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The Isotype of Autoantibodies Influences the Phagocytosis of Antibody-Coated Platelets in Autoimmune Thrombocytopenic Purpura

M. Hoemberg*†, D. Stahl‡§, P. Schlenke*, W. Sibrowski*, U. Pachmann¶ & U. Cassens**

*Institute of Transfusion Medicine and Transplantation Immunology, University Hospital Muenster, Germany; †Department of Pediatric Oncology and Hematology, University Children's Hospital, Cologne, Germany; ‡University Clinic for Blood Group Serology and Transfusion Medicine, Paracelsus Medical University, Salzburg, Austria; §Haema AG, Leipzig, Germany; ¶Center of Transfusion Medicine, Bayreuth, Germany; and **Institute of Transfusion Medicine, Laboratory Medicine and Medical Microbiology, Klinikum Dortmund, Dortmund, Germany

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Correspondence to: M. Hoemberg, Institute of Transfusion Medicine and Transplantation Immunology, University Hospital Muenster, Domagkstrasse 11, D-48149 Münster, Germany. E-mail: m.hoemberg@uni-muenster.de

Abstract

Autoimmune thrombocytopenic purpura (AITP) is an acquired autoimmune bleeding disorder, characterized by isolated thrombocytopenia because of destruction of auto-antibody-coated platelets by Fc-receptor-mediated phagocytosis. The destruction of autoantibody-sensitized platelets by FcγR-bearing phagocytic cells and the following antigen presentation are considered to play a key role for the pathophysiology of AITP. Although different isotypes of AITP-mediating autoantibodies, e.g. IgG, IgM and IgA, are frequently found in AITP patients, their role in the pathophysiology of AITP remains unclear. Using a flow cytometric monocyte-based phagocytosis assay, we investigated the impact of disease-associated autoantibody isotype in antibody-mediated phagocytosis of platelets. Platelets, labelled with 5-chloromethyl fluorescein diacetate (CMFDA), were incubated with AITP patients' serum characterized by pure IgG or IgM antiplatelet autoantibodies. Labelled platelets were incubated with monocytes. Phagocytosis was defined as the product of percentage of CMFDA-positive monocytes and mean fluorescence intensity of CMFDA. Adherence of platelets to monocytes was quantified by anti-CD61-PerCp in a $CMFDA^+$ $CD14^+$ gate. IgG-coated platelets showed a significantly higher phagocytic index than IgM-coated platelets (mean 796 ± 157 versus 539 ± 78, $P < 0.01$). There were no significant differences regarding platelet adherence to monocytes. The isotype of autoantibodies influences the quantity of *in vitro* phagocytosis of autologous platelets by monocytes. Therefore, the AITP-mediating autoantibody isotype should be considered more carefully in pathophysiologic models and furthermore in diagnostic, therapeutic and prognostic approaches in AITP.

Introduction

Autoimmune thrombocytopenic purpura (AITP) is an acquired autoimmune bleeding disorder, characterized by isolated thrombocytopenia because of destruction of autoantibody-coated platelets by Fc-receptor-mediated phagocytosis in the reticuloendothelial system [1] and suppression of platelet production [2, 3]. Patients usually develop mucocutaneous bleeding signs. In rare cases, life-threatening events like intracranial haemorrhage may occur [4]. The annual ITP incidence in adults has been estimated at 3.2 per 10.000 [5]. With respect to the clinical onset and the duration of symptoms, AITP is classified in an acute (<6-month duration) and a chronic (more than 6 months) disorder. Children are more frequently affected by acute AITP, while adults usually suffer from chronic courses of thrombocytopenia [1, 6]. The onset of acute AITP in children is often 2–3 weeks following an immunological event like immunization or a viral infection, and the disease is often self-limiting. In contrast, adult AITP has usually no preceding event, shows more often an insidious onset and has a chronic course [6]. T cell-related and cytokine abnormalities could be shown in chronic AITP [7, 8]. Acute and chronic AITP, although both are immune mediated, might be different disease entities with different underlying pathophysiologic mechanisms [9].

The diagnosis of AITP remains one of exclusion [1]. Usually, secondary forms (e.g. in association with systemic lupus erythematosus or lymphoproliferative

disorders) should be excluded. No clinical signs or laboratory findings that may lead to a different diagnosis like marked splenomegaly, altered bone marrow aspiration samples, blood count or blood smear apart from thrombocytopenia are found. Several assays were designed to detect anti-platelet antibodies and show a sensitivity of 49–66% and an estimated specificity of 78–92% [1, 10, 11]. Though, a negative test result cannot rule out the diagnosis of AITP [12].

