Thrombosis Research 183 (2019) 69–75

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Thalidomide prevents antibody-mediated immune thrombocytopenia in mice

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ARTICLE INFO

Keywords:
Thalidomide
Immune thrombocytopenia
Macrophage
Cell viability, cell cycle
Cyclin E2

ABSTRACT

Immune thrombocytopenia (ITP) is a heterogeneous autoimmune disorder characterized by immune-mediated platelet destruction, leading to lower platelet count. Thalidomide is considered as a novel immunomodulatory drug for treating several autoimmune diseases. Whether thalidomide can ameliorate ITP remains unclear. This study aims to evaluate the effect of thalidomide on ITP mouse model. ITP mouse model was established through intraperitoneal injection of rat anti-mouse integrin GPIIb/CD41 immunoglobulin. Thalidomide (10, 20 or 50 mg/kg body weight) was intraperitoneally injected into mice followed by antibody injection. Then, peripheral blood and plasma was isolated for analysis of platelet count and the level of IFN-γ and IL-17 in plasma. Meanwhile, spleen was extracted to measure the expression of CD68, a macrophage marker. In addition, macrophage cell line RAW264.7 was cultured and treated with thalidomide followed by analysis of cell viability, apoptosis as well as cell cycle. Thalidomide prevented antiplatelet antibody-mediated platelet destruction in ITP mouse model. Compared with vehicle (phosphate-buffered saline), thalidomide significantly inhibited the secretion of IFN-γ and IL-17 in ITP mouse and reduced the expression of CD68 in spleen. After thalidomide treatment, the cell viability of RAW264.7 cell was significantly reduced and the cell number in S phase was also significantly decreased. In addition, the expression of cyclin E2 was significantly reduced. In conclusion, thalidomide prevents antiplatelet antibody-mediated platelet destruction in ITP mouse possibly through reducing the number of macrophages, suggesting that it might be a novel approach for treating ITP.

1. Introduction

Immune thrombocytopenia (ITP) is a heterogeneous autoimmune disorder, characterized by antiplatelet autoantibodies-mediated decreased platelet production and increased platelet destruction, resulting in a low platelet count (thrombocytopenia), putting patients on a risk of bleeding [1,2]. The bleeding manifestations of ITP are highly heterogeneous with intracranial hemorrhage being the most feared complication [3,4].

The development and pathogenesis of ITP is complex, involving multiple factors. Previous studies indicate that the main cause of ITP is the immunoglobulin G (IgG) autoantibodies against platelets. These IgG autoantibodies bind to platelet to form an antigen-antibody complex, which will then be phagocytosed and destroyed by macrophages in the reticuloendothelial system in the spleen through engagement of Fc receptor (FcR) [5,6]. Beyond antiplatelet autoantibodies, cellular immune dysregulation has also been demonstrated to play roles in ITP pathogenesis, including T cells, B cells, or antigen presenting cells [5,7]. As an immune response regulator, abnormal differentiation and function of T cells (Th1, Th2, Th17, Th22 cells) or related cytokines (IFN-γ, IL-4, IL-17 or IL-22) have been shown to be closely associated with ITP [8–11]. In addition, our previous study showed that Th9 cells and IL-9 plasma level was increased in newly diagnosed ITP patients, and reduced in ITP patients with remission [12], suggesting Th9/IL-9 might

Abbreviations: IFN-γ, Interferon-γ; IL-17, Interleukin-17; ITP, Immune thrombocytopenia; PHA, phytohaemagglutinin

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https://doi.org/10.1016/j.thromres.2019.09.035
Received 15 May 2019; Received in revised form 6 August 2019; Accepted 18 September 2019
Available online 22 October 2019
0049-3848/

also be involved in the pathogenesis of ITP.

Thalidomide, a synthetic glutamic acid derivative first marketed in 1954, was originally prescribed as a sedative and antinausea medicine in pregnant women [13], but was withdrawn from the market due to severe birth defects which were observed in patients after administration of thalidomide [14,15]. After being withdrawn from market > 4 decades, thalidomide was approved by the US Food and Drug Administration (FDA) in 1998 as it exerted anti-inflammatory effect during the treatment of erythema nodosum leprosum, a painful inflammatory complication of leprosy [16]. In recent years, thalidomide and its analogues (CC5013 and CC-4047) are widely used as novel immunomodulatory drugs for treating several diseases, such as lupus erythematosus [17], inflammatory bowel disease [18], solid tumors [19], as well as hematologic malignancies [20]. The molecular mechanism by how thalidomide modulates immune response is complex and thought to be through cytokine elaboration, anti-inflammatory effect on TNF-α, IL-1β or others, anti-angiogenesis, or regulation of the function of immune cells, such as T cells, natural killer cells [21].

