In vitro recovery of Th1/Th2 balance in PBMCs from patients with immune thrombocytopenia through the actions of IL-18BPa/Fc

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A R T I C L E   I N F O

Article history:
Received 15 February 2011
Accepted 11 July 2011
Available online 10 September 2011

Keywords:
Immune thrombocytopenia
Interleukin 18
Interleukin 18 binding protein
Cytokines
Apoptosis
Proliferation

A B S T R A C T

To determine the effects of IL-18BPa/Fc on the cytokine production and survival of peripheral blood mononuclear cells (PBMCs) of immune thrombocytopenia (ITP), PBMCs isolated from patients with ITP and healthy donors were treated with or without of IL-18BPa/Fc. The production of IFN-γ, IL-2, tumor necrosis factor (TNF)-α, IL-4, IL-5 and IL-10 was measured by ELISA, and mRNA expression of IFN-γ and IL-18 was evaluated by RT-PCR. Besides, flow cytometric analysis of cell apoptosis was performed by staining with annexin V-FITC/Propidium Iodide (PI). The proliferation rate of PBMCs was examined by CCK-8 assay. IL-18BPa/Fc at 10 ng/ml significantly stimulated IL-10 secretion from PBMCs in patients with ITP and healthy donors, while it decreased IFN-γ release. Further, IL-18BPa/Fc enhanced dexamethasone (DEX) reduction of PHA-induced IFN-γ production by an additional 38.9%(DEX 20 nmol/l) and 49.9%(DEX 50 nmol/l) in ITP patients. Interestingly, the treatment of PBMCs with IL-18BPa/Fc increased the percentage of early apoptotic cells in patients with ITP. In conclusion, IL-18BPa/Fc, via neutralizing the biologic activity of mature IL-18, accelerates lymphocyte apoptosis and downregulates IFN-γ, while permitting the production of Th2 cytokine IL-10. These observations suggest a role of IL-18BPa/Fc in the recovery of Th1/Th2 balance, as well as its therapeutic potential in the treatment of ITP.

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Introduction

Immune thrombocytopenia (ITP), is an autoimmune-mediated acquired disease characterized by transient or persistent decrease of the platelet count and, depending upon the degree of thrombocytopenia, increased risk of bleeding [3]. It was long suspected that ITP is mediated by platelet antibodies that accelerate platelet destruction and inhibit their production [2,6]. Apart from phagocytosis, destruction mechanisms include complement activation and cellular mechanisms, such as the Th1 bias [18,25], the platelet destruction by cytotoxic T cells (CTL) [17,23,33], the decreased number or defective suppressive function of regulatory T cells [24,32].

The cause for these abnormalities remains poorly understood, however, uncontrolled Th-1 lymphocyte activation appear to be important mechanisms of the disease. Interleukin(IL)-18, a member of IL-1 cytokine family, is widely expressed in monocytes/macrophages, CD4+ T cells, NK cells, adipocytes and Kupffer cells in the liver, pancreas, kidney, skeletal muscle, lung, osteoblasts and keratinocytes [4,15]. Furthermore, IL-18 promotes Th1 responses through stimulating IFN-γ production by T lymphocytes and natural killer (NK) cells via IL-18R, which dominate during ITP [26], rheumatoid arthritis [8], Crohn disease [19], and multiple sclerosis [30]. Our previous study confirmed that the plasma and mRNA levels of both IFN-γ and IL-18 in active ITP patients were increased significantly compared with the normal controls. The balance of IL-18/IL-18BP plays a role in progression of ITP [27,28]. Since IL-18 is an important cytokine in Th-1 immune activation, the important pathogenic mechanisms in ITP, inhibition of IL-18 would be an efficient approach in the treatment of ITP patients. Although soluble receptors for IL-18 exist, IL-18 shows greater affinity for IL-18BP than for IL-18R, and its binding to IL-18BP is essentially irreversible [5]. Therefore, IL-18BP may potentially be employed to block IL-18 signaling and reduce inflammation and platelet destruction.

In this study, we examined the expression of IFN-γ, IL-2, TNF-α, IL-4, IL-5, IL-10 levels and IL-18 in PBMCs from the ITP patients and controls after IL-18BPa/Fc administration. Furthermore, effects of IL-18BPa/Fc on the proliferation and apoptosis of PBMCs were investigated. Finally, the responsiveness of IL-18R to IL-18BP stimulation was also observed.
Material and methods

Patients

The patients (Table 1) (6 females and 4 males, age range 16–50 years, median 34.5 years), were from the Department of Hematology, Qilu Hospital, Shandong University, China and were newly diagnosed as having ITP, according to the international criteria [22]. None of them had been treated with glucocorticosteroid prior to first sampling. A control group consisted of 10 adult healthy volunteers (6 women and 4 men; range: 23–62 years; median age 44 years). The study was approved by the Medical Ethical Committee of Qilu Hospital, Shandong University, and informed consent was obtained from all patients.

