International Immunopharmacology 67 (2019) 287–293

Contents lists available at ScienceDirect

International Immunopharmacology

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

Effect of recombinant human thrombopoietin on immune thrombocytopenia in pregnancy in a murine model

Yang Liu[a,1], Rui Wang[a,1], Panpan Han[a], Yajing Zhao[a], Guijie Li[b], Guosheng Li[a], Mu Nie[a], Lingjun Wang[a], Jian Chen[c], Xuena Liu[d,*], Ming Hou[a,e,f,**]

[a] Department of Hematology, Qilu Hospital, Shandong University, Jinan, China
[b] Department of Interventional Diagnosis and Treatment, Shandong Provincial Qianfoshan Hospital Affiliated to Shandong University, Jinan, China
[c] Department of Hematology, Jining No. 1 People's Hospital, Jining, China
[d] Department of Rheumatology, Qilu Hospital, Shandong University, Jinan, China
[e] Shandong Provincial Key Laboratory of Immunohematology, Qilu Hospital, Shandong University, Jinan, China
[f] Leading Research Group of Scientific Innovation, Department of Science and Technology of Shandong Province, Qilu Hospital, Shandong University, Jinan, China

ARTICLE INFO

Keywords:
Recombinant human thrombopoietin
Immune thrombocytopenia in pregnancy
Murine model
Treg
TGF-β1

ABSTRACT

Primary immune thrombocytopenia (ITP) is a serious medical disorder that has the potential for maternal and fetal mortality. Corticosteroids, intravenous immunoglobulin, or both are the first-line treatments for ITP in pregnancy, but choices are limited if patients fail to respond. Recombinant human thrombopoietin (rhTPO) has been proved effective and safe in management of chronic ITP. However, the efficacy and safety of rhTPO for pregnant ITP patients still need to be explored. Here we developed an ideal murine model that simulated human ITP in pregnancy and evaluated the efficacy and safety of rhTPO in management of ITP in pregnancy. Model mice were subcutaneously administered with 0, 150, 1,500 and 15,000 U/kg rhTPO for 14 days. Significant higher platelet counts were noted in rhTPO-treated groups on Day 7, 10 and 14. On Day 20, half the maternal mice were sacrificed. Frequencies of Tregs in $CD4^+$ T cells in rhTPO-treated groups were statistically higher than control. Significant higher plasma levels of TGF-β1 were observed in rhTPO-treated groups than control. There was no significant abnormality in gross or visceral examination of fetuses. The remaining half maternal mice and their pups were observed for at least three weeks to assess vital signs. No abnormal signs were noted.

Furthermore, we investigated the underlying mechanisms. Results showed that Tregs were negative for c-Mpl and rhTPO had no direct effect on Tregs. Additionally, the Treg frequency in splenic $CD4^+$ T cells in LY2109761-treated model mice was statistically lower than control. Thus, rhTPO may be a safe and effective option for treatment of pregnant ITP patients.

1. Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune disease characterized with a low platelet count and mucocutaneous bleeding [1,2]. The incidence of ITP in the adults is 5–10/100,000, while among pregnant women the incidence increases to 10–100/100,000 [3–5]. Thrombocytopenia affects 6% to 10% of all pregnant women and is the most common hematologic disorder in pregnancy other than anemia [6]. Although only about 3% of these cases are due to ITP [7], ITP in pregnancy affects maternal, fetal or neonatal outcomes, so it is important to accurately diagnose and appropriately manage ITP in pregnancy.

The pathologic mechanism of ITP in pregnancy is the same with ITP in adults, which is primarily due to autoantibody-mediated platelet destruction [8]. A growing body of evidence suggests that impaired production of platelets also contributes to ITP [9,10]. The anti-platelet antibodies can directly bind to megakaryocytes, causing destruction of megakaryocytes and decrease of platelet production [11–13]. Thrombopoietin (TPO) is the main factor that promotes the production of platelets. It binds to its receptor c-Mpl on the megakaryocyte membrane to stimulate megakaryocyte proliferation and differentiation as well as platelet production [14–18]. However, instead of compensative increase, TPO keeps at an inappropriately low level in ITP patients, which may further contribute to the impaired platelet production [19–22].

* Correspondence to: X. Liu, Department of Rheumatology, Qilu Hospital, Shandong University, Jinan 250012, China.
** Correspondence to: M. Hou, Department of Hematology, Qilu Hospital, Shandong University, Jinan 250012, China.
E-mail addresses: clxy1986@126.com (X. Liu), qlhouming@sina.com (M. Hou).
[1] Y.L. and R.W. contributed equally to this study.

https://doi.org/10.1016/j.intimp.2018.12.032
Received 25 August 2018; Received in revised form 4 December 2018; Accepted 13 December 2018
Available online 17 December 2018

These findings suggest that TPO has the potential to become a treatment option for ITP in pregnancy.

The management of ITP in pregnancy is quite complex, and to reach optimum therapeutic effect requires collaboration of obstetricians, hematologists and neonatologists. Primary treatment options for ITP in pregnancy are similar to those for adult ITP patients [23]. Corticosteroids, intravenous immunoglobulin, or both are the first-line treatments for ITP in pregnancy, but choices are limited if the patients fail to respond.

A series of domestic researches have confirmed the efficacy and safety of recombinant human full-length glycosylated TPO (rhTPO) in management of chronic ITP [24]. And rhTPO has been recommended for the treatment of chronic adult ITP patients by the experts in China for many years [25,26]. There are some related reports on rhTPO in management of ITP in pregnancy, which demonstrated it is effective, fast-onset and has no side effects on mother and fetus [27,28]. However, the efficacy and safety of rhTPO in management of ITP in pregnancy still need reliable support by study on murine model.

In this study, we established a murine model of ITP in pregnancy and treated with different doses of rhTPO for 14 consecutive days. We evaluated the health conditions of dams and pups, the teratology of fetuses and monitored the platelet counts of both dams and pups. In addition, many recent studies have reported that, besides their direct role in stimulating platelet production from megakaryocytes, TPO-receptor agonists (RAs) have demonstrated additional effects on immune-regulation [29,30]. Regulatory T cell (Treg) and regulatory B cell (Breg) activity has been identified to be remarkably enhanced in TPO-RA treated ITP patients [31,32]. We previously observed a considerable increase of transforming growth factor (TGF)-β1 in TPO-RAs treated ITP patients [33]. Thus we further examined Tregs in spleen as well as TGF-β1 levels in plasma of the murine model. Additionally, the underlying mechanism was investigated.