Considering an autoimmune disorder of ITP and the immunomodulatory effects of thalidomide, whether thalidomide can prevent antibody-mediated ITP remains poorly understood. In this study, we aimed to investigate the effect of thalidomide on ITP mouse model, which was established through injection of antiplatelet antibody.

2. Materials and methods

2.1. Animals

C57BL/6 mice, aged 8–12 weeks and weighed 24–28 g, were bought from SLAC LABORATORY ANIMAL (Shanghai, China). The mice were housed in SPF grade environment in the Experimental Animal Center of Xuzhou Medical University with free access to food and water. All experimental procedures were complied with the ARRIVE guidelines and approved by the Ethnic Committee of Xuzhou Medical University (Xuzhou, China).

2.2. Establishment of ITP mouse model and treatment

ITP mouse model was induced as previously described [22]. In brief, mice ($n = 8$) received intraperitoneal injection of anti-platelet monoclonal antibody (rat anti-mouse integrin GPIIb/CD41 immunoglobulin, clone MWReg30) (BD Biosciences) at a dose of 0.1 mg/kg body weight to establish ITP model. At different time point after antibody injection, whole blood samples were collected from tail vein for measuring platelet count using an automatic hematology analyzer (BC-5300, Mindray, Shenzhen, China).

Different concentrations of thalidomide (10, 20 or 50 mg/kg body weight) was intraperitoneally injected into mice ($n = 8$) followed by antibody injection to induce ITP model. Normal mice receiving injection of vehicle (phosphate-buffered saline, PBS) were served as control group.

2.3. Plasma isolation

Peripheral blood was drawn from the retro-orbital venous plexus at 6 h post antibody injection and mixed with anticoagulant solution (EDTA). Blood was centrifuged at 2000 rpm for 20 min at room temperature and the supernatant (plasma) was collected and then stored at −80 °C until further analysis.

2.4. ELISA

Secretion level of IFN-γ and IL-17 in plasma was measured by commercial ELISA kit (eBioscience) according to manufacturer's instructions.

2.5. Immunohistochemical staining

CD68 expression in spleen was measured by immunohistochemical staining as described previously [23]. Spleen was isolated and fixed with formaldehyde solution, dehydrated, waxed, and sliced into 4 μm thickness by RM2126 microtome. Slices were then incubated with 3% H_2O_2 at room temperature and blocked with 5% goat serum. Then the slices were incubated with anti-CD68 antibody (Abcam, Cambridge, MA, USA) and subsequently incubated with HRP-conjugated secondary antibody. Color was developed with 3,3′-diaminobenzidine. The CD68 expression (%) was calculated as the ratio of CD68 positive cells to total cells in each field. Four random fields were selected.

2.6. Cell line

Macrophage cell line RAW264.7 cells were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.7. Analysis of cell viability by CCK-8 assay

CCK-8 assay (Dojindo, Laboratories, Japan) was performed to analyze cell viability as described previously [24]. In brief, RAW264.7 cells were seeded into 96-well plates and treated with different concentrations of thalidomide (0, 1, 10 50 and 100 μg/ml) for 48 h in the RPMI 1640 medium containing 10% FBS followed by addition of CCK-8 to measure the absorption value at a wavelength of 450 nm in a microplate reader.

2.8. Measurement of cell apoptosis

Cell apoptosis was measured using 7AAD and Annexin-V-PE (Ebioscience, San Diego, CA, USA) as described previously [24]. Briefly, RAW264.7 cells were seeded into 24-well plates and treated with different concentrations of thalidomide (0, 1, 10 50 and 100 μg/ml) for 48 h. Then, cells were collected, resuspended in 100 μl binding buffer and incubated with 5 μl Annexin V for 15 min at room temperature under dark. After that, cells were incubated with 5 μl 7-AAD for 10 min and then cell apoptosis was measured by flow cytometry (Calibur, BD, USA).