PBMC preparation

Fifteen milliliters of heparinized venous peripheral blood were collected from each patient and control. PBMCs were isolated from heparinized blood by gradient centrifugation on Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden), then were adjusted to a density of 1×10⁶ cells/well in a 48-well culture plate and incubated in RPMI-1640 culture medium (Invitrogen, Carlsbad, USA), cultured at a density of 1×10⁶ cells/well in a 48-well culture plate and incubated in RPMI-1640 culture medium (Invitrogen, Carlsbad, USA). Supernatants of culture were determined using a commercial ELISA according to manufacturer's (Bender MedSystems, Burlingame, USA) instructions. The lower detection limit of these assays were 2 pg/ml, 4 pg/ml, 2 pg/ml and 7 pg/ml.

Cytokines determination by ELISA

IFN-γ and IL-4 in supernatant of culture were measured by a commercial ELISA according to manufacturer’s (Bender MedSystems, Burlingame, USA) instructions. The lower detection limit of the assays were both 2 pg/ml. No significant cross-reactivity with a multitude of recombinant cytokines has been observed. IL-2, TNF-α, IL-5 and IL-10 in supernatant of culture were measured using ELISA method. PBMCs (1×10⁶/ml) with PHA (10 µg/ml) were cultured in RPMI 1640 medium supplemented with IL-18BPα/Fc (10 ng/ml) in the presence of IL-18BPα/Fc (0 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml) and cell supernatants were harvested after 48 h for quantifying IFN-γ using the ELISA and RT-PCR method.

Table 1

Clinical characteristics of active ITP patients.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex/Age (year)</th>
<th>Bleeding symptoms</th>
<th>Platelet counts (×10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/28</td>
<td>PT, GH</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>M/31</td>
<td>GH</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
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<td>ECE, GH</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>M/50</td>
<td>EC, GH</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>F/43</td>
<td>PT, GH</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>F/39</td>
<td>EP, GH</td>
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<td>8</td>
<td>F/48</td>
<td>EP</td>
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<td>M/22</td>
<td>NONE</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>F/19</td>
<td>PT, EC</td>
<td>12</td>
</tr>
<tr>
<td>Median</td>
<td>34.5</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>(min–max)</td>
<td>(16–50)</td>
<td></td>
<td>(1–16)</td>
</tr>
</tbody>
</table>

PT = Petechiae, EC = ecchymoses, EP = epistaxis, GUH = genitourinary hemorrhage, GH = gingival hemorrhage

Quantitative real-time polymerase chain reaction analysis

Total RNA was isolated by Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The amount of RNA was determined using the Eppendorf Biophotometer (Brinkmann Instruments, Westbury, NY, USA) and normalized to 1 µg/ml for each subsequent real-time quantitative polymerase chain reaction (RT-PCR) process on a ABI PRISM®7500 Sequence Detection System (Applied Biosystems Foster City CA, USA) by using SYBR® Green (Toyobo, Osaka, Japan) as a double-strand DNA-specific binding dye. The primers and annealing temperatures used for the amplification were listed in Table 2.

The PCR reactions were cycled 40 times after initial denaturation (95°C, 5 min) with the following parameters: denaturation 95°C, 15 s; annealing 60°C (IFN-γ and β-actin) / 62°C (IL-18R) 15 s; extension 72°C, 35 s with temperature transition rates of 20°C/s. Fluorescence was acquired at extension 72°C below the product melting temperature (Tm) and holding for 5 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction. Fluorescence was acquired every 0.1 s. All reactions were carried out in triplicate. Controls included RNA subjected to RT-PCR without reverse transcription, RT-PCR with water replacing RNA, and PCR with water replacing cDNA, and all these controls gave a threshold cycle (Ct) value of 40, indicating no detectable PCR product under these cycle conditions. ABI Sequence Detection System software version 1.0 (PE Applied Biosystems, Warrington, UK) was used to determine the cycle number at which fluorescence emission crossed the automatically determined Ct value. All experiments were conducted in triplicate.