2. Materials and methods

2.1. Mice

All the mice were purchased from the Laboratory Animal Center of Shandong University. Eight-week-old female wild type mice (C57BL/6J background) weighing about 20 g were mated with the same-age male wild type mice (C57BL/6J background). The presence of sperm in the vaginal smear and/or a mating plug were considered evidence of successful mating, and the day was recorded as Day 0 of gestation. The rest of female mice remained in cohabitation with males until the desired number (40 per group) of mated females was reached. Another 30 model mice were used for assessment of ITP murine model and investigation of rhTPO's effect on Tregs. Animal studies were approved by the Shandong University Ethics Committee for Animal Experiments.

2.2. Induction of immune thrombocytopenia in pregnant mice

We established the passive ITP pregnant mouse model by administering rat anti-CD41 antibody (MWReg30) (BD PharMingen; CA, USA) at a dose that was adjusted depending on the platelet counts to achieve a relatively stable platelet destruction mouse model [34]. Briefly, the ITP group received tail intravenous injection of MWReg30 diluted in 200 μL of phosphate buffer saline (Yuan da jing mei; Jinan, China) every other day. The dose of MWReg30 was 5 μg/mouse on Day 0 and 2, 7.5 μg/mouse on Day 4 and 6, and 10 μg/mouse from Day 8 to Day 14. The isotype control group received injection of an equal volume and dose of rat IgG1 (BD PharMingen; CA, USA).

2.3. Treatment regimen of rhTPO on mice

The rhTPO (3SBIO; Shenyang, China) was diluted in normal saline. The pregnant mice with ITP were randomly assigned to four groups receiving the following treatments: the control groups were treated with normal saline; the low-dose group were treated with 150 U/kg rhTPO; the mid-dose group were treated with 1500 U/kg rhTPO; and the high-dose group were treated with 15,000 U/kg rhTPO. The test items were administered subcutaneously for 14 consecutive days beginning from Day 1 on which day platelet counts reached nadir. Platelet counts were determined in maternal mice on Day 0, 1, 3, 7, 10, 14 and after delivery, as well as in pups on post-natal day 21. On Day 20, half the maternal mice of each group were sacrificed for gross examination and bone narrow samples were acquired for smear and reticular fiber staining. The whole blood and spleen samples of sacrificed maternal mice in each group were acquired for further analysis. The fetuses were checked for external and visceral abnormalities as previously described [35]. The remaining half maternal mice and their pups were observed for at least three weeks to assess their vital signs.

2.4. Identification of Tregs by flow cytometry

Single cell suspensions of spleen from the sacrificed maternal mice on Day 20 were prepared. To determine the frequency of $CD4^+CD25^{hi}Foxp3^+$ Tregs, the freshly obtained splenocytes from the mice (1×10^6/tube) were incubated with fluorescein isothiocyanate (FITC)-labeled monoclonal anti-CD4 and allophycocyanin (APC)-labeled anti-CD25 (Biolegend; CA, USA). Then the samples were fixed, permeabilized, and stained for intracellular Foxp3 (clone PCH101) and an isotype control using the phycoerythrin (PE) anti-mouse/rat/human FOXP3 Flow kit (Biolegend; CA, USA) following the manufacturer's instructions. Tregs were examined on a FACS 3GalliosTM flow cytometer (Beckman Coulter; CA, USA) and analyzed using Flowjo software. $CD25^{hi}Foxp3^+$ cells gated in the $CD4^+$ cell fraction were recorded as Tregs.

2.5. Detection of TGF-β1 plasma levels

The peripheral blood from the sacrificed pregnant mice were centrifuged at 3000 ×*g* for 10 min to obtain the plasma. The TGF-β1 plasma levels of the samples were determined by enzyme-linked immunosorbent assays (ELISA) using a Mouse/Rat/Porcine/Canine TGF-β1 Immunoassay kit (R&D systems; MN, USA) following the manufacturer's instructions. The lower detection limit was 15.4 pg/mL.

2.6. Investigation into the effect of rhTPO on Tregs in model mice

We detected the presence for c-Mpl, the receptor of TPO, on $CD4^+$ T cells. Single-cell suspension of model mice spleen was incubated with FITC labeled anti-CD4, and then stained with anti-mouse c-Mpl biotin (IBL; Gunma, Japan) and PE anti-biotin antibody (Biolegend; CA, USA) for flow cytometry analysis.

Mouse splenic $CD4^+$ T cells were sorted using CD4 magnetic beads (Miltenyi Biotec; Bergisch Gladbach, Germany) and cultured with or without 2 μg/L rhTPO for 3 days. Afterwards, the frequency of Tregs was assessed by flow cytometry.

Model mice were administered with a TGF-β receptor inhibitor (LY2109761) (AbMole BioScience; TX, USA) at a dose of 100 mg/kg or vehicle (0.5% carboxymethyl cellulose sodium solution) (Sigma Aldrich; MO, USA) by gavage for 14 days beginning from Day 1. At the same time, all the mice were injected with 1500 U/kg rhTPO subcutaneously. Fourteen days later, the model mice were sacrificed. The platelet count was recorded and the proportion of Tregs in splenic $CD4^+$ T cells was detected by flow cytometry.

2.7. Statistical analysis

All data were expressed as mean ± standard deviation (SD). The statistical significance among different groups was determined by one-way ANOVA test followed by Tukey's multiple comparisons test, unless

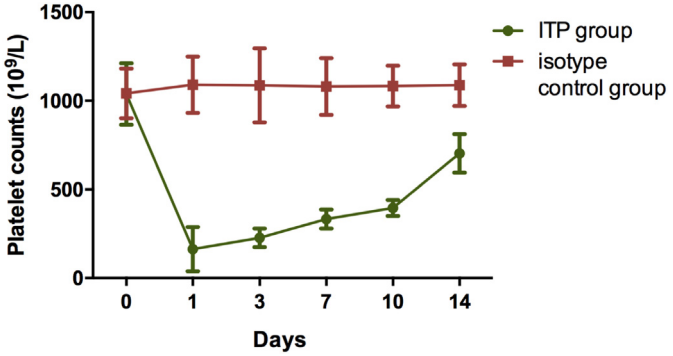


Fig. 1. Platelet counts in pregnant mice during antibody/isotype administration. Pregnant mice were injected with MWReg30 (●) or the same volume of IgG1 (■). The platelet counts of the MWReg30 group decreased to the lowest level on day 1, and kept at a quite low level until Day 10 ($P < 0.01$).

the data was not normally distributed, in which case the Kruskal-Wallis test followed by Dunn's multiple comparison test was used. The difference in platelet counts in model mice of the four groups over time was assessed by two-way ANOVA test followed by Tukey's multiple comparisons test. For comparison between two groups, *t*-test (for normally distributed variables) or Mann-Whitney *U* test (for non-normally distributed variables) was used. All tests were performed by SPSS 19.0. *P* value < 0.05 was considered statistically significant.

