Plasma levels and expression of interleukin-37 in patients with immune thrombocytopenia

FENG ZHANG¹, XIAO-JUAN ZHU¹, XIAO-JING ZHU², YAN-XIA LIU¹, TING YUAN¹ and QING-MIN YAO¹

¹Department of Hematology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021; ²Department of Orthopedics, Chinese Medicine Hospital of Linyi City, Linyi, Shandong 276003, P.R. China

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Abstract. Interleukin (IL)-37 has an important role in autoimmune diseases by suppressing immunity and inflammation; however, the role of IL-37 in immune thrombocytopenia (ITP) has remained largely elusive. The present study aimed to investigate the expression of IL-37 and its potential role in the pathogenesis of ITP. The plasma levels and expression of IL-37 in the peripheral blood mononuclear cells of patients with active ITP, ITP patients in remission and healthy controls were measured by ELISA and reverse transcription-quantitative PCR, respectively. The levels of IL-37 in patients with ITP treated with and without glucocorticoids were also determined by ELISA. Specific anti-platelet glycoprotein (GP)IIb/IIIa and/or GPIb/IX autoantibodies were assayed by modified monoclonal antibody-specific immobilization of platelet antigens. The mean value of plasma IL-37 in ITP patients was slightly higher than that in healthy controls, but this was not statistically significant. There was no correlation between IL-37 and anti-platelet autoantibodies, and no significant difference in the IL-37 concentration was identified between patients treated with and without glucocorticoids. In addition, the correlation between IL-37 and the platelet count was analyzed, with no statistical significance observed. It was therefore concluded that IL-37 may not have a pivotal role in the development of ITP. However, the lack of significant differences may be due to the limited number of patients in different groups. A larger number of ITP patients should be enrolled in the future work and achieve more accurate results.

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a low platelet count with mucocutaneous or other types of bleeding. The pathogenesis of ITP remains poorly understood, while immune disorders are thought to have an important implication. The presence of anti-platelet glycoprotein (GP) antibodies is also considered to be critically involved in ITP (1). In addition, a complex dysregulation of cellular mechanisms has been reported, including the imbalance of the type 1 T-helper cell (Th1)/Th2 ratio (2), increased number of Th17 cells (3), decreased number or functional deficit of regulatory T cells (4,5) and increased cytotoxic T lymphocyte-mediated cytotoxicity (6).

Interleukin (IL)-37, a novel anti-inflammatory cytokine previously known as interleukin-1 family member 7 before it was renamed, has a pivotal role in the suppression of immune responses (7-10). Alternative splicing of the human gene for IL-37 gives rise to 5 different isoforms: IL-37a, b, c, d and e, among which IL-37b is the most likely one to be biologically functional. IL-37 is widely expressed in several types of cells, tissues and organs, including peripheral blood mononuclear cells (PBMCs) (7). The major role of IL-37 is to decrease excessive inflammation in innate and adaptive immune diseases, mainly by inhibiting the expression, production and function of pro-inflammatory cytokines, including IL-1α, IL-6, tumor necrosis factor (TNF) and macrophage inflammatory protein-2. The abundance of these cytokines has been reported to increase with the silencing of endogenous IL-37 in human blood cells (7,11). In vivo, IL-37-transgenic mice exhibited markedly reduced manifestations of endotoxemia, dextran sulphate sodium colitis, ischemia-reperfusion injury and obesity-induced inflammation (12-14). Furthermore, treatment with recombinant IL-37 in wild-type mice has been demonstrated to exert protective effects in several models of inflammation and injury (15-17).

Aberrant expression of IL-37 has been observed in several inflammatory and autoimmune diseases, including rheumatoid arthritis (RA) (18-21), systemic lupus erythematosus (SLE) (22,23), inflammatory bowel disease (IBD) (24,25), ankylosing spondylitis (AS) (26) and Graves' disease (GD) (27). However, the role of IL-37 in ITP has remained elusive. To
investigate the expression of IL-37 and its potential role in the pathogenesis of ITP, the levels of IL-37 in ITP patients were measured and their correlation to disease activity was determined.

