Contributions of TRAIL mediated megakaryocyte apoptosis to impaired megakaryocyte and platelet production in immune thrombocytopenia

TRAIL mediated megakaryocyte apoptosis in ITP

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Abstract

Recent in vitro studies provide evidence for autoantibody-induced suppression of megakaryocytopoiesis, which studies show a reduction in megakaryocyte production and maturation in the presence of immune thrombocytopenia (ITP) plasma. Here, we will present CD34+ cells from healthy umbilical cord blood mononuclear cells cultured in medium containing thrombopoietin, stem cell factor, interleukin-3 and 10% plasma from either ITP patients or healthy subjects. The quantity, quality and apoptosis of megakaryocytes were measured. We observed that most ITP plasma boosted megakaryocyte quantity, but impaired quality, resulting in significantly less polyploidy cells (N≥4) and platelet release. In these megakaryocytes, we found a lower percentage of cell apoptosis, a lower expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and a higher expression of Bcl-xL. Furthermore, there was a decrease of sTRAIL in ITP plasma and in cell culture supernatants of this group when compared with the control group. Our findings suggest that decreased apoptosis of megakaryocytes also contributes to in vitro dysmegakaryocytopoiesis and reduced platelet production. The abnormal expression of sTRAIL in plasma, TRAIL and Bcl-xL in megakaryocytes may play a role in the pathogenesis of impaired megakaryocyte apoptosis in ITP.
Introduction

Immune thrombocytopenia (ITP) is an immune-mediated bleeding disorder in which platelets are opsonized by autoantibodies and destroyed by phagocytic cells in the reticuloendothelial system.\textsuperscript{1-4} Although autoantibodies produced by autoreactive B cells against self-antigens, specifically IgG antibodies against GPIIb/IIIa and/or GPIb/IX, are considered to play a crucial role, T-cell mediated immune abnormalities such as Th1 bias,\textsuperscript{5,6} the decreased number or defective function of regulatory T cells\textsuperscript{7,8} and the platelet destruction by cytotoxic T cells (CTL)\textsuperscript{9,10}, have been considered equally important in the pathogenesis of ITP. As a result of the accelerated destruction, platelet production is thought to compensatorily increase. However, platelet turnover studies in the 1980s reported that two-thirds of ITP patients showed decreased or normal platelet production, suggesting that impaired platelet production may also contribute to thrombocytopenia.\textsuperscript{11}

Recent in vitro studies, showing reduced megakaryocyte production and maturation in the presence of autoantibodies against platelet glycoproteins in ITP plasma, have provided evidence for autoantibody-induced suppression of megakaryocytogenesis.\textsuperscript{12,13} However; reduced platelets cannot be ascribed to decreased megakaryocyte production in ITP patients with normal or increased bone marrow megakaryocytes.

It has been known that platelets are formed from mature megakaryocytes and arise from the development of long and thin cytoplasmic extensions called proplatelets.\textsuperscript{14-16} Several reports indicate a close relationship among apoptosis, megakaryocyte maturation, and platelet release. Zauli et al\textsuperscript{17} found that the onset of megakaryocyte apoptosis coincides with the maximum in the high ploidy megakaryocyte fraction and the number of platelets released in the culture. De Botton et al\textsuperscript{18} elegantly provided evidence that proplatelet formation was a consequence of local caspase activation during megakaryocyte differentiation. Therefore, we suppose abnormal megakaryocyte apoptosis may be responsible for persistent thrombocytopenia in ITP.
In the present study, we observed that most ITP plasma inhibited megakaryocyte apoptosis, boosted megakaryocyte mass and depressed platelet production in vitro, suggesting a possible role of megakaryocyte apoptosis in the pathogenesis of ITP.

**Materials and methods**

**Patients and controls**

Whole blood was collected from 49 patients (28 females and 21 males) with chronic ITP, all of whom did not undergo any form of therapy for at least three weeks prior to blood sampling (Table 1). The diagnosis in all patients, based on the criteria for chronic ITP as previously described,\(^9\) exhibited isolated thrombocytopenia for more than 12 months, normal or increased bone marrow megakaryocytes, normal spleen size, and no secondary immune or nonimmune abnormality that could account for the thrombocytopenia. Patients with systemic lupus erythematosus (SLE) and/or antiphospholipid syndrome (APS) were excluded from this study, as were pregnant patients or those with concomitant human immunodeficiency or hepatitis C virus infection. Median age (range) and median platelet count (range) at the time of enrollment was 33 years (16-70) and 19×10\(^9\)/L (2-68), respectively. Control blood was obtained from 22 healthy blood donors (13 females and 9 males; age range 23-65 years, median 36 years) with no history of blood transfusions or pregnancies. Platelet counts ranged from 159 to 287×10\(^9\)/L, with the median count of 208×10\(^9\)/L. Enrollment took place between March 2007 and August 2009 at the Department of Hematology, Qilu Hospital, Shandong University. Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Ethical approval for the study was obtained from the Medical Ethical Committee of Qilu Hospital, Shandong University.