3. Results

3.1. The murine model of ITP in pregnancy

As shown in Fig. 1, platelet counts of the pregnant mice administered with MWReg30 sharply declined to the lowest level on Day 1, and remained at a relatively stable low level until Day 10. However, the platelets of control mice which were injected with IgG1 remained stable at the baseline level throughout the study. Although the platelet counts recovered slowly, on Day 14 the platelet counts of the model group were still significantly lower than the control group ($P < 0.05$), which indicated that we successfully established the murine model of ITP in pregnancy.

3.2. Efficacy and safety of rhTPO on the murine model of ITP in pregnancy

The platelet counts of mice in rhTPO-treated group were significantly elevated compared with normal saline-treated group. The effect of platelet elevating was enhanced with the increased rhTPO dosage, and the platelet counts in all the three rhTPO-treated groups were statistically higher than control group on Day 7, 10 and 14. After delivery, statistical significance was only observed between high-dose group and control group ($P < 0.05$). (Fig. 2).

The general condition, appetite and mental condition of the female mice in the four groups had no obvious differences during the rhTPO injection as well as the withdrawal period. There was no redness or swelling at the local injection site. No abnormality was noted in external and visceral examination of fetal mice in rhTPO-treated groups. There was no significant difference in the results of biochemistry and bone marrow fibrosis between the maternal mice of normal saline group and the three rhTPO-treated groups. No statistical effect of rhTPO on the average litter size was noted. No abnormality of pups was observed within 3 weeks after birth (data not shown).

There was no statistical difference in platelet counts of pups among the four groups on post-natal day 21 ($P > 0.05$; Fig. 3).

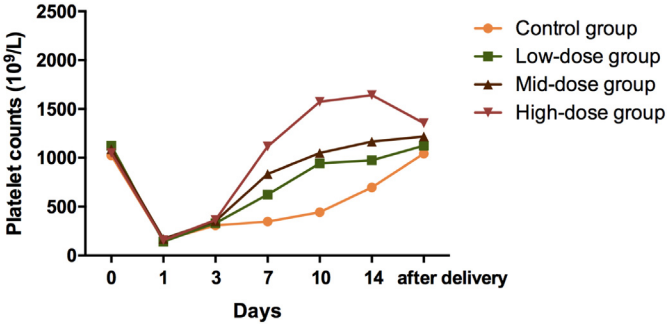


Fig. 2. Platelet counts in pregnant ITP murine models treated with different doses of rhTPO. Platelet counts were recorded in pregnant mice with ITP treated with normal saline (n = 20, ●), low-dose rhTPO (n = 20, ■), mid-dose rhTPO (n = 20, ▲) or high-dose rhTPO (n = 20, ▼). The platelet counts of all mice showed an increasing tendency during rhTPO treatment period. The data is expressed as platelet count ($\times 10^9$/L; mean) over time (days).

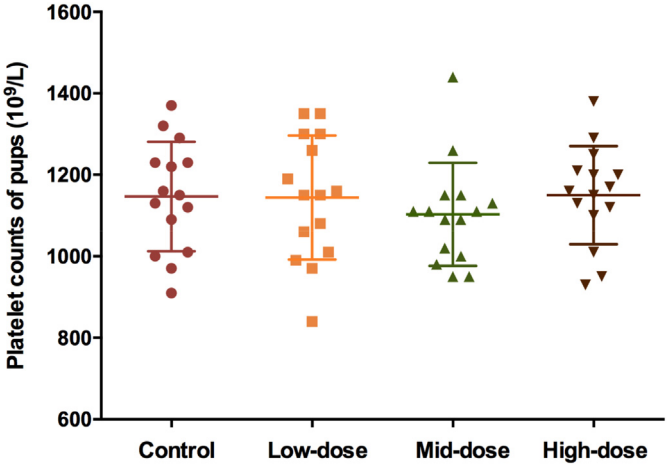


Fig. 3. Platelet counts of the pups in the four experimental groups on post-natal day 21. No statistical differences in platelet counts of pups among the groups (normal saline (n = 16, ●), low-dose rhTPO (n = 16, ■), mid-dose rhTPO (n = 16, ▲) and high-dose rhTPO (n = 16, ▼)) were seen ($P > 0.05$).

3.3. Frequency of Tregs in model mice splenic $CD4^+$ T cells

As shown in Fig. 4, the frequency of Treg cells in splenic $CD4^+$ T cells in the three rhTPO-treated groups was statistically higher than control group (control group: 5.47 ± 1.48; low-dose group: 11.22 ± 2.30, $P < 0.001$; mid-dose group: 12.28 ± 2.36, $P < 0.001$; high-dose group: 11.35 ± 2.57, $P < 0.001$).

3.4. Plasma levels of TGF-β1

The plasma levels of TGF-β1 in the maternal mice after treatment were determined by ELISA. As indicated in Fig. 5, the plasma levels of TGF-β1 were significantly elevated in rhTPO-treated groups when compared with control group (control group: 685.73 ± 226.07; low-dose group: 1248.27 ± 275.87, $P < 0.001$; mid-dose group: 1186.53 ± 311.46, $P < 0.001$; high-dose group: 254.07 ± 404.81, $P < 0.001$). However, there was no statistical difference in the TGF-β1 levels among the three rhTPO-treated groups.

3.5. The effect of rhTPO on Tregs in model mice

Flow cytometry analysis showed that $CD4^+$ T cells in spleen were negative for c-Mpl (Fig. 6A). After 3-day culture of sorted splenic $CD4^+$ T cells with administration of 2 μg/L rhTPO, the proportions of Tregs

