Multifactorial aspects of antibody-mediated blood cell destruction
Kapur, R.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
C-Reactive Protein enhances antibody-mediated platelet destruction


1Department of Experimental Immunohematology, Sanquin Research, Amsterdam, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands; 2Department of Pediatric Hematology-Oncology, University Medical Center Utrecht/Wilhelmina Children’s Hospital, Utrecht, Netherlands; 3Department of Thrombocyte and Leukocyte Serology, Sanquin Diagnostic Services, Amsterdam, Netherlands; 4Department of Blood Cell Research, Sanquin Research, Amsterdam, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands; 5Medical Cell Biophysics, MIRA institute, University of Twente, Enschede, The Netherlands.

Submitted for publication. [Parts were orally presented at ASH 2011].
Abstract

Immune-mediated platelet destruction can be caused by allo- or autoantibodies via Fcγ-receptor (FcγR)-dependent phagocytosis. Disease severity can be predicted neither by antibody isotype nor by titer, indicating other factors to play a role. Here we show that the acute-phase protein C-Reactive Protein (CRP), a ligand for Fc-receptors on phagocytes, enhances antibody-mediated platelet destruction in vitro by human phagocytes, and in vivo in mice. Without anti-platelet antibodies, CRP was inert towards platelets, but bound to phosphorylcholine exposed after oxidation triggered by anti-platelet antibodies, enhancing platelet phagocytosis. Levels of CRP were significantly elevated in patients with allo- and autoantibody-mediated thrombocytopenias. Within a week, IVIg-treatment in children with newly diagnosed immune thrombocytopenia led to significant decrease of CRP levels, increased platelet numbers and clinically decreased bleeding severity. These data suggest CRP to amplify platelet destruction and explain the aggravation of thrombocytopenias upon infections. Hence, targeting of CRP could offer new therapeutic opportunities.
Introduction

Fetal or neonatal alloimmune thrombocytopenia (FNAIT) and auto-immune thrombocytopenia (ITP) are both antibody-mediated disorders in which platelets are destroyed mainly through activating IgG-Fc-receptors on phagocytes in the spleen and liver, eventually resulting in thrombocytopenia. FNAIT is a potentially destructive disease in pregnancies, with intracerebral hemorrhage of the fetus or neonate as the most feared complication, resulting in perinatal death in 1-7% or in severe neurological impairments in 14-26% of affected pregnancies. FNAIT is caused by maternal IgG-platelet alloantibodies that cross the placenta and destroy the platelets of the fetus or newborn. In Caucasians these antibodies are mainly directed against anti-human platelet antigen (HPA)-1a, as found in about 85% of FNAIT-cases. Immunization against HPA-1a occurs approximately in 1:450 random pregnancies, resulting in FNAIT in 1:1200 and in severe FNAIT (<50 x 10⁹ platelets/L) in 1:1800 pregnancies. Similarly, ITP is characterized by a typical history of acute development of purpura and bruising in an otherwise healthy child, due to development of platelet autoantibodies, with an incidence of about 5 in 100,000 children. Most children with newly diagnosed ITP will not suffer from serious bleedings and will recover within twelve months. In about 60% of ITP, there is a history of a prior infection.

Although it has been suggested that platelet decrement is determined by the antibody titer in FNAIT, this correlation is not strict, as cases with low titers and very low platelet counts, as well as cases with high titers and yet with normal platelet counts, are frequently observed. Therefore, we investigated other potential co-factors playing a role in the pathogenesis of immune-mediated thrombocytopenia. Here we identified C-Reactive Protein (CRP), a member of the pentraxin family of highly conserved calcium-dependent ligand-binding proteins, to amplify IgG-mediated platelet destruction. CRP is composed of five identical, non-glycosylated 206 amino acids protomers forming a non-covalently-linked annular symmetrical pentamer. CRP was originally described by Tillett et al. in 1930 as a substance present in the sera of patients with acute inflammation, precipitating with cell-wall (C) polysaccharide (CWPS) of Streptococcus pneumoniae. CRP is now well established as a major acute-phase protein and is utilized in daily clinical practice as a sensitive biomarker for infection and inflammation, with its level increasing from less than 0.05 mg/L to more than 500 mg/L after acute infections. CRP is produced by hepatocytes, in response to inflammatory cytokines such as IL-6 and IL-1, with serum concentrations rising above 5 mg/L after 6 hours, peaking after about 48 hours. In healthy young adult volunteer blood donors, the median concentration of CRP was found to be around 0.8 mg/L.