**Materials and methods**

**Patients and controls.** A total of 34 patients with ITP were enrolled in the present study (Table I), consisting of 18 newly diagnosed ITP patients with active disease (11 females and 7 males; age range, 19-62 years; median age, 39.89 years) and 16 patients in remission (10 females and 6 males; age range, 19-62 years; median age, 37.25 years). The platelet count of patients with active ITP was 1.30±10^9/l (median, 12.28±10^9/l), which was significantly lower than that of patients in remission (1.01-3.05±10^9/l; median, 163±10^9/l; P<0.001). Out of the 34 patients with ITP, 12 were treated with glucocorticoids, including high-dose dexamethasone (HD-DEX) 40 mg daily for 4 day severy 4 weeks and predinsone 1.0 mg/kg daily, which was then tapered.

The control group consisted of 15 healthy volunteers admitted to the hospital for routine physical examination (9 females and 6 males; age range, 16-66 years; median, 39.67 years). The platelet count was 128-325×10^9/l (median, 231×10^9/l).

The enrollment of patients and healthy volunteers took place between November 2016 and January 2018 at the Department of Hematology of the Shandong Provincial Hospital affiliated to Shandong University (Jinan, China). All cases met the diagnostic criteria of ITP, as previously described (28). Patients complicated with diabetes, hypertension, cardiovascular diseases, pregnancy, active or chronic infection or connective tissue diseases were excluded from the study.

**PBMC preparation.** Plasma samples were separated by centrifugation and stored at -20˚C to be used for the determination of IL-37 and anti-platelet autoantibodies.

PBMCs were isolated from heparinized blood samples by Ficoll-Hypaque density gradient centrifugation at 780 x g for 20 min at 20˚C, and stored at -80˚C for future use.

**ELISA of IL-37.** The plasma concentration of IL-37 was measured by a commercial ELISA kit, according to the manufacturer's protocols (AdipoGen). The lower detection limit of this assay was 16 pg/ml.

**Determination of the mRNA expression of IL-37.** For reverse transcription (RT), total RNA was extracted from PBMCs using TRIzol reagent (Thermo Fisher Scientific, Inc.). The RNA was then reverse transcribed to complementary DNA using the PrimeScript™ RT Reagent kit (Takara Bio, Inc.), according to the manufacturer's protocol. Quantitative qPCR for IL-37 was performed on an ABI PRISM_7500 Sequence Detection System (Thermo Fisher Scientific, Inc.) using SYBR Green (ToyoBo Life Science) according to the manufacturer's protocol. β-Actin was used as the endogenous control. The sequences of specific primers were as follows: IL-37 forward, 5'-AGACCTTACGCACTGGGACATC-3' and reverse, 5'-TCTTGGTATTGCAAGTGTGAGGATCCA-3'; β-actin forward, 5'-TTGCCGCACAGGTACGAA-3' and reverse, 5'-GCCGATCCACCGGAGTACT-3'. The relative expression levels between IL-37 and β-actin were compared using the 2^⁻ΔΔCq method (29).

**Anti-platelet autoantibody determination.** The specific anti-platelet autoantibodies to GPIIb/IIIa and/or GPIb/IX were analyzed by modified monoclonal antibody-specific immobilization of platelet antigens, as previously described (30).

**Statistical analysis.** Values are expressed as the mean ± standard deviation. Statistical significance between two groups was determined using an unpaired Student's t-test. For multiple comparisons, one-way analysis of variance followed by Bonferroni's post-hoc test was used. The correlation analysis was performed using Pearson's correlation. All statistical analyses were performed using SPSS 13.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**IL-37 expression in ITP patients and controls.** Fig. 1A presents the plasma concentration of IL-37 in the ITP and control groups. The mean plasma level of IL-37 in the ITP patients (90.4±32.56 pg/ml) was higher than that in normal controls (75.62±27.52 pg/ml), but this was not statistically significant (P>0.05). In Fig. 1B, the mRNA levels of IL-37 in PBMCs determined using the 2^⁻ΔΔCq method are presented as the fold change in gene expression normalized to an endogenous reference gene (β-actin) and relative to normal controls. The relative mRNA expression of IL-37 in untreated patients was 1.07 times that in the normal controls, with no statistically significant difference observed (P>0.05; Fig. 1B).