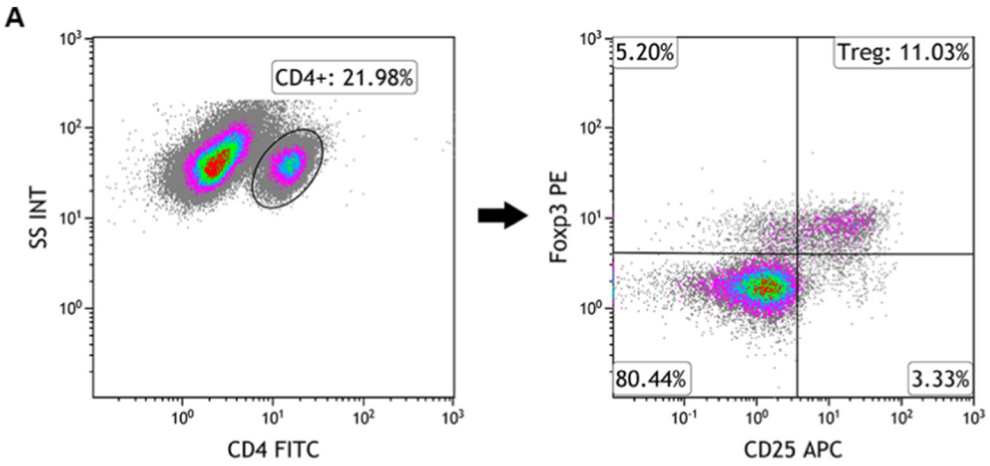
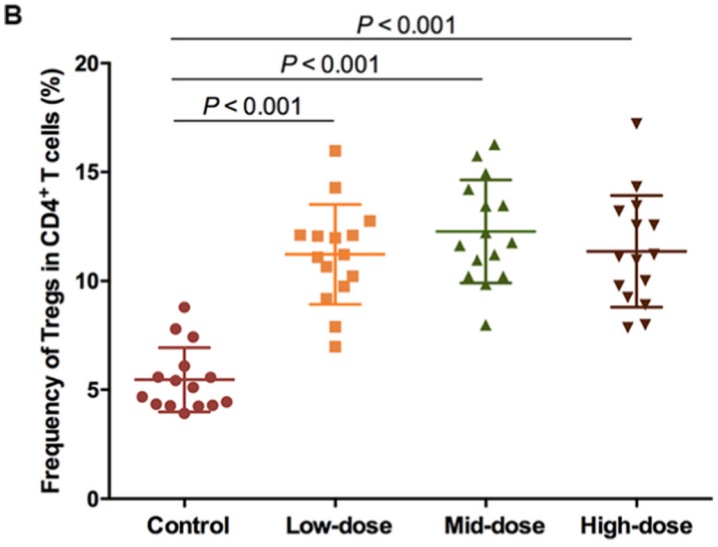


Fig. 4. Frequency of $CD4^+CD25^{hi}Foxp3^+$ Tregs in splenic $CD4^+$ T cells. Splenocytes from the mice treated with normal saline or different doses of rhTPO were stained with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25, followed by intracellular staining using PE-conjugated anti-Foxp3. (A) Representative dot plots demonstrating the gating of $CD4^+CD25^{hi}Foxp3^+$ Tregs. The cells were first gated on the $CD4^+$ lymphoid population in splenocytes, then the frequency of $CD25^{hi}Foxp3^+$ cells was measured based on the shown gating strategy. (B) Percentage of $CD25^{hi}Foxp3^+$ cells in the $CD4^+$ population in model mice treated with normal saline (n = 15, ●), low-dose rhTPO (n = 15, ■), mid-dose rhTPO (n = 15, ▲) and high-dose rhTPO (n = 15, ▼). No statistical difference was noted among rhTPO-treated groups. However, the frequencies of Tregs in all the three rhTPO-treated groups were significantly higher than that in control mice treated with normal saline (all $P < 0.001$). Data are shown as mean ± SD.



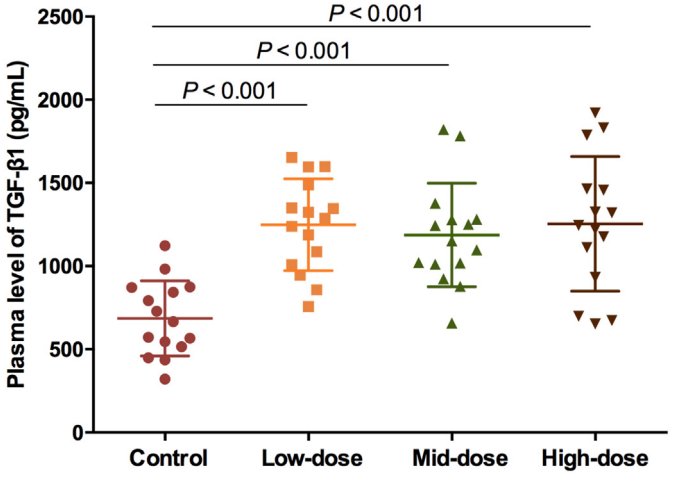


Fig. 5. Plasma levels of TGF-β1 in the maternal mice on Day 20. Plasma samples from mice treated with normal saline (n = 15, ●), low-dose rhTPO (n = 15, ■), mid-dose rhTPO (n = 15, ▲) and high-dose rhTPO (n = 15, ▼) were collected to assess the concentration of plasma TGF-β1 by ELISA. The plasma levels of TGF-β1 in all the three rhTPO-treated groups were significantly higher than that in control mice treated with normal saline (all $P < 0.001$). Data are shown as mean ± SD.

remained comparable with untreated $CD4^+$ T cells (Fig. 6B; $P > 0.05$). Additionally, after subcutaneous administration of rhTPO and intragastric administration of TGF-β receptor inhibitor (LY2109761) or vehicle for 14 days, the frequency of Tregs in splenic $CD4^+$ T cells in model mice was analyzed by flow cytometry and platelet counts were recorded. Results showed no significant difference in platelet count between the two groups (Fig. 6C; $P > 0.05$). Notably, the Treg frequency in splenic $CD4^+$ T cells in LY2109761-treated model mice were significantly lower than control mice (Fig. 6D; $P < 0.05$).

4. Discussion

The goal of medical treatment for ITP in pregnancy is to minimize the risk of hemorrhage during delivery associated with thrombocytopenia. Current consensus guidelines recommend that, except for the delivery period, treatment indications for pregnant women are similar to those recommended for adult ITP patients [1,26,36]. At the time of delivery, the minimum platelet counts recommended are 80×10^9/L for epidural anesthesia and 50×10^9/L for cesarean delivery [37]. Corticosteroids, intravenous immunoglobulin, or both are the first-line treatments for ITP in pregnancy. Treatment should be adapted to the individual patient, taking into account the occurrence and severity of bleeding, the desired increase speed of platelet count, and possible side effects to mother or fetus. Once failing first-line therapy, few other therapeutic options could be chosen.