2.9. Detection of cell cycle

After treatment with different concentrations of thalidomide, cells were collected, washed with PBS and fixed in 70% ice cold ethanol for 2 h at −20 °C. After washing once, cells were resuspended in 250 μl PBS containing 10 μl PI (1 mg/ml) and 10 μl RNase A (1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 min under dark. At last, cellular DNA was assessed by flow cytometry as described previously [24]. Cell cycle distribution was analyzed using ModFit LT 3.3 software.

2.10. Western blot

Total protein was isolated from RAW264.7 cells after thalidomide treatment using RIPA lysis buffer, separated on 10% SDS-PAGE and transferred to an NC membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membranes were then blotted with anti-cyclin D1 (Cell Signaling Technology, Danvers, MA, USA) or cyclin E2 antibody (Affinity Biosciences, Cincinnati, OH, USA). Bound antibody was visualized after incubation with HRP-conjugated secondary antibody and subsequent enhanced chemiluminescence.

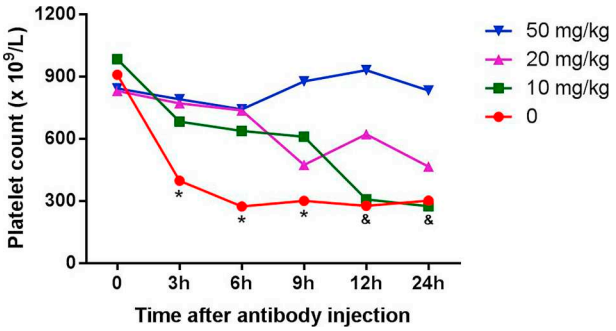

Fig. 1. Platelet count in ITP mouse model. Mice received treatment of different concentrations of thalidomide followed by intraperitoneal injection of anti-platelet antibody. Peripheral blood was extracted at different time point for measuring platelet count. Data were presented as mean. Compared with 10, 20 and 50 mg/kg, $^{*}P < 0.05$; Compared with 20 and 50 mg/kg, $^{\&}P < 0.05$.

2.11. Statistical analysis

Data were processed using GraphPad Prism software and presented as mean ± standard error (SE). Student *t*-test was used to compare the difference between two groups. For comparison of difference among different groups, data were assessed by one-way ANOVA. *P* value < 0.05 was considered to be statistically significant.

3. Results

3.1. Thalidomide ameliorates thrombocytopenia in ITP mouse model

To investigate whether thalidomide affects ITP, mice were treated with different doses of thalidomide (0, 10, 20 or 50 mg/kg) followed by injection of anti-platelet GPIIb/IIIa antibody to trigger antibody-mediated platelet destruction, which is similar to platelet destruction in ITP patients. As seen in Fig. 1, mice without thalidomide treatment showed a dramatic decrease of platelet count after administration of anti-platelet GPIIb/IIIa antibody and reached a nadir at 6 h post injection followed by a steady level until 24 h. However, thalidomide treatment (10 and 20 mg/kg) significantly delayed antibody-mediated the decrease of platelet count and higher dose of thalidomide (50 mg/kg) could prevent antiplatelet antibody-induced thrombocytopenia. Taken together, these data showed that thalidomide inhibited antiplatelet antibody-induced immune thrombocytopenia in mice model.

3.2. Decreased IFN-γ and IL-17 level after thalidomide treatment in ITP mouse

A previous study showed that thalidomide significantly inhibited pro-inflammatory cytokine IFN-γ secretion from phytohaemagglutinin (PHA)-stimulated human peripheral blood mononuclear cell cultured in vitro [25]. As IFN-γ is shown to play roles in the pathogenesis of ITP, we next measured whether thalidomide affects IFN-γ secretion in ITP mouse and showed a significantly higher level of IFN-γ in ITP mouse model (26.81 ± 2.82 pg/ml) compared with control mice (13.58 ± 2.00 pg/ml) ($P < 0.05$) (Fig. 2A), which was consistent with elevated IFN-γ level in ITP patients. However, thalidomide treatment significantly reduced IFN-γ level in ITP mouse (16.62 ± 1.66 pg/ml) compared with that in ITP mouse treated with vehicle (Fig. 2A). As IL-17 was reported to be involved in ITP [5], we also detected IL-17 level after thalidomide treatment. Similar to the expression pattern of IFN-γ, a significantly increased IL-17 level was observed in ITP mouse model and was dramatically reduced after treatment with thalidomide (Fig. 2B). These data indicated that thalidomide exerts anti-inflammatory effects on ITP mouse.

