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Thalidomide induce response in patients with corticosteroid-resistant or relapsed ITP by upregulating Neuropilin-1 expression

Zhi-gang Yang[a,*,1], Rui-ting Wen[b,1], Yu-ming Zhang[b], Guo-cai Wu[a], Wei-juan Chen[c], Yu-feng Wang[c], Zhi-yun Weng[c], Si-si Wen[c], Xiao-jun Zhang[c], Mei-hua Guan[a]

[a] Affiliated Central People's Hospital of Zhanjiang of Guangdong Medical University, Zhanjiang, Guangdong 524045, PR China
[b] Department of Hematology, Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong 524001, PR China
[c] Guangdong Medical University, Zhanjiang, Guangdong 524023, PR China

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ABSTRACT

Background: Immune thrombocytopenia (ITP) is an immune-mediated acquired autoimmune hemorrhagic disease. About one-third of patients are unresponsive to first-line therapies. Thalidomide (THD) as an immunomodulatory agent is now used to treat several autoimmune disorders. Therefore, we assessed the safety and efficacy of THD in corticosteroid-resistant or relapsed ITP patients, and preliminarily explore its mechanism.

Methods: 50 newly-diagnosed ITP patients and 47 healthy volunteers were enrolled in this study. Additionally, 17 corticosteroid-resistant or relapsed ITP patients were recruited, with 7 cases in the rhTPO + THD group and 10 cases in the THD monotherapy group. Overall response rate at 6, 12, and 24 months were assessed. Levels of Neuropilin-1(NRP-1), regulatory T cells (Tregs) and regulatory B cells (Bregs) were detected.

Results: Expression of NRP-1, Tregs and Bregs were reduced in newly-diagnosed ITP patients. In vitro, THD treatment upregulated expression of NRP-1and Tregs only in ITP patients. As for corticosteroid-resistant or relapsed ITP patients, overall response rate at 6, 12, and 24 months was 85.7%, 57.1% and 100% in the rhTPO + THD group and 60%, 75% and 83.3% in the THD group, respectively. Additionally, rhTPO plus THD or THD therapy significantly increased the levels of NRP-1, Tregs and Bregs in responders.

Conclusions: Our study shows for the first time that NRP-1 is involved in the pathogenesis of ITP, THD could induce response in ITP patients by upregulating NRP-1 expression and restoring the proportion of Tregs and Bregs. THD might be served as a novel therapeutic agent in corticosteroid-resistant or relapsed ITP patients.

1. Introduction

Immune thrombocytopenia (ITP) is an immune-mediated acquired autoimmune hemorrhagic disease characterized by increased platelet destruction and reduced platelet production [1]. The pathogenesis of ITP is complicated, and so far, the exact etiology and pathogenesis remain unclear. Antiplatelet antibodies produced by B cells play a key role in the pathogenesis of ITP [2]. B cell-depleting therapy has been used as a second-line treatment for chronic ITP [3]. The dysregulation of B cells development has also been associated with ITP. It was recently shown that a new subset of immunosuppressive cells, known as regulatory B cells (Bregs), also play an important role in the pathogenesis of ITP. Impaired function and reduced frequency of Bregs were observed in ITP patients [4]. Although autoreactive B lymphocytes secreting antiplatelet antibodies are thought as the primary immunologic defect in ITP, dysfunction of T cells is also considered to play a critical role in the pathogenesis of ITP [5]. Accumulating evidence suggest that autoreactive T cells, including T-helper (Th)1, Th17 and Th22 cell subsets, are increased in ITP patients, whereas regulatory T cells (Tregs), a protective subset, is deficient in both numbers and function [6–9]. Therefore, numerical and functional defects in Tregs and Bregs may contribute to impaired self-tolerance in ITP patients. Increasing the number of Tregs and Bregs or restoring their function may become a promising therapeutic strategy for ITP.

Neuropilin-1 (NRP-1) is a type 1 transmembrane protein, originally identified for its role in neuronal development [10]. Recently, it has been showed that NRP-1 could be a useful surface marker to differentiate between natural-Tregs (nTregs) and induced-Tregs (iTregs)

* Corresponding author at: Affiliated Central People's Hospital of Zhanjiang of Guangdong Medical University, #236 Yuanzhu Road, Chikan District, Zhanjiang, Guangdong 524045, PR China.
E-mail address: yangzg@gdmu.edu.cn (Z.-g. Yang).
[1] These authors contributed equally to this work and share co-first authorship.

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[11,12]. NRP-1 is essential for proper maintenance of peripheral tolerance and its absence can result in unchecked autoreactive responses, leading to autoimmune diseases [13]. Decreased expression of NRP-1 as a novel key factor contributed to peripheral microvasculopathy and defective angiogenesis in systemic sclerosis [14]. However, the role of NRP-1 in ITP has not been reported, and the interaction between NRP-1 and Tregs in ITP remains unclear. Therefore, in the present study, we preliminarily investigated the role of NRP-1 in the pathogenesis of ITP.

Corticosteroids and intravenous immunoglobulin are recommended as the first-line therapies for ITP. However, approximately one-third of patients are unresponsive to treatment, or relapse after corticosteroid tapering or discontinuation. Additionally, the optimal second-line treatment remains uncertain [15]. Thalidomide (THD) is used as an immunomodulatory agent, which has been shown to have immunomodulatory and anti-inflammatory activity and is now used to treat several autoimmune disorders [16]. In 2004, Falco et al. successfully managed ITP with THD in a patient with multiple myeloma [17]. In vitro, it has been showed that THD could correct impaired mesenchymal stem cell function in patients with ITP [18]. According to these findings, we hypothesized that THD may be a viable treatment option for ITP patients. In the present study, to elucidate the pathogenesis of ITP and explore the efficacy and mechanism of THD in ITP, we analyzed the changes of NRP-1, Tregs and Bregs in newly-diagnosed ITP patients, and investigated the effects of THD on these indicators of ITP patients in vitro. In addition, we also assessed the safety and efficacy of THD in corticosteroid-resistant or relapsed ITP patients.