More recently, with the development of study on pathogenesis of

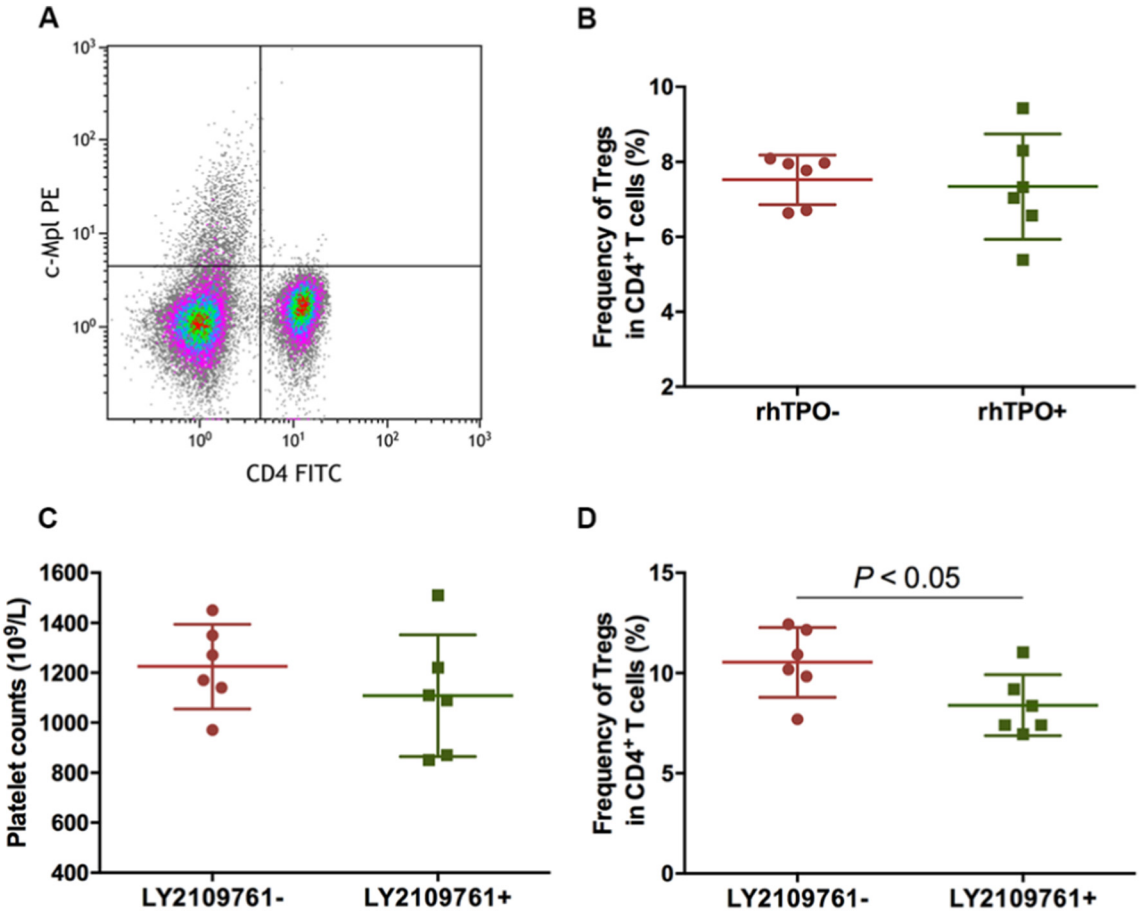


Fig. 6. The effect of rhTPO on Tregs in model mice. (A) $CD4^+$ T cells in spleen were negative for c-Mpl detected by flow cytometry. (B) After 3-day culture of sorted splenic $CD4^+$ T cells with administration of 2 μg/L rhTPO, the proportions of Tregs remained comparable with untreated controls ($P > 0.05$). (C) No significant difference in platelet count was noted between LY2109761-treated model mice (n = 6) and control mice (n = 6) ($P > 0.05$). (D) The Treg frequency in splenic $CD4^+$ T cells in LY2109761-treated model mice (n = 6) were significantly lower than control mice (n = 6) ($P < 0.05$).

ITP, the relative decrease of TPO was found to play a crucial role in ITP [19–22]. A number of thrombopoietic agents have been developed and shown to be highly effective in the treatment of ITP [27,31,36,38,39]. rhTPO has been used for the treatment of corticosteroid-resistant and relapsed ITP patients in China for many years [24,26]. However, whether rhTPO can be used safely and effectively for pregnant patients is still unknown.

In this study we successfully established a murine model of ITP in pregnancy which simulated the process of ITP induced by anti-platelet antibody. Then we studied the safety and efficacy of rhTPO using the murine model by subcutaneously administration of rhTPO for 14 consecutive days. Our result showed that rhTPO could increase the platelet count effectively without evident side effects to maternal, fetal or neonatal mice.

The immune-pathogenesis of ITP has been extensively investigated in the last decade, and it appears that one of the major contributing factors in the development of this disorder is Treg deficiency [40–44]. Tregs are a subpopulation of T cells marked with $CD4^+CD25^{hi}Foxp3^+$ which modulate the immune system, maintain tolerance to self-antigens, and prevent autoimmune disease [45]. Many autoimmune diseases, such as rheumatoid arthritis, type I diabetes and multiple sclerosis, are associated with Treg deficiencies [38,39,46]. The reasons for Treg deficiency in active ITP are still unknown, but several therapies that successfully raise platelet counts have been shown to be associated with normalization of Treg deficiency [42,47].

c-Mpl is the receptor for TPO and the expression of c-Mpl is restricted to cells of the megakaryocytic lineage, $CD34^+$ hematopoietic progenitors and stem cells [48]. Consistent with the results in our study, $CD4^+$ T cells were negative for c-Mpl. Moreover, sorted splenic $CD4^+$ T cells cultured with rhTPO showed no significant difference in frequencies of Tregs compared with untreated control. These results suggest that rhTPO cannot act directly on $CD4^+$ T cells.

It was reported that thrombopoietic agents treatment rescued the peripheral splenic Treg deficiencies and elevated the serum levels of TGF-β1 [31,49]. TGF-β1 acts as a mediator of feedback signal from platelets, the end product of megakaryocyte, and it is highly concentrated in platelets [50,51]. TGF-β1 can be released by platelet activation/degranulation [50,52]. TGF-β1 plays an integral role in regulating the immune response, especially in adaptive immunity. TGF-β1 also contributes to the suppression of the T cell responses by regulating Tregs [47]. The percentage of Tregs and serum level of TGF-β1 were found decrease in a passive murine model of ITP [53]. The data in our recent study suggested that the frequency of Tregs in the splenocytes and the serum levels of TGF-β1 both increased after thrombopoietic agents treatment [54]. In this study, rhTPO-treated model mice had a significantly higher plasma level of TGF-β1 and frequency of Tregs in splenocytes as well as a higher platelet count compared with untreated control group. TGF-β1 was reported to play an important role in the peripheral induction of Tregs [55–57]. We speculated that elevated TGF-β1 released by increased platelets contributed to the induction of Tregs in model mice.

