C-Reactive Protein Enhances IgG-Mediated Phagocyte Responses and Thrombocytopenia

Supplementary Methods, Table and Figures
Kapur et al, Supplementary Table 1

<table>
<thead>
<tr>
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<th>ITP patients (n=78)</th>
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<tbody>
<tr>
<td>Male</td>
<td>42 (53.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>36 (46.2%)</td>
</tr>
<tr>
<td>Average age in years</td>
<td>5.4 (± 4.2)</td>
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<tr>
<td>Treated with IVIG</td>
<td>38 (48.7%)</td>
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<tr>
<td>Non-treated</td>
<td>40 (51.3%)</td>
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<td>Bleeding score diagnosis</td>
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</tr>
<tr>
<td>1</td>
<td>11 (14.1%)</td>
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<tr>
<td>2</td>
<td>34 (43.6%)</td>
</tr>
<tr>
<td>3</td>
<td>30 (38.5%)</td>
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<tr>
<td>Average platelet count at diagnosis (x 10*9/L)</td>
<td>7.4 (±5.0)</td>
</tr>
<tr>
<td>Average CRP at diagnosis (mg/L)</td>
<td>1.7 (±2.5)</td>
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**Supplementary Table 1.** Characteristics of ITP patients from the TIKI trial, which are included in this study. (n=78). Number between brackets represents either % as indicated, or SD.
Supplementary Figure 1. Phagocytosis of IgG-opsonized platelets. Phagocytosis of platelets by PMN using pHrodo-labeled platelets (only fluorescent at acid pH found in phagosomes), in PBS, HEPES, Normal Human Sera (NHS), or sera from fetal or neonatal alloimmune thrombocytopenia (FNAIT). Phagocytosis of platelets was measured by FACS and expressed as mean fluorescent intensities (MFI) of PMN’s. Statistical comparison: one-way ANOVA with Tukey’s post-test, **: p≤0.01; ***: p≤0.001.

Supplementary Figure 2. Complement has no effect on respiratory burst towards platelets opsonized with FNAIT sera. Respiratory burst activity of PMN towards platelets opsonized with FNAIT sera, either without treatment or heat-inactivated. No significant effect was observed by heat inactivation. Data are representative of three independent experiments, showing mean ± standard deviation. Statistical comparison: one-way ANOVA with Tukey’s post-test, **: p≤0.01; NS: non-significant.

Supplementary Figure 3. Enhanced respiratory burst towards IgG1-opsonized platelets was not observed with all sera, independent of time. Respiratory burst of PMN towards anti-HPA-1a IgG1 (B2G1)-opsonized platelets – an example of two sera at different times after initiation of the respiratory burst. NHS 1 and 2 correspond to NHS 1 and 2 from Fig. 2b, respectively. Data are representative of three independent experiments, showing mean ± standard deviation. Statistical comparison: two-way anova with Bonferroni post-test. *: p≤0.05; **: p≤0.01.

Supplementary Figure 4. Platelet FcγRIIa does not prime platelets through co-crosslinking of anti-HPA1a. Platelets, with or without pre-incubation with anti-CD32-Fab antibodies, and after subsequent washing, were opsonized with B2G1-antibody, washed and resuspended in NHS or PBS (negative control) together with PMN to monitor the respiratory burst. Data are representative of three independent experiments, showing mean ± standard deviation. Statistical comparison: one-way ANOVA with Tukey’s post-test, **: p≤0.01; NS: non-significant.
Supplementary Figure 5. Blocking anti-FcγRIIa Fab inhibits FcγRIIa-dependent platelet aggregation. The heparin induced platelet activation (HIPA) test\(^1\) was used for assessment of the anti-CD32 Fab (clone 7.3) blocking function. Patient serum containing IgG-antibodies specific for platelet factor 4 (PF4) and heparin complex, was added at different dilutions to platelets together with heparin, which normally results in co-crosslinking of FcγRIIa, leading to aggregation, and platelet-response was monitored using an absorbance microplate reader. (a) The platelets were first incubated with suspension buffer (Tyrode buffer, see materials and methods) or collagen, inducing aggregation as measured by the absorbance microplate reader (b) The same experimental setup, but with blocking FcγRIIa-Fab, indicating no FcγRIIa-involvement for platelets through the collagen receptor (GPVI). Patient sera, however, caused massive aggregation (c) that was completely blocked by the anti-FcγRIIa Fab (d).