2. Materials and methods

2.1. Patients and healthy volunteers

Fifty newly-diagnosed ITP patients (29 females and 21 males, age range, 18–60 years; median age, 43 years) were enrolled in this study. Enrollment occurred between January 2015 and June 2017 in the Department of Hematology of Affiliated Hospital of Guangdong Medical University (Zhanjiang, China). The platelet counts ranged from 1×10^9/L to 28×10^9/L, with a median count of 12×10^9/L. The diagnosis of ITP patients was based on previously reported criteria [19]. Forty-seven healthy volunteers (HVs) were included (26 females and 21 males, age range, 18–55 years; median age, 45 years). The platelet counts of HVs ranged from 128×10^9/L to 291×10^9/L, with a median count of 193×10^9/L.

Seventeen corticosteroid-resistant or relapsed ITP patients (11 females and 6 males; age range, 18–65 years; median age, 30 years) were recruited in this study. Recruitment took place between June 2015 and June 2018 at the Department of Hematology of Affiliated Hospital of Guangdong Medical University (Zhanjiang, China). The clinical parameters of the corticosteroid-resistant or relapsed ITP patients are shown in Table 1. Inclusion criteria were described as follows: (1) eligible ITP patients were age 18 years or older; (2) relapse after conventional glucocorticoids administration or no response to glucocorticoids therapy; (3) relapse or ineffective after the second-line drugs therapy include cyclosporine, rituximab, and danazol; (4) ITP patients with platelet counts $< 20 \times 10^9$/L were enrolled in recombinant human thrombopoietin (rhTPO) plus THD group ($n = 7$), platelet counts $\geq 20 \times 10^9$/L and absence of bleeding were enrolled in THD monotherapy group ($n = 10$). None of the included patients had been treated with rhTPO or THD. Exclusion criteria included drug-induced thrombocytopenia, virus-induced thrombocytopenia (such as HIV, hepatitis B virus, or hepatitis C virus), connective tissue diseases, malignancy, active infection, pregnancy or lactation, liver and kidney function impairment, hypertension, diabetes, cardiovascular diseases, history of thrombosis and allergy to THD. This study was approved by the ethical committee of the Affiliated Hospital of Guangdong Medical University and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

2.2. Treatment regimens

rhTPO (3SBIO, Shenyang, China) was given at a daily dose of 300 U/kg subcutaneously for 14 days. The dose frequency of rhTPO was adjusted as follows: (1) rhTPO was given once a day when platelet count $< 100 \times 10^9$/L; (2) rhTPO was administered every other day when platelets count ranged from 100×10^9/L to 300×10^9/L; (3) rhTPO was discontinued if platelets count were $^{>}300 \times 10^9$/L in 14 days.

THD (Changzhou Pharmaceutical Factory, Changzhou, China) was administered orally in a dosage of 2.5 mg/kg daily for 3 months. For patients reaching complete response (CR), THD was reduced in a dosage of 50 mg/monthly and discontinued during 3 months. For patients reaching response (R), THD was reduced in a dosage of 25 mg/monthly and discontinued during 6 months.

2.3. Response evaluation criteria

The criteria for response were defined as follows [19]: (1) CR: platelet count $\geq 100 \times 10^9$/L and absence of bleeding, (2) R: platelet count $\geq 30 \times 10^9$/L and at least 2-fold increase the baseline count and absence of bleeding, and (3) No response (NR): platelet count $< 30 \times 10^9$/L or $<$ 2-fold increase of the baseline platelet count or bleeding. Platelet counts were confirmed on 2 separate occasions at least 7 days apart when defining CR or R. Time to response (TTR) was defined as the time from starting treatment to time of achievement of CR or R. Both of CR and R were considered to be effective. Relapse: platelet count below 100×10^9/L or bleeding (from CR) or below 30×10^9/L or $<$ 2-fold increase of baseline platelet count or bleeding (from R).

Time to relapse (duration of response) was measured from the achievement of CR or R to relapse. Platelet counts were monitored weekly during the first month of treatment, then at biweekly intervals up to the third month and monthly thereafter. Adverse events were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0.

2.4. Flow cytometry (FCM) assay

The proportion of Tregs ($CD4^+CD25^+Foxp3^+$ cells), Bregs ($CD19^+CD24^{hi}CD38^{hi}$ cells) and expression of NRP-1 on Tregs ($CD4^+CD25^+$ T cells) were detected with FCM (Becton Dickinson, Franklin Lakes, NJ, USA). Briefly, peripheral blood (PB) were collected in sterile EDTA tubes and mononuclear cells (MNCs) were separated by density gradient centrifugation with Ficoll (TBD Science, Tianjin, China). Isolated peripheral blood mononuclear cell (PBMC) were then resuspended in phosphate-buffered saline (PBS) at a concentration of 1×10^6 cells/mL. The proportion of Tregs, Bregs and expression of NRP-1 on Tregs were examined according to the manufacturer's protocol. The following antibodies were used: phycoerythrin (PE)- NRP-1, PE-CD24, fluorescein isothiocyanate (FITC)-CD4, FITC-CD38, allophycocyanin (APC)-CD19, APC-CD25, and phycoerythrin-cyanin 7 (PE-Cy7) -FoxP3. All of these antibodies and its isotype controls were purchased from eBioscience (San Diego, CA, USA), except for PE-NRP-1 and corresponding isotype controls, which were obtained from Miltenyi Biotech (Bergisch Gladbach, Germany).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Plasma samples were collected from participants and were stored at −80 °C. The expression of NRP-1 (R&D Systems, Minneapolis, MN, USA) was detected by ELISA according to the manufacturers' instructions.

2.6. Quantitative PCR (qPCR) assay

Total RNA was extracted from PBMCs with Trizol reagent (TaKaRa,

Table 1
Baseline characteristics and results of 17 corticosteroid-resistant or relapsed ITP patients treated with rhTPO plus THD or THD alone.