In the present paper we describe how CRP interacts directly with platelets, and functions as a novel pathogenic co-factor in IgG-mediated platelet destruction by phagocytes, ultimately leading to platelet destruction in patients that can, at least partially, be overcome by treatments aiming at lowering CRP levels.
Methods

Sera
The following sera were used in the study: maternal FNAIT sera (Sanquin, Amsterdam, The Netherlands), 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 and other reagents
Antibodies used in the study included: human monoclonal antibody against anti-HPA1a; B2G1\textsuperscript{15} (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. Purified CRP was purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands. Isotype human IgG1 used was recombinant TA99 anti mouse-GP75,\textsuperscript{16} cloned, produced and purified as described previously.\textsuperscript{17}

Other reagents: Diphenylene Iodonium (DPI), Glucose Oxidase from Aspergillus Niger and D+Glucose (Sigma-Aldrich, Zwijndrecht, The Netherlands).

Cell isolations
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).
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/10^8 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 10^8 platelets/ml.

Platelets were opsonized by resuspending a pellet of platelets with 100 μl serum or antibody solutions for every 10^8 platelets, and subsequent incubation for 30 minutes at RT. Hereafter platelets were washed twice and resuspended at 10^9/ml in the indicated medium, in case of NHS, this was pre-diluted 1:3 in PBS.

**Phagocytosis and respiratory burst assays**

Phagocytosis and respiratory burst experiments were carried out using freshly isolated human PMN, freshly isolated platelets or freshly sorted CD16+ monocytes. For phagocytosis experiments, platelets were labeled with pHrodo as described above, while for respiratory burst experiments unlabeled platelets were used. Equal volumes of 2.0*10^6/ml PMN and 1.0*10^8/ml platelets were mixed in a total volume of 100 μl in 1.4 ml U-bottom tubes (Micronic, Lelystad, The Netherlands), for 20 minutes in a shaking incubator at 37°C. Respiratory burst experiments were carried out in exactly the same way, except that Dihydrorhodamine 123 (Invitrogen, Molecular Probes, Eugene, Oregon, USA), at a final concentration of 1 μM was added to PMN before mixing with platelets, and the incubation at 37°C was carried out for 45 minutes unless otherwise indicated. When platelets were used without pre-opsonization, they were incubated directly in the indicated media or 1:10 diluted sera. After the reaction, the cells were kept on ice and washed in cold PBS. Samples were measured in a flow cytometer (LSRII, BD Bioscience, San Jose, CA, USA) and analyzed using FacsDIVA software (BD Bioscience, San Jose, CA, USA).

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

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. 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: 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 albumine (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%/Ca²⁺ 2mM and the temperature was set to 25°C. The same buffer was used for the platelets. Three samples were made containing 1.0*10⁹/ml platelets opsonized with B2G1 and also including CWPS (500 µg/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 19. 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 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 20. In total, 12 spots were created with both the B2G1 antibody. Additionally complement protein C1q, both in 5-fold dilutions (starting from 400 nM to16 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.

**CRP labeling for FACS**

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. 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.

**Induction of thrombocytopenia in vivo**

6-week old female BALB/c mice (strain BALB/cOlaHsd) were obtained from Harlan (Harlan, Netherlands) and were housed in our animal facility for one week, before initiating experiments. Thrombocytopenia was induced in the mice, based on our previously described model. Briefly, rat anti-mouse CD41; clone MWReg30, was injected intra-peritoneally. 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). Following experiments were done with 0.75 ug/ml of antibody with co-injection of various concentrations of CRP and platelet counts were determined as mentioned.