Supplementary Figure 6. CRP does not directly interact with IgG. Human IgG1 (B2G1 anti-HPA1a epitope), anti-C1q, and anti-CRP were coupled to the biosensors array on different spots and binding of CRP and C1q to those spots monitored as indicated by the different sensorgrams. CRP was injected before C1q (panel A), after C1q (panel B) and together with C1q (panel C). (a-c) As expected, C1q clearly bound human IgG1 (B2G1, blue lines), and CRP (red lines). A slight binding of C1q was observed to anti-CRP (b, red line), probably due to incomplete regeneration from a previous run as this was not observed on fresh sensor chips (data not shown). However, no binding of CRP to IgG1 was detected (blue lines, a-c), despite ample spotting confirmed by specific binding after injection of anti-human IgG (d). Data are representative of three independent experiments.
Supplementary Figure 7. CWPS binds to CRP. CRP was spotted with the CFM to the Easy2Spot P-type chips at a concentration of 800nM. Both anti-CRP and CWPS, injected over the sensor chip at different concentrations, bound to the spotted CRP. Data are representative of three independent experiments.

Supplementary Figure 8. Binding of B2G1-opsonized platelets to CRP was further investigated by cellular surface plasmon resonance (cSPR) imaging, with CRP spotted to three sensor surface spots and three BSA control spots. Specific response was observed for platelets to the CRP spots, which was enhanced if the platelets were opsonized with anti-HPA-1a B2G1, but blocked by CWPS, indicating that platelet-phosphorylcholine is the ligand for CRP. Statistical comparison was made using one-way ANOVA with Tukey’s post-test. *: p≤0.05; **: p≤0.01; ***: p≤0.001.

Supplementary Figure 9. Binding of B2G1-opsonized platelets versus B2G1-F(ab’2)–opsonized platelets to CRP by cellular surface plasmon resonance (cSPR) imaging, to test the Fc-dependency. CRP was spotted to three sensor surface spots and three BSA control spots were also included. A specific response was observed for platelets to the CRP spots, which was enhanced if the platelets were opsonized with the full length B2G1 compared to B2G1-F(ab’2), demonstrating Fc-dependency. Statistical comparison was made using one-tailed paired student t-test. *: p<0.05
Supplementary Figure 10. 78 Newly diagnosed ITP patients, randomized to observation or to IVIg-treatment (0.8 g/Kg), one week after treatment. Most patients receiving IVIg, displayed an elevated number of platelets, to more than 100*10^9/mL. Statistical comparison was made using student t-test. ***: p<0.0001

Supplementary Figure 11. CRP levels were significantly decreased in individuals that had normalized their platelet counts (>100*10^9 platelets/L) after one week from diagnosis in newly diagnosed ITP patients. Statistical comparison was made using student t-test. *: p<0.05
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Clinical trial of intravenous immunoglobulin (IVIg)-treatment in newly diagnosed ITP in children