N	Age, y/sex	Group	Previous therapies	Baseline PLT count (10^9/L)	Time to R/CR (week)	Month 6	Month12	Month 24	Duration of response (Month)	Relapse	Follow-up (Month)	Current status
1	18/F	rhTPO + THD	GC	13	−/1	CR	CR	CR	37	–	37	CR
2	23/M	rhTPO +THD	GC, RTX	17	−/1	CR	CR	CR	27	–	27	CR
3	65/M	rhTPO + THD	GC	8	4/−	R	R	–	9	Yes	18	Death
4	18/F	rhTPO + THD	GC, CsA, DNZ	16	1/−	R	Relapse	–	3	Yes	21	R
5	24/M	rhTPO + THD	GC	8	1/24	CR	Relapse	CR	4	Yes	24	CR
6	18/F	rhTPO + THD	GC	5	−/1	Relapse	Relapse	–	3	Yes	18	Relapse
7	32/F	rhTPO + THD	GC	2	1/4	CR	CR	CR	24	–	24	CR
8	34/M	THD	GC, CsA, DNZ	55	−/32	NR	CR	CR	36	–	43	CR
9	55/F	THD	GC, DNZ	30	12/96	NR	NR	CR	24	Yes	43	CR
10	57/F	THD	GC, CsA	52	−/48	NR	CR	Relapse	1	Yes	38	CR
11	28/M	THD	GC, CsA, DNZ	60	−/4	CR	CR	CR	24	–	24	CR
12	63/M	THD	GC, CsA	36	12/96	R	R	CR	20	–	31	Relapse
13	35/F	THD	GC	20	4/−	R	R	R	6	Yes	38	R
14	35/F	THD	GC, CsA	42	8/−	R	Relapse	–	7	Yes	12	Relapse
15	36/F	THD	GC, CsA	23	2/36	R	CR	–	12		12	CR
16	63/F	THD	GC	33	8/−	R	–	–	3	–	7	R
17	18/F	THD	GC, DNZ	37	−/−	NR	–	–		–	7	NR

THD, thalidomide; rhTPO, recombinant human thrombopoietin; GC, glucocorticoids; RTX, rituximab; CsA, Cyclosporine A; DNZ, danazol; F, female; M, male; N, patient number; NR, no response; R, response; CR, complete response.

Tokyo, Japan), according to the manufacturer's instructions. Subsequently, cDNA synthesis and quantitative PCR (qPCR) were performed by using a reverse transcriptase kit (TaKaRa) and an SYBR Premix Ex TaqTM II kit (TaKaRa), respectively, each according to the manufacturer's instructions. The primer of NRP-1 was synthesized by Invitrogen (Carlsbad, CA, USA). The primers for NRP-1 were as follows: NRP-1 forward, 5′-CTC TGT CTC CCG CTC ATC TT-3′ and reverse, 5′-CAA CAC ACA CCA AAG CCA AT-3′; GAPDH forward, 5′-GTC AGT GGT GGA CCT GAC CT-3′ and reverse, 5′-TGA GCT TGA CAA AGT GGT CG-3′. Expression level of NRP-1 was quantified according to the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method.

2.7. In vitro assay

PBMCs obtained from 15 newly-diagnosed ITP patients and seven HVs were incubated for 24 h with (PBMC + THD group) or without (PBMC group) 4 μg/ml THD (Sigma-Aldrich, St. Louis, MO, USA) in 1640 complete medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), respectively. Subsequently, the proportion of Tregs, Bregs and expression of NRP-1 on Tregs were detected with FCM as previously described. NRP-1 mRNA expression was measured by quantitative real-time PCR according to previously described.

2.8. Statistical analysis

Results exhibiting a normal distribution are presented as mean values ± standard deviation (SD). To evaluate differences between patients and HVs, the independent-samples *t*-test was applied. Data from paired samples were analyzed by using the paired-samples t-test. SPSS 19 statistical software (IBM, Armonk, NY, USA) was used to perform statistical analyses, with *P*-values < 0.05 considered statistically significant.

3. Results

3.1. Decreased proportion of Tregs, Bregs and expression of NRP-1 on Tregs in newly-diagnosed ITP patients

The proportion of Tregs, Bregs and expression of NRP-1 on Tregs were detected with FCM in blood samples collected from 50 patients with newly-diagnosed ITP patients and 47 HVs. The proportion of Tregs in newly-diagnosed ITP patients was lower than in HVs (1.28 ± 0.87% vs. 2.47 ± 1.44%, respectively; $P < 0.0001$) (Supplementary Fig. 1A and B). Expression of NRP-1 on the Tregs from the newly-diagnosed ITP patients was also significantly lower compared to the levels of NRP-1 expression on the Tregs from the HVs (0.81 ± 0.73% vs. 1.98 ± 1.65%, respectively; $P < 0.0001$) (Fig. 1A and B). The proportion of Bregs in newly-diagnosed ITP patients was also lower than in HVs (2.35 ± 1.91% vs. 7.98 ± 3.76%, respectively; $P < 0.0001$) (Supplementary Fig. 1C and D).

3.2. Reduced NRP-1 plasma level and NRP-1 mRNA expression in newly-diagnosed ITP patients

Plasma levels of NRP-1 were detected by ELISA and were found to be lower in a group of 30 newly-diagnosed ITP patients (142.31 ± 57.67 ng/ml) compared with the plasma levels of NRP-1 in 28 HVs (280.98 ± 73.40 ng/ml) ($P < 0.0001$, Fig. 2A). Expression levels of *NRP-1* mRNA were also decreased in 30 newly-diagnosed ITP patients $(0.76 \pm 0.68) \times 10^{-4}$ compared to the 28 HVs $(1.79 \pm 0.72) \times 10^{-4}$ ($P < 0.0001$, Fig. 2B).