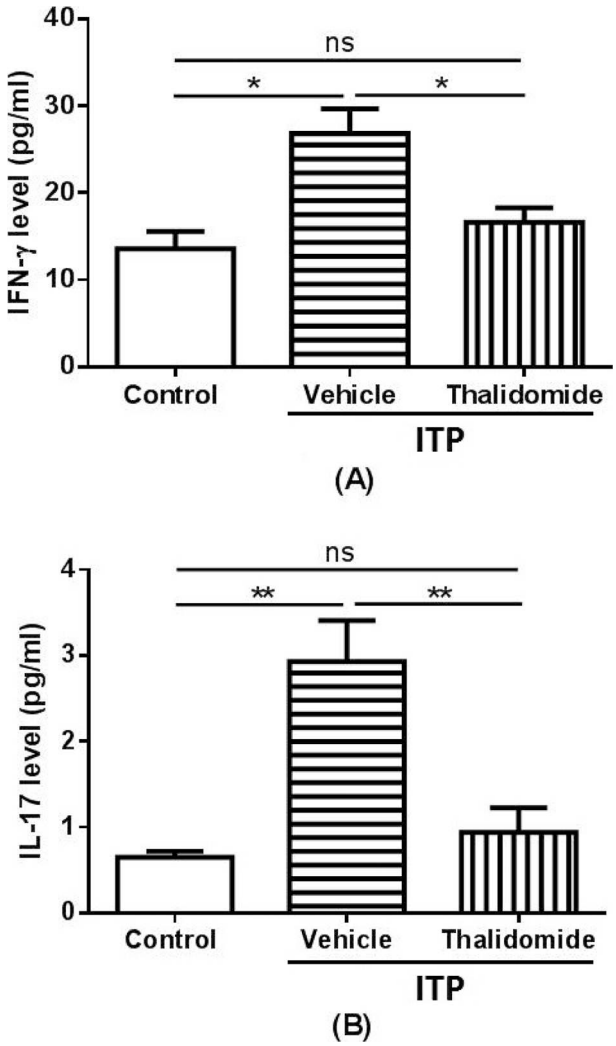

Fig. 2. Level of IFN-γ and IL-17 in the plasma. Plasma was isolated from control normal mice or ITP mouse treated with vehicle or thalidomide for measuring the level of IFN-γ (A) and IL-17 (B) by ELISA. $^{*}P < 0.05$; $^{**}P < 0.01$.

3.3. Thalidomide reduces the number of macrophages in ITP mouse

As the thrombocytopenia in ITP mouse was induced by antiplatelet antibody, we next evaluated whether thalidomide affected macrophages as they are the main cells responsible for the phagocytosis of antiplatelet antibody-opsonized platelets in ITP mouse, leading to lower platelet count. Through measuring the expression of CD68 in the spleen, a macrophage marker, by immunohistochemical staining, we found that CD68 expression was significantly increased in ITP mouse model compared with control mice (Fig. 3), indicating antibody induced the increase of macrophages, leading to accelerated phagocytosis and subsequently lower platelet count. However, CD68 expression was significantly reduced after thalidomide treatment (50 mg/kg), suggesting that thalidomide reduced antibody-induced the increase of the number of macrophages.