In order to further investigate the underlying mechanisms of rhTPO's effect on Tregs, we treated model mice with subcutaneous administration of rhTPO and intragastric administration of LY2109761

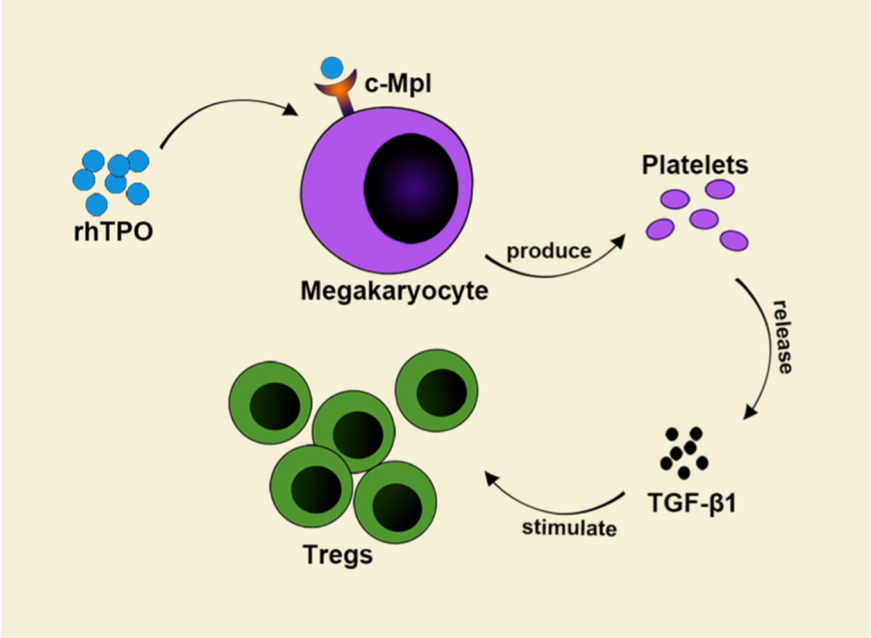


Fig. 7. The pathway of rhTPO resulting in Treg stimulation. rhTPO binds to its receptor c-Mpl on megakaryocyte and promote platelet production, resulting in an elevated plasma TGF-β1 level, which results in Tregs stimulation and may possess tolerance-inducing effects.

or vehicle for 14 days. Platelet counts at Day 14 showed no significant difference between LY2109761-treated group and control group, while the Treg frequency in splenic $CD4^+$ T cells was significantly lower in LY2109761-treated group, which indicated that TGF-β was important for Tregs induction.

Taken together, we conclude that rhTPO binds to its receptor c-Mpl on megakaryocyte and promote platelet production, resulting in an elevated TGF-β1 level, which results in Tregs stimulation and may possess tolerance-inducing effects (Fig. 7).

In conclusion, rhTPO can effectively increase the platelet counts of ITP in pregnancy murine models without evident side effects. Besides, rhTPO may have immune tolerance-inducing effect associated with Treg induction. However, the long-term safety and efficacy of rhTPO in treatment of ITP in pregnancy still need to be studied in further researches.

Acknowledgements

The authors wish to acknowledge the assistance of Shuang Wang for editing the manuscript. This work was supported by grants from National Natural Science Foundation of China, China (No. 81770114, No. 81470284, No. 81800157), Major Research Plan of Natural Science Foundation of Shandong Province, China (ZR2016QZ008), Key R & D project of Shandong Province, China (2015GGH318006), State Key Clinical Specialty of China for Hematological Diseases, China and Taishan Scholar of Shandong Province, China.

Declaration of interest

None.

References

[1] D. Provan, R. Stasi, A.C. Newland, V.S. Blanchette, P. Bolton-Maggs, J.B. Bussel, B.H. Chong, D.B. Cines, T.B. Gernsheimer, B. Godeau, J. Grainger, I. Greer, B.J. Hunt, P.A. Imbach, G. Lyons, R. McMillan, F. Rodeghiero, M.A. Sanz, M. Tarantino, S. Watson, J. Young, D.J. Kuter, International consensus report on the investigation and management of primary immune thrombocytopenia, Blood 115 (2) (2010) 168–186.

[2] G. Li, S. Wang, N. Li, Y. Liu, Q. Feng, X. Zuo, X. Li, Y. Hou, L. Shao, C. Ma, C. Gao, M. Hou, J. Peng, Proteasome inhibition with bortezomib induces apoptosis of long-lived plasma cells in steroid-resistant or relapsed immune thrombocytopaenia, Thromb. Haemost. 118 (10) (2018) 1752–1764.

[3] R.F. Burrows, J.G. Kelton, Fetal thrombocytopenia and its relation to maternal thrombocytopenia, N. Engl. J. Med. 329 (20) (1993) 1463–1466.

[4] R. Sukenik-Halevy, M.H. Ellis, M.D. Fejgin, Management of immune thrombocytopenic purpura in pregnancy, Obstet. Gynecol. Surv. 63 (3) (2008) 182–188.

[5] E. Verdy, V. Bessous, M. Dreyfus, C. Kaplan, G. Tchernia, S. Uzan, Longitudinal analysis of platelet count and volume in normal pregnancy, Thromb. Haemost. 77 (4) (1997) 806–807.

[6] K.R. McCrae, Thrombocytopenia in pregnancy, Hematology Am. Soc. Hematol. Educ. Program 2010 (2010) 397–402.

[7] D.B. Cines, L.D. Levine, Thrombocytopenia in pregnancy, Hematology Am. Soc. Hematol. Educ. Program 2017 (1) (2017) 144–151.

[8] V. Labarque, C. Van Geet, Clinical practice: immune thrombocytopenia in paediatrics, Eur. J. Pediatr. 173 (2) (2014) 163–172.

[9] P.J. Ballem, G.M. Segal, J.R. Stratton, T. Gernsheimer, J.W. Adamson, S.J. Slichter, Mechanisms of thrombocytopenia in chronic autoimmune thrombocytopenic purpura. Evidence of both impaired platelet production and increased platelet clearance, J. Clin. Invest. 80 (1) (1987) 33–40.

[10] H. Louwes, O.A. Zeinali Lathori, E. Vellenga, J.T. de Wolf, Platelet kinetic studies in patients with idiopathic thrombocytopenic purpura, Am. J. Med. 106 (4) (1999) 430–434.

[11] E.J. Houwerzijl, N.R. Blom, J.J. van der Want, M.T. Esselink, J.J. Koornstra, J.W. Smit, H. Louwes, E. Vellenga, J.T. de Wolf, Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura, Blood 103 (2) (2004) 500–506.

[12] R. McMillan, L. Wang, A. Tomer, J. Nichol, J. Pistillo, Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP, Blood 103 (4) (2004) 1364–1369.

