Original Article
Recognition of the human antibody-mediated platelet destruction in adult ITP patients by C-reactive protein

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Abstract: Immune thrombocytopenia purpura (ITP) is characterized by destruction of circulating platelets and the presence of antiplatelet IgG antibodies, which opsonize platelets for splenic clearance resulting in low levels of circulating platelets, and the disease severity can be predicted neither by antibody isotype nor by titer, indicating that other factors also play a role. Although the main cause of ITP remains unclear, but its relationship with some infection was demonstrated, including viral or bacterial infections. C-reactive protein (CRP), a member of the pentraxin family, is a major acute-phase protein in humans and is a clinical marker of infection. We aimed to investigate the correlation between the levels of CRP and the presence of antiplatelet IgG antibodies in adults with newly diagnosed ITP. CRP levels and platelet counts were measured in the blood samples from a 60 ITP patient (with confirmed anti-GPIIb/IIIa antibodies), 60 infection patients (all without anti-GPIIb/IIIa antibodies) and 60 normal individuals. The bleeding score, recover time of intravenous immune globulin (IVIg) therapy and the number of megakaryocytes in bone marrow were recorded in ITP patients. The platelet count, bleeding score, recover time of intravenous immune globulin (IVIG) therapy and the number of megakaryocytes in bone marrow and CRP concentrations were compared in ITP group using Spearman's correlation coefficient. We examined the influence of intraperioneal CRP administration on antibody-mediated platelet destruction in mice. There were no statistical differences in gender, age and body mass index among the three groups (P>0.05). Though CRP levels are significantly elevated in ITP patients and infection patients (P<0.05), the platelet count was markedly lower only in ITP patients. We found that CRP was inert toward platelets without antiplatelet antibodies in this study. There are a significant correlation between CRP levels and platelet counts, bleeding severity and the number of megakaryocytes in bone marrow aspiration (r=-0.5079, r=0.5498, r=0.4172, P<0.001, respectively). Moreover, a significant correlation was observed between the recovery time of platelet count and CRP levels (r=-0.5569, P<0.001). In mice, platelet count was lower in Anti-CD41 (0.75 µg)+, CRP (200 µg) group as compared with Anti-CD41 (0.75 µg)+, CRP(-) group and Anti-CD41 (0.75 µg)-, CRP (200 µg) group (P<0.05). In summary, this study indicated that CRP levels are significantly elevated in ITP patients all with confirmed anti-GPIIb/IIIa antibodies, which is able to predict the clinical bleeding severity of ITP patients. The slower CRP levels reduction after IVIg treatment predicted slower platelet count recovery in ITP.

Keywords: C-reactive protein, immune thrombocytopenic purpura, antiplatelet antibodies

Introduction

Immune thrombocytopenia purpura (ITP) is characterized by destruction of circulating platelets and the presence of antiplatelet IgG antibodies, which opsonize platelets for splenic clearance resulting in low levels of circulating platelets. Although the main cause of ITP remains unclear, but its relationship with some infection was demonstrated including viral or bacterial infections [1-4]. The ensuing low plate-let counts result in bleeding symptoms [5] that range from mild, common events, such as petechiae and bruising, to rare, serious events, such as intracranial hemorrhage [6]. Antibody-mediated platelet destruction in ITP occurs primarily through engagement of immunoglobulin IgG opsonized platelets with activating Fc receptors (FcγRs) on the surface of phagocytes in the spleen and liver, resulting in phagocytosis and thrombocytopenia [7]. Autoantibodies against the major membrane glycoproteins (GP)

can be identified in about 80% of patients with ITP [8, 9] and the majority of these antibodies target epitopes on GPIIb/IIIa (CD41/CD61) [10].

Although platelet decrement is related to antibody titer in ITP [11, 12], this correlation is not strict, as cases with low titers and very low platelet counts, as well as cases with high titers and normal platelet counts, are frequently observed. Recently we found that this discrepancy is partially due to the differences in the functional quality of these antibodies, determined by its Fc glycosylation, in particular the level of core fucosylation [13]. However, the data indicated that additional cofactors may also be involved.