3.3. Thalidomide upregulated NRP-1 expression and increased the proportion of Tregs and Bregs in PBMCs from ITP patients in vitro

PBMCs obtained from 15 newly-diagnosed ITP patients and seven HVs were incubated for 24 h with THD (4 μg/ml). The percentage of Tregs increased following THD treatment (0.58 ± 0.53% vs. 1.13 ± 1.10%, respectively; $P = 0.015$) (Fig. 3A). Similarly, expression of NRP-1 on the Tregs also increased following THD treatment (2.51 ± 1.69% vs. 3.40 ± 2.24%, respectively; $P = 0.041$) (Fig. 3B). However, the percentage of Bregs did not change significantly following THD treatment (4.30 ± 2.38% vs. 4.21 ± 2.09%, respectively; $P = 0.854$) (Fig. 3C). In addition, levels of *NRP-1* mRNA were also found to increase following THD treatment (1 vs. 2.29 ± 1.33, respectively; $P = 0.002$) (Fig. 3D). The percentages of Tregs, Bregs and expression of NRP-1 on Tregs in healthy volunteers did not change significantly after culture with THD (Supplementary Fig. 2).

3.4. Efficacy and safety of THD in corticosteroid-resistant or relapsed ITP patients

As shown in Table 1, a total of 17 corticosteroid-resistant or

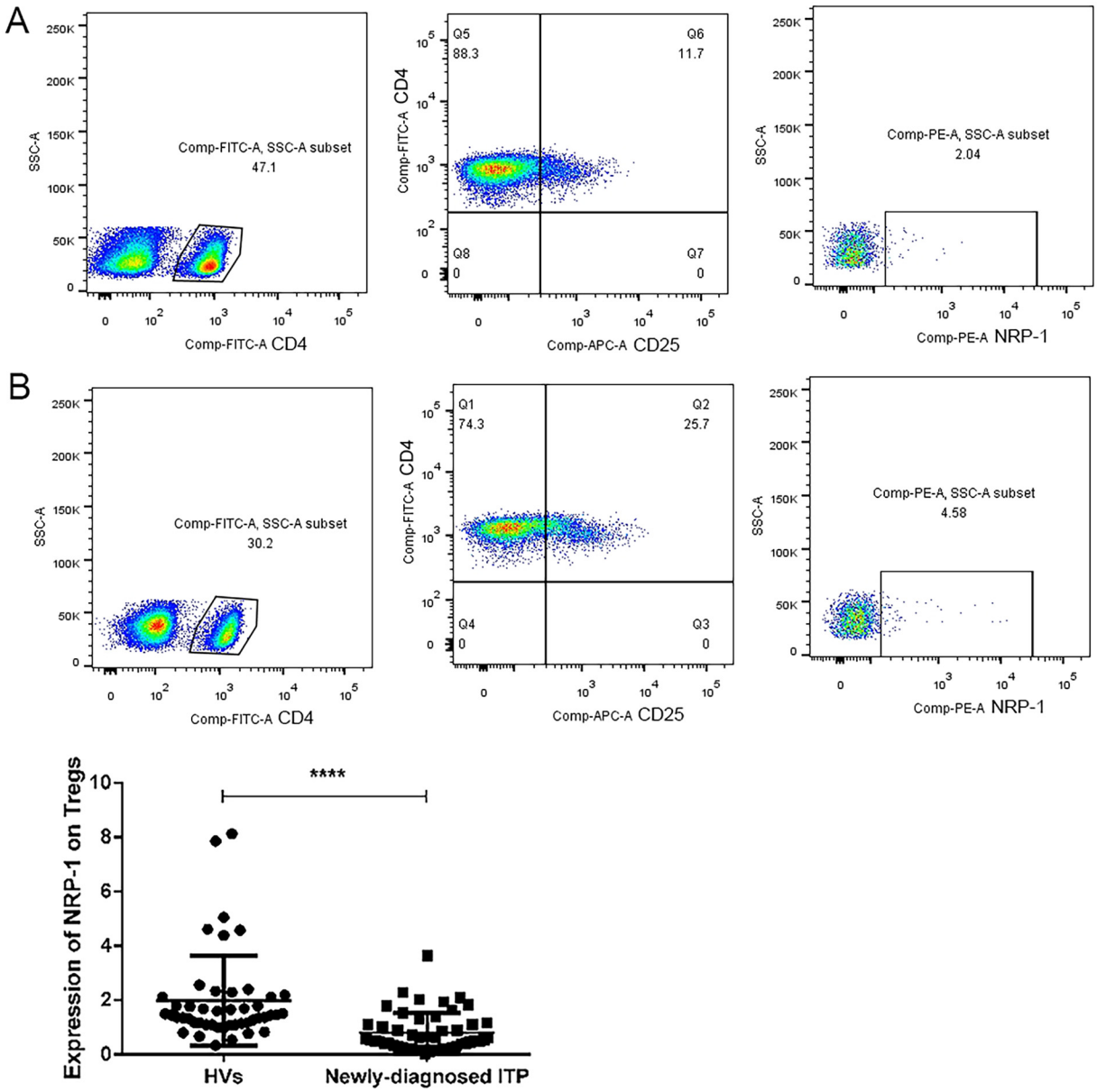

Fig. 1. Decreased expression of NRP-1 on Tregs in newly-diagnosed ITP patients. (A) Representative flow cytometry plots for NRP-1 expression on Tregs ($CD4^+CD25^+$ T cells) in newly-diagnosed ITP patients; (B) Representative flow cytometry plots for NRP-1 expression on Tregs in HVs. HVs, healthy volunteers; **** denotes $P < 0.0001$.

relapsed ITP patients were evaluated, with 7 cases in the rhTPO + THD group and 10 cases in the THD monotherapy group. The median age of patients in the rhTPO + THD group and the THD group was 23 (range 18–65) years and 35.5 (range 18–63) years, respectively. The median platelet count at the time of enrollment was 8×10^9/L (range 2–17 $\times 10^9$/L) in the rhTPO + THD group and 36.5 $\times 10^9$/L (range 20–60 $\times 10^9$/L) in the THD group, respectively. The median follow-up time in the rhTPO + THD group and the THD group was 24 (range

