Regular Article

TLR7 promotes Th1 polarization in immune thrombocytopenia

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Keywords: Immune thrombocytopenia, Toll-like receptor, T helper cell, Macrophage, Cytokine

A B S T R A C T

Introduction: T helper 1 cell (Th1) polarization persists in the autoimmune response found in immune thrombocytopenia (ITP), Toll-like receptor 7 (TLR7) expression, which also plays an important role in autoimmune diseases, was verified to increase in ITP. However, the exact role of TLR7 in ITP is not well elucidated. Here, we explored the hypothesis that TLR7 participates in the pathophysiology of ITP by affecting Th1 polarization.

Materials and methods: Twenty-two ITP patients and twenty-one controls were enrolled in this study. We examined the cytokine secretion of macrophages in ITP patients and controls using both TLR7 agonist (imiquimod) and antagonist (IRS 661). The influence of macrophage secretion from these groups and its effects on Th1/Th2 differentiation were subsequently studied. Effects of TLR7 on Th1/Th2 balance and platelet counts were also studied in vivo using a thrombocytopenic mouse model.

Results: In in vitro assays, imiquimod enhanced interleukin (IL)-12 secretion in macrophages from ITP patients inducing Th1 differentiation. However, IRS 661 had the exact opposite effect and skewed Th differentiation towards the Th2 subset in ITP. Results from our in vivo studies indicated that injection of imiquimod in ITP mice resulted in elevated plasma levels of IFN-γ and decreased platelet counts. Nevertheless, injection of IRS 661 resulted in elevated plasma levels of IL-4 and platelet counts.

Conclusion: These findings indicate that TLR7 promotes Th1 polarization and may contribute thus in the pathogenesis of ITP.

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phosphorothioate oligonucleotides of TLR7 and negative control
32×10^9/L, median 9×10^9/L) were enrolled in this study (Information of
Patients
oligonucleotides.

Materials and methods

Reagents

Imiquimod, TLR7 agonist, was purchased from InvivoGen (San Diego,
USA), IRS 661 (5′-TCTTCTGCAAGCTTGCAAGCA-3′), specific inhibitory phosphorothioate oligonucleotides of TLR7 and negative control oligonucleotides (5′-TCTTCGAGTTAAGT-3′) were prepared by Biosune (Shanghai, China) and determined to have less than 5 endotoxin U/mg oligonucleotides.

Patients

Twenty-two patients with active ITP disease (14 females and 8 males, age range 18–65 years, median 34 years, platelets range 1–32×10^9/L, median 9×10^9/L) were enrolled in this study (Information of the patients enrolled in our study will be supplied on request). All of the cases met the diagnosis criteria of ITP as previously described [21]. None of them had been treated with glucocorticosteroid prior to sampling. Twenty-one healthy controls (14 females and 7 males, age range 19–51 years, median 33 years, platelets range 104–310×10^9/L, median 180×10^9/L) matched for sex and age with the study population were voluntarily blood donors. Enrollment took place between March 2009 and January 2010 at the Department of Hematology, Qilu Hospital, Shandong University, Jinan, China. Informed consent was obtained from each patient and healthy controls. Ethical approval for the study was obtained from the Medical Ethical Committee of Qilu Hospital, Shandong University.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using 1.077 g/ml Ficoll-Hypaque (TBD, Tianjin, China). After washing with phosphate-buffered saline solution (PBS), PBMCs were suspended in RPMI-1640, dispensed in 6-well plates and incubated for 3 h at 37 °C in a humidified atmosphere containing 5% CO2. Then the adherent cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 200 U/ml rhGM-CSF (Peprotech, Rocky Hill, USA) for cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 200 U/ml rhGM-CSF (Peprotech, Rocky Hill, USA).

After four immunizations, the experiment mice with platelet counts reaching a minimum were called ‘ITP mice’ and were divided into three groups. Group I received rat platelet injection once (regarded as ITP mice), group II received rat platelets one time plus an intraperitoneal injection of 25 μg imiquimod every other day (regarded as ITP mice plus imiquimod), and group III received rat platelets one time plus an intraperitoneal injection of 40 μg IRS 661 every other day (regarded as ITP mice plus IRS 661). The blood and plasma samples were collected from all groups including control mice one week later (i.e. four weeks after first immunization).