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

The HIPA test was performed as originally described in detail. 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).
Patients included from clinical trial of IVIg treatment in newly diagnosed ITP in children

Children aged 3 months to 16 years with newly diagnosed ITP, a platelet count below 20 x 10^9/L and a bleeding score according to Buchanan et al^25 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. 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 webbased case record form (CRF).

Study approval

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.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 software for Windows (GraphPad Software, San Diego, CA). P-values less than 0.05 were considered statistically significant.

Results

A factor in normal human serum (NHS) is required for phagocyte responses towards IgG-opsonized platelets

In vitro handling of platelets (e.g. labeling for phagocytosis experiments) can lead to platelet activation, resulting in phagocytosis of non-opsonized platelets (Supplementary Fig. 1). By measuring respiratory burst (nicotinamide adenine dinucleotide phosphate, NADPH, oxidase activity) of neutrophils by flow cytometry, and thereby minimizing platelet
handling, we observed minimal background signals originating from normal human serum (NHS) or buffered saline solutions, whereas platelets opsonized with anti-Human Platelet Antigen-1a (anti-HPA-1a) IgG1-containing FNAIT serum, displayed a strong and specific phagocyte response (Fig. 1a). We then tested whether the phagocyte responses observed with HPA1a-specific FNAIT sera could be simulated with a human recombinant HPA1a-specific monoclonal IgG1 antibody (B2G1). No respiratory burst was observed, unless NHS was added during the assay (Fig. 1b).

**Figure 1** Normal human sera contain a factor required for phagocyte responses towards IgG-opsonized platelets.

(a) Respiratory burst activity of PMN towards platelets in NHS or anti-HPA-1a antibody-containing FNAIT sera or saline solutions, analyzed by the conversion of dihydrorhodamine-1,2,3 to fluorescent rhodamine-1,2,3, measured by FACS. (b-c) PMN-respiratory burst activity towards IgG-opsonized platelets was only observed in NHS. (b) Platelets were opsonized with the human monoclonal anti-HPA1a IgG1 antibody; B2G1, subsequently washed, and resuspended in either PBS, HEPES or NHS together with PMN to initiate phagocyte responses. (c) Platelets were pre-incubated with either NHS (unopsonized) or opsonized with FNAIT serum, washed with PBS and re-suspended in the indicated media before addition to PMN, then the respiratory burst was measured after 45 minutes at 37°C. Data are representative of three independent experiments, showing mean ± standard deviation. Statistical comparisons were performed as follows: (a) one-way ANOVA with Tukey’s post-test, for (b) and (c) a two-way ANOVA with Bonferroni post-test. ***: p≤0.001.
The same was true for platelets washed after opsonization with maternal FNAIT sera containing anti-HPA-1a antibodies, as no antibody-dependent respiratory burst was observed, unless NHS was added after washing (Fig. 1c). This indicated that, besides platelet-specific antibodies, a factor present in NHS was required for antibody-mediated phagocyte activity.

**The serum factor is heat-resistant, found at variable levels in NHS and requires Ca\(^{2+}\) for platelet-binding**

To investigate whether this serum factor was complement, sera were heat-inactivated at 56 °C for 30 minutes. This had no significant effect, ruling out the requirement of complement (Fig. 2a, Supplementary Fig. 2).

![Figure 2](image)

**Figure 2** Enhancing effect of serum is not complement, varies between NHS, requires divalent cations for interaction with platelets.