Children aged 3 months to 16 years with newly diagnosed ITP, a platelet count below $20 \times 10^9$/L and a bleeding score according to Buchanan et al.\(^1\) based on the overall extent of bleeding ranging from 0 (no bleeding) to 5 (life-threatening or fatal). Patients with a bleeding score of less than 4 were eligible for inclusion in the Treatment with or without IVIG in Kids with acute ITP (TIKI) study. Patients were excluded if they had received immunomodulating drugs within one month before diagnosis or suffered from conditions with a contraindication for IVIg. The patient characteristics are summarized in Supplementary Table 1. Within 72 hours after diagnosis, patients were randomized to receive either a single infusion of IVIg (0.8 g/kg) or careful observation and treatment to raise platelet count only in case of severe bleeding. At diagnosis, after 1 week, 1 month, 3 months, 6 months and 12 months laboratory studies were performed and clinical data were gathered. At each time point, the highest bleeding score since the last study visit was noted by the patients’ pediatrician and entered in a web based case record form (CRF). All use of human material was in compliance with the local medical ethical committee, and the use of experimental animals was conducted after examination and approval by the local animal ethic committee. The TIKI study was approved by the institutional review board of the University Medical Center Utrecht and the Competent Authority of The Netherlands. Patients were recruited by pediatricians from 60 participating Dutch hospitals. All parents and patients aged 12 years and older gave written informed consent before inclusion. The study was
registered in the Dutch Trial register (www.trialregister.nl; study ID number NTR TC1563) and conducted in accordance with Good Clinical Practice guidelines.

**Induction of thrombocytopenia in vivo**

Blood was collected from the retro-orbital plexus 5 minutes prior to antibody-injection \((t=0)\), and 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 haematology system using murine-BALB/c parameters (Siemens Medical Solutions Diagnostics, Erlangen, Germany).

**Sera, antibodies and other reagents**

The following sera were used in the study: maternal FNAIT sera (Sanquin, Amsterdam, The Netherlands, 3 different sera), containing anti-human platelet antigen (HPA)1a alloantibodies, as determined by monoclonal antibody-specific immobilization of platelet antigens (MAIPA), neonatal FNAIT sera (Sanquin, Amsterdam, The Netherlands), cord blood sera, newly diagnosed childhood immune thrombocytopenia (ITP) sera, serum samples from healthy children, Normal Human Sera (NHS), type A\(^+\)B\(^+\), from healthy donors, clinically tested and not containing any platelet-reactive antibodies, HIPA sera: sera of patients diagnosed with heparin-induced thrombocytopenia, containing antibodies directed against platelet factor 4 (PF4) and heparin.

Antibodies used in the study included: human monoclonal antibody against anti-HPA-1a; B2G1\(^2\) (kindly provided by Dr. W. Ouwehand and C. Ghevaert, University of Cambridge, NHS Blood and Transplant, Cambridge, UK), polyclonal anti-D antibody (K1120, Sanquin,
Amsterdam, The Netherlands), monoclonal anti-human FcγRII (CD32) Fab antibody; clone 7.3 (Ancell corporation, Bayport, USA), mouse anti-human CRP (Roche, Roche Diagnostics GmbH, Mannheim, Germany), rat anti-mouse CD41; clone MWReg30 (BD Pharmingen), polyclonal goat anti-human IgG (Thermo Scientific, Rockford, IL, USA), polyclonal chicken anti-human C1q and monoclonal mouse Anti-human CRP antibody [C6] (Abcam, Cambridge, UK), anti-human CD16 IgG2a antibody GRM1 and isotype anti-TNP IgG2a antibody (Sanquin Diagnostics, Amsterdam, the Netherlands), both antibodies were conjugated to Pacific Blue by using the DyLight 405 Amine-Reactive Dye (Thermo Scientific, Waltham, MA), according to the manufacturer’s protocol. Isotype human IgG1 was recombinant TA99 anti mouse-GP75,3 cloned produced and purified as described previously.4 B2G1-F(ab’2) fragments were generated by using FabRICATOR (Genovis AB, Lund, Sweden), according the manufacturer’s protocol.

Other reagents: CRP purified from human plasma (>99% purity by SDS-Electrophoresis and tested negative for infectious agents) was purchased from Sigma-Alderich, Zwijndrecht, The Netherlands, Diphenylene Iodonium (DPI), Rotenone, Glucose Oxidase from Aspergillus Niger and D+Glucose (Sigma-Aldrich, Zwijndrecht, The Netherlands).