C-reactive protein (CRP) [14], a member of the pentraxin family, is a major acute-phase protein in humans and is a clinical marker of infection. CRP, a known ligand for FcRs produced by the liver in response to inflammation due to various stimuli, has been shown to bind and activate Fcγ receptors (FcγR) on monocytes and macrophages [15-18]. In addition, CRP suppressed immune complex mediated nephron toxic nephritis in a mouse model [19]. Despite their distinct folds, both antibody and pentraxins bind FcγR in a 1:1 stoichiometry, obligating pathogen opsonization or immune complex formation as the mechanism for receptor clustering and activation [18, 20, 21]. Moreover, they share an overlapping binding site on FcγR, predicting a mutually exclusive FcγR association between antibodies and pentraxins. CRP levels are useful as a clinical diagnostic tool for infection, and it is a common knowledge that ITP is triggered by viral infection that precedes the clinical picture of ITP by a few days to a few weeks [22]. Therefore, we have been particularly interested in the role of CRP which interacts directly with antiplatelet IgG antibodies and functions as a novel pathogenic cofactor in IgG mediated platelet destruction.

In this study, we aimed to explore the correlation between the levels of CRP and the presence of antiplatelet IgG antibodies in adults with newly diagnosed ITP. We sought to investigate the hypothesis of CRP as a novel pathogenic cofactor in IgG-mediated platelet destruction by phagocytes, ultimately leading to platelet destruction in ITP patients. We also established a mouse model in which CRP was intraperitoneally injected and observed whether the elevated CRP levels directly contribute to antibody-mediated platelet destruction in vivo.

Materials and methods

Patients

We prospectively enrolled 60 patients with newly diagnosed ITP, 60 infection patients whose diagnosis included upper respiratory tract infection (31 cases), pneumonia (17 cases), and periodontitis (12 cases) and 60 age-gender matched healthy individuals for the study from January 2015 to February 2017 at the Second Affiliated Hospital of Harbin Medical University. The study was approved by the ethics committee of our hospital, and informed consent was signed by all individuals.

The inclusion criteria: ITP was diagnosed based on the guidelines proposed by the American Society of Hematology [23]. Only patients aged >18 years who had platelet counts <100×10^9/L with confirmed anti-GPIIb/IIIa antibodies and no history of other clinical conditions that can cause thrombocytopenia were included. Both sixty infection patients and normal controls without anti-GPIIb/IIIa antibodies. The exclusion criteria in ITP patients included, pseudo-thrombocytopenia; clinical or serologic evidence of associated conditions or factors that can cause thrombocytopenia, such as systemic lupus erythematosus, lymphoproliferative disorders, liver cirrhosis, or therapy with drugs such as heparin or quinidine; and previous treatment with corticosteroids or splenectomy.

Mice

Six-week-old BALB/c mice (strain BALB/cOlaHsd) were obtained from the animal experiment center of Harbin Medical University and the use of experimental animals was conducted after examination and approval by the local animal ethic committee.

Baseline definitions and measurements

Peripheral blood and bone marrow smears were examined and the number of megakaryocytes in bone marrow aspiration was recorded for ITP patients. The ITP patients were carefully examined for the presence of clinical signs of bleeding and the score according to Buchanan et al [24] based on the overall extent of bleed-

ing ranging from 0 (no bleeding) to 5 (life threatening or fatal). All infection patients were evaluated as to the existence of a site of infection using clinical and microbiological data. Additional blood samples were from 60 normal individuals who underwent routine health check-ups at the hospital. The body weight and height were recorded and subsequently body mass index (BMI) was calculated ITP patients (34 cases) with platelet counts $<20\times10^9$/L were placed on intravenous immuneglobulin (IVIg) therapy and platelet counts returned to normal in all patients. Intravenous immuneglobulin (IVIg) was administered at a dose of 400 mg/kg/day for 5 days. Laboratory studies were performed and clinical data (including platelet counts and CRP levels) were collected on day 2 or 3, 4-6, 7-9, and 10-14 after treatment.