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5′→3′)</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-bet</td>
<td>CATGCGGCTGACGTCCTACC GATGGGTGTCACAGATGTCGACGAGGACCTAC TCTGTCGTCGTCGTCGTCG</td>
<td>121</td>
</tr>
<tr>
<td>GATA3</td>
<td>AGAGATCGGACGACGACGAGGACCTAC TCTGTCGTCGTCGTCGTCG</td>
<td>171</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TTGCGCAATTCTTCTGGCAACTC CGCTACATGCTACAGATGTCGACGAGGACCTAC TCTGTCGTCGTCGTCG</td>
<td>120</td>
</tr>
<tr>
<td>IL-4</td>
<td>AGAGATCGGACGACGACGACGAGGACCTAC TCTGTCGTCGTCGTCGTCG</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>TTGCGGACAGGATCAGAGAAA GGGCATCCACAGAGGACTC G</td>
<td>110</td>
</tr>
</tbody>
</table>

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instruction. The amount of RNA was determined using ultraviolet spectrophotometer DU800 (Beckman, Palo Alto, USA) and then normalized to 1 μg/ml. Reverse transcription of total RNA into cDNA was performed using PrimerScript™ RT reagent kit (Takara, Kyoto, Japan). Multiple real-time PCR was performed for IL-4, IFN-γ, T-box expressed in T cells (T-bet), GATA-binding protein 3 (GATA3) and endogenous control β-actin on Lightcycler 2.0 (Roche, Mannheim, Switzerland) using SYBR Premix Ex Taq (Takara, Kyoto, Japan). The sequences of the amplification primers are listed in Table 1.

The PCR reactions were conducted with an initial denaturing at 95 °C for 10 s, and then involved 40 cycles of 60 °C 5 s, 72 °C 10 s, and terminated by a cooling step 40 °C 30 s. The melting-curve analysis was performed to confirm the absence of primer dimers in specific PCR products. Controls included RNA subjected to RT-PCR without reverse transcriptase, RT-PCR with water replacing RNA, and PCR with water replacing cDNA, and all these controls gave no signal. Each experiment was carried out in triplicate and the data were analyzed using LightCycler Software 4.0 (Roche, Mannheim, Switzerland). All PCR products were visualized by 2% agarose gel electrophoresis stained with ethidium bromide. The mRNA expression of the target (i.e., T-bet, IFN-γ, GATA3 and IL-4) was normalized to β-actin relative to control using the 2^ΔΔCt method [23].
ELISA

The macrophage supernatant was used to determine the levels of IL-12 and IL-4. Plasma samples from CBA mice of different groups were obtained from peripheral blood after centrifuging at 2000 g for 20 min to measure the levels of IFN-γ and IL-4. The cytokine levels above were detected by commercial ELISA kits according to manufacturer's instructions (Jingmei, Shenzhen, China).

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis were performed using Statistical Package for the Social Sciences (SPSS, version 13.0) by one-way analysis of variance (ANOVA). P value less than 0.05 was considered statistically significant.

Results

Effects of TLR7 on secretion of IL-12 and IL-4 by macrophages

The levels of IL-12 in the macrophage supernatant were measured by ELISA. Compared with healthy controls, secretion of IL-12 by macrophages in ITP patients was significantly increased (ITP: 54.63 pg/ml, control: 33.22 pg/ml, P < 0.001). Imiquimod significantly increased secretion of IL-12 while IRS 661 inhibited secretion of IL-12 in ITP. The levels of IL-12 secreted by ITP macrophages from group II and group III were 149 ± 8.94 pg/ml and 20.83 ± 5.15 pg/ml, respectively. However, no obvious changes were observed in the three groups of macrophages from controls (Fig. 1A). The levels of IL-4 in the macrophage supernatant were below the detectable limit of the assay used. The macrophages were harvested to analyze relative mRNA expression of IL-4 by real-time PCR. Relative mRNA expression of IL-4 in ITP macrophages was 0.55 ± 0.06 when compared to controls. Imiquimod appeared to decrease expression of IL-4 in ITP though not significantly. IRS 661 significantly increased expression of IL-4 in ITP macrophages (4.69 ± 0.48, P < 0.001) but not in controls (Fig. 1B). Taken together, these results demonstrate that secretion of IL-12 is increased in ITP patients, stimulation of TLR7 enhances the increase of IL-12 while inhibition of TLR7 rescued the increase of IL-12 and increased expression of IL-4 in ITP.