(a) Respiratory burst activity of PMN towards B2G1-opsonized platelets in NHS was not diminished after heat-inactivation of complement. NHS, deficient in mannann-binding lectin (MBL) was also capable of enhancing phagocyte responses to B2G1-opsonized platelets. NHS served as a negative control. (b) The respiratory burst activity of PMN towards B2G1-opsonized platelets was enhanced by some, but not all 14 different NHS compared to unopsonized platelets. (c) Chelation of calcium from NHS with EDTA (5 mM), ablates the ability of serum to induce respiratory burst of PMN triggered by B2G1-opsonized platelets. (d) The serum factor, enhancing IgG-specific PMN respiratory-burst activity towards platelets binds directly to platelets in a calcium-dependent manner. Platelets were pre-incubated with NHS or anti-HPA-1a FNAIT sera, washed with HEPEs with or without Ca\(^{2+}\), and resuspended in HEPEs with or without Ca\(^{2+}\) as indicated. Data are representative of three independent experiments, showing mean ± standard deviation. Statistical comparisons were performed as follows: (a) one-way ANOVA with Tukey’s post-test, (b) paired t-test, (c) and (d) two-way ANOVA with Bonferroni post-test. *: ps0.05; **: ps0.01; ***: ps0.001.
Also, B2G1-opsonized platelets still demonstrated the serum-enhancing effect in NHS deficient for mannan-binding lectin (NHS MBL -/-) (Fig. 2a). When testing 14 different healthy donors, we observed a variable degree of serum-dependent respiratory burst activity towards B2G1-opsonized platelets (Fig. 2b), also evident in a time-dependent fashion (Supplementary Fig. 3), suggesting the responsible serum component to be present at different levels in NHS. Next, we tested whether this factor required divalent cations for its function. Addition of EDTA to NHS completely abolished the serum-enhancing effect on B2G1-mediated respiratory burst (Fig. 2c). No IgG-specific respiratory burst was observed when the opsonizing FNAIT serum was washed away without Ca²⁺, even if Ca²⁺ was re-added after washing during the respiratory burst. An IgG-specific anti-platelet response was only observed if Ca²⁺ was present during

![Figure 3](image)

**Figure 3** C-reactive protein (CRP) enhances phagocyte responses of PMN towards IgG-opsonized platelets.

(a) B2G1-specific respiratory burst activity of 14 NHS correlates significantly with the CRP concentrations in the sera. (b) Both CRP and calcium were required to induce IgG-specific respiratory burst. B2G1-opsonized platelets were incubated in HEPES supplemented with or without calcium and in the presence or absence of CRP, before addition of PMN to measure the respiratory burst. (c) Depletion from NHS with phosphocholine-coated beads neutralized its ability to enhance IgG1-mediated respiratory burst towards B2G1-opsonized platelets, but re-supplementing CRP restored this capacity. (d) CRP addition to NHS with low CRP (0.07 mg/L) rendered the serum capable of inducing IgG-specific respiratory burst towards B2G1-opsonized platelets. (e) Serial increments of CRP to the same sera resulted in increasing respiratory burst response that correlated significantly with the amount of CRP added. Data are representative of three independent experiments, showing mean ± standard deviation. Statistical comparisons were performed as follows: (a) and (e) Pearson correlation, (b-d) two-way ANOVA with Bonferroni post-test, *: p≤0.05; **: p≤0.01; ***: p≤0.001.
the washing step (Fig. 2d), indicating that the responsible serum factor reacts directly and reversibly with anti-HPA-1a-opsonized platelets in a calcium-dependent manner.

**Identification of CRP as the unknown serum factor**

Due to the variable presence of the unknown serum factor in different NHS and its calcium dependency, we hypothesized that CRP could be this factor. Further analysis of the previously tested 14 NHS indeed demonstrated a significant correlation between the IgG-specific respiratory burst activity and CRP concentration of the NHS (Fig. 3a). We then tested whether addition of CRP and calcium enabled phagocyte responses towards B2G1-opsonized platelets. Only with both CRP and calcium (either component alone was not sufficient) did we observe enhanced respiratory burst towards IgG-opsonized platelets (Fig. 3b). Depletion of CRP from NHS using phosphorylcholine beads (Fig. 3c) also resulted in reduction of the B2G1-induced respiratory burst activity. After re-adding purified CRP to the CRP-depleted NHS, the B2G1-specific effect was restored (Fig. 3c). Also, addition of CRP to NHS with very low CRP levels (0.07 mg/L), resulted in a significant increase of the respiratory burst (Fig. 3d), in a concentration-dependent manner (Fig. 3e). These data indicate that CRP in the serum was responsible for enhancing phagocyte responses toward IgG-opsonized platelets.