The monoclonal antibody immobilization of platelet antigens assay (MAIPA)

The MAIPA technique described by Kiefel et al (1987), with modifications (Morel-Kopp and Kaplan 1994) [25]. Briefly, 1×10^8 platelets from healthy blood group "O" donors were sensitized with 100 μL plasma of patients or controls, washed, and solubilized in Tris-buffered saline containing 1% Triton X-100 and 0.1 mg/mL leupeptin. Microtiter plates were coated with affinity-purified goat anti-mouse IgG (Immunotech, Marseille, France), and next incubated with GPIIb/IIIa mAb (P2 clone) for 60 min at room temperature. After washing, the sensitized platelet lysate was added in duplicates to each well and incubated for another 60 min. IgG bound to the captured GPIIb/IIIa was detected using alkaline-phosphatase-conjugated goat anti-human IgG (Fc specific; Sigma Chemical Co, Saint Louis, MI, USA). p-Nitrophenyl-phosphate was used as the substrate, and the plates were read on an automated microtiter plate reader (Thermo-Multiskan Mk3; Hudson, NH, USA) using dual wavelength (405 and 492 nm). A positive result was defined as absorbance beyond mean + 3 SD normal controls.

CRP determination

In all individuals, blood specimens were obtained on the day newly diagnosed. In ITP patients blood specimens were also obtained and on the day when platelet counts returned normal. The samples were obtained at approxi-

mately the same time each morning. Plasma samples were collected in EDTA tubes, centrifuged at 3000 rpm for 10 minutes at 4°C, labelled and stored at -80°C till analysis. Serum CRP levels were detected with a high sensitivity assay (Sekisui Medical Co., Tokyo, Japan) using an auto-analyzer (Hitachi 7600-210; Hitachi Medico, Tokyo, Japan). In this study, CRP≤1 mg/L was defined as negative (-); CRP>1 mg/L was defined as positive (+).

Induction of thrombocytopenia in vivo

The mice were divided into three groups: CRP (200 μg) group, antiplatelet MWReg30 IgG (0.75 μg) group, and CRP (200 μg) + antiplatelet MWReg30 IgG (0.75 μg) group. Blood was collected from the retro-orbital plexus 16 hours after injection from cardiac puncture (t=16), into lithium-heparin tubes (Microvette, Sarstedt). Subsequently, the blood was diluted in PBS/EDTA and the number of platelets was immediately counted using the ADVIA 2120 hematology system using murine-BALB/c parameters (Siemens Medical Solutions Diagnostics, Erlangen, Germany).

Statistical analysis

Continuous variables were reported as the mean and the standard deviation (SD). The clinical and demographic variables were compared using the student's t test for continuous variables with a normal distribution. Comparisons between groups were subjected to one-way ANOVA followed by post-hoc Tukey's test parameter fault (GraphPad InStat Version 3.06). Values of $P<0.05$ were considered to be statistically significant. Spearman's rank correlation coefficient was used to evaluate the significance of the correlation between CRP levels and platelet counts, bleeding severity, number of megakaryocytes, and recover time in ITP patients. An association was considered significant if $P<0.05$. All analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics of patients in the study

The study enrolled 60 patients (23 males and 37 females, 28-54 y (average 41 y) who were

Table 1. Clinical characteristics of health controls, infection patients and ITP patients

Variables	Health (n=60)	Infection (n=60)	ITP (n=60)
Male/female	29/31	32/28	23/37
Age (years)	42 ± 12	40 ± 14	41 ± 13
BMI (kg/m)	27.68 ± 3.27	27.98 ± 3.31	27.66 ± 3.24
Hemoglobin (mg/dL)	13.02 ± 1.63	12.86 ± 1.71	12.98 ± 1.75
Platelets (×10^9/L)	182.9 ± 72.6	172.3 ± 71.3	18.4 ± 23.3**
CRP (mg/dL)	0.61 ± 0.88	3.99 ± 1.64*	3.38 ± 1.59*

Data are means ± SD. Variable are compared using student's t test. *P<0.05; **P<0.01. BMI: body mass index; WBC: white blood cell; CRP: C-reactive protein.