**Cell isolations and labeling**

Human platelets were obtained from a buffy coat (Sanquin, Amsterdam, The Netherlands) using platelet-rich plasma (PRP), obtained after centrifugation at 400 g for 10 minutes. The platelets were washed once in phosphate-buffered saline (PBS) containing EDTA as well as 100 ng/mL prostaglandin E1 (PGE1, Sigma-Aldrich, Zwijndrecht, The Netherlands) in order to minimize activation, and resuspended in 1*10^8 /ml PBS. Human PMN were isolated from peripheral blood obtained from healthy laboratory volunteers (Sanquin, Amsterdam, The Netherlands) using a
Ficoll 1.077 density gradient (Pharmacia Biotech) followed by hypotonic lysis of residual red blood cells on ice for several minutes. CD16+ monocytes were isolated from a buffy coat, lymphocyte-depleted using CD3- and CD19-microbeads (Miltenyi Biotech, Leiden, The Netherlands), and labeled with anti-human Pacific Blue-labeled anti-CD16. Subsequently, CD16+ monocytes were purified on a FACSARIA II cell sorter (BD Biosciences, Erembodegem, Belgium).

Platelets were labeled with pHrodo by resuspending a platelet pellet in 0.23 mM pHrodo succinimidyl ester (100 μl/10⁸ platelets) (Invitrogen, Molecular Probes, Eugene, Oregon, USA), in 100 ng/mL PGE1, for 45 minutes in the dark at room temperature (RT). Finally, the platelets were washed twice and re-suspended in PBS/EDTA/PGE1 at 10⁸ platelets/ml.

Platelets were opsonized by resuspending a pellet of platelets with 100 μl serum or antibody solutions for every 10⁸ platelets, and subsequent incubation for 30 minutes at RT. Hereafter platelets were washed twice and resuspended at 10⁸/ml in the indicated medium, in case of NHS, this was pre-diluted 1:3 in PBS.

**CRP labeling, addition, neutralization, depletion, measurement and NHS incubations**

CRP was labeled with FITC (Sigma-Aldrich, Zwijndrecht, The Netherlands) at a final molar ratio of FITC to protein of approximately 1.5, as described previously.⁵,⁶ PRP was incubated together with the opsonizing antibody for 30 minutes at 4°C, after which CRP-FITC was added to the cells in platelet-free plasma (PFP), supplemented with or without Ca²⁺, for 45 minutes at either 4 or 37°C. Subsequently, cells were washed with PFP with or without Ca²⁺, followed by flow cytometry analysis.
CRP present in NHS was neutralized by addition of cell wall polysaccharide (CWPS, 10 µg/ml) from *Streptococcus pneumoniae* (Statens Serum Institut, Copenhagen, Denmark). CRP was depleted from NHS as described by Weiser et al.\(^7\) In short, NHS was incubated for 30 minutes at 4°C with an equal volume of immobilized phosphoryl choline-coupled agarose beads (Pierce Chemical Co, Thermo Scientific, Rockford, IL), which were washed in 0.02 M Tris (pH 7.2), 0.15 M NaCl, and 10 mM CaCl\(_2\). CRP was measured from sera using an immunoturbidimetric assay according to the manufacturer’s protocol (Roche, Roche Diagnostics GmbH, Mannheim, Germany). In short, human CRP from serum samples is allowed to agglutinate when incubated with latex particles coated with monoclonal mouse anti-human CRP antibodies. Subsequently, the aggregates are determined turbidimetrically using the Cobas 8000 (Roche, Roche Diagnostics GmbH, Mannheim, Germany).

