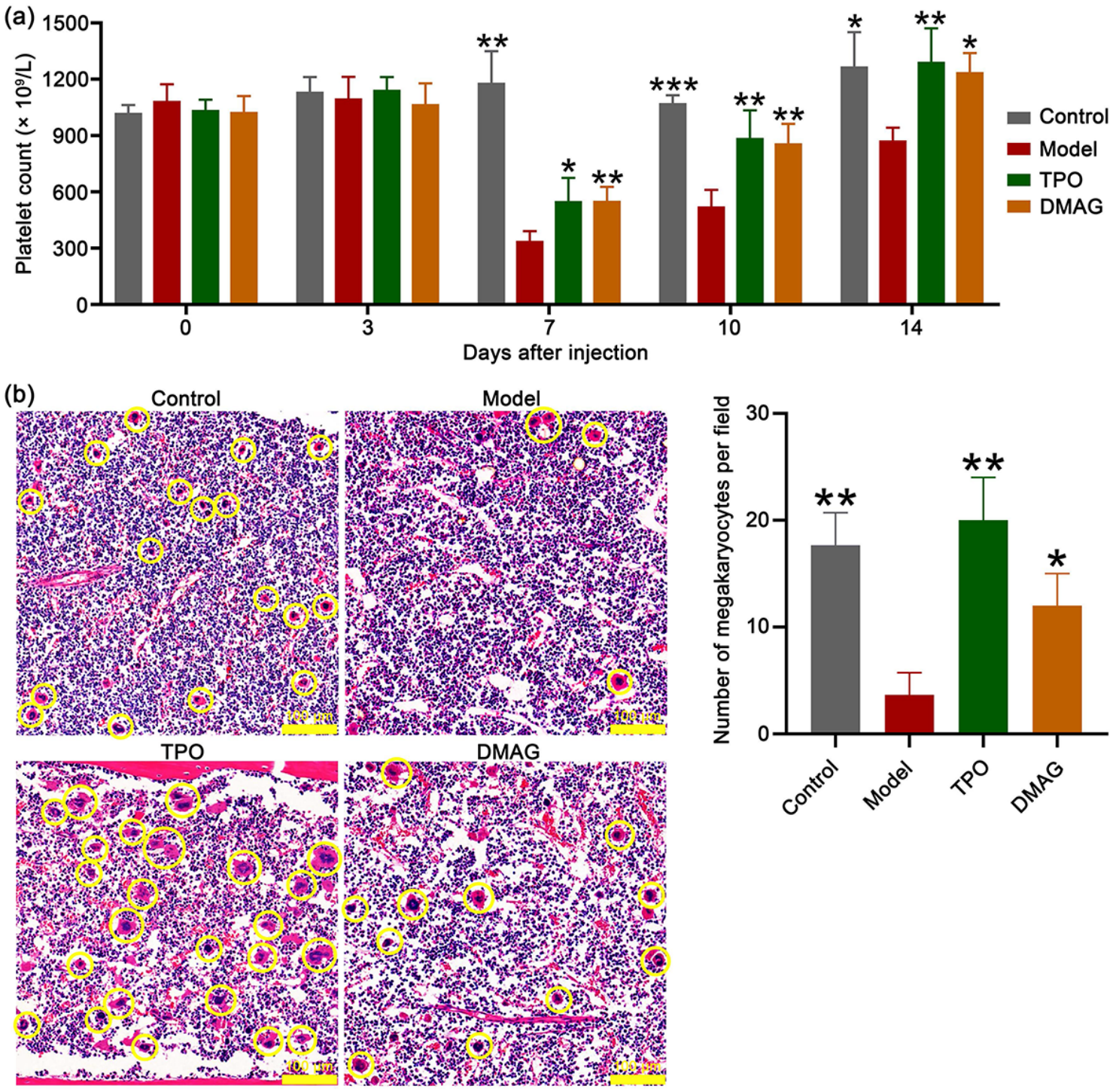

## Figure 1

Morphological changes of HEL cells influenced by DMAG. (a) Morphological changes induced by DMAG (10, 20 and 40 μM). Scale bar: 100 μm. (b) Giemsa staining showed the multinucleation of HEL cells treated with or without DMAG (10, 20 and 40 μM) for 6 days. Scale bar: 500 μm. (c) Phalloidin staining of HEL cells treated with or without DMAG (10, 20 and 40 μM) for 6 days. Scale bar: 100 μm.