Table 2. Characteristics of ITP patients

	ITP patients n=60 (%)
Number of a history of a prior infection	32 (53.3%)
UTI*	5 (8.3%)
Pneumonia	4 (6.7%)
Diarrhea	8 (13.3%)
Upper respiratory tract infection	12 (20.0%)
Periodontitis	3 (5.0%)
Bleeding score diagnosis	
0	4 (6.7%)
1	13 (21.6%)
2	12 (20.0%)
3	15 (25.0%)
4	13 (21.7%)
5	3 (5.0%)
Treated with IVIG**	34 (56.7%)
Non-treated	26 (43.3%)

Number between brackets represents %. *UTI: Urinary Tract Infection; **IVIG: intravenous immunoglobulin.
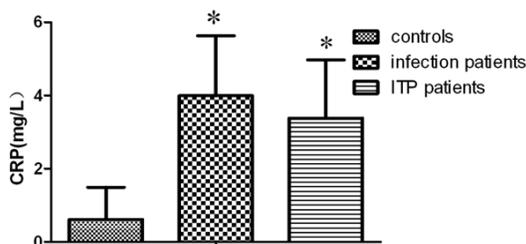
Figure 1. CRP levels in different groups. Figures represent mean ± SD, ANOVA followed by post hoc Tukey's test. *P<0.05 as compared to control group.

newly diagnosed with ITP (all with confirmed anti-GPIIb/IIIa antibodies)and 60 infection patients (32 males and 28 females, 26-54 y (average 40 y) (all without anti-GPIIb/IIIa antibodies) whose diagnosis included upper respiratory tract infection (31 cases), pneumonia (17 cases), and periodontitis (12 cases) and control blood samples were from 60 normal individuals (29 males and 31 females, 30-54 y (average 42 y) who underwent routine health check-ups at the hospital. The general characteristics of the study groups were included in **Table 1**. The three groups did not differ statistically in terms of age, female/male ratio, body mass index (BMI) or hemoglobin (P>0.05).

The ITP patient characteristics are summarized in **Table 2**. ITP patients who had a history of a prior infection were 32 (53.3%), whose diagnosis included UTI (urinary tract infection) 5 cases (8.3%), pneumonia 4 cases (6.7%), diarrhea 8 cases (13.3%), upper respiratory tract infection 12 cases (20.0%), periodontitis 3 cases (5.0%). The bleeding score according to Buchanan et al based on the overall extent of bleeding ranging from 0 (no bleeding) to 5 (life-threatening or fatal) on the day of enrollment was "0" in 4 patients, "1" in 13 patients, "2" in 12 patients, "3" in 15 patients, and "4" in 13 patient, "5" in 3 patient in **Table 2**. Within 72 hours after diagnosis, the 34 (56.7%) patients whose platelet count below 20×10^9/L received a single infusion of IVIg (0.4 g/kg/day).

Serum CRP levels

To examine the relevance of CRP levels in adult ITP patients, we measured the plasma CRP level. The ITP and infection patients exhibited much higher levels of CRP (ITP, 3.38 ± 1.59 mg/dL; Infection, 3.99 ± 1.64 mg/dL) than healthy controls 0.61 ± 0.88 mg/dL (**Table 1** and **Figure 1**).