**Platelet activation exposes CRP ligands, recognized by CRP phosphorylcholine-binding site**

We then investigated whether the binding of anti-HPA-1a antibody led to platelet activation and exposure of CRP ligands after co-crosslinking with platelet-FcγRIIa (the Kurlander phenomenon\(^\text{26}\)). Blocking anti-FcγRII (clone 7.3)-Fab had no effect on respiratory burst induced by platelets opsonized with FNAIT-derived IgG (Fig. 4a) or with B2G1 (Supplementary Fig. 4). FcγRIIA-blocking capacity of 7.3-Fab was verified in the FcγRIIA-dependent Heparin-Induced Platelet Activation (HIPA) assay\(^\text{24,27}\) (Supplementary Fig. 5). We next sought to determine whether CRP bound directly to IgG-opsonized platelets as suggested by Fig. 2d. When FITC-labeled CRP was incubated with B2G1-opsonized platelets, weak but significant binding of CRP was observed at 37°C in the presence of calcium, but not at 0°C or in the absence of calcium (Fig. 4b). This indicated the calcium-dependent binding domains of CRP were involved in the binding to the platelets, but also platelet-activation to be required. Furthermore, CRP did neither bind IgG1 directly, as detected by SPR (Supplementary Fig. 6a), nor did the C1q binding to spotted IgG1 facilitate an additional CRP binding (Supplementary Figure 6b-c). Captured C1q by spotted anti-C1q antibodies also did not provide a platform for CRP binding (Supplementary Fig. 6b-c), suggesting an alternative ligand for CRP to be exposed on the IgG-opsonized platelets. To determine the binding site of CRP on platelets, NHS was pre-incubated with pneumococcal CWPS, providing native calcium-dependent phosphorylcholine-ligand for CRP\(^\text{28}\). This resulted in a reduced IgG-specific respiratory burst
Figure 4 Cellular activation exposes phosphorylcholine after oxidation, enabling CRP binding and phagocytosis.

(a) Platelet-FcγRIIa was not involved in the opsonization of platelets with FNAIT serum. Platelets were pre-treated with blocking anti-CD32-Fab antibody (clone 7.3), opsonized with anti-HPA-1a-containing FNAIT sera (NHS as negative control), washed and resuspended in PBS (negative control) or NHS. Thereafter, the platelets were added to PMN and the respiratory burst was measured. (b) CRP-FITC bound directly to B2G1-opsonized platelets and this process required both calcium and platelet activity at 37 °C, as measured in the FACS. (c) Neutralization of CRP with pneumococcal cell wall polysaccharide (CWPS) in NHS disabled CRP-induction of the respiratory burst towards B2G1-opsonized platelets. (d) Binding of opsonized platelets to CRP was further investigated by cellular surface plasmon resonance (SPR) imaging(21), with CRP spotted to three sensor surface spots and three BSA control spots. A 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. (e) Binding of platelets to CRP was inhibited by diphenylene iodonium (DPI), an NADPH oxidase inhibitor. (f) Conversely, stimulation of platelet oxidation by glucose and glucose oxidase enhanced platelet binding to CRP. Each line in (d-f) represents the average sensorgrams and standard deviation from three spots monitored simultaneously in real time. Statistical comparisons were performed as follows: (g) The CRP enhancement of IgG-mediated phagocytosis of platelets by neutrophils was inhibited by DPI. Data are representative of three independent experiments, showing mean ± standard deviation. (a) and (c-f) one-way ANOVA with Tukey’s post-test, (b,g) two-way ANOVA with Bonferroni post-test. *: p≤0.05; **: p≤0.01; ***: p≤0.001.
C-Reactive Protein Enhances Antibody-Mediated Platelet Destruction