**Plasma preparation**

Plasma samples were obtained from ethylenediaminetetraacetic acid (EDTA) anticoagulated blood by centrifugation at 3000 g for 30 min at 20°C to remove platelets, and stored at -80°C prior to use.

**IgG purification**

IgG antibody was purified from plasma by affinity chromatography using 4HiTrap
Protein A HP column which was operated with AKTA explorer 100 chromatography system (Amersham Co., Sweden) according to the manufacturer’s instructions. The IgG concentration was adjusted to 1.2 mg/ml. The final IgG preparations were dialyzed overnight with culture medium.

**Adsorption of autoantibodies from ITP plasma**

ITP patient plasma (0.5 ml) was mixed with the washed control platelets (1×10⁹/ml plasma) and incubated at 4°C for 1 hour. After centrifugation at 3000g for 5 minutes, the supernatant plasma was again adsorbed with fresh platelets at 4°C for 1 to 1.5 hours. The adsorbed plasma was then analyzed by modified monoclonal antibody specific immobilization of platelet antigens (MAIPA) for the presence of autoantibodies as previously described in detail by Hou et al.²⁰

**Megakaryocyte culture**

CD34⁺ cells were purified from healthy umbilical cord blood mononuclear cells (MNCs) obtained from Ficoll-Hypaque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) by using a magnetic cell separation method (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity was subsequently checked and always exceeded 92%.

Colonies forming unit - megakaryocytes (CFU-MKs) were quantitated using a commercially available kit (MegaCult®-C; Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. 5×10³ CD34⁺ cells were planted in each chamber slide, supplemented with thrombopoietin (TPO, 50 ng/ml), IL-3 (10 ng/ml) and IL-6 (10 ng/ml) (Stem Cell Technologies) in 1.5ml volume containing 10% plasma from ITP patients or healthy controls for 12 days of incubation at 37°C in a humidified atmosphere of 5% CO₂. After dehydration, fixation, and immunocytochemical staining with mouse anti-human GPIIb/IIIa antibody and biotin-conjugated goat anti-mouse IgG, megakaryocyte colonies were defined as groups of three or more GPIIb/IIIa-positive cells.

CD34⁺ cells were cultured at 2×10⁵/well in 24-well plates in the presence of 100μl plasma from patients or controls and 900μl serum-free medium (SFEM, Stem Cell Technologies) supplemented with 100ng recombinant human thrombopoietin...
(rhTPO, Sansheng Pharmacy, Shenyang, China), 100ng stem cell factor (SCF, Diao Pharmacy, Chengdu, China) and 10ng recombinant human interleukin-3 (rhIL-3, Biosouth Research Laboratories, New Orleans, USA). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 8~15 days.

**Flow cytometric analysis**

Thereafter, cells were labeled with phycoerythrin-Cyano dyes 5(PEcy5)-conjugated CD41a mAb (BD Biosciences, San Jose, CA, USA), PEcy5-conjugated IgG1 (BD Biosciences) used as an isotype control, according to the manufacturer’s instructions, and then analyzed with a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). CD41a⁺ cells were gated and recognized as megakaryocytes, and the number of megakaryocytes was determined [cell number (determined by direct counting of each culture) ×%megakaryocytes (determined from FACS data)].

To measure ploidy distribution, the megakaryocytes were identified after labeled with PEcy5-conjugated CD41a mAb and incubated with 500µl propidium iodide (PI, BD ParMingen, San Diego, CA, USA) containing RNase. CD41a⁺ cells were gated and ploidy distribution was assessed by the intensity of the PI fluorescence.

Detection of platelets produced in culture was performed as previously described. In brief; cultured cells were centrifuged at 350g for 15 minutes and incubated with PEcy5-conjugated CD41a mAb. After incubation, each sample was diluted to 300µl and collected at median rate for 50 seconds by flow cytometry. An analytical gate was determined according to scatter properties of normal blood platelets treated similarly using a log scale for FSC and SSC which excluded large contaminating cells (megakaryocytes) and small debris or microparticles.

Megakaryocyte apoptosis was measured using the Annexin V-FITC Apoptosis Detection Kit (Jingmei biotech co., Ltd., Beijing, China) according to the manufacturer’s instructions. Cells were labeled with PEcy5-conjugated CD41a mAb, incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI, and subsequently analyzed by flow cytometry. CD41a⁺ cells were gated and apoptotic megakaryocytes were Annexin V⁺PI cells within that population.
To analyze the expression of certain proteins (Cyclin B1/Cyclin D3, Bcl-2/Bcl-xL) in megakaryocytes, PEcy5-conjugated CD41a mAb labeled cells were incubated with FITC-conjugated Mouse Anti-Human Cyclin B1/FITC-conjugated Mouse IgG1 (BD Bioscience), FITC-conjugated Mouse Anti-Human Cyclin D3/FITC-conjugated Mouse IgG1κ (BD Bioscience), phycoerythrin (PE)-labeled mouse anti-human Bcl-2/PE-labeled mouse IgG1 (BD Bioscience) or FITC-labeled mouse anti-human Bcl-xL/FITC-labeled mouse IgG3 (Southern Biotech, Birmingham, Alabama, USA). The labeled cells were resuspended after washing and analyzed within two hours using flow cytometer.