Correlations between CRP levels and platelet counts, bleeding severity, number of megakaryocytes in bone marrow aspiration in ITP patients

To further investigate whether the level of CRP affects ITP patients, we examined the correlation of CRP and platelet counts, bleeding ten-
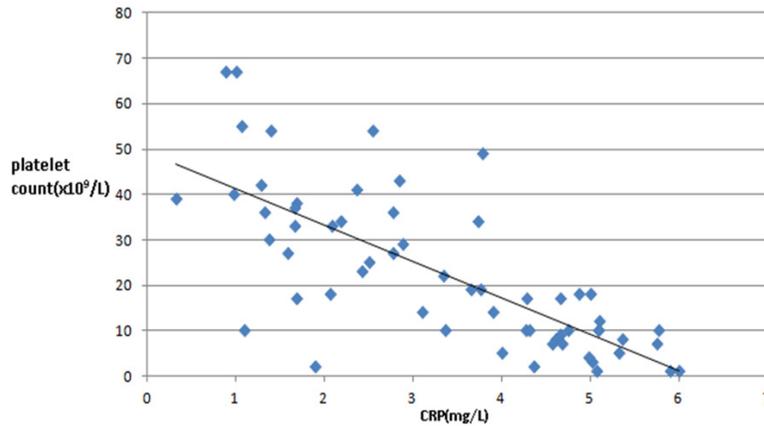
Figure 2. Correlation between CRP levels and platelet counts. (r=-0.5079, P<0.001 by Spearman's rank correlation coefficient).
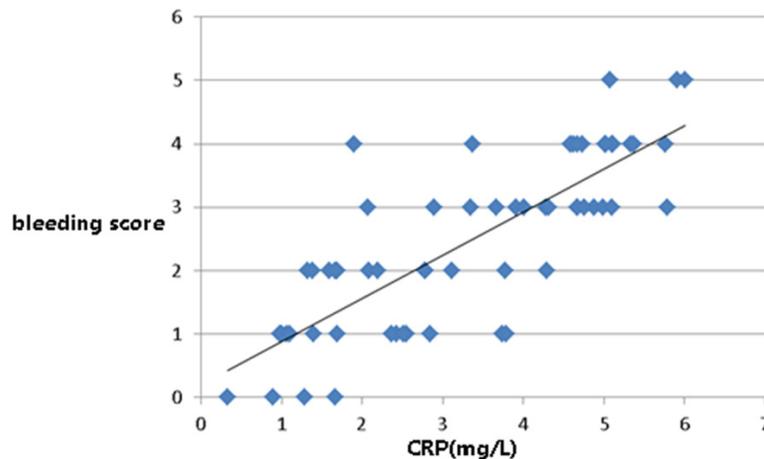
Figure 3. Correlation between CRP levels and bleeding score. (r=0.5498, P<0.001 by Spearman's rank correlation coefficient).

dency, and number of megakaryocytes in ITP patients by Spearman's test. A significant negative correlation between CRP levels and platelet count was found in ITP patients (all with confirmed anti-GPIIb/IIIa antibodies (r=-0.5079, P<0.001) (**Figure 2**). A significant positive correlation between CRP levels and bleeding severity, number of megakaryocytes in ITP patients was found (r=0.5498, P<0.001; r=0.4172, P<0.001, respectively) (**Figures 3**, **4**).

Treatment and response in ITP patients

To further analyzed the effect of intravenous immunoglobulin (IVIg) treatment on adult patients with newly diagnosed ITP in a clinical trial investigation, Spearman's test was used to evaluate the relationship of the decrease of CRP levels from onset to platelet recover normal and platelet recover time. We found that IVIg treatment led to a significant increased platelet counts. A significant correlation between the decrease of CRP levels and platelet recover time was found in ITP patients (all with confirmed anti-GPIIb/IIIa antibodies (r=-0.5569, P<0.001) (**Figure 5**). Patients with slower CRP decay took more time to platelet counts, indicating that monitor of CRP levels may be used to predict the response of patients to treatments.