(Fig. 4c). To investigate the binding of CRP to platelets more closely, we developed a new and sensitive cellular label-free multiplex surface plasmon resonance (SPR) method for platelets, as we recently described for red blood cells. B2G1-opsonized, and to a lesser extent non-opsonized platelets, bound to CRP-conjugated spots, but not to control BSA-conjugated spots (Fig. 4d). The interaction of opsonized platelets with spotted CRP was blocked with CWPS (Fig. 4d), which bound to the CRP spots (Supplementary Figure 7). Taken together, the data suggest CRP to bind membrane phosphorylcholine residues on IgG-opsonized platelets exposed after platelet activation through GPIIbIIIa-triggering with HPA-1a-specific antibodies.

**Platelet-oxidation exposes CRP ligands**

As oxidized Low-Density Lipoprotein (LDL) particles and apoptotic cells have been shown to expose phosphatidylcholine residues, and because anti-GPIIIa (containing the HPA-1a epitope, the target of the FNAIT sera as well as the B2G1 antibody, used in this study) antibodies have been shown to induce oxidation of platelets, we investigated whether platelet-oxidation contributed to CRP binding. Pre-treatment of platelets with diphenylene iodonium (DPI), inhibiting NADPH-oxidase, resulted in less binding of opsonized platelets to CRP compared to untreated opsonized platelets (Fig. 4e). In addition, stimulation of oxidation of opsonized platelets after treatment with glucose oxidase (Fig. 4f), resulted in enhanced binding of opsonized platelets to CRP spots compared to untreated opsonized platelets.

As CRP also enhanced antibody-mediated phagocytosis, measuring complete ingestion of B2G1-opsonized platelets (Fig. 4g), we were able to investigate the role of oxidation in antibody-mediated platelet ingestion. In agreement with the finding that oxidation was required for binding of CRP to platelets, CRP-mediated enhancement of phagocytosis was completely inhibited by DPI-treatment (Fig. 4g). Curiously, although CRP clearly enhanced phagocytosis, we also noticed that B2G1 was able to mediate phagocytosis without CRP (Fig. 4g), unlike for the respiratory burst where CRP seemed to be indispensable for the IgG-specific response (Fig. 3b), underlining the different thresholds and different cellular mechanisms required for activation of these different processes.

**CRP is elevated in FNAIT and newly diagnosed ITP patients, is associated with platelet counts, and clinical bleeding severity**

To examine the relevance of CRP in patient sera, we measured the CRP concentration in neonatal FNAIT patient samples and in sera from children with newly diagnosed ITP, and compared this to normal cord blood or sera of healthy children, respectively. CRP levels were increased in both FNAIT and newly diagnosed ITP patient populations compared to control samples (Fig. 5a). To further investigate if the level of CRP affects the bleeding tendency in these patients, we made use of the ongoing “Treatment with or without IVIG in Kids with acute
ITP" (TIKI) trial. In this trial patients with newly diagnosed immune thrombocytopenia were randomized upon diagnosis to the observation arm or to those receiving 0.8 g/Kg IVIg and followed in time. At inclusion all patients had <20*10^9 platelets/L. One week after receiving IVIg, most patients had normalized their number of platelets, while this was not the case for the observation group (Supplementary Figure 8). The elevated CRP levels seen in these patients (Fig. 5a) also became significantly lower in the IVIg group, but not in the control group (Fig. 5b-c). Both the number of platelets and their bleeding tendencies correlated significantly with the CRP levels in these patients (Fig. 5d-e, respectively). For both groups as a whole, those individuals who had normalized their number of platelets to the international...
C-Reactive Protein Enhances Antibody-Mediated Platelet Destruction

consensus level (>100*10^9/L) also had significantly lower CRP levels compared to those with thrombocytopenia (<100*10^9/L, Supplementary Figure 9). This suggests CRP to be an important amplifier of antibody-mediated platelet destruction in immune thrombocytopenic patients.