**IL-37 expression in patients with active ITP and in remission.** To investigate any potential correlation between IL-37 and disease activity, the concentration of plasma IL-37 and IL-37 mRNA were further analyzed in patients with active ITP, patients in remission and healthy controls. The concentration of plasma IL-37 in the different groups was as follows: 98.75±36.85 pg/ml (active ITP patients), 81.04±24.83 pg/ml (ITP patients in remission) and 75.62±27.52 pg/ml (normal controls); no significant differences were observed among active ITP patients, patients in remission and healthy controls (P>0.05; Fig. 2A). Despite not reaching statistical significance, the P-value for the comparison of plasma levels of IL-37 between active ITP patients and controls was 0.107, as determined by Bonferroni's post-hoc test. Similarly, no significant difference in the IL-37 mRNA expression was observed among the three groups (P>0.05; Fig. 2B).

**Plasma IL-37 concentration in anti-platelet autoantibody positive/negative patients.** Since anti-platelet autoantibodies have an important role in the pathogenesis of ITP, the concentration of IL-37 was further compared in anti-platelet autoantibody-positive and -negative patients. Fig. 3A presents the concentration of IL-37 in anti-GPIIb/IIIa-positive and -negative ITP patients. No significant difference was identified between anti-GPIIb/IIIa-positive (83.06±22.02 pg/ml) and -negative ITP patients (93.93±36.47 pg/ml; P>0.05), or in
the plasma levels of IL-37 between anti-GPIIb/IIIa-positive and -negative ITP patients, as compared with that in normal controls (75.62±27.52 pg/ml; P>0.05). Similar results were obtained for anti-GPIb/IX-positive/negative ITP patients (P>0.05; Fig. 3B).

**Influence of glucocorticoids on plasma IL-37 concentration in ITP patients.** Glucocorticoids are used as a first-line therapy for ITP. In order to investigate any potential difference between patients treated with and without glucocorticoids, the concentration of plasma IL-37 was analyzed in different groups. The concentration of IL-37 was as follows: ITP patients treated without glucocorticoids (97.06±36.45 pg/ml), ITP patients treated with glucocorticoids (78.23±19.84 pg/ml) and normal controls (75.62±27.52 pg/ml); no significant difference was observed (P>0.05; Fig. 4).

**Correlation between IL-37 and platelet count in ITP patients.** The correlation between plasma levels of IL-37 and platelet count in patients with active ITP was assessed by Pearson's correlation analysis and no significant correlation was identified (P>0.05).

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Groups: Cases no. 1-18, ITP patients with active disease; cases no. 19-34, patients in remission. M, male; F, female; EC, ecchymoses; EP, epistaxis; GH, gingival hemorrhage; GUH, genitourinary hemorrhage; PT, petechiae; HD-DEX, high-dose dexamethasone; IVIG, intravenous gamma globulin; Plt, platelet (infusion); Pred, prednisone; R, rituximab; GP, glycoprotein.
Discussion

Autoimmune diseases, including ITP, are characterized by impaired function caused by an immune response, in which abnormal antibodies are produced and attack the body itself. IL-37, a novel member of the IL-1 family, has been recognized as an important anti-inflammatory cytokine expressed by immune cells. Abnormal expression of IL-37 has been reported...
in various autoimmune diseases, including SLE, RA, IBD, GD and AS (18-27), as a potential negative factor influencing the development of these disorders. According to Ye et al (22), increased IL-37 expression was associated with SLE disease activity, and IL-37 levels were significantly higher in patients with renal disease. Xia et al (20) indicated that the expression of IL-37 was markedly increased in RA patients, and a significant correlation was identified between IL-37 levels and disease activity. In addition, patients with active AS and GD had higher levels of IL-37 than those with inactive AS and GD and healthy controls (26,27). All aforementioned data suggest that the expression of IL-37 is associated with disease activity of the above autoimmune diseases. Furthermore, inflammatory cytokine expression was higher in patients with active disease, as compared with that in patients with inactive disease and healthy controls. Functional analysis indicated that certain pro-inflammatory cytokines, including IL-1/6/10 and TNF-α, may be involved in promoting the expression of IL-37, while high IL-37 expression may inhibit the overproduction of pro-inflammatory cytokines in autoimmune diseases through a negative feedback mechanism (31).