Association of CRP levels with anti-GPIIb/IIIa antibodies

We aimed to explore how CRP levels influence the platelet counts in ITP patients. We investigated the hypothesis of CRP as a novel pathogenic cofactor in IgG-mediated platelet destruction by phagocytes, ultimately leading to platelet destruction. To further investigate the relevance of CRP levels, we measured the platelet counts and the CRP concentration in sera from patients with newly diagnosed ITP (all with anti-GPIIb/IIIa antibodies) and compared with infection patients (all without anti-GPIIb/IIIa antibodies) and healthy controls. We found that the platelet count in ITP patients group shows a significant reduction compared to control groups (P<0.05) (**Figure 6**). However, the platelet counts were comparable between infection patients and healthy controls (**Figure 6**). Although the infection patients group had higher levels of CRP than ITP patients group, the platelet counts in patients without antiplatelet antibodies did not cause significant reduction. Without antiplatelet antibodies, CRP was found to be inert toward platelets.
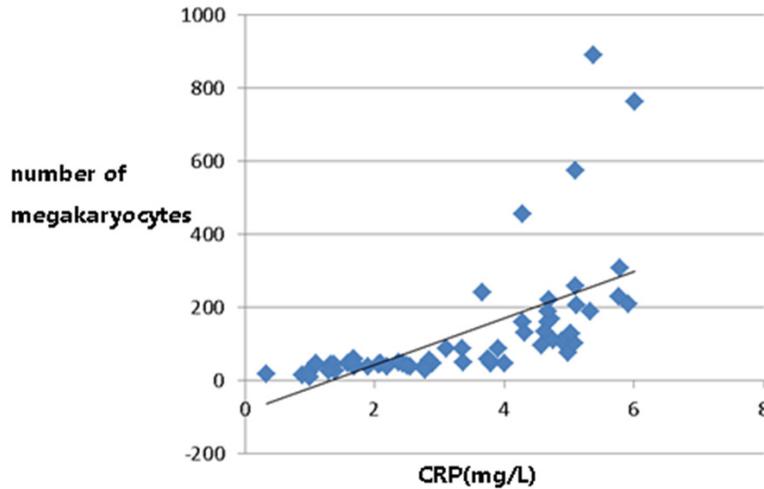
Figure 4. Correlation between CRP levels and number of megakaryocytes. (r=0.4172, P<0.001 by Spearman's rank correlation coefficient).
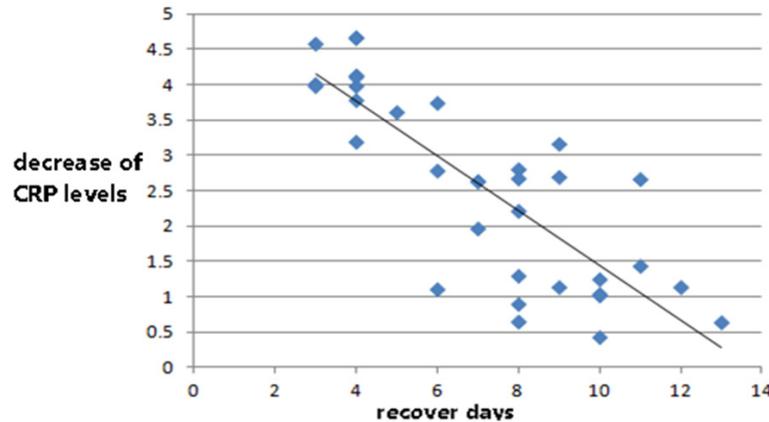
Figure 5. Correlation between decrease of CRP levels from onset to platelet recover normal and the recovery time of platelet counts. (r=-0.5569, P<0.001 by Spearman's rank correlation coefficient).
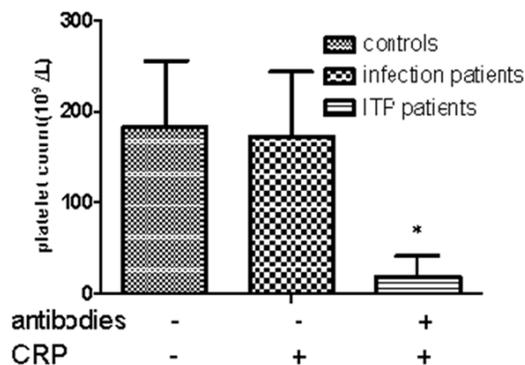
Figure 6. CRP was found to be inert toward platelets without antiplatelet antibodies. CRP: C-reactive protein. Bars represent mean and SD values. Statistical comparison was performed with ANOVA followed by post hoc Tukey's test. *P<0.05.