CRP enhances anti-platelet IgG-mediated destruction of platelets in vivo

We next investigated if the elevated CRP levels observed in the immune thrombocytopenic patients, could indeed contribute to platelet degradation in vivo. As monocytes and macrophages have been generally proposed to be more relevant effector cells for clearance of the platelets in the spleen, we first tested whether CRP also enhances monocyte-mediated phagocytosis of platelets. In line with the results described above, monitoring the respiratory burst and phagocytosis by PMN, we found CRP also to stimulate phagocytosis of B2G1-opsonized platelets by monocytes (Fig. 6a). We next examined whether the observed effects of CRP in antibody-mediated platelet destruction also occurred in vivo, using our previously established mouse model for immune-mediated thrombocytopenia. Co-administration of 200 µg CRP, together with a limiting dose of 0.75 µg of anti-platelet IgG, significantly decreased the mean platelet counts compared to injection of 0.75 µg of anti-platelet IgG alone, while administration of 200 µg CRP alone had no effect on platelet counts (Fig. 6b). Co-administration of a lower amount of CRP (20 µg) did not result in a significant difference between platelet counts in these mice. Taken together, we conclude that CRP can potentiate the degree of thrombocytopenia induced by anti-platelet antibodies in vivo.
Discussion

In this study, we describe a previously unrecognized role for CRP in IgG-mediated cellular destruction.

We observed that anti-HPA-1a antibodies induce exposure of CRP-ligands on platelets due to oxidative damage. This is in agreement with studies showing that anti-GPIIIa antibodies, the glycoprotein expressing the HPA-1a epitope, induce cellular activation through oxidative damage initiated by the platelet NADPH-oxidase. More importantly, this is also in line with studies describing oxidation-dependent lipid changes of both LDL, as well as apoptotic cells, to expose phosphatidylcholines to the extracellular milieu for recognition by anti-PC antibodies and CRP. Choline-containing CWPS blocked binding of CRP to platelets and prevented CRP-mediated enhancement of anti-platelet antibody-mediated respiratory burst and phagocytosis. Moreover, NADPH-oxidase inhibition reduced binding of CRP to opsonized platelets. Conversely, stimulation of platelet oxidation increased binding to CRP. While the platelet NADPH oxidase is apparently capable of inducing CRP deposition, suggesting at least the initial oxidation to be platelet-derived, it is likely that the more powerful NADPH-oxidase system of the phagocyte also provides for additional oxidized CRP-ligands, further enhancing platelet phagocytosis.

These results might seem in contrast with a previous study, showing that CRP inhibits ITP in a mouse model. However, this effect was shown to be indirect, caused by ex vivo interaction of CRP with macrophage/monocyte FcγR, leading to upregulation of the inhibiting FcγRIIb. In contrast to our study, CRP was washed away before the injection of the antibody and was therefore not present together with CRP. Here, we investigated a completely different mechanism, namely the direct interaction of CRP with platelets leading to enhanced phagocytosis.