*Surface plasmon resonance (SPR)*

A pre-activated P-type sensor chip (Ssens bv, Enschede, The Netherlands) was spotted using a Continuous Flow Microspotter (Wasatch Microfluidics, Salt Lake City, UT, USA). Regarding the cell-flow based SPR experiments (cSPR): CRP was spotted onto the sensor chip in 10 mM MES buffer, pH 6.0, in replicates at a concentration of 800 nM. For reference purposes bovine serum albumin (BSA) was spotted. Deactivation of the sensor chip was carried out with 100 nM Ethanolamine, pH 8, and subsequently the system was loaded with HEPES/BSA 1%/Ca\(^{2+}\) 2mM and the temperature was set to 25°C. The same buffer was used for the platelets. Three samples were made containing 1.0*10\(^9\)/ml platelets opsonized with B2G1 and also including CWPS (500 ug/ml), platelets opsonized with B2G1 but without addition of CWPS, and unopsonized platelets without any additions. The platelet-samples were loaded into the IBIS and at injection of the
sample onto the sensor chip, the flow was stopped for 10 minutes, allowing specific binding to occur as described for red blood cell measurements. Slight refractive index changes (due to temperature or buffer composition) during the measurement can influence the sensorgram, therefore the readout of the BSA spots was set as a reference. For oxidation experiments, untreated platelets as well as platelets pre-treated with DPI (10 µM), or Rotenone (100 µM) or Glucose oxidase (20 Units/ml oxidase in HEPES containing 60 mM D-glucose, pH 5.5, for 20 min at 37°C, followed by washing of cells with HEPES containing BSA and calcium at pH 7.4), were injected over the Easy2Spot P-type chip, containing spotted CRP at a concentration of 800 nM.

In order to test the binding of CRP to IgG, a pre-activated G-type sensor chip (Ssens bv, Enschede, Netherlands) was used, and measurements carried out in an IBIS MX96 (IBIS Technologies, Enschede, Netherlands) as described by de Lau et al. In total, 12 spots were created with both the B2G1 antibody. Additionally complement protein C1q, both in 5-fold dilutions (starting from 400 nM to 16 nM) in 10 mM MES buffer, pH 6.0 was spotted. Positive and negative control spots contained anti-CRP antibody, anti-C1Q antibody, BSA and sodium acetate buffer as reference spot. Both anti-CRP and anti-C1q antibodies were spotted in 5-fold dilutions (starting from 100 nM to 4 nM in sodium acetate buffer pH 5). BSA was spotted with a concentration of 200 nM in 10 mM sodium acetate buffer pH 4. Subsequently, a series of CRP and C1q samples were injected over the chip surface and the multiplex interaction on the various spots were monitored simultaneously in the IBIS MX96. First 100 nM CRP was injected followed by a second injection of 100 nM C1q. Regeneration was carried out with acid buffer (Gly-HCl, 10mM, pH 2.0) for 2 minutes. Hereafter, the analyses were repeated with a lower concentration of CRP (50nM). Subsequently, the order of injections was reversed by first
injecting C1q and then CRP. Also a mixture of C1q and CRP was co-injected. The covalent coupling of B2G1 was confirmed by injecting anti-human IgG.

**Heparin induced platelet activation (HIPA) test**

The HIPA test was performed as originally described in detail.\(^{10}\) In short, patient serum containing IgG-antibodies specific for platelet factor 4 (PF4) and heparin complex, was added to washed platelets together with heparin. Subsequently, the FcγRII (CD32)-response was recorded with and without prior incubation of platelets with an anti-CD32-Fab blocking antibody. Incubation with suspension buffer (Tyrode buffer: aquadest containing 2.5 ml bicarbonate buffer, 0.5 ml 10% glucose solution and 0.875 ml BSA 22%, 1 ml calciumchloride (0.196M) and 0.5 ml magnesiumchloride (0.212M)) was used as a negative control and collagen was used as a positive control. Data was measured using SpectraMax Plus384 Absorbance Microplate Reader (Sunnyvale, California, USA).
Reference List