Detection of IL-18R by flow cytometry

Analysis for surface immunofluorescence were performed on FACScan flow cytometry (Becton Dickinson, New Jersey, USA). PBMCs (1×10⁶ cells/ml) in the presence of IL-18BPα/Fc (10 ng/ml) and PHA (10 µg/ml) to be analysed for immunofluorescence were performed on FACScan flow cytometry (Becton Dickinson, New Jersey, USA). PBMCs (1×10⁶ cells/ml) in the presence of IL-18BPα/Fc (10 ng/ml) and PHA (10 µg/ml) to be analysed for immunofluorescence were incubated with saturating 10 µl of FITC-anti-human IL-18R monoclonal and PE-anti-human CD3 monoclonal (ebioscience, San Diego, CA, USA) for 30 minutes at 37°C, washed twice in the diluent and resuspended in PBS containing 1% formaldehyde and 0.5% sodium azide. Non-specific binding was subtracted using appropriate controls.

Table 2

Primers and conditions for the RT-PCR experiments performed in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ → 3’)</th>
<th>T (°C)</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18R</td>
<td>TCTGAACGTGCGCCGCAGATA</td>
<td>62</td>
<td>313</td>
</tr>
<tr>
<td>IL-18R</td>
<td>CACACCCACATTCTTACCTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TGGCTATATCTCTGAAAAG</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>β-actin</td>
<td>TCCGCGACGGTATCGGAAAGAA</td>
<td>60</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>GCCATACACCAGAGCTACT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
harvested after 48 hours and incubated with 5 μl FITC-conjugated annexin V (Invitrogen) for 15 minutes and were analyzed within 1 hour by FACS.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical significance was determined by ANOVA, and difference between two groups was determined by non-parametric test. All data had a normal distribution, which was tested by Skewness-Kurtosis. The numerical results of RT-PCR results were analysed using REST software. P value less than 0.05 was considered statistically significant.

Results

Suppression of IFN-γ level in PBMCs after IL-18BPa/Fc administration

The expression of IFN-γ in supernatant of PBMCs cultures with PHA (10 ng/ml) from ITP patients (169.4 ± 60.5) pg/ml were higher than those in normal control group (135.9 ± 44.8) pg/ml (P = 0.002) (Fig. 1). In normal controls, a dose–response curve revealed that IL-18BPa/Fc as low as 10 ng/ml was able to significantly modulate IFN-γ production. IL-18BPa/Fc untreated control PBMCs secreted (135.9 ± 44.8) pg/ml IFN-γ into the supernatant. Addition of IL-18BPa/Fc resulted in a 29.9% decrease (95.5 ± 30.3) pg/ml (P < 0.0001, IL-18BPa/Fc 10 ng/ml (Fig. 1A)). Similar results were obtained in cultures of PBMCs derived from ITP patients, where IL-18BPa/Fc (10 ng/ml) reduced IFN-γ secretion from (169.4 ± 60.5) pg/ml to (85.5 ± 35.1) pg/ml (P < 0.0001, Fig. 1B). Using the REST software, the data were presented as the fold change in gene expression normalized to an endogenous reference gene and relative to healthy controls. The relative amount of IFN-γ mRNA was decreased 12.5-fold after IL-18BPa/Fc (P = 0.029) treated (Fig. 1C). However, there was no detectable protein and mRNA level of IFN-γ when incubating cells without PHA in the present study.

The levels of TNF-α were (72.9 ± 36.2) pg/ml and (52.8 ± 22.3) pg/ml in PHA stimulated culture supernatant of ITP patients and healthy volunteers (P = 0.043). Although the level of TNF-α in PHA stimulated culture supernatant were suppressed after IL-18BPa/Fc administration in ITP individuals but it was not significant. However, there was no difference in IL-2 levels in culture supernatant of PBMCs of ITP individuals and controls after IL-18BPa/Fc treatment.

The synergistic effects of IL-18BPa/Fc and DXM in PBMCs

To further investigate reinforcement effects of IL-18BPa/Fc with DXM, PBMCs were treated with IL-18BPa/Fc (0 ng/ml, 10 ng/ml) in combination with DXM (0 nmol/l, 20 nmol/l, 50 nmol/l) (Fig. 2). DXM (20 nmol/l) alone decreased PHA (10 ng/ml)-induced IFN-γ by 14.9% in control PBMCs, co-incubation with IL-18BPa/Fc (10 ng/ml) further enhanced the inhibition of DXM by another 40.8% (P = 0.0003). And IL-18BPa/Fc enhanced dexamethasone (50 nmol/l) reduction of PHA-induced IFN-γ production by an additional 40.7% (P < 0.0001). Similar results were obtained in cultures of PBMCs derived from ITP patients, where IL-18BPa/Fc (10 ng/ml) enhanced dexamethasone (DEX) reduction of PHA-induced IFN-γ production by an additional 38.3% (DEX 20 nmol/l) and 49.9% (DEX 50 nmol/l) in ITP patients (P < 0.0001).