To observe the expression of certain external apoptosis pathway proteins (Fas/FasL, TRAIL/TRAIL-R2, caspase-3/caspase-8) in megakaryocytes, PEcy5-conjugated CD41a mAb labeled cells were incubated with FITC-conjugated Mouse Anti-Human CD95/FITC-conjugated Mouse IgG1κ (BD Bioscience), PE-conjugated Mouse Anti-Human CD178/PE-conjugated Mouse IgG1 (BD Bioscience), PE-conjugated Mouse Anti-human TRAIL/PE-conjugated Mouse IgG1 (BD Bioscience) or PE-conjugated Mouse Anti-human DR5(TRAIL-R2)/PE-conjugated Mouse IgG1κ (eBiosciences, San Diego, CA, USA). And we used CaspGLOW™ Fluorescein Active Caspase-3/Caspase-8 Staining Kit (BioVision, CA, USA) to analyze the active caspase-3/caspase-8 expression in PEcy5-conjugated CD41a mAb labeled megakaryocyte cells according to the manufacturer’s instructions. The labeled cells were resuspended after washing and analyzed within two hours using flow cytometer.

We also measured the expression of TRAIL on the surface of produced platelets. Cultured cells were centrifuged at 350g for 15 minutes and incubated with PEcy5-conjugated CD41a mAb and PE-conjugated mouse anti-human TRAIL. After incubation, each sample was diluted to 300µl and collected at median rate for 50 seconds by flow cytometry as described above.

**ELISA for TPO, IL-11, sFas and sTRAIL in plasma**

The levels of plasma TPO and IL-11 in controls and patients were measured by sandwich ELISA kits (R&D, Oxon, United Kingdom) according to the manufacturer’s
instructions. In addition, we measured the plasma levels of sFas (Bender Medsystems GmbH Campus Vienna, Austria) and sTRAIL (Diaclone, Besancon cedex, France) in controls and ITP patients by sandwich ELISA kits.

**Statistical analysis**

All data is presented as mean ± standard deviation (SD). Differences among four groups were assessed using analysis of variance (ANOVA) and differences among four groups at different measuring time were assessed using repeated measures of ANOVA analysis. Because some of the data had heterogeneity of variance, comparison between these numbers was analyzed by Kruskal-Wallis test. P value less than 0.05 was considered statistically significant. Statistical analysis was performed by the SPSS 13.0 (SPSS Inc., Chicago, USA) statistical software programs.

**Results**

**Effects of ITP plasma on megakaryocyte production, maturation and platelet release**

As shown in Figure 1A, ITP patients can be divided into three groups according to the effect that their plasma had on megakaryocyte production after 15 days co-culture. Compared with healthy controls [mean±SD, (5.10±0.90) ×10^5], the number of megakaryocytes in group A (26 ITP patients) was significantly increased [(7.13±0.86) ×10^5, P<0.0001], while that in group B (14 ITP patients) was markedly decreased [(2.83±1.02) ×10^5, P<0.0001]. However, the plasma of 9 ITP patients in group C did not significantly affect the number of megakaryocytes [(5.20±0.66) ×10^5, P=0.776].

CFU-MK formation in group A, B, C and controls were 11.89±2.93, 11.50±2.62, 10.56±2.35 and 12.05±2.72, respectively. There was no remarkable differences among the four groups (P=0.553), suggesting that increased megakaryocyte counts are not mediated by accelerated proliferation of megakaryocyte progenitors (Figure 1B).

To rule out the possibility that TPO and IL-11, the known positive regulators of megakaryopoiesis, might be elevated in ITP plasma from group A compared with controls and other groups, we assayed the plasma levels of TPO and IL-11 by ELISA. Consistent with previous reports, TPO levels in plasma did not differ significantly
among ITP patients in group A [(71.14±12.15) pg/ml], group B [(68.24±17.25) pg/ml],
group C [(66.36±15.21) pg/ml] and controls [(57.08±24.04) pg/ml]. Plasma IL-11
congestion in group A [(126.74±44.23) pg/ml] was remarkably higher than that in
controls [(31.19±9.20) pg/ml] (P<0.0001), but there was no significant difference
among group A [(126.74±44.23) pg/ml], B [(130.03±40.16) pg/ml] and C
[(134.15±38.03) pg/ml].

The percentage of megakaryocyte polyploidy (N≥4) in group A, B, C and controls
were 16.35±4.90%, 16.11±5.66%, 24.66±2.49% and 24.57±2.83%, respectively. The
platelet release in group A, B, C and control cultures were (7.51±2.41)×10³,
(7.21±2.45)×10³, (10.12±1.91)×10³ and (11.21±1.82)×10³, respectively (Figure 1C).
The megakaryocyte polyploidy and platelet counts in group A and group B were
significantly lower than those in controls (P<0.0001).