## Figure 2

DMAG induces the differentiation and maturation of HEL cells. (a) The expression of CD41 and CD61 after the cells treated with DMAG (10, 20 and 40 μM) for 6 days. (b) High Content Screening analysis of DNA ploidy after the cells treated with or without DMAG (10, 20 and 40 μM) for 6 days. (c) Flow cytometry analysis of DNA ploidy after the cells treated with or without DMAG (10, 20 and 40 μM) for 6 days. The data represent the mean ± SD of three independent experiments, **p < 0.01, ***p < 0.001, compared to the control group.




## Figure 3

DMAG administration enhances recovery of platelet level in mice with thrombocytopenia. (a) Platelet counts of normal or irradiated mice after injecting with normal saline, TPO (3000 U/kg), or DMAG (5mg/kg), respectively. (b) Representative images of H&E staining of BM from normal, model, TPO and DMAG groups. Scale bar: 100 μm. (c) Quantification of BM megakaryocytes in each group. The data represent the mean ± SD of three independent experiments, *p < 0.05, **p < 0.01, compared to the model group.

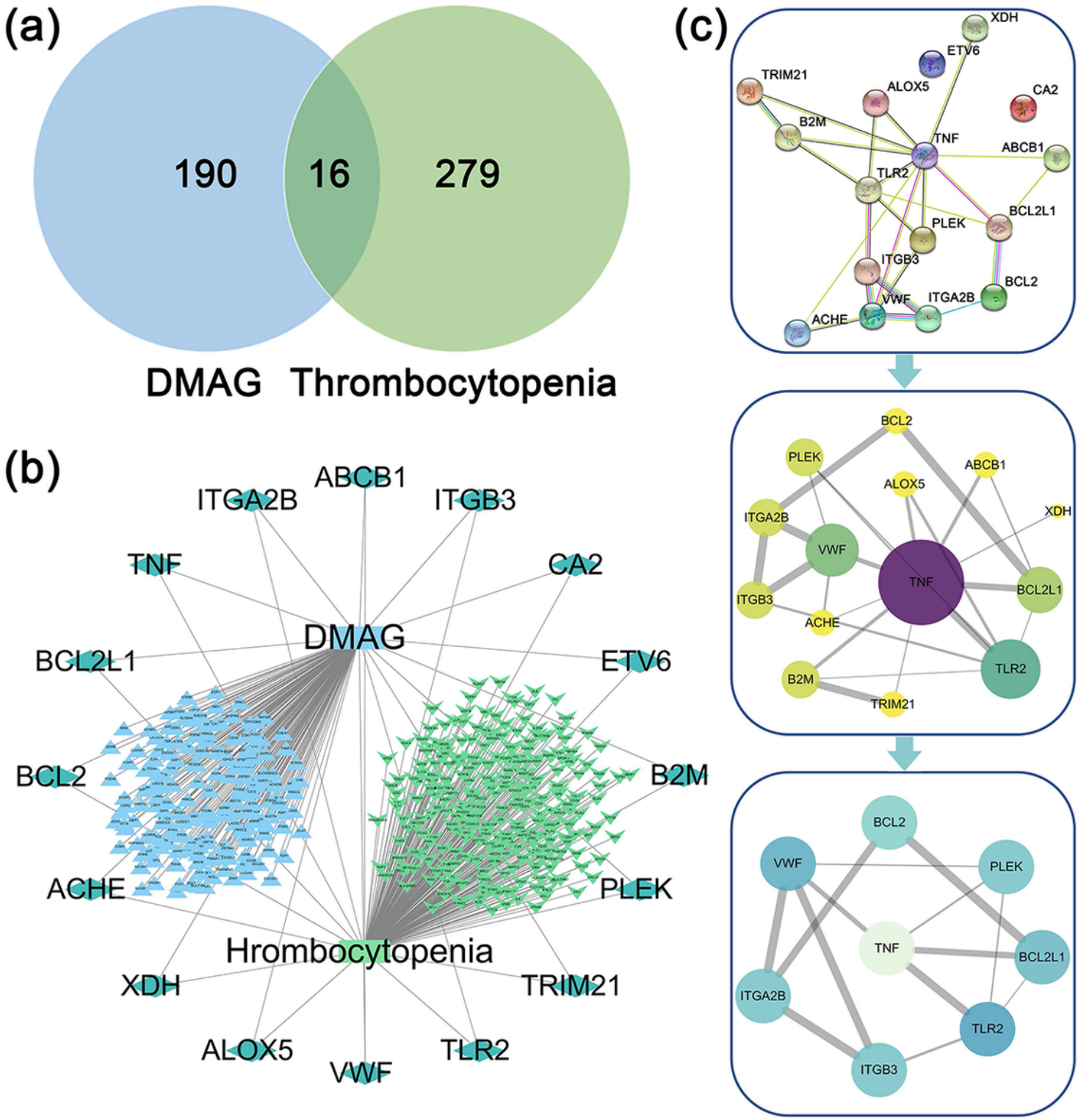
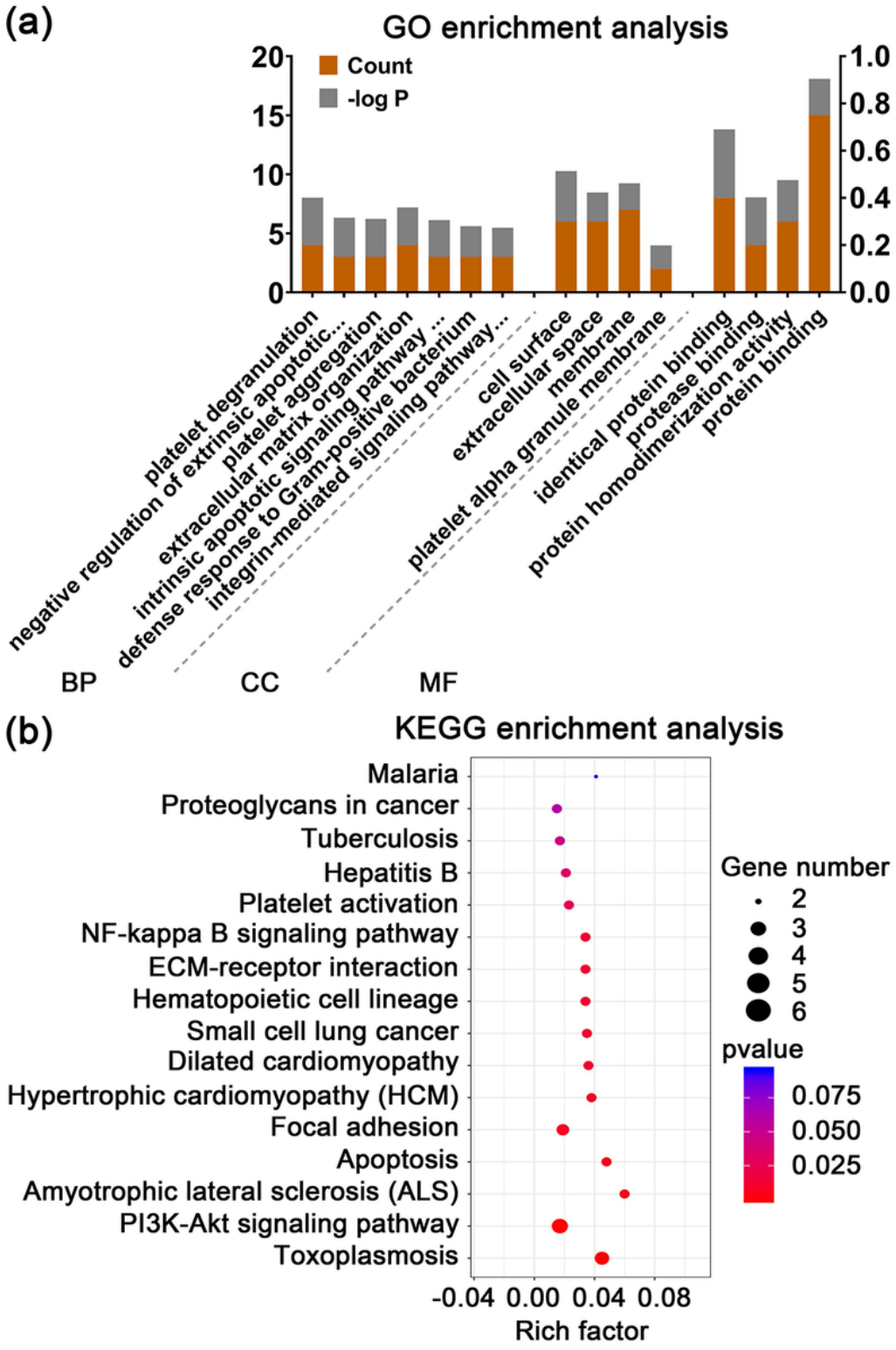