Infections, viral or bacterial, are known to promote the initiation of ITP or enhance platelet clearance, but the exact underlying mechanisms are unknown. Previously, one of the possible mechanism suggested is the molecular mimicry between platelet antigens and various viral and bacterial antigens, giving rise to cross-reactive autoantibodies. The gram-negative bacterial endotoxin lipopolysaccharide (LPS) has also been shown to enhance FcγR-mediated phagocytosis of IgG-opsonized platelets in vitro, and to exert a strong synergistic effect in vivo. LPS may therefore be one factor explaining why in ITP patients the thrombocytopenia worsens during gram-negative infections, but also why symptoms are alleviated after the infection is resolved. Although the mechanisms of this LPS effect remains unknown, it has been suggested that LPS triggers either phagocyte- and/or platelet- toll-like receptor 4 (TLR4), inducing synergistic signaling through FcγR, and thereby enhanced platelet clearance. The work presented here provides an alternative explanation, suggesting endotoxin exposure to increase CRP levels through the acute phase responses. The endotoxin itself, low levels of anti-platelet antibodies, or the combination, may lead to platelet activation, followed by oxidation and subsequent phosphorylcholine exposure, providing a binding platform for CRP. Low-level IgG-
opsonization of platelets, not sufficient to initiate phagocytosis on its own, can then be enhanced by CRP, providing a ligand for additional phagocyte FcγR, but also FcαRI (the IgA receptor), as both receptor types have been shown to bind CRP, further marking it for phagocyte destruction. These observations are also in line with recent reports indicating that oxidative stress correlates with prognosis in ITP patients. Curiously, we found that a widespread treatment used in ITP, IVIg, which has been proposed to exert its action through numerous ways, also induced reduction in the CRP levels—and those levels significantly correlated with both the number of platelets as well as clinical bleeding severity. A more direct evidence for the causality between increased CRP levels and enhanced platelet degradation was obtained in mice, where CRP alone was inert against platelets, but enhanced thrombocytopenia together with anti-platelet antibodies—also in line with results obtained from phagocytosis of platelets.

In summary, we conclude that CRP functions as a previously unknown pathogenic co-factor that contributes to antibody-mediated platelet destruction (a model is provided in Supplementary Figure 10). CRP levels dropped after IVIg-treatment, accompanied by normalization of platelet counts and decreased clinical bleeding severity. As such, CRP sheds light on why relapses occur on the onset of infections in ITP, indicating that CRP may serve as an important biomarker for severities of IgG-mediated thrombocytopenias. Importantly, FNAIT and/or ITP patients may benefit from interventions aimed at reducing CRP levels or inhibiting its function.

Acknowledgements

We thank Prof. dr. René van Lier for critically reading the manuscript, Cedric Ghevaert and Prof. dr. Willem Ouwehand for the generous gift of purified B2G1 antibody, and Anton Tool for his supply of the oxidation inhibitor DPI used in the study. R.K. was supported by Sanquin PPOC-09-025.

Author Contributions

R.K. performed most experiments, together with A.E.H.B. and R.V.; M.C.A.B. and K.H. supervised the clinical trial; R.K and G.V. wrote the manuscript; and all authors conceived of or aided with experiments, analyzed data, edited the manuscript and prepared figures; G.V. initiated and supervised the study.

Competing Financial Interests

The authors declare no competing financial interest.
Supporting Information

Supplementary figures

Supplementary Figure 1 Phagocyte responses towards 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 thrombo-cytopenia (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, showing mean ± standard deviation.

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, showing mean ± standard deviation.
**Supplementary Figure 8** Normalization of platelet counts in newly diagnosed ITP patients one week after treatment with IVIG.

78 Newly diagnosed ITP patients were 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 $100 \times 10^9$/mL. Statistical comparison was made using student t-test. ***: $p<0.0001$

**Supplementary Figure 9** CRP levels were significantly decreased in individuals that had normalized their platelet counts ($>100 \times 10^9$ platelets/L) after one week of diagnosis in newly diagnosed ITP patients.

Statistical comparison was made using student t-test. *: $p<0.05$
**Supplementary Figure 10** Illustration of the proposed role for CRP in IgG-mediated platelet destruction.

Anti-platelet IgG-antibodies from serum bind to platelets (1). Platelet oxidation results in phosphorylcholine exposure, independently from FcγRII (2). Subsequently CRP, present in the serum, binds to platelet-phosphorylcholine in a calcium-dependent manner (3). This potentiates the uptake and degradation of the platelets via Fc-receptors of the phagocyte (4). CRP alone is insufficient to mediate this response (see main text).
C-Reactive Protein Enhances Antibody-Mediated Platelet Destruction

References

C-Reactive Protein Enhances Antibody-Mediated Platelet Destruction