**Inhibited apoptosis and overexpressed Bcl-xL in megakaryocytes cultured with
group A ITP plasma**

As shown in Figure 1D, reduced megakaryocyte apoptosis was observed in group A
(21.88%±3.53%) compared with that of controls (29.43%±3.80%)( P<0.0001) on day
15. Megakaryocyte apoptosis did not differ significantly among group B
(27.36%±4.31%), group C (28.21%±4.02%) and controls. Meanwhile, megakaryocytes
in each group cultures all showed a decreased expression of Bcl-xL during the culture
process with a significantly higher level in group A cultures. At day 8 and 15, Bcl-xL
expression in group A (82.29%±5.02%, 72.57%±5.28%) was significantly higher than
in controls (51.02%±4.77%, 47.34%±5.87%)(P<0.0001), while the expression of Bcl-2
was relatively stable and showed no significant difference in megakaryocytes among
the four groups.

**Effects of patient IgG on megakaryocyte yield, maturation, apoptosis and platelet
release**

To evaluate the role of autoantibody on the quantity and quality of megakaryocytes, we
studied the effects of patient and control IgG on megakaryocyte production, ploidy
distribution, platelet release and megakaryocyte apoptosis.

A significant reduction in megakaryocyte production, ploidy distribution and
platelet release was seen in cultures containing IgG from both group A \([(3.15\pm0.93)\times10^5, 14.12\%\pm6.09\%, (5.95\pm2.27)\times10^3]\) and group B \([(3.02\pm1.01)\times10^5, 15.68\%\pm5.98\%, (6.15\pm2.37)\times10^3]\) plasma in comparison with cultures containing IgG from group C \([(4.57\pm0.78)\times10^5, 23.14\%\pm2.27\%, (9.85\pm1.61)\times10^3]\) and control plasma \([(4.90\pm0.48)\times10^5, 23.98\%\pm2.23\%, (10.97\pm1.92)\times10^3]\) (Figure 2A, 2C). On the other hand, no significant difference was seen in megakaryocyte apoptosis and CFU-MK among cultures containing IgG from group A, B, C and control plasma, indicating megakaryocyte apoptosis and CFU-MK are not affected by autoantibodies in ITP plasma (Figure 2B, 2D).

After adsorption of autoantibody from patient plasma, megakaryocyte number, polyploidy percentage and platelet release rose to control level and even more megakaryocytes were present in cultures with adsorbed group A plasma \([(7.85\pm1.30)\times10^5]\) than those cultured with control plasma \([(5.29\pm0.74)\times10^5]\) (Figure 3A, 3C). There was no apparent effect of adsorbed plasma on CFU-MK among the four groups (Figure 3B). However, after adsorption, megakaryocyte apoptosis in group A (22.44\%\pm3.56\%) was still lower than that in controls (30.24\%\pm3.96\%) (P<0.0001) (Figure 3D) and almost showed no remarkable differences between autoantibody adsorbed and unadsorbed plasma. These results indicate that there must be factors, other than autoantibody in ITP plasma, which might influence in vitro megakaryocyte apoptosis and platelet release.

**Abnormal expression of cyclin B1/D3 in megakaryocytes cultured with patient IgG**

Several lines of evidence show that cyclin B1 and cyclin D3 are two very important proteins participating in megakaryocytic endomitosis and high ploidy formation. To investigate whether the inhibition of in vitro megakaryocyte polyploidization by ITP IgG was mediated by abnormal expression of these cyclins, we examined the effect of IgG on the expression of cyclin D3 and cyclin B1 in megakaryocytes. Cultured with control IgG, megakaryocytes expressed cyclin B1 with a reduction from 44.3% at day 5 to 21.6% at day 9 and a limited expression of cyclin D3 (3.5%) at day 9. Whereas when cultured with patient IgG (from group A and group B), megakaryocytes
expressed cyclin B1 at a persistently low level (20.5%) and almost did not express cyclin D3 during the whole culture period (Figure 4A). These results indicate that the abnormal expression of cyclin B1 and cyclin D3 might contribute to megakaryocyte dysmaturity when cultured with ITP patient IgG.

Abnormal expression of TRAIL, caspase-8 and caspase-3 in megakaryocytes cultured with group A patient plasma
In order to investigate the mechanism of impaired megakaryocyte apoptosis as well as whether inhibition of in vitro megakaryocyte apoptosis by ITP plasma was mediated by abnormal expression of certain proteins in the apoptosis pathway, we examined the expression of crucial key factors (Fas/FasL, TRAIL/TRAIL-R2, caspase-3/caspase-8) in megakaryocytes at day 6, 10, 14 of the culturing process. As a result, at day 6, TRAIL, caspase-3 and caspase-8 expression in megakaryocytes did not differ significantly among the four groups. While at day 10 and 14, the expression of TRAIL, caspase-3 and caspase-8 in group A were remarkably lower than that of controls, but there was no significant difference amongst group B, group C and the controls (Figure 4B-D). On the other hand, the decreased expression of TRAIL, caspase-3 and caspase-8 in group A were consistent with inhibited megakaryocyte apoptosis and decreased platelet production at day 10 and 14 of the culture process. However, the expression of Fas and FasL were low and showed no significant difference on the surface of megakaryocytes among the four group cultures at any detecting points. The expression of TRAIL-R2 on megakaryocytes and the expression of TRAIL on produced platelets were relatively stable and showed no significant difference among the four groups at any detecting points.