Effects of TLR7 on Th1/Th2 differentiation through macrophages in vitro

To evaluate effects of TLR7 on Th1/Th2 differentiation, Th cells were cocultured with autologous macrophages. After three days of coculture, Th cells were harvested for RNA isolation and real-time PCR analysis. The data were presented as relative mRNA expression normalized to β-actin and relative to controls. The relative amounts of T-bet and IFN-γ in ITP Th cells were increased to 3.39 ± 0.28 (P < 0.001) and 3.84 ± 0.43 (P < 0.001), respectively, when compared to controls. Imiquimod significantly increased whereas IRS 661 remarkably decreased the expression of T-bet and IFN-γ only in ITP. The relative expression of T-bet in group II and group III of ITP Th cells were 9.97 ± 0.39 and 0.13 ± 0.02, respectively. The relative expression of IFN-γ in group II and group III of ITP Th cells were 33.36 ± 3.58 and 0.13 ± 0.03, respectively. No significant changes were found in relative amounts of T-bet and IFN-γ among different groups of Th cells from controls. The changes in expression of GATA3 and IL-4 in Th cells were just different from those of T-bet and IFN-γ. The relative amounts of GATA3 and IL-4 in ITP Th cells were decreased to 0.37 ± 0.02 (P = 0.014) and 0.31 ± 0.04 (P = 0.001), respectively, when compared to controls. Remarkably, imiquimod enhanced the decrease of IL-4 (P = 0.001) only in ITP. However, IRS 661 significantly increased expression of GATA3 only in ITP and expression of IL-4 in both ITP and controls. The relative expression of GATA3 in group II and group III of ITP Th cells were 0.18 ± 0.06 and 7.23 ± 0.66, respectively. The relative expression of IL4 in group II and group III of ITP Th cells were 0.1 ± 0.01 and 6.05 ± 0.12, respectively (Fig. 2). Together, our results indicate that there is a Th1-phenotype response in ITP in vitro and stimulation of TLR7 enhances Th1 polarization. Then we proceeded to investigate the effects of TLR7 on Th1/Th2 differentiation in vivo.

Effects of TLR7 in vivo

A thrombocytopenia mouse model described by Musaji [24] was used. The experiment mice developed gradual thrombocytopenia and reached the maximum (only 49% of the platelet counts before immunization) 3 weeks after the first rat platelet administration (data not shown) just as Musaji described [24]. Then the experiment mice were called 'ITP mice'. The ITP mice were divided into three groups and treated as mentioned above. Plasma samples from all groups including controls were obtained and the levels of IFN-γ and IL-4 were determined by ELISA. The plasma levels of IFN-γ in ITP mice were significantly increased compared to controls. Imiquimod injection significantly increased the plasma levels of IFN-γ whereas IRS 661 injection significantly decreased the plasma levels of IFN-γ in ITP mice. The plasma levels of IFN-γ in controls, ITP mice, ITP mice plus imiquimod and ITP mice plus IRS 661 were 10.38 ± 1.52 ng/ml, 22.42 ± 1.66 ng/ml, 32.34 ± 2.15 ng/ml and 5.75 ± 0.71 ng/ml, respectively (Fig. 3A). The plasma levels of IL-4 in ITP mice were significantly decreased compared with controls. Imiquimod injection remarkably decreased the plasma levels of IL-4 whereas IRS 661 injection remarkably increased the plasma levels of IL-4 in ITP mice. The mean ± SD of IL-4 levels in controls, ITP mice, ITP mice plus imiquimod and ITP mice plus IRS 661 were 10.42 ± 0.51 ng/ml, 7.08 ± 0.76 ng/ml, 3.65 ± 0.59 ng/ml and 13.52 ± 0.58 ng/ml, respectively (Fig. 3B). These results suggest that TLR7 plays a direct role in Th1/Th2 differentiation by inducing Th1 polarization in ITP. In order to explore the role of Th1 polarization induced by TLR7 in the pathology of ITP, we detected
platelet counts in ITP mice using both imiquimod and IRS 661. The change of platelet counts was expressed as relative platelet count, i.e. the ratio of the platelet counts after immunization to the platelet counts before immunization of the same mouse. After five times of immunization (i.e. four weeks after first immunization), the relative platelet counts of ITP mice were significantly decreased compared to controls (P = 0.009). Imiquimod injection significantly decreased the relative platelet counts whereas IRS 661 rescued the changes in relative platelet counts in ITP mice. The relative platelet counts in controls, ITP mice, ITP mice plus imiquimod and IRS mice plus IRS 661 were 1.01 ± 0.09, 0.75 ± 0.03, 0.56 ± 0.06 and 1.18 ± 0.09, respectively (Fig. 3C). These results showed that activation of TLR7 contributes to the pathology of ITP. Together, our in vivo study indicate that TLR7 promotes Th1 polarization and may contribute thus in the pathogenesis of ITP.

Discussion

In this study, we have demonstrated that in ITP patients, activation of TLR7 increased secretion of IL-12. However, inhibition of TLR7 exerted the contrary effects by increasing expression of IL-4. It is of interest to note that secretion of IL-12 was increased in macrophages from ITP patients compared with healthy controls. More importantly, activation of TLR7 remarkably enhanced up-regulation of IL-12 in ITP. These results were consistent with early studies showing that TLR7 activation resulted in increased production of IL-12 both in vitro and in vivo [25–28] and that TLR7(−/−) mice had a reduced IL-12 response after viral infection [29,30]. There are conflicting data concerning about the influence of TLR7 on secretion of IL-4. In various animal models, ligation of TLR7 down-regulated serum levels of IL-4 [31–33]. Normally in in vitro analysis of the secretion of IL-4, cells are usually pretreated with stimulators including mitogen or anti-CD3 antibody (mainly for T cells) as described in the user manual of ELISA kit. However, in order to exclude the influence of other stimulating factors, we treated macrophages from ITP patients with TLR7 agonist or antagonist alone. The lack of pretreatment of our macrophages may account for the failure to detect the secretion of IL-4. Despite of an undetectable IL-4 in macrophage supernatant, we showed a decrease of mRNA expression of IL-4 in ITP macrophages (though not significant) and TLR7 negatively regulated mRNA expression of IL-4 in ITP.