3.4. Thalidomide reduces cell viability of macrophage in vitro

To further investigate the effects of thalidomide on macrophage, we cultured mouse macrophage cell line RAW264.7 in the presence or absence of different doses of thalidomide followed by measuring cell viability by CCK-8 assay. As seen in Fig. 4A, cell viability of macrophage was significantly reduced after thalidomide treatment in a dose-dependent manner. To rule out the non-specific effect of thalidomide,

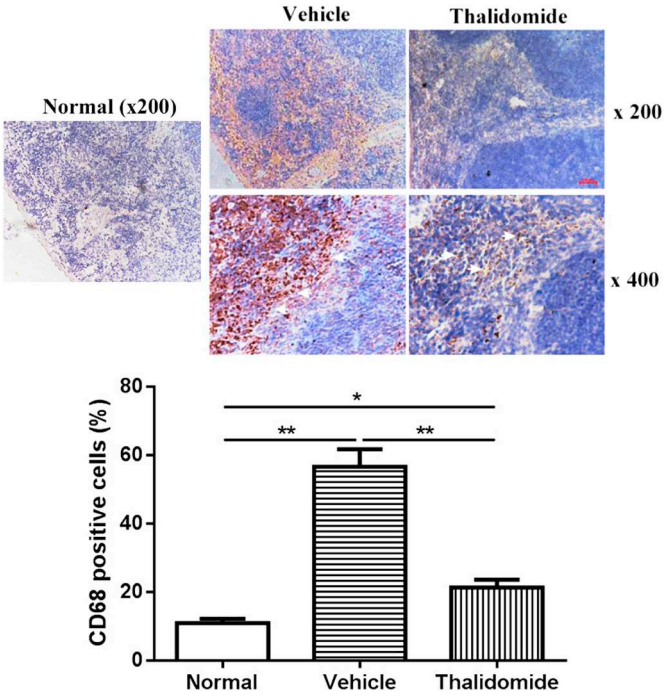

Fig. 3. Immunohistochemical staining analysis of CD68 expression in the spleen. Spleen was extracted from ITP mouse model treated with vehicle or thalidomide followed by measuring the expression of CD68 by immunohistochemical staining. Scale: 100 μm for ×200 and 50 μm for ×400. Arrow indicated the positive staining of CD68 cells. *$P < 0.05$; **$P < 0.01$.

we also measured the thalidomide's effect on viability of mouse fibroblast cell line L929 cells and found no significant changes of viability of L929 cells after thalidomide treatment (data not shown). Next, to evaluate whether reduced cell viability was due to cell apoptosis, we measured apoptosis of RAW264.7 cell after thalidomide treatment and found no significant difference of apoptotic rate of thalidomide-treated RAW264.7 cells compared with vehicle-treated cells (Fig. 4B).

3.5. Thalidomide affects the cell cycle of macrophage

As thalidomide reduces viability of RAW264.7 cells, we next investigated the effect of thalidomide on cell cycle of RAW264.7 cells and found that the cell number in S phase was significantly decreased after thalidomide treatment in a dose-dependent manner (Fig. 5), which was consistent with reduced cell viability of RAW264.7 cells after thalidomide treatment.

3.6. Thalidomide reduces the expression of cyclin E2 in macrophage

As thalidomide affects the cell cycle of macrophage, we next measured the expression of cyclin D1 and E2, which are important players in the regulation of cell cycle. As seen in Fig. 6, thalidomide treatment significantly decreased the expression of cyclin D1 (only for the higher concentration of thalidomide) and cyclin E2, indicating that thalidomide reduces the percentage of macrophages in S phase through reducing the expression of cyclin E2, which was consistent with the role of cyclin E2 in the regulation of progression of cells through G1 to S phase as demonstrated by the decreased percentage of cells in G1 and increased cells in S-phase after overexpression of cyclin E2 in human osteosarcoma cell line Saos-2 cells [26].