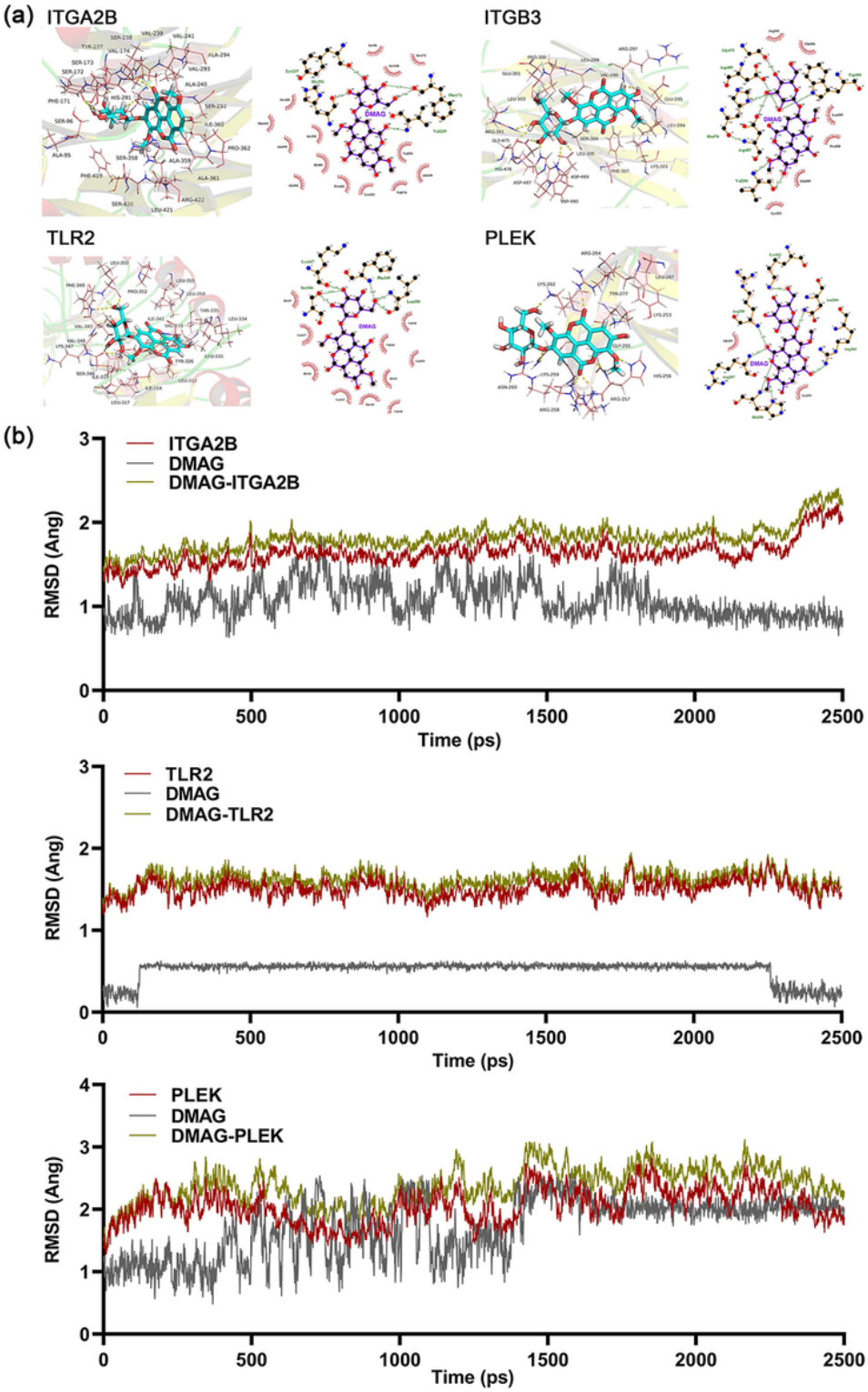

**Figure 4**

Prediction of the targets of DMAG against thrombocytopenia by network phar-macology. (a) Venn diagram shows the common targets of DMAG and thrombocytopenia. (b) The DMAG-targets-thrombocytopenia network. (c) PPI network for identifying core targets of DMAG against thrombocytopenia.