CRP enhances antiplatelet IgG-mediated destruction of platelets in mice

We then investigated whether the elevated CRP levels directly contribute to platelet degradation in vivo. We examined whether the observed effects of CRP in antibody-mediated platelet destruction also occurred in vivo, using our previously mouse model for immune-mediated thrombocytopenia. Co-administration of 200 μg CRP, together with a limiting dose of 0.75 μg of antiplatelet MWReg30 IgG (GPIIb), significantly decreased the mean platelet counts compared with injection of 0.75 μg of antiplatelet IgG alone, whereas administration of 200 μg CRP alone had no effect on platelet counts (**Figure 7**). These data are in line with the clinical manifestation that the platelet counts of patients without antiplatelet antibodies did not cause significant reduction.

Discussion

In our study, we described a previously unrecognized role of CRP in IgG-mediated platelet destruction in immune thrombocytopenia. We observed that CRP levels were significantly elevated in patients with autoantibody-mediated thrombocytopenias as compared to healthy controls and significantly correlated with platelet counts, bleeding severity, number of megakaryocytes in bone marrow aspiration. Intriguingly, the slower CRP levels decrease after IVIg treatment, the longer it took before stable platelet counts were reached in ITP patients. The present study showed an association between CRP and antiplatelet antibodies, which CRP alone had no effect on the platelet counts in infection patients. We also established a mouse model and observed that elevated CRP levels directly contribute to antibody-mediated platelet destruction in vivo.
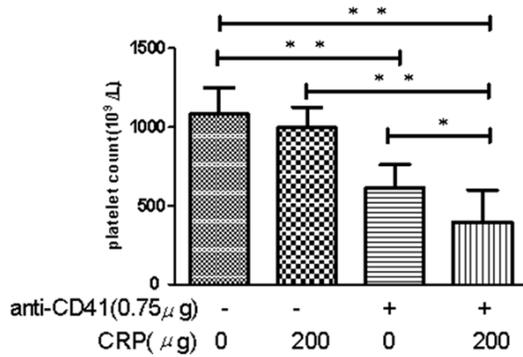
Figure 7. CRP enhances antiplatelet IgG-mediated destruction of platelets in mice. BALB/c mice developed thrombocytopenia 16 hours after intraperitoneal injection of the platelet- and megakaryocyte-specific rat anti-mouse CD41 IgG at 0.75 μg. Coinjection of 200 μg CRP with 0.75 μg rat anti-mouse CD41 IgG resulted in aggravated thrombocytopenia after 16 hours, whereas 200 μg CRP alone had no effect. Data are representative of 2 independent experiments; each data symbol represents 1 mouse (7 per group). Statistical comparisons were performed by one-way ANOVA with Tukey's posttest. *P<0.05; **P<0.01.

Studies in the past decade suggested that CRP can be a good risk predictor for cardiovascular disease [26-30]. This acute phase reactant is currently considered as a key biomarker of systemic inflammation. It is mainly synthesized by hepatocytes in response to inflammation and tissue damage. It can also be produced locally by arterial tissue [29, 31-33]. Furthermore, serum CRP levels on admission may serve as an important diagnostic tool and may also indicate that short term prognosis was demonstrated by duration of hospitalization. In our study, we found that CRP level was higher in adult patients with ITP. This is in line with Kapur and colleagues' study [34] in which they revealed a significant and previously unknown association between the CRP and the antiplatelet antibodies in fetal or neonatal alloimmune thrombocytopenia (FNAIT) and ITP. Our study showed that CRP levels negatively correlated with platelet counts, and positively correlated with the bleeding score and the number of megakaryocytes in ITP patients. This suggests CRP levels maybe an alternative prognostic bleeding risk marker for immune thrombocytopenia. This is in line with some studies [35, 36] which have reported bleeding events to be associated with increased levels of CRP including in acute coronary syndromes and ICH. CRP

levels were even found to predict early hematoma outgrowth after ICH [37]. CRP may seem to be useful to assess and monitor the individual bleeding risk in adult ITP patients, but has to be evaluated and validated in a larger cohort.