IL-18BPa/Fc enhances IL-10 production by PBMCs stimulated with PHA

As depicted in Fig. 3, addition of IL-18BPa/Fc (0 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml) to cultures of human PBMCs activated with PHA from healthy and ITP patients resulted in a significant enhancement of IL-10 production. This effect was concentration-dependent, with more than a 3-fold induction of IL-10 in ITP PBMCs and a 2-fold in control PBMCs (IL-18BPa/Fc 10 ng/ml). However, stimulation with IL-18BPa/Fc did not result in changes on IL-10 mRNA levels (data not shown). The level of IL-4 and IL-5 was below the detectable limit of the assay used.

Effects of IL-18BPa/Fc on apoptosis of PBMCs

Furthermore, we investigated the effects of IL-18BPa/Fc on the apoptosis of lymphocytes from healthy and ITP patients. Compared with healthy controls, the percentage of early apoptotic cells was significantly decreased in ITP patients (annexin V%: ITP: 10.9 ± 2.1%, controls: 10.9 ± 2.1%) (P = 0.02). IL-18BPa/Fc (10 ng/ml) significantly increased apoptosis of lymphocytes in ITP patients (P = 0.04) but not in controls (P = 0.96) (Fig. 4).

IL-18BPa/Fc has no effects on the proliferation of PBMCs

After cultured with different concentrations of IL-18BPa/Fc (0 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml) for 48 h, proliferation in active ITP patients and controls was assayed by CCK-8. As expected, significant difference was not found between the treated and the untreated group for the growth stimulating activity.
results showed IL-18BPa/Fc indeed had no direct effect of stimulating cells proliferation.

Responsiveness of human IL-18R to IL-18BPa/Fc

After 48 h culture with IL-18BPa/Fc, the expression of IL-18R protein and mRNA (Fig. 1C) in PBMCs of ITP patients and controls was unaffected. Responsiveness of human IL-18R to IL-18BPa/Fc was obvious and reproducible. These observations revealed modulation of IL-18R expression was possibly irrelevant to IFN-γ downregulation induced by IL-18BPa/Fc.

Discussion

ITP is characterized by a Th-1 lymphocyte stimulation with a prominent cytokine response, consisting of elevated levels of circulating IFN-γ, IL-2, IL-18, TNF-α and IL-6 [18,26–28]. Our previous study confirmed that the levels of IL-18 and IFN-γ in active ITP patients were increased. Despite the moderate high levels of IL-18BP in ITP, it is still insufficient to bind most of the IL-18. As a result, it is likely that IL-18 reflects an imbalance favoring a pro-Th-1 and inflammatory state. In addition, a significant correlation was observed between concentrations of IL-18 and IFN-γ, which has been linked to the pathogenesis of ITP.

IL-18 is known to participate in host defense against infection and cancer through stimulation of NK and CD8 cytotoxicity as well as IFN-γ and TNF-α secretion and Th-1 lymphocyte activations [4,9,13,20]. The latter mechanisms obviously appear to be fully active in ITP. Therefore, inhibition of IL-18 would be an efficient approach in the treatment of ITP patients. IL-18BP has been identified as a specific inhibitor of IL-18 [1,14]. There is no significant similarity between IL-18BP and IL-18 receptor components. Since it lacks a transmembrane domain, IL-18BP appears to exist only as a soluble, circulating protein. Its major function is to regulate the inflammatory activity of IL-18 by acting as its soluble decoy receptor. Human IL-18BPa and IL-18BPc isoforms are capable of binding to and neutralizing IL-18. The affinity of IL-18BPc isoform is 10 fold less than IL-18BPa isoform [10]. IL-18BPa/Fc used in this study is a disulfide-linked homodimeric protein, transcribed from a DNA sequence encoding amino acid residues 1–192 of human IL-18BP and fused to the Fc region of human IgG1 via a polypeptide linker. IL-18BP is a novel candidate for anticytokine therapy that proved to be effective in collagen-induced arthritis, rheumatoid arthritis, nephritic syndrome and other autoimmune diseases [12,14].