## Figure 5

GO (a) and KEGG (b) enrichment analysis of the core targets of DMAG against thrombocytopenia. BP: biological process. CC: cellular component. MF: molecular function.




## Figure 6

Molecular docking (a) and molecular dynamics simulation (b) shows the interaction and the binding ability between DMAG and its core targets.

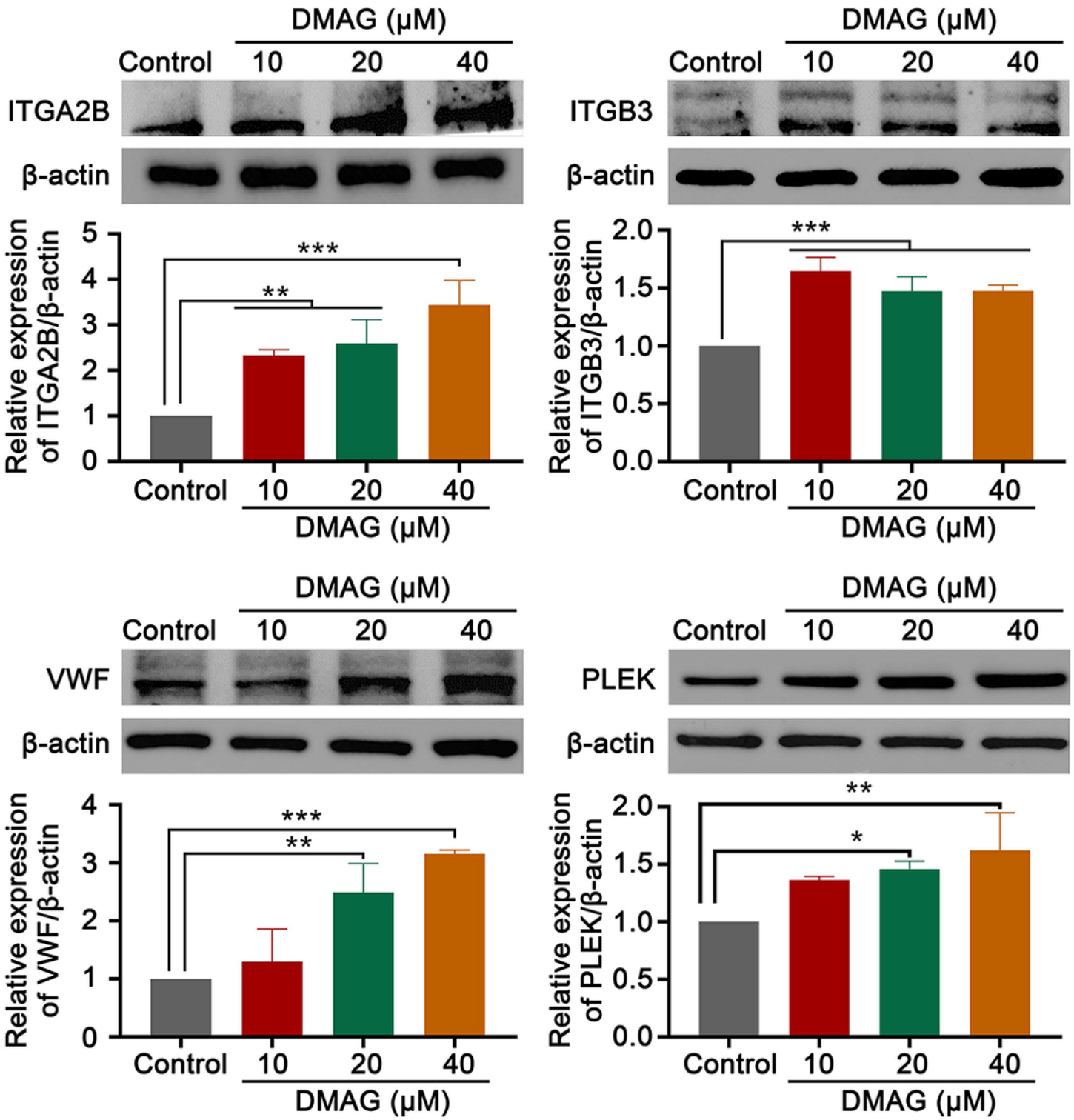



**Figure 7**

Western blot analysis of expression of core targets of DMAG. The data represent the mean ± SD of three independent experiments, *p < 0.05, **p < 0.01, ***p < 0.001, compared to the control group.

## Supplementary Files

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