Decreased sTRAIL expression in plasma and cell culture supernatants of group A
We measured sFas and sTRAIL in plasma and cell culture supernatants to see if these two factors contributed to abnormal megakaryocyte apoptosis. Figure 5A show the plasma sFas levels of different groups. The plasma levels of sFas in group A, B, C and controls were 130.83±36.05, 119.55±31.86, 113.59±31.54 and 117.40±10.71, respectively and showed no significant difference. While the sFas levels in cell culture supernatant of group A [(127.38±18.28, 137.72±25.67) pg/ml] were significantly lower
than those in control supernatants \([(163.75\pm23.21, 178.68\pm17.40) \text{ pg/ml}] \) at day 6 and 10 \((P<0.0001)\). However, no difference was found at day 14. There were no remarkable differences among group A, group B and group C at any detecting points (Figure 5B). In addition, plasma sTRAIL concentration in group A \([(2343.24\pm1155.81) \text{ pg/ml}] \) was remarkably lower than that of controls \([(5653.37\pm1583.32) \text{ pg/ml}] \) \((P<0.0001)\) (Figure 5C). And at day 6, 10 and 14, sTRAIL concentration in cell culture supernatants of group A \([(1200.67\pm321.49, 585.59\pm277.98, 373.65\pm272.51) \text{ pg/ml}] \) were remarkably lower than those in controls \([(2347.18\pm479.40, 1257.13\pm329.24, 852.32\pm307.32) \text{ pg/ml}] \) \((P<0.0001)\) (Figure 5D). sTRAIL levels in plasma and cell culture supernatants showed no remarkable differences among group B, group C and controls at any detecting points.

**Discussion**

In the early 1980s, studies of autologous platelet survival showed that in the majority of ITP patients, platelet turnover was either reduced or normal. It would be expected that platelet destruction was not the sole pathogenic mechanism, and impaired platelet production might also contribute to persistent thrombocytopenia. Chang et al\(^{12}\) reported that production of megakaryocytes was significantly reduced in the presence of ITP plasma with detectable antiplatelet GPIb autoantibodies. McMillan et al\(^{13}\) recently documented that ITP plasma with detectable anti-GPIIb/IIIa, anti-GPIb or both not only reduced the yield of megakaryocytes, but also suppressed megakaryocyte maturation.

Our present results in patients with chronic ITP are similar, in some way, to previous studies mentioned above. We showed that some (group B) ITP plasma suppressed megakaryocytopoiesis and autoantibody from some ITP plasma exerted suppression on megakaryocyte production, maturation and platelet release. Autoantibody adsorption further confirmed that the autoantibody may be partly responsible for the reduced yields of megakaryocytes and platelets. Several possible mechanisms may contribute to the suppression of megakaryocyte production by autoantibodies. First, megakaryocytes express GPIIb/IIIa or GPIb/IX on their surfaces
during maturation as well as platelets, autoantibodies binding to megakaryocytes and platelets could mediate megakaryocyte and platelets destruction by phagocytic cells.\textsuperscript{28} Second, autoantibody induced activation of complement may play a role in megakaryocyte apoptosis.\textsuperscript{29} Unfortunately, in our study, a significant difference in megakaryocyte apoptosis was not detected between cultures containing antibody-adsorbed and unadsorbed plasma. It is unlikely that autoantibody affects megakaryocyte apoptosis in this way. In addition, para-apoptosis was found in 83\% of megakaryocytes and 64\% of megakaryoblasts from ITP marrows.\textsuperscript{30} Houwerzijl et al also reported that morphology compatible with para-apoptosis could be induced in cultured megakaryocytes with ITP plasma.\textsuperscript{31} Whether para-apoptosis mediated megakaryocyte destruction was induced by autoantibody requires further investigation.

Moreover, as with two important cyclins in megakaryocyte development and high ploidy formation,\textsuperscript{32} the absence of cyclin D3 during the whole culture and low expression of cyclin B1 at early stage may have been the mechanism by which patient IgG inhibited megakaryocyte polyploidization. As a result, IgG in ITP plasma destructed megakaryocytes and affected megakaryocyte polyploidy, leading to less megakaryocyte ploidy and platelet release. However, even when cultured with autoantibody-adsorbed plasma, more megakaryocytes were not reasonably accompanied with more polyploidy cells and platelet production, indicating that autoantibody was not the sole factor in impairing megakaryocyte maturation and platelet production.