IVIg has been proposed to exert its action through numerous ways in treating ITP [38]. We found that IVIg was also associated with a reduction in the CRP levels in ITP, and the decrease of CRP levels significantly correlated with the number of platelets. Hence, lowering CRP levels could offer new therapeutic opportunities for these patients.

The present study showed an association between CRP and antiplatelet antibodies, which CRP alone had no effect on the platelet counts in infection patients. The direct evidence for the causality between increased CRP levels and enhanced platelet degradation was obtained in our clinical studies. We found that although the infection patients group had higher levels of CRP than ITP patients group, the platelet counts of patients without antiplatelet antibodies did not cause significant reduction. More direct evidences for the causality between increased CRP levels and enhanced platelet degradation were obtained in mice, where CRP alone was inert against platelets but enhanced thrombocytopenia together with antiplatelet antibodies, also in line with results obtained from phagocytosis of platelets. We also observed that the elevated CRP levels directly contribute to antibody-mediated platelet destruction in a mouse model. Our findings are in line with previous studies [39, 40] which demonstrate an intriguing correlation between CRP concentrations and antibody-mediated phagocytic activation against platelets through FcRs. The mechanisms of which these factors mediate phagocytic responses are currently incompletely understood, but they are likely to play an initial role in the development of ITP. Importantly, this still needs to be investigated in detail using a panel of antiplatelet antibodies against various GPIIb/IIIa epitopes, ITP sera containing various combinations of antibodies, and against other platelet proteins.

The data suggest that CRP may be an important factor that could explain the frequently observed aggravation of ITP on infections. Infections are known to promote the initiation of ITP or enhance platelet clearance, although

the main cause of ITP remains unclear, its relationship with some infection was demonstrated. The association between ITP and some infections have been reported previously [41-43]. Several studies [44-46] have investigated one of the possible mechanisms that is the molecular mimicry between platelet antigens and various viral and bacterial antigens, giving rise to cross-reactive antibodies. In our studies, the role of increased CRP may directly enhance IgG-mediated responses against platelets. Some other studies [47, 48] elucidate the mechanism responsible for platelet recovery in ITP patients after the successful eradication of H. pylori. The findings demonstrate that the platelet recovery observed in ITP patients after H.pylori eradication is associated with modulation of the monocyte Fcγ receptor balance toward the inhibitory Fcγ receptor IIB (FcγRIIB). This suggests that treatment of the underlying cause of the inflammation in addition to treatment of the thrombocytopenia itself would be beneficial for the patient. These data also suggested a possible mechanism for the relapse of ITP associated with infections. Therefore, the treatment of adult ITP has focused on lowering CRP levels. As infections may be associated with an increase in CRP levels, CRP may serve as an important biomarker for monitoring severities of IgG-mediated thrombocytopenias. Therefore, we suggest that prevention and early adequate treatment of infection are warranted.

In conclusion, CRP could be considered as a novel pathogenic cofactor to enhance IgG-mediated phagocytic responses resulting in thrombocytopenia. Increased CRP levels at diagnosis predicted lower platelet count and higher clinical bleeding severity in ITP. The slower the CRP levels dropped after IVIg treatment, predicted the slower platelet count recovery in ITP. As such, these data also suggested a possible mechanism for the exacerbation or relapse of ITP associated with infections, as infections may be associated with an increase in CRP, indicating that CRP may serve as an important biomarker for monitoring severities of IgG-mediated thrombocytopenias. These results provide insight into the mechanism of CRP regulatory activity in autoimmunity and identify new therapeutic targets in the prevention and treatment of ITP. Future studies may expand these observations and further investi-gate CRP affected by IgG-mediated phagocytosis in other diseases such as those associated with anti-red blood cell antibodies.

Disclosure of conflict of interest

None.

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