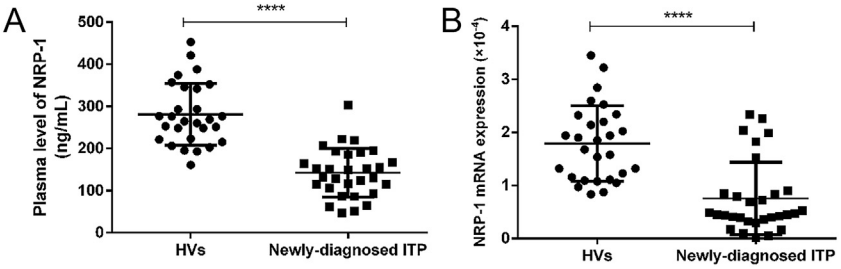

Fig. 2. Reduced NRP-1 plasma level and *NRP-1* mRNA expression in newly-diagnosed ITP patients. (A) Plasma levels of NRP-1 in newly-diagnosed ITP patients and HVs. (B) Expression of *NRP-1* mRNA in newly-diagnosed ITP patients and HVs. HVs, healthy volunteers; **** denotes $P < 0.0001$.

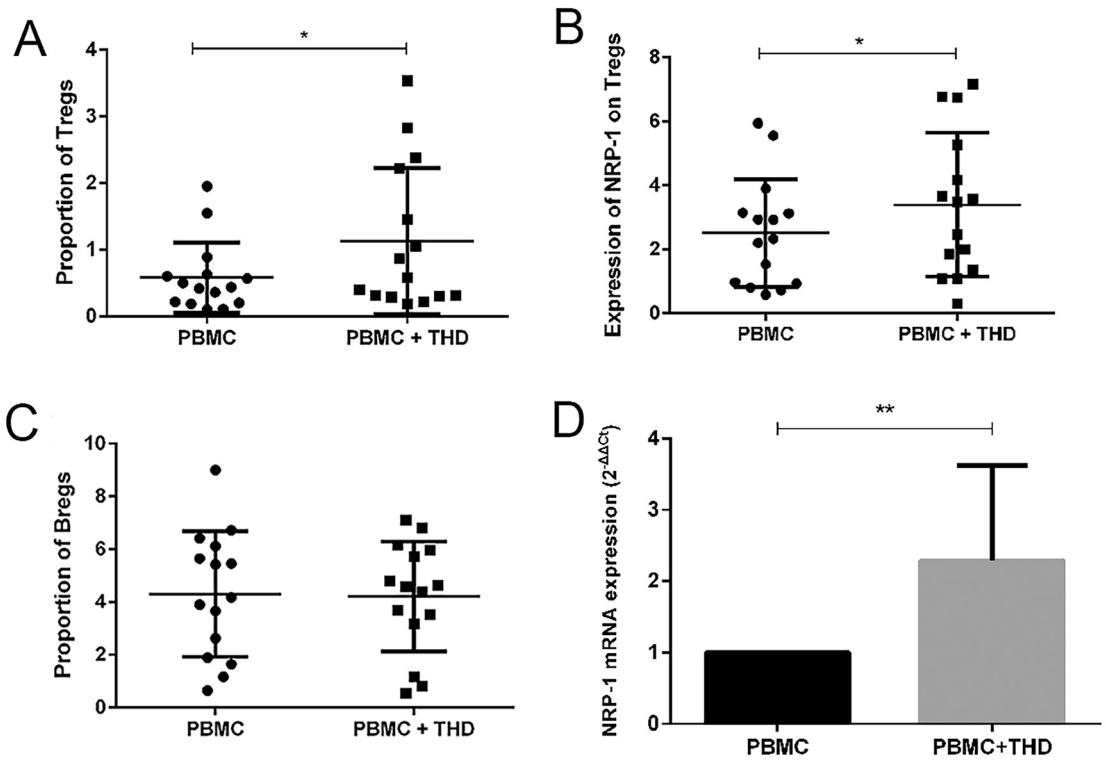

Fig. 3. Thalidomide increased proportion of Tregs and expression of NRP-1 on Tregs in PBMCs from ITP patients in vitro. (A) Proportion of Tregs in PBMCs from ITP patients. (B) Expression of NRP-1 on Tregs in PBMCs from ITP patients. (C) Proportion of Bregs in PBMCs from ITP patients. (D) Expression of NRP-1 mRNA in PBMCs from ITP patients. PBMC, peripheral blood mononuclear cell; THD, Thalidomide; * denotes $P < 0.05$. ** denotes $P < 0.01$.

18–37) months and 27.5 (range 7–43) months, respectively. All patients were followed for at least 7 months. Most patients completed the trial with a minimal dose of corticosteroids (5–10 mg prednisone).

Median TTR in the rhTPO + THD group and the THD group was 1 (range 1–4) week and 8 (range 2–48) weeks, respectively. Median duration of response in the rhTPO + THD group and the THD group was 9 (range 3–37) months and 12 (range 1–36) months, respectively. At 6 months' follow-up, the overall response rate was 85.7% (of which CR was 57.1%) in the rhTPO + THD group, whereas the overall response rate was 60% (of which CR was 10%) in the THD group. Overall response rate at 12 months in the rhTPO + THD group and the THD group was 57.1% (including 42.9% with CR) and 75% (including 50% with CR), respectively. At 24 months' follow-up, overall response rate was achieved in 100% (All 4 evaluated cases achieved CR) of patients in the rhTPO + THD group and 83.3% (including 66.7% with CR) patients in the THD group. In 17 corticosteroid-resistant or relapsed ITP patients, overall response rate at 6, 12, and 24 months was 70.6%, 66.7% and 90%, respectively. During the follow-up period, relapse rate in the rhTPO + THD group and the THD group was 57.1% and 44.4%, respectively. From initial treatment to 24 months follow-up, changes in platelet counts are shown in Fig. 4.