In the present work, we were surprised to find that the group with the most ITP plasma (group A) boosted megakaryocyte counts, while impairing megakaryocyte maturation and its ability to produce platelets. It was compatible with early morphologic studies of ITP bone marrow showing normal or increased number of megakaryocytes.\textsuperscript{33,34} This was somewhat inconsistent with the findings of Chang's and McMillan's. Possible reasons for these differences could be: (1) Most of the patients enrolled in Chang's study were diagnosed as acute ITP, while the patients in our group were chronic. (2) In McMillan's paper, they mainly investigated patients whose plasma inhibited megakaryocyte production and did not analyze other patients with increased
megakaryocytes. (3) In vitro megakaryocyte culturing time was 8 or 10 days in their studies, while we prolonged our study to 15 days, to preferably investigate the role of megakaryocyte apoptosis in chronic ITP. (4) We used cord blood derived megakaryocytes in our research. Compared with megakaryocytes derived from peripheral blood or adult bone marrow, megakaryocytes derived from cord blood yielded the higher numbers of megakaryocytes, but these cells were smaller with less endomitosis, showing reduced polyploidization and platelet number. Therefore, different sources of megakaryocytes may influence the results in the study. Since increased megakaryocyte counts was not a result of an accelerated proliferation of megakaryocyte progenitors or elevated levels of TPO and IL-11, the two positive regulators of megakaryocytopoiesis in our experiment, there must be some other reasons leading to increased megakaryocyte mass with impaired maturation and platelet production.

Several reports have indicated a close relationship among megakaryocyte maturation, apoptosis, and platelet release. Local caspase activation can lead to proplatelet formation and thus platelet release. Houwerzijl et al have reported that the inhibited megakaryocyte apoptosis may contribute to thrombocytopenia and augment of dysfunctional megakaryocytes. We found reduced megakaryocyte apoptosis in the boosted megakaryocyte group, indicating that the reduced megakaryocyte apoptosis could be associated with a boosted megakaryocyte mass, but an impaired megakaryocyte maturation and reduced platelet production. It would be interesting and informative to further investigate the causative factors leading to impaired megakaryocyte apoptosis in ITP patients.

The role of the apoptosis inducing ligands of the TNF family and their death receptors has been explored for years, especially the role of Fas/FasL pathway. Shenoy et al found that 25% pediatric patients with chronic hematologic autoimmunity had profound defects in lymphocyte death via the Fas pathway. Although it has been shown that the Fas/FasL system may accelerate megakaryocytic apoptosis and platelet release, Fas and FasL are mainly expressed on T and B lymphocytes, inducing cellular apoptosis disorder associated with cellular and humoral immune reaction.
Yoshimura C et al\textsuperscript{37} reported that there were two groups of ITP patients, one with elevated levels of sFas/sFasL in their plasma and the other with normal levels, suggesting that the pathogenesis of some ITP patients might include an alteration of the Fas/FasL pathway. In our study, the sFas levels in cell culture supernatant of group A were significantly lower than those in control supernatants at day 6 and 10, which was consistent with the data from Yoshimura. However, no difference was found at day 14, the surface expressions of Fas and FasL on megakaryocytes were low in both ITP patients and controls, and sFas levels in plasma showed no difference between ITP patients and controls. These findings indicate that Fas/FasL interaction is important in lymphocyte apoptosis, but plays little role in decreased megakaryocyte apoptosis in ITP patients. The sFas in plasma seems not to be the factor contributing to decreased platelet production in ITP.

Several studies indicate that TRAIL is involved in the pathogenesis of some autoimmune diseases including systemic lupus erythematosus (SLE), Sjogren syndrome and autoimmune thyroid diseases.\textsuperscript{38-40} Previous studies\textsuperscript{41-43} show that TRAIL can promote the maturation and apoptosis of megakaryocytes. Human megakaryocytes cultured in vitro express TRAIL and TRAIL-R2 on their surfaces. Similarly, platelets also express TRAIL, which may be obtained from megakaryocytes. We found that TRAIL expression on megakaryocytes and sTRAIL concentrations in plasma and cell culture supernatants of group A patients were prominently decreased when compared with the other three groups. Therefore, decreased sTRAIL might be a contributing factor to impaired megakaryocyte apoptosis. As we expected, both the expression of caspase-8 and caspase-3 decreased in group A megakaryocytes. We inferred that decreased TRAIL expression may have led to less caspase-8 and caspase-3 activation, thus triggering less megakaryocyte apoptosis. In addition, although TRAIL expression on the surface of platelets was almost equal, platelets produced in group A were much lower than controls. In a word, the total TRAIL expression on the platelet surface of group A might be in a much lower level, which may enhance the role of TRAIL in impaired megakaryocyte apoptosis through the TRAIL/TRAIL-R2 pathway. Recently,
Sedger et al have reported that FasL and TRAIL double deficient mice develop extreme lymphoproliferative disease (LPD) and fatal autoimmune thrombocytopenia, which is a little different from the results of our study. However, severe LPD is due to maximal resistance of lymphocytes to activation-induced cell death (AICD) and dysregulated lymphocyte homeostasis resulted in the production of anti-platelet IgM and IgG causing thrombocytopenia. As a result, spontaneous thrombocytopenia in these mice is secondary to LPD. This is somewhat different from the mechanism of primary immune thrombocytopenia.