[13] R. Takahashi, N. Sekine, T. Nakatake, Influence of monoclonal antiplatelet glycoprotein antibodies on in vitro human megakaryocyte colony formation and proplatelet formation, Blood 93 (6) (1999) 1951–1958.

[14] F.J. de Sauvage, P.E. Hass, S.D. Spencer, B.E. Malloy, A.L. Gurney, S.A. Spencer, W.C. Darbonne, W.J. Henzel, S.C. Wong, W.J. Kuang, et al., Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand, Nature 369 (6481) (1994) 533–538.

[15] K. Kaushansky, S. Lok, R.D. Holly, V.C. Broudy, N. Lin, M.C. Bailey, J.W. Forstrom, M.M. Buddle, P.J. Oort, F.S. Hagen, et al., Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin, Nature 369 (6481) (1994) 568–571.

[16] S. Lok, K. Kaushansky, R.D. Holly, J.L. Kuijper, C.E. Lofton-Day, P.J. Oort, F.J. Grant, M.D. Heipel, S.K. Burkhead, J.M. Kramer, et al., Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo, Nature 369 (6481) (1994) 565–568.

[17] D.F. Sabath, K. Kaushansky, V.C. Broudy, Deletion of the extracellular membrane-distal cytokine receptor homology module of Mpl results in constitutive cell growth and loss of thrombopoietin binding, Blood 94 (1) (1999) 365–367.

[18] F. Wendling, E. Maraskovsky, N. Debili, C. Florindo, M. Teepe, M. Titeux, N. Methia, J. Breton-Gorius, D. Cosman, W. Vainchenker, cMpl ligand is a humoral regulator of megakaryocytopoiesis, Nature 369 (6481) (1994) 571–574.