Most of the adverse effects were mild to moderate. All adverse events had been previously described or reported. In the rhTPO + THD group, adverse reactions included rashes (2 patients), lower extremity venous thrombosis (2 patients) and constipation (1 patient). The incidence of adverse reactions was lower in THD group, including 2 cases of rash and 1 case of constipation. These side effects were treated successfully or disappeared after drug reduction. During the follow-up period, one patient death occurred in the rhTPO + THD group in the eighteenth month with a platelet count of 38×10^9/L. The chest computed tomography angiography (CTA) from this patient showed pulmonary embolism cannot be excluded. The relationship between his death and the treatment was uncertain.

3.5. Increased NRP-1 expression and the proportion of Tregs and Bregs in corticosteroid-resistant or relapsed ITP responders after rhTPO + THD or THD treatment

The proportion of Tregs, Bregs and expression of NRP-1 on Tregs were detected in 11 CR/R patients and five NR/relapse patients before and after rhTPO + THD or THD treatment for 6 months. RhTPO + THD or THD treatment significantly increased the proportion of Tregs (0.80 ± 0.75% vs. 3.25 ± 1.57%; $P = 0.0002$), Bregs (1.90 ± 1.88% vs. 7.47 ± 2.93%; $P = 0.0001$) and expression of NRP-1 on Tregs (0.40 ± 0.29% vs. 2.40 ± 1.08%; $P < 0.0001$) in CR/R patients (Fig. 5A). In contrast, the proportion of Tregs, Bregs and expression of NRP-1 on Tregs in NR/relapse patients did not differ following rhTPO + THD or THD treatment (Fig. 5B).

Before rhTPO + THD or THD treatment, the proportion of Tregs and Bregs were no significant difference between CR/R patients and NR/relapse patients (0.80 ± 0.75% vs. 1.17 ± 0.64%; $P > 0.05$), (1.90 ± 1.88% vs. 1.95 ± 1.85%; $P > 0.05$). While, the expression of NRP-1 on Tregs from the NR/relapse patients was higher compared to the levels of NRP-1 expression on the Tregs from the CR/R patients before rhTPO + THD or THD treatment (1.27 ± 0.75% vs. 0.40 ± 0.29%, respectively; $P = 0.004$) (Fig. 5C). When compared to the expression of NRP-1 on Tregs from the HVs, there were no significant differences (1.27 ± 0.75% vs. 1.98 ± 1.65%, $P > 0.05$).

3.6. Elevated NRP-1 plasma level in corticosteroid-resistant or relapsed ITP responders after rhTPO + THD or THD treatment

Plasma level of NRP-1 was detected in nine CR/R patients and five NR/relapse patients before and after rhTPO + THD or THD treatment for 6 months. RhTPO + THD or THD treatment significantly increased NRP-1 level (241.61 ± 129.46 ng/ml vs. 446.11 ± 132.71 ng/ml; $P = 0.008$) in CR/R patients (Fig. 6A). In contrast, the plasma level of NRP-1 in NR/relapse patients did not differ following rhTPO + THD or

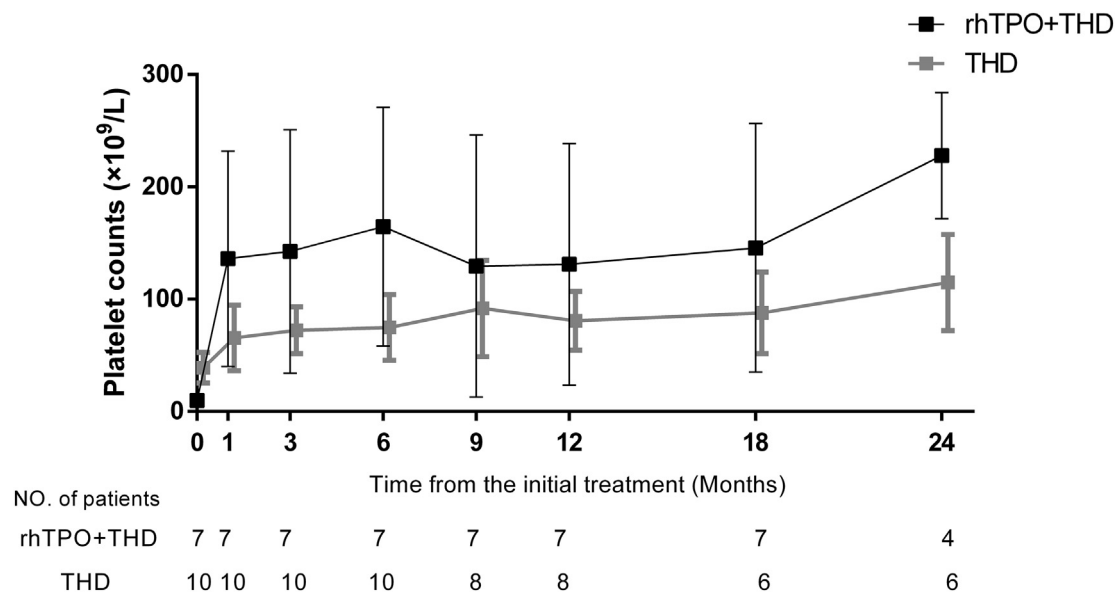

Fig. 4. Changes in platelet counts from initial treatment to 24 months follow-up.

THD treatment (Fig. 6B). Before rhTPO + THD or THD treatment, the plasma level of NRP-1 was no significant difference between CR/R patients and NR/relapse patient (Fig. 6C).