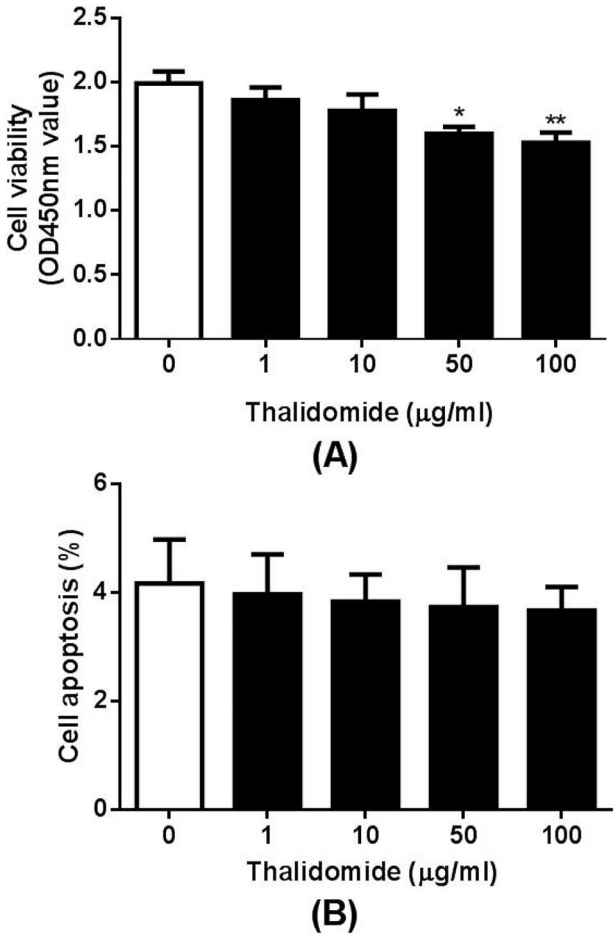

Fig. 4. Cell viability and apoptosis of RAW264.7 after thalidomide treatment. RAW264.7 cells were treated with different concentrations of thalidomide followed by analysis of cell viability by CCK-8 assay (A) and cell apoptosis by flow cytometry (B). Data were presented as mean ± SE ($n = 6$ for each group). Compared with 0, *$P < 0.05$; **$P < 0.01$.

4. Discussion

Immune thrombocytopenia (ITP) is an autoimmune disorder and characterized by lower platelet counts resulting from accelerated platelet destruction or impaired platelet production [2]. The pathogenesis of ITP is complex, involving several factors, among whose macrophage-mediated phagocytosis and subsequent destruction of anti-platelet antibody-opsonized platelets in the reticuloendothelial system in the spleen through engagement of Fc receptors plays a critical role in the development of ITP [5,6]. However, except Fc receptors, other factors might also be involved in the pathogenesis of ITP [5]. Thalidomide has been widely used as immunomodulatory drugs for treating several autoimmune diseases through regulation of inflammatory cytokines or immune cells [21]. Since ITP is an autoimmune disorder, whether thalidomide could be used for treating ITP remains poorly understood. In the present study, through establishing passive ITP mouse model, we investigated whether thalidomide plays a role in platelet destruction in vivo and showed that thalidomide prevents antiplatelet antibody-mediated platelet destruction or clearance in ITP mouse model.

Apart from commonly used in treating several cancers such as multiple myeloma and myelodysplastic syndrome (MDS), thalidomide have also been applied in the treatment of various inflammatory or