Recent studies showed that Bcl-xL is up-regulated during megakaryocyte differentiation but is absent from senescent megakaryocytes. Over expression of Bcl-xL could decrease platelet formation. Therefore, Bcl-xL, as an anti-apoptosis factor, should play a role in megakaryocyte differentiation and apoptosis, as well as in platelet formation. Lei Zhang et al discovered that Bcl-xL is down-regulated early during in vitro differentiation of megakaryocytes from essential thrombocythemia (ET) patients, leading to overproduction of megakaryocytes and platelets. In the current research, we found that megakaryocytes in group A had higher Bcl-xL expression when compared with other groups on day 8, prior to the peak day of high ploidy (day10-12) (data not shown). Thus, we conclude that overexpression of Bcl-xL in immature megakaryocytes might suppress megakaryocyte maturation and platelet production by inhibiting apoptosis.

Studies have demonstrated that CD8+T cell-induced lysis of platelets in chronic ITP may be involved in the pathogenesis of this disorder. A new murine model of severe ITP induced by both antibody and CD8+T cells has further confirmed the T cell mediated immune disorder in ITP. Li S et al discovered that activated CD8+T cells in bone marrow of patients with chronic ITP might suppress megakaryocyte apoptosis and lead to impaired platelet production in vitro. sFas, derived from activated CD8+T cells, might act as a paracrine regulator of the megakaryocytic apoptosis. In the present study, we found ITP plasma inhibited megakaryocyte apoptosis and we could not rule out the function of CD8+T cells or their secretory factors in ITP plasma on in vitro megakaryocyte culture.
Taken together, the observation that suppressed megakaryocyte apoptosis by some ITP plasma led to boosted megakaryocyte mass and reduced platelet production in vitro, may interpret the increased bone marrow megakaryocytes with decreased peripheral platelets in vivo. The abnormal expression of sTRAIL in plasma, TRAIL and Bcl-xL in megakaryocytes may play a role in the pathogenesis of impaired megakaryocyte apoptosis in ITP. Further studies on promoting megakaryocyte apoptosis and blocking anti-apoptotic proteins should be performed to further clarify the role of megakaryocyte apoptosis in the pathogenesis of ITP, and thereby provide a new stratagem for ITP treatment.

Acknowledgments

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Authorship

Contribution: Y.L. performed research, analyzed data and wrote the manuscript, W.L. performed research and wrote the manuscript, Z.C. performed research and analyzed data, Z.X. analyzed data, H.Y. analyzed data, P.J. analyzed data and wrote the
manuscript, H.M. designed the research and wrote the manuscript.

Conflict of interest disclosure: The authors declare no competing financial interests.

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### Table 1. Patients with chronic ITP

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*Plasma of Patients 1~26 increased megakaryocyte production (group A); plasma of Patients 27~40 suppressed megakaryocyte production (group B); plasma of Patients 41~49 did not affect megakaryocyte production (group C).
†Antiplatelet antibodies were assayed by modified monoclonal antibody specific immobilization of platelet antigens (MAIPA). P, positive; N, negative.
‡Pred, prednisone; Dex, dexamethasone; IVIG, intravenous gamma globulin; CSA, cyclosporine; VCR, vincristine; MTX, methotrexate.
§PT, petechiae; EC, ecchymoses; EP, epistaxis; GUH, genitourinary hemorrhage; GH, gingival hemorrhage.
FIGURE LEGENDS

Figure 1. Effects of whole plasma on in vitro megakaryocyte production, CFU-MK, platelet release and megakaryocyte apoptosis. (A) ITP plasma of Group A significantly increased the yield of megakaryocytes on day 15 when compared with control cultures ([7.13±0.86]×10⁵ vs [5.10±0.90]×10⁵, P<0.0001). ITP plasma of Group B significantly reduced the yield of megakaryocytes on day 15 when compared with control cultures ([2.83±1.02]×10⁵ vs [5.10±0.90]×10⁵, P<0.0001). Significant difference in megakaryocyte counts was not seen between group C and control cultures ([5.20±0.66]×10⁵ vs [5.10±0.90]×10⁵, P=0.776). (B) In the presence of whole plasma, in vitro CFU-MK formation in group A, B, C and controls were 11.89±2.93, 11.50±2.62, 10.56±2.35 and 12.05±2.72, respectively. There was no remarkable differences among the four groups (P=0.553). (C) In the presence of whole plasma, platelet release in group A was significantly lower than controls ([7.51±2.41]×10³ vs [11.21±1.82]×10³, P<0.0001). Platelet release in group B was significantly lower than controls ([7.21±2.45]×10³ vs [11.21±1.82]×10³, P<0.0001). There was no remarkable differences between group C and controls ([10.12±1.91]×10³ vs [11.21±1.82]×10³, P=0.214). (D) In the presence of whole plasma, group A cultures showed decreased apoptotic megakaryocytes (21.88%±3.53%) compared with group B (27.36%±4.31%), group C (28.21%±4.02%) and control cultures (29.43%±3.80%, P<0.0001). All data were presented as mean ± SD.