4. Discussion

Corticosteroids have been widely used as the first-line therapy for patients with ITP. However, approximately one-third of patients are unresponsive to treatment, or relapse after tapering or discontinuation. The second-line therapeutic drugs for ITP include cyclosporine, danazol, rituximab, thrombopoietin, romiplostim and eltrombopag, but they are only effective in some patients, and each of these treatments has its limitations [15]. Recent studies have reported that THD as an immunomodulatory agent, has been used to treat several autoimmune disorders [16]. ITP is also an autoimmune disease, the pathogenesis of which is complicated. Accumulating evidence suggests that abnormalities of Tregs and Bregs play a crucial role in the pathogenesis of ITP [4,9]. Our results show that the proportion of Tregs and Bregs in newly-diagnosed ITP patients is decreased compared to the HVs, which consistent with the findings of previous studies [4,20,21]. To the best of our knowledge, this is the first study to report that the expression of NRP-1 on Tregs is downregulated, and mRNA and plasma levels of NRP-1 are also decreased in ITP patients, thereby we speculate that the dysfunction of NRP-1 may be involved in the pathogenesis of ITP. Recent studies have reported THD could correct impaired mesenchymal stem cell function in patients with ITP [18]. In that way, whether THD could affect the changes of Tregs, Bregs and NRP-1 in ITP patients. In vitro, we found THD treatment upregulated expression of Tregs and NRP-1 only in ITP patients, but not in HVs. Our findings provide the first evidence that THD may be a promising therapeutic regimen for ITP by upregulating NRP-1 expression and increasing the proportion of Tregs.

To validate these findings, seventeen corticosteroid-resistant or relapsed ITP patients who received THD or THD plus rhTPO were enrolled in this study. Patients can achieve a rapid response with rhTPO treatment but usually the response is not sustained once the regime was withdrawn, making it difficult to achieve a long-term remission for ITP [22]. Therefore, in our study, ITP patients with platelet counts $< 20 \times 10^9$/L were enrolled in rhTPO plus THD group, when platelet counts greater than or equal to 20×10^9/L were enrolled in THD monotherapy group, to evaluate the efficacy and safety of THD in corticosteroid-resistant or relapsed ITP patients. Our results showed that the overall response rate at 6, 12, and 24 months in the rhTPO + THD group was 85.7%, 57.1% and 100%, respectively. In the THD group, overall response rate at 6, 12, and 24 months was 60%, 75% and 83.3%, respectively. In addition, the combination of rhTPO and THD yields a significantly shorter TTR. Furthermore, we also found rhTPO plus THD or THD therapy could significantly increase NRP-1 expression and the proportion of Tregs and Bregs in responders, but not in the non-responders. Our results demonstrated for the first time that THD could induce response in patients with corticosteroid-resistant or relapsed ITP by elevating the levels of NRP-1, Tregs and Bregs. Simultaneously, we also assessed the treatment-related adverse effects, most of which were mild and consistent with those previously reported, such as rash, constipation and thrombosis [23–26]. During the follow-up period, one patient death occurred in 7 patients with rhTPO plus THD therapy, which may result from pulmonary embolism. Therefore, it should be noted that rhTPO combined with THD may increase the risk of thrombosis.

Furthermore, it was also come to our notice that THD treatment in vitro could up-regulate the expression of NRP-1 and increase the number of Tregs in ITP patients, but had no effect on Bregs. However, the levels of NRP-1, Tregs and Bregs in patients who responded to treatment increased significantly after THD therapy. These findings indicate that THD may directly affect NRP-1, up-regulate its expression and increase the number of Tregs, while the increase of Bregs after treatment may be the indirect effect of THD. The expression level of NRP-1 in non-responders before rhTPO plus THD or THD therapy was higher than that in responders, and there was no significant difference when compared to HVs. The results further indicate that the response of ITP patients induced by THD may be mainly by upregulating NRP-1 expression. NRP-1 may be an effective therapeutic target for ITP patients who respond to THD treatment, but the pathogenesis of ITP patients who do not respond to THD treatment may not involve NRP-1.

Although our study shows encouraging results, there are still some limitations. First, a small sample size in corticosteroid-resistant or relapsed ITP patients, and the lack of a randomized controlled design. Second, concurrent medication was inevitable in this study. Most patients with corticosteroid-resistant or relapsed ITP completed the trial with a minimal dose of corticosteroids. It was difficult to analyze whether there was drug synergism or interactions that affected the outcome. Third, we used a dose of THD that is currently approved for use in human beings according to previous reports and our experience, the optimum dosage and duration of medication still need to be further