While most studies have reported an increased IL-37 expression in autoimmune diseases, decreased IL-37 expression was also identified in other autoimmune conditions, including Behcet's disease (32), asthma (33), Vogt-Koyanagi-Harada disease (34) and allergic rhinitis (35). The inconsistent expression of IL-37 among different autoimmune diseases may be due to differences in their immunological mechanisms. As mentioned above, IL-37 levels frequently exhibit a positive correlation with disease activity. This means that infection or inflammation increases IL-37 expression, while a high level of IL-37 may help to reduce the disease severity through a negative feedback mechanism. Conversely, low levels of IL-37 may indicate more severe inflammation.

To date, the role of IL-37 in ITP patients has remained elusive. In the present study, the plasma concentration of IL-37 and its mRNA expression in PBMCs of ITP patients was determined for the first time by using ELISA and RT-qPCR, respectively. The results indicated no significant difference in IL-37 levels between ITP patients and controls. In addition, no correlation was identified between IL-37 and anti-platelet autoantibodies. The correlation between IL-37 and the platelet count was also analyzed, with no statistical significance observed. However, the mean value of plasma IL-37 in ITP, particularly active ITP, was much higher than that in the controls. However, the P-value for the comparison of plasma IL-37 between patients with active ITP and controls was 0.107, as determined by Bonferroni's post-hoc test. Further studies with a bigger sample size are required to confirm these results, which may obtain a higher statistical significance.

It has been reported that IL-37 is able to translocate into the nucleus and downregulate pro-inflammatory cytokines (36), suggesting that IL-37 may also have an intracellular, in addition to its extracellular, function. Further studies examining the expression of intracellular IL-37 by methods including flow cytometry may be required.

Glucocorticoids, including conventional prednisone and HD-DEX, have been recommended as first-line therapy for ITP patients. Song et al (23) reported that glucocorticoids are able to downregulate the increased expression of IL-37 in SLE. The concentration of IL-37 was analyzed in ITP patients treated with and without glucocorticoids, with no significant difference observed between the two groups, which indicates that glucocorticoids may not regulate IL-37 in ITP patients. There are several explanations for this. First, the patients included in the present study were treated with glucocorticoids days, months or years ago, and thus, the glucocorticoid medication or blood sample collection time was inconsistent. Furthermore, the patients treated with or without glucocorticoids were not the same patients, and in a future study, assessment of the concentration of IL-37 in the same patients prior to and after glucocorticoid treatment may be more meaningful.

Accumulating evidence suggests that IL-37 has a pivotal role in autoimmune diseases. In the present study, the expression of IL-37 in ITP patients was evaluated for the first time, but no significantly abnormal expression of IL-37 was identified in these patients. It was therefore concluded that IL-37 may not have a pivotal role in the development of ITP. However, the lack of significant differences may be due to the limited...
number of patients in different groups. Larger number of ITP patients should be enrolled in the future work and achieve more accurate results.

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Availability of data and materials
All data generated or analyzed during the present study are included in this published article.

Authors' contributions
FZ contributed to experimental design, data analysis and manuscript writing; X-JuZ contributed to case collection, literature search and manuscript writing; X-JiZ and TY performed the experiments and statistical analysis; YL contributed to case collection, data interpretation and figure creation; QY was responsible for data interpretation, literature search, paper revision and submission.

Ethics approval and consent to participate
The study was approved by the Medical Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University (Jinan, China). Written informed consent was obtained from all patients and/or their guardians.

Patient consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

References