Our results showed that the neutralization of endogenous IL-18 by IL-18BPa/Fc reduced the PHA-induced IFN-γ production, suggesting a role for endogenously produced IL-18 in IFN-γ production, which is in accord with previous reports [7,21]. It is of particular relevance that IL-18BPa/Fc was still active at low concentrations (10 ng/ml), a fact that underscores its therapeutic potential. In addition, the effect of IL-18BP was much stronger in ITP, probably because ITP patients produced more endogenous IL-18 and responded more strongly to IL-18BP, which implies the possibility of neutralization of endogenous IL-18 by IL-18BP in ITP. Using PHA-activated PBMCs from healthy donors, we show that IL-18BPa/Fc further amplified dexamethasone inhibition of IFN-γ release by 49.9%. A similar effect was observed in PBMCs from patients with clinically active ITP.

Beyond induction of the central proatherogenic factor IFN-γ, IL-18 also changed the production of various other proinflammatory cytokines. PBMCs from ITP patients produced higher levels of IFN-γ, IL-6 and TNF-α, whereas the production of IL-10 was lower compared to healthy controls [11]. These findings are consistent with the hypothesis that IL-18 is a key regulator of the proinflammatory cytokine response in ITP. Administration of IL-18BPa/Fc in vivo was shown to reduce the levels of IFN-γ, IL-6 and TNF-α and increase the levels of IL-10 in ITP patients [21]. These observations support the potential use of IL-18BP as a therapeutic agent in ITP.
IL-2, TNF-α and lower IL-10 than did cells from healthy controls. In accordance with previous reports, the IFN-γ/IL-2/TNF-α: IL-10 ratio, which reflects the TH1/TH2 cytokine balance in blood, was higher in ITP patients. Administration of IL-18BPa/Fc to PBMCs resulted in elevation of IL-10 reduction and IFN-γ levels, causing a shift in the TH1/TH2 cytokine balance. IL-18BPa/Fc may play a therapeutic role in ITP by down-regulation of IFN-γ and other TH1 cytokines while permitting the production of TH2 cytokines (IL-10) via neutralizing the biologic activity of mature IL-18 in vitro. 

To further illuminate the mechanisms between imbalance of IL-18/IL-18BP and immune dysfunction in ITP, we detected the effects of IL-18BPa on the apoptosis of lymphocytes. Lymphocyte development, selection and education represent tightly controlled immune processes that normally prevent autoimmunity. Lymphocyte development likely induces cellular selection through apoptosis to remove potentially autoreactive cells [11]. Dysregulated lymphocyte apoptosis and altered self-antigen processing and presentation of apoptotic platelets leads to the activation of immune response and development of autoantibodies in ITP patients. It's of interest to note that the percentage of early apoptotic cells was enhanced in patients with active disease (P=0.02) compared with normals after stimulated with PHA for 48 hours, in accordance with previous reports [16,17,29,31]. More importantly, IL-18BPa/Fc only remarkably promoted the survival of lymphocytes in ITP patients. Aberration of lymphocytic apoptosis plays important roles in immune pathologic injury and immune regulation dysfunction in ITP patients. The present data demonstrate that IL-18BPa/Fc may play a pathogenic role in ITP by accelerating the apoptosis of activated lymphocytes, the precise mechanisms await further elucidation.

In addition, the expression of IL-18R in PBMCs of ITP patients and pathogenic role in ITP by accelerating the apoptosis of activated patients. The present data demonstrate that IL-18BPa/Fc may play a role as a cytokine that induces IFN-γ production by T cells. Nature 1995;378:81–91.


References


Acknowledgements

This work was supported by the Tai Shan Scholar Foundation, National Natural Science Foundation of China (grants 8110334, 81070396, 81070411, 81070407, 81070408, 30570779, 30600259, 30770922, 30800491, 30801258, and 30971278), the National 973 Basic Research Program of China (grant 2011CB503906), Foundation for the Author of National Excellent Doctoral Dissertation of PR China (grant 200561), Program for New Century Excellent Talents in University (grant NCET-07-0514), Key Project of Chinese Ministry of Education (grant 109097), Key Clinical Research Project of Public Health Ministry of China 2010–2012, National Science Foundation of Shandong Province (grant ZR2009CM001, ZR2010HQ002), Clinical Medicine Center Foundation of Shandong Province, Leading Medical Professionals Foundation of Shandong Province, Outstanding Young Scientist Research Award Foundation of Shandong Province (grant 2008BS03009), Independent Innovation Foundation of Shandong University (grant 2009T15053), State Program of National Natural Science Foundation of China for Innovative Research Group (grant 81021001).

Conflict of Interest Statement

The authors reported no potential conflicts of interest.