Figure 2. Effects of IgG on in vitro megakaryocyte production, CFU-MK, platelet release and megakaryocyte apoptosis. (A) In the presence of patient IgG from both group A and B, most cultures showed reduced megakaryocyte yield compared with cultures containing control IgG ([3.15±0.93]×10⁵, [3.02±1.01]×10⁵ vs [4.90±0.48]×10⁵, P<0.0001). There was no remarkable differences between group C and controls (P=0.811). (B) In the presence of IgG from ITP patients or healthy controls, significant difference in CFU-MK was not seen among group A, B, C and controls (P=0.568). (C) In the presence of IgG, platelet release in group A and group B were significantly lower than controls ([5.95±2.27]×10³, [6.15±2.37]×10³ vs [10.97±1.92]×10³, P<0.0001). There was no remarkable differences between group C and controls (P=0.184). (D) In the presence of IgG from ITP patients or healthy controls, significant difference in megakaryocyte apoptosis was not seen among group A, B, C and controls (P=0.353). All
data were presented as mean ± SD.

**Figure 3.** Effects of antibody adsorbed plasma on in vitro megakaryocyte production, CFU-MK, platelet release and megakaryocyte apoptosis. (A) After adsorption of autoantibody from patient plasma, megakaryocyte number rose to control level and even more megakaryocytes were present in cultures with adsorbed group A plasma than those cultured with control plasma ([7.85±1.30]×10^5, [5.29±0.74]×10^5, P<0.0001). (B) After antibody adsorption, significant difference in CFU-MK was not seen among group A, B, C and controls (P=0.816). (C) After adsorption, significant difference in platelet release was not seen among group A, B, C and controls (P=0.061). (D) After adsorption, megakaryocyte apoptosis in group A was still lower than that in controls (22.44±3.56% vs 30.24±3.96%, P<0.0001). All data were presented as mean ± SD.

**Figure 4.** Abnormal expression of cyclin B1/D3, TRAIL, caspase-8 and caspase-3 in megakaryocytes. (A) Cultured with control IgG, megakaryocytes expressed cyclin B1 with a reduction from 44.3% at day 5 to 21.6% at day 9 and a limited expression of cyclin D3 (3.5%) at day 9. Whereas cultured with patient IgG (from group A and group B), megakaryocytes expressed cyclin B1 at a persistently low level (20.5%) and almost did not express cyclin D3 during the whole culture time. (B) Cultured with whole plasma, TRAIL expression on the surface of megakaryocytes did not differ significantly among ITP patients in group A, B, C and controls at day 6. At day 10 and 14, TRAIL expression in group A was remarkably lower than that in controls (P<0.0001), but there was no significant difference among group B, group C and control group. (C) Cultured with whole plasma, caspase-8 expression in megakaryocytes did not differ significantly among ITP patients in group A, B, C and controls at day 6. At day 10 and 14, caspase-8 expression in group A was remarkably lower than that in controls (P=0.015 and P=0.004), but there was no significant difference between group B, C and controls. (D) Cultured with whole plasma, caspase-3 expression in megakaryocytes did not differ significantly among ITP patients in group A, B, C and controls at day 6. At day 10 and 14, caspase-3 expression in group A was remarkably lower than that in controls (P=0.023 and P<0.0001), but there was no significant difference among group B, C and controls.

**Figure 5.** sFas and sTRAIL levels in plasma and cell culture supernatants of ITP patients and
healthy controls. (A) Plasma sFas did not differ significantly among group A, B, C and controls (P=0.126). (B) sFas levels in cell culture supernatants of group A were significantly lower than those in control supernatants at day 6 and 10 (P<0.0001). At day 14, sFas levels in cell culture supernatants did not differ significantly between ITP patients in group A and controls and there were no remarkable differences among group A, group B and group C at any detecting time. (C) Plasma sTRAIL concentration was deceased in group A ITP patients compared to healthy controls (P<0.0001). There were no remarkable differences among group B, group C and controls. (D) At day 6, 10 and 14, sTRAIL concentration in cell culture supernatants of group A were remarkably lower than those in controls (P<0.0001). There were no remarkable differences among group B, group C and controls at any detecting time.
Figure 1

(A) Megakaryocyte count (10e5) for Control, Group A, Group B, and Group C. Differences are statistically significant (p<0.0001).

(B) CFU-MK for Control, Group A, Group B, and Group C. No significant differences are observed.

(C) Platelet count (10e3) for Control, Group A, Group B, and Group C. Differences are statistically significant (p<0.0001).

(D) Megakaryocyte apoptosis (%) for Control, Group A, Group B, and Group C. Differences are statistically significant (p<0.0001).
Figure 3

(A) Megakaryocyte count ($10^5$)
(B) CFU-MK
(C) Platelet count ($10^3$)
(D) Megakaryocyte apoptosis (%)
Figure 5

(A) sFAS level in plasma (pg/ml)

(B) sFAS level in cell culture supernatant (pg/ml)

(C) sTRAIL level in plasma (pg/ml)

(D) sTRAIL level in cell culture supernatant (pg/ml)
Contributions of TRAIL mediated megakaryocyte apoptosis to impaired megakaryocyte and platelet production in immune thrombocytopenia

Lei Yang, Lin Wang, Chun-hong Zhao, Xiao-juan Zhu, Yu Hou, Jun Peng and Ming Hou