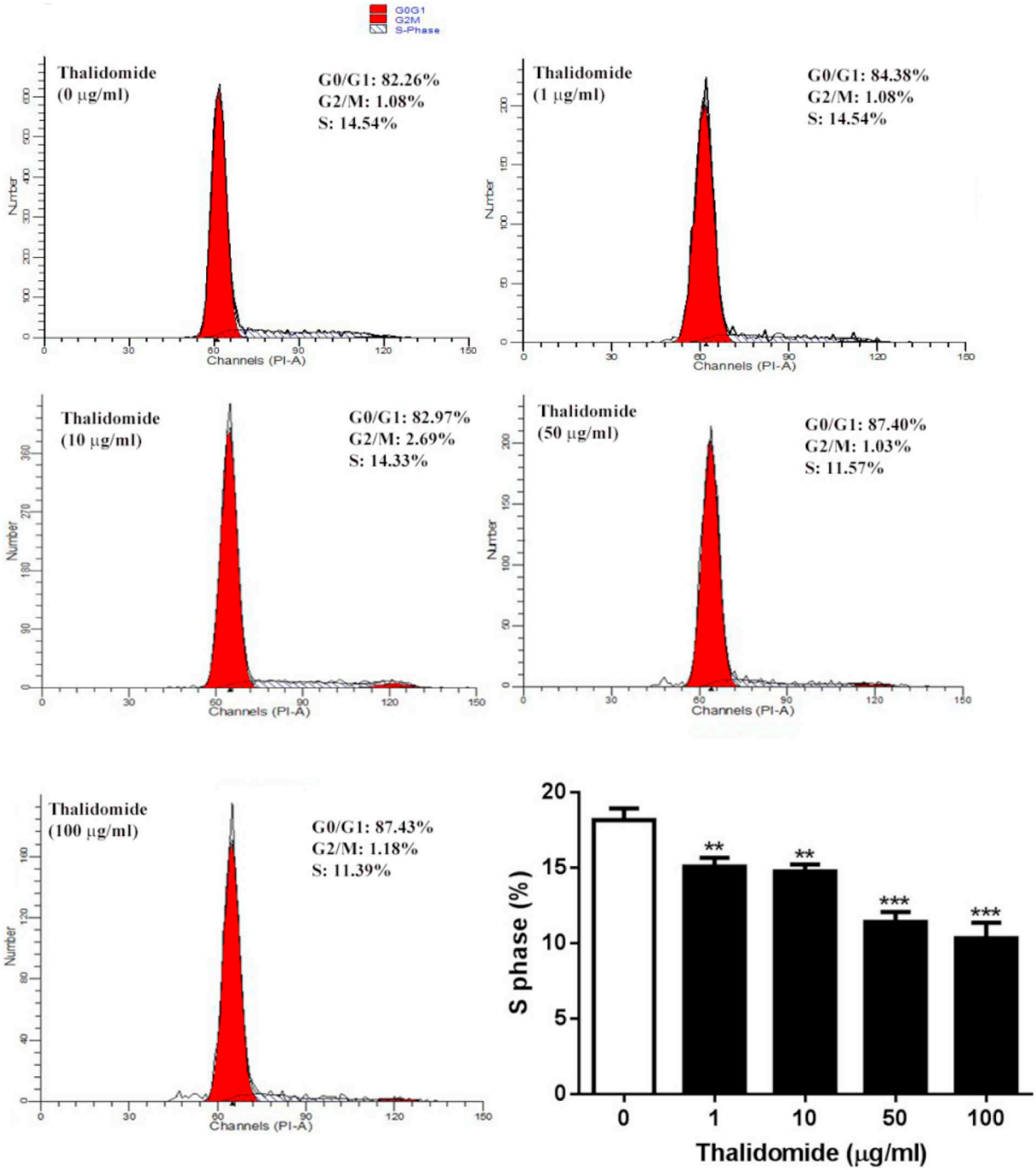

Fig. 5. Cell cycle analysis after thalidomide treatment. RAW264.7 cells were treated with different concentrations of thalidomide and cell cycle was measured by flow cytometry. Data were presented as mean ± SE ($n = 6$ for each group). Compared with 0, $^{*}P < 0.05$; $^{**}P < 0.01$.

autoimmune diseases, such as skin pathology associated with Hansen's disease/leprosy, systemic lupus erythematosus and inflammatory bowel disease [27]. The main mechanism by how thalidomide exerts anti-inflammatory effects might be through inhibition of tumor necrosis factor and NF kappa B activation in response to stimulation of inflammatory agents, as well as downregulation of cell adhesion molecules LFA-l and ICAM-l [28,29]. As several inflammatory cytokines have also been demonstrated to play critical roles in the pathogenesis of ITP, such as IFN-γ and IL-17 [7], we also investigated whether thalidomide could inhibit the inflammation in ITP mouse. Firstly, we evaluated whether antiplatelet antibody injection into mice stimulates the secretion of proinflammatory cytokines which are seen in ITP patients and found a significantly higher level of IFN-γ and IL-17 in ITP mouse model compared with control mice. However, thalidomide treatment significantly reduced antiplatelet antibody-induced the increase of IFN-γ secretion in ITP mouse model. In addition, IL-17 level was also significantly inhibited after thalidomide treatment in ITP mouse, which was consistent with previous studies showing that treatment of cord and adult blood samples with thalidomide (100 mg/ml) reduced the intracytoplasmic pro-inflammatory cytokine production from neonatal monocytes and the IFN-γ production from neonatal lymphocytes [30] or peripheral blood mononuclear cells [25]. This study suggests that thalidomide can exert anti-inflammatory effects on ITP mouse model which is possibly through inhibiting the secretion of IFN-γ and IL-17.