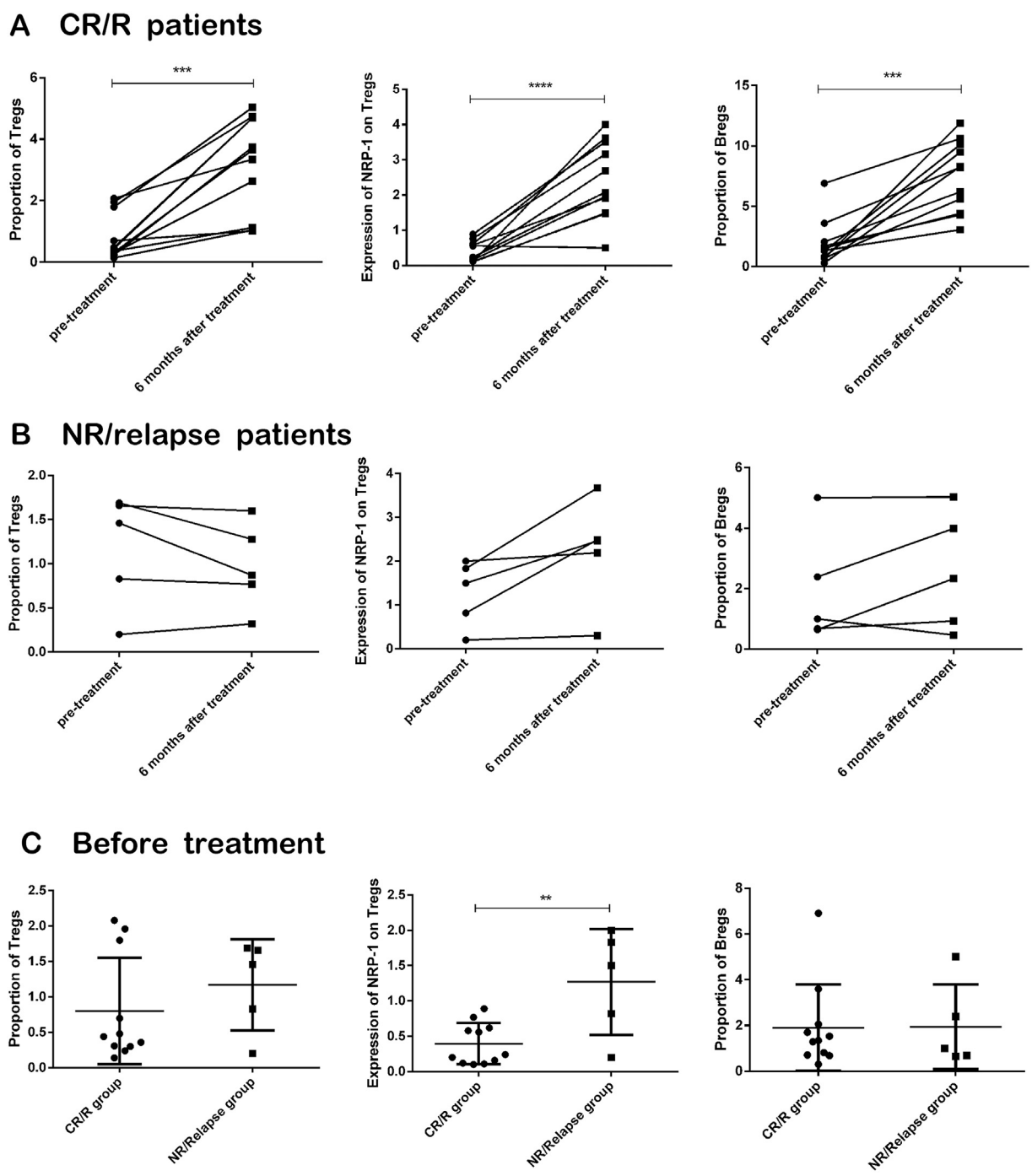

Fig. 5. The proportion of Tregs, Bregs and expression of NRP-1 on Tregs in CR/R patients and NR/relapse patients before and after rhTPO + THD or THD treatment for 6 months. (A) The proportion of Tregs, Bregs and expression of NRP-1 on Tregs in CR/R patients. (B) The proportion of Tregs, Bregs and expression of NRP-1 on Tregs in NR/relapse patients. (C) The proportion of Tregs, Bregs and expression of NRP-1 on Tregs in CR/R patients and NR/relapse patients before rhTPO + THD or THD treatment. **** denotes $P < 0.0001$; *** denotes $P < 0.001$; ** denotes $P < 0.01$.

explored.

In conclusion, our study shows for the first time that NRP1 is involved in the pathogenesis of ITP, and THD could induce response in patients with corticosteroid-resistant or relapsed ITP by upregulating NRP-1 expression and restoring the proportion of Tregs and Bregs. The preliminary results of our study suggest that THD might be served as a novel therapeutic agent in ITP patients. However, multicenter, randomized, controlled, large-sample clinical trials are still required.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.04.041.

List of abbreviations

ITP	immune thrombocytopenia
THD	thalidomide
rhTPO	recombinant human thrombopoietin
Tregs	regulatory T cells
Bregs	regulatory B cells
NRP-1	Neuropilin-1
HVs	healthy volunteers
R	response
CR	complete response

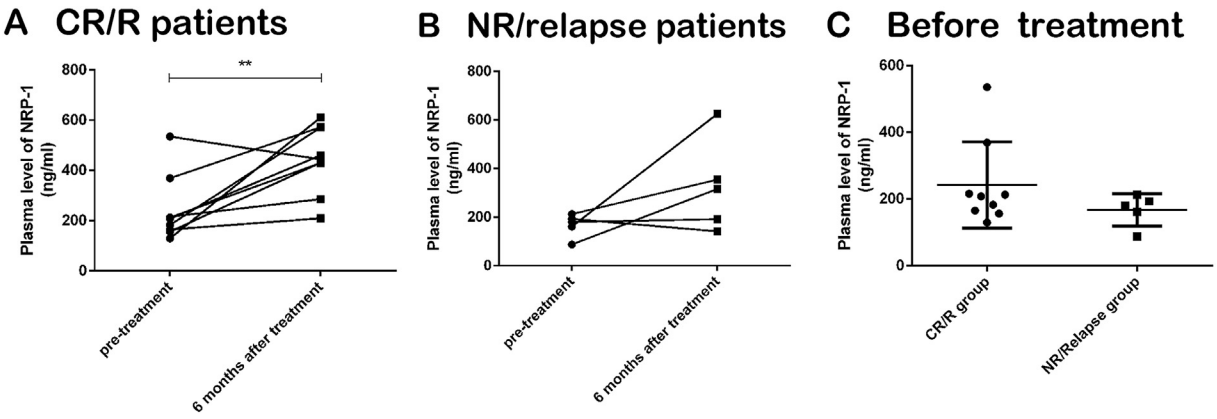

Fig. 6. Plasma level of NRP-1 in CR/R patients and NR/relapse patients before and after rhTPO + THD or THD treatment for 6 months. (A) Plasma level of NRP-1 in CR/R patients. (B) Plasma level of NRP-1 in NR/relapse patients. (C) Plasma level of NRP-1 in CR/R patients and NR/relapse patients before rhTPO + THD or THD treatment. ** denotes $P < 0.01$.

NR	no response
TTR	time to response
PBMC	peripheral blood mononuclear cell

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Authors' contributions

Zhigang Yang designed the study. Ruiting Wen, Weijuan Chen, Yufeng Wang, Zhiyun Weng and Sisi Wen performed laboratory correlative experiments. Zhigang Yang, Ruiting Wen and Xiaojun Zhang analyzed the data. Yuming Zhang, Guocai Wu and Meihua Guan implemented the treatment program. Zhigang Yang and Ruiting Wen drafted the manuscript.

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