[19] M. Chang, Y. Suen, G. Meng, J.S. Buzby, J. Bussel, V. Shen, C. van de Ven, M.S. Cairo, Differential mechanisms in the regulation of endogenous levels of thrombopoietin and interleukin-11 during thrombocytopenia: insight into the regulation of platelet production, Blood 88 (9) (1996) 3354–3362.
[20] R.V. Emmons, D.M. Reid, R.L. Cohen, G. Meng, N.S. Young, C.E. Dunbar, N.R. Shulman, Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction, Blood 87 (10) (1996) 4068–4071.
[21] M.C. Kappers-Klunne, M. de Haan, P.C. Struijk, H.H. van Vliet, Serum thrombopoietin levels in relation to disease status in patients with immune thrombocytopenic purpura, Br. J. Haematol. 115 (4) (2001) 1004–1006.
[22] S. Kosugi, Y. Kurata, Y. Tomiyama, T. Tahara, T. Kato, S. Tadokoro, M. Shiraga, S. Honda, Y. Kanakura, Y. Matsuzawa, Circulating thrombopoietin level in chronic immune thrombocytopenic purpura, Br. J. Haematol. 93 (3) (1996) 704–706.
[23] D. Sun, N. Shehata, X.Y. Ye, S. Gregorovich, B. De France, D.M. Arnold, P.S. Shah, A.K. Malinowski, Corticosteroids compared with intravenous immunoglobulin for the treatment of immune thrombocytopenia in pregnancy, Blood 128 (10) (2016) 1329–1335.
[24] H. Zhou, M. Xu, P. Qin, H.Y. Zhang, C.L. Yuan, H.G. Zhao, Z.G. Cui, Y.S. Meng, L. Wang, F. Zhou, X. Wang, D.Q. Li, K.H. Bi, C.S. Zhu, C.S. Guo, X.X. Chu, Q.C. Wu, X.G. Liu, X.Y. Dong, J. Li, J. Peng, M. Hou, A multicenter randomized open-label study of rituximab plus rhTPO vs rituximab in corticosteroid-resistant or relapsed ITP, Blood 125 (10) (2015) 1541–1547.
[25] T.S.o.P.C.M.A. Subspecialty Group of Hematology, C.J.o.P, Editorial Board, Recommendations for diagnosis and treatment of primary immune thrombocytopenia in children, Zhonghua Er Ke Za Zhi 51 (5) (2013) 382–384.
[26] Thrombosis, H.S.C.M.A. Hemostasis Group, Consensus of Chinese experts on diagnosis and treatment of adult primary immune thrombocytopenia (version 2016), Zhonghua Xue Ye Xue Za Zhi 37 (2) (2016) 89–93.
[27] S. Wang, R. Yang, P. Zou, M. Hou, D. Wu, Z. Shen, X. Lu, Y. Li, X. Chen, T. Niu, H. Sun, L. Yu, Z. Wang, Y. Zhang, N. Chang, G. Zhang, Y. Zhao, A multicenter randomized controlled trial of recombinant human thrombopoietin treatment in patients with primary immune thrombocytopenia, Int. J. Hematol. 96 (2) (2012) 222–228.
[28] Y. Huang, X. Liu, F. Xue, Y. Chen, W. Liu, R. Fu, L. Zhang, R. Yang, Efficacy and safety of rhTPO in the treatment of pediatric primary immune thrombocytopenia, Zhonghua Xue Ye Xue Za Zhi 36 (6) (2015) 511–514.
[29] G. Cheng, Eltrombopag, a thrombopoietin- receptor agonist in the treatment of adult chronic immune thrombocytopenia: a review of the efficacy and safety profile, Ther. Adv. Hematol. 3 (3) (2012) 155–164.
[30] K.P. Garnock-Jones, S.J. Keam, Eltrombopag, Drugs 69 (5) (2009) 567–576.
[31] W. Bao, J.B. Bussel, S. Heck, W. He, M. Karpoff, N. Boulad, K. Yazdanbakhsh, Improved regulatory T-cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents, Blood 116 (22) (2010) 4639–4645.
[32] X. Li, H. Zhong, W. Bao, N. Boulad, J. Evangelista, M.A. Haider, J. Bussel, K. Yazdanbakhsh, Defective regulatory B-cell compartment in patients with immune thrombocytopenia, Blood 120 (16) (2012) 3318–3325.
[33] M.M. Qu, X.N. Liu, X.G. Liu, Q. Feng, Y. Liu, X. Zhang, S. Liu, L. Zhang, G.S. Li, Y.Y. Zhu, M.Y. Lv, J. Peng, M. Hou, Cytokine changes in response to TPO receptor agonist treatment in primary immune thrombocytopenia, Cytokine 92 (2017) 110–117.
[34] P. Trepanier, I. St-Amour, R. Bazin, Cationized IVIg as a potential substitute to IVIg for the treatment of experimental immune thrombocytopenia, Int. Immunopharmacol. 16 (4) (2013) 409–413.
[35] Y. Liu, S. Qiu, L. Wang, N. Zhang, Y. Shi, H. Zhou, X. Liu, L. Shao, X. Liu, J. Chen, M. Hou, Reproductive and developmental toxicity study of caffeic acid in mice, Food Chem. Toxicol. 123 (2018) 106–112.
[36] C. Neunert, W. Lim, M. Crowther, A. Cohen, L. Solberg Jr., M.A. Crowther, H. American Society of, The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia, Blood 117 (16) (2011) 4190–4207.
[37] R.M. Kaufman, B. Djulbegovic, T. Gernsheimer, S. Kleinman, A.T. Tinmouth, K.E. Capocelli, M.D. Cipolle, C.S. Cohn, M.K. Fung, B.J. Grossman, P.D. Mintz, B.A. O'Malley, D.A. Sesok-Pizzini, A. Shander, G.E. Stack, K.E. Webert, R. Weinstein, B.G. Welch, G.J. Whitman, E.C. Wong, A.A. Tobian, Aabb, platelet transfusion: a clinical practice guideline from the AABB, Ann. Intern. Med. 162 (3) (2015) 205–213.
[38] M.E. Brunkow, E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, F. Ramsdell, Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse, Nat. Genet. 27 (1) (2001) 68–73.
[39] S. Sakaguchi, M. Miyara, C.M. Costantino, D.A. Hafler, FOXP3+ regulatory T cells in the human immune system, Nat. Rev. Immunol. 10 (7) (2010) 490–500.
[40] M. Aboul-Fotoh Lel, M.M. Abdel Raheem, M.A. El-Deen, A.M. Osman, Role of CD4+CD25+ T cells in children with idiopathic thrombocytopenic purpura, J. Pediatr. Hematol. Oncol. 33 (2) (2011) 81–85.
[41] D.Y. Chang, J. Ouyang, R.F. Zhou, J.Y. Xu, B. Chen, Y.G. Yang, Q.G. Zhang, X.Y. Shao, C.Y. Guan, Y. Xu, Profiles of different subsets of CD(4)(+) T cells in chronic idiopathic thrombocytopenic purpura, Zhonghua Nei Ke Za Zhi 49 (3) (2010) 213–216.
[42] X.P. Wang, Y.S. Qiu, G.P. Hao, L. Zhu, Levels of regulatory T cells in peripheral blood of children with idiopathic thrombocytopenic purpura, Zhongguo Dang Dai Er Ke Za Zhi 13 (4) (2011) 282–284.
[43] X.L. Zhang, J. Peng, J.Z. Sun, J.J. Liu, C.S. Guo, Z.G. Wang, Y. Yu, Y. Shi, P. Qin, S.G. Li, L.N. Zhang, M. Hou, De novo induction of platelet-specific CD4(+)CD25(+) regulatory T cells from CD4(+)CD25(−) cells in patients with idiopathic thrombocytopenic purpura, Blood 113 (11) (2009) 2568–2577.
[44] H. Zhao, W. Du, D. Wang, D. Gu, F. Xue, J. Ge, T. Sui, R. Yang, The expression of IFN-gamma, IL-4, Foxp3 and perforin genes are not correlated with DNA methylation status in patients with immune thrombocytopenic purpura, Platelets 21 (2) (2010) 137–143.
[45] N.T. Le, N. Chao, Regulating regulatory T cells, Bone Marrow Transplant. 39 (1) (2007) 1–9.
[46] C.L. Bennett, J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, H.D. Ochs, The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3, Nat. Genet. 27 (1) (2001) 20–21.
[47] M.A. Travis, D. Sheppard, TGF-beta activation and function in immunity, Annu. Rev. Immunol. 32 (2014) 51–82.
[48] S. Ziegler, K. Burki, R.C. Skoda, A 2-kb c-mpl promoter fragment is sufficient to direct expression to the megakaryocytic lineage and sites of embryonic hematopoiesis in transgenic mice, Blood 100 (3) (2002) 1072–1074.
[49] B. Liu, H. Zhao, M.C. Poon, Z. Han, D. Gu, M. Xu, H. Jia, R. Yang, Z.C. Han, Abnormality of CD4(+)CD25(+) regulatory T cells in idiopathic thrombocytopenic purpura, Eur. J. Haematol. 78 (2) (2007) 139–143.
[50] R.K. Assoian, A. Komoriya, C.A. Meyers, D.M. Miller, M.B. Sporn, Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization, J. Biol. Chem. 258 (11) (1983) 7155–7160.
[51] M.T. Mitjavila, G. Vinci, J.L. Villeval, N. Kieffer, A. Henri, U. Testa, J. Breton-Gorius, W. Vainchenker, Human platelet alpha granules contain a nonspecific inhibitor of megakaryocyte colony formation: its relationship to type beta transforming growth factor (TGF-beta), J. Cell. Physiol. 134 (1) (1988) 93–100.
[52] N.O. Fortunel, A. Hatzfeld, J.A. Hatzfeld, Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis, Blood 96 (6) (2000) 2022–2036.
[53] G. Zhang, P. Zhang, H. Liu, X. Liu, S. Xie, X. Wang, Y. Wu, J. Chang, L. Ma, Assessment of Th17/Treg cells and Th cytokines in an improved immune thrombocytopenia mouse model, Hematology 22 (8) (2017) 493–500.
[54] X.G. Liu, S. Liu, Q. Feng, X.N. Liu, G.S. Li, Z. Sheng, P. Chen, Y. Liu, Y. Wei, X.Y. Dong, P. Qin, C. Gao, C. Ma, L. Zhang, M. Hou, J. Peng, Thrombopoietin receptor agonists shift the balance of Fcgamma receptors toward inhibitory receptor IIb on monocytes in ITP, Blood 128 (6) (2016) 852–861.
[55] D. Haribhai, X. Luo, J. Chen, S. Jia, L. Shi, J.A. Schroeder, H. Weiler, R.H. Aster, M.J. Hessner, J. Hu, C.B. Williams, Q. Shi, TGF-beta1 along with other platelet contents augments Treg cells to suppress anti-FVIII immune responses in hemophilia a mice, Blood Adv. 1 (2) (2016) 139–151.
[56] S.W. Lee, H. Choi, S.Y. Eun, S. Fukuyama, M. Croft, Nitric oxide modulates TGF-beta-directive signals to suppress Foxp3+ regulatory T cell differentiation and potentiate Th1 development, J. Immunol. 186 (12) (2011) 6972–6980.
[57] F.J. Quintana, H.L. Weiner, Environmental control of Th17 differentiation, Eur. J. Immunol. 39 (3) (2009) 655–657.