Usually, uncommitted Th cells can be induced to differentiate towards Th1 and Th2 phenotypes according to the local cytokine milieu. This differentiation process is initiated by stimulation with antigen and the cytokines IL-12 and IL-4, respectively. Both IL-12 and IL-4 play important roles in Th differentiation. Therefore, we evaluated the effects of TLR7 on Th1/Th2 balance by coculturing Th cells with autologous macrophages. Th1 polarization of the immune response was observed in ITP in vitro in accordance with our and others previous reports [19,34,35], whereas contradictory findings were described elsewhere [36,37]. The mRNA expression of T-bet and IFN-γ were increased while GATA3 and IL-4 were decreased in ITP Th cells. The presence of IL-12, signaling through signal transducers and activators of transcription (STAT)4 resulted in induction and maintained expression of the specific transcription factor T-bet [38,39]. Previous studies have suggested that T-bet is the master regulator of Th1 lineage commitment, recruiting transcription factors such as NFAT (nuclear factor of activated T cells)-1 to IFN-γ promoter and promoting expression of IFN-γ [40–42]. The committed Th1 cells have been implicated in the immunopathology of autoimmune diseases whereas a role as regulators has been suggested for Th2 cells [43]. IL-4 signaling through STAT6 initiates Th2 differentiation and induces expression of GATA3 (specific transcription factor for Th2 cells). GATA3 significantly down-regulates IFN-γ production from developing Th1 cells and induces IL-4 and IL-5 levels, thus playing an important role in Th2 differentiation and phenotype maintenance [44,45]. Dereegulation of T-bet/GATA3 is evident in patients with various diseases including MS [46], SLE [47] and rheumatoid arthritis [48]. In our study, further coculture experiments showed activation of TLR7
promoted increased mRNA expression of the Th1-associated transcription factor and cytokines in ITP indicating that TLR7 promotes Th1 polarization in ITP in vitro.

To confirm the results obtained from experiments in vitro, studies in vivo were also performed using a thrombocytopenic mouse model first described by Musaji [24]. After four immunizations of rat platelets, experiment mice with the minimum of platelet counts were called ‘ITP mice’. Autoantibodies to the platelet glycoprotein (GP) Iib were detected in ITP mice indicating that the drop in platelet counts was due to an autoimmune response [24]. This is consistent with the phenomenon that autoantibodies, especially IgG antibodies against GPIb/IIIa and/or GPIb/IX, are the frequently clinical detectable autoantibodies in ITP patients [49]. Besides the presence of autoantibodies, our result showed increased plasma levels of IFN-γ in ITP mice and a decrease of IL-4, suggesting there are cellular immune abnormalities in ITP mice. This is similar to the pathophysiology of ITP patients. IFN-γ, a Th1 cytokine, has been shown to be pivotal to the development of autoimmune. Patients undergoing therapy with IFN-γ or animals upon administration of IFN-γ develop varied autoimmune syndromes [43,50]. However, Th2 cytokines (e.g., IL-4, TGF-β) have been shown to have a protective inhibitory effect on autoimmunity and play direct role in the induction or maintenance of tolerance. Our results showed that stimulation of TLR7 enhanced the increase of IFN-γ and decrease of IL-4 in ITP mice suggesting the positive impact of TLR7 on Th1 polarization and autoimmune response in ITP in vivo. Otherwise, activation of TLR7 decreased the relative platelet counts in ITP mice while inhibition of TLR7 corrected the decrease of platelet counts in ITP mice. These findings correlate Th1 polarization induced by TLR7 to the pathology of ITP.

Taken together, TLR7 stimulation increased secretion of IL-12 while TLR7 inhibition increased expression of IL-4 in macrophages from ITP patients. TLR7-stimulated macrophages promoted Th differentiation towards Th1 phenotype in vitro. In the thrombocytopenic mouse model, activation of TLR7 also promoted Th1 polarization and decreased platelet counts. These results suggest that TLR7 plays a direct role in Th1/Th2 differentiation and participates in platelet destruction of ITP by promoting Th1 polarization.

Conflict of interest

None.

Acknowledgements

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