In ITP patients, antiplatelet autoantibodies bind to platelet to form an immune complex and is phagocytosed or cleared by macrophages in

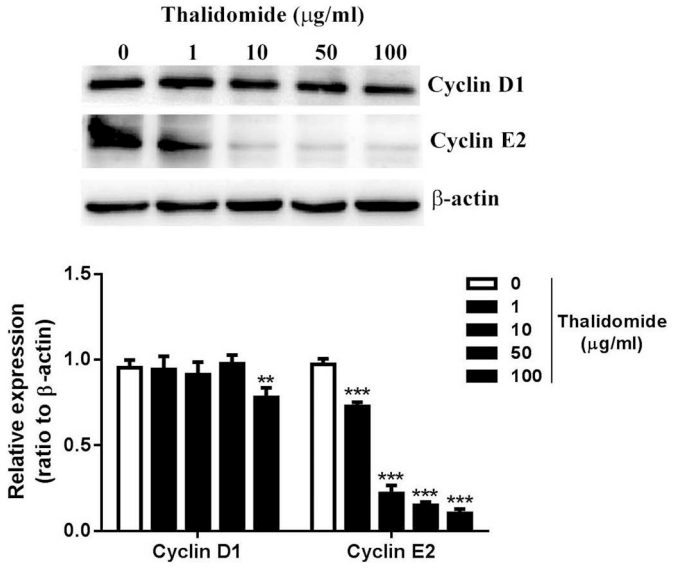

Fig. 6. Expression of cyclin D1 and E2 in RAW264.7 cells. After treated with thalidomide, protein was extracted from RAW264.7 cells followed by analysis of the expression of cyclin D1 and E2 by western blot. Data were presented as mean ± SE ($n = 6$ for each group). Compared with 0, $^{**}P < 0.01$; $^{***}P < 0.001$.

the spleen, leading to a lower platelet count, suggesting that macrophages play an important role in the pathogenesis and development of ITP [6]. A previous study showed that thalidomide had a potent suppressive effect on alternative activation of macrophages in vitro and in asthma mouse model [31]. However, whether thalidomide affect macrophage function in ITP mouse model remains unclear. In the present study, we established passive ITP mouse model through injection of antiplatelet antibody to investigate the effect of thalidomide on ITP and showed that thalidomide treatment prevents antiplatelet antibody-mediated platelet destruction in ITP mouse model. Considering the role of macrophage in platelet clearance, we next assessed the effect of thalidomide on macrophage and found that thalidomide treatment significantly reduced the number of macrophage in the spleen of ITP mouse model as demonstrated by decreased immunohistochemical staining of CD68, a macrophage marker. To further investigate the effect of thalidomide on macrophage, we cultured RAW264.7 cells in the presence or absence of different concentrations of thalidomide and found that cell viability was significantly reduced after thalidomide treatment in a dose-dependent manner. In addition, thalidomide treatment reduced the number of cells in S phase and the expression of cell cycle-related molecule cyclin E2, indicating that thalidomide reduces the viability of macrophage possibly through decreasing cyclin E2 expression.

There are limitations in our present study. One is that our data are partially transferable as the mouse model is a passively induced ITP model and only immunological changes caused by passively induced thrombocytopenia are considered. Another limitation is that whether thalidomide plays a role or affects the existing immune thrombocytopenia remains unclear as thalidomide was administrated prior to antibody injection.

In conclusion, our study demonstrates that thalidomide prevents antiplatelet antibody-mediated platelet destruction through reducing the viability of macrophage, suggesting that it might be used as a novel approach for treating ITP in clinic.

Declaration of competing interest

All authors have no conflict of interest to declare.

Acknowledgement

This research was supported by National Natural Science Foundation of China (grant no. 81400082, 81570096, 81500097, 81641151, 81700178 and 81970124), the Natural Science Foundation of Jiangsu Province (grant no. BK20140219), the funding for the Distinguished Professorship Program of Jiangsu Province, the Shuangchuang Project of Jiangsu Province, the Six Talent Peaks Project in Jiangsu Province (WSN-133), the 333 Projects of Jiangsu Province (BRA2017542), Key University Science Research Project of Jiangsu Province (18KJA320010 and 17KJA320008), Jiangsu Province's Key Provincial Talents Program (ZDRCA2016054), Jiangsu Province's Graduate Scientific Research Innovation Program (KYCX18-2186, KYCX19-2231 and KYCX19-2234) and Youth Science and Technology Innovation Team of Xuzhou Medical University.

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