Current diagnostic procedures usually focus on detection of IgG antibodies and the platelet-specific glycoprotein target of the disease-mediating antibody. Concerning diagnostic and pathophysiological aspects of AITP, autoantibodies of the IgM class and other isotypes of antiplatelet antibodies are often neglected.

The principal aim in the treatment of AITP is to raise platelet count to prevent the patient from severe and life-threatening bleeding. Because bleeding in AITP patients is rare and platelet function is preserved, many patients do not need treatment. The risk of bleeding should face the side effects and toxicity of treatment. Corticosteroids and IVIg remain the most common first-line treatment for AITP. Anti-rhesus antibody (anti-D) is also effective in Rh-D-positive individuals. Splenectomy might be considered for adult patients with chronic AITP. Rituximab, azathioprine, cyclosporin, cyclophosphamide, mycophenolate mofetil and etanercept are also used in AITP treatment, usually as second-line treatment [4]. New treatment options as thrombopoietin receptor agonists, monoclonal anti-Fcγ antibodies and inhibitors of syk kinase are currently evaluated [13, 14].

Many concepts for the immune pathophysiology of AITP put the destruction of autoantibody-sensitized platelets by FcγR-bearing phagocytic cells in the centre of interest [1, 6, 15]. At least three classes of FcγRs are expressed on phagocytic effector cells in the reticuloendothelial system. High-affinity activating FcγRI binds monomeric and immune-complexed IgG, while low-affinity activating receptors FcγRIIA and FcγRIIIA bind only immune-complexed IgG. Additionally, inhibitory FcγRIIB has recently been found [15]. In 1986, Clarkson *et al.* [16] showed that blocking of the receptors leads to reduced platelet destruction in AITP patients. Furthermore, FcγR polymorphisms that can significantly alter antibody binding are significantly overexpressed in a population with childhood AITP compared with healthy controls [17], and thus, FcγR polymorphisms may influence disease occurrence and severity [18]. Much work has been carried out to clarify the role of different Fc receptors in the pathogenesis of AITP, and today, low-affinity IgG receptors FcγRIIA and FcγRIIIA seem to be primarily responsible for removal of opsonized platelets [15, 19]. Specific inhibitors of phagocyte-mediated consumption of platelets are now investigated for new treatment options of AITP [13].

Little is known about the pathophysiological differences of AITP-mediating autoantibody isotype and the corresponding Fc receptor classes for phagocytosis of antibody-coated platelets, but they might be of high relevance for a better understanding of the disease. It is surprising that although much work has been carried out to identify the IgG receptor-subclass and the AITP-mediating autoantibody IgG subclasses, little work has been addressed to clarify the role of the disease-mediating autoantibody isotype.

Therefore, we used the model of AITP to investigate the impact of disease-mediating antibody isotype on immune phagocytosis of autologous platelets.

Material and methods

Patients and controls. Plasma samples from 11 adult patients with AITP were obtained. Additionally, immunoglobulin from the platelet membrane of eight AITP patients could be eluted by acid elution technique as used in standard procedures [20]. Material obtained by the latter procedure is referred to as eluates. All patients presented with clinical bleeding signs because of thrombocytopenia at the time point of investigation, and the diagnosis of AITP was made on the basis of verification of platelet-reactive autoantibodies. None of the patients had received treatment prior to the collection of blood samples. No information was available on other clinical diagnoses. Healthy adults were enrolled as controls. Studies were approved by the ethics commission at the University of Muenster, Germany.

Immunohaematological and biochemical investigations. Autoantibody isotype in plasma and eluate samples of AITP patients was determined by the platelet immunofluorescence test [20]. Results were confirmed using the monoclonal antibody-specific immobilization of platelet antigens method in case of IgG antibodies [21] and by commercially available enzyme-linked immunosorbent assay (GTI, Brookfield, WI, USA) in the case of IgM antibodies. The ELISA was performed according to the manufacturer's specifications with the exception that we used anti-IgM as secondary antibody instead of a mixture of anti-IgM, anti-IgG and anti-IgA. Anti-human leukocyte antigen (HLA) antibodies in plasma were excluded by the lymphocyte cytotoxicity assay [20]. The concentrations of IgM and IgG in plasma and in eluates were determined by quantitative alkaline phosphatase sandwich ELISA. The antibody concentrations were compared to standard immunoglobulin solutions and checked with negative and precision controls.

Characterization of patients and controls with regard to plasma immunoglobulin concentrations and anti-platelet autoantibody isotype. The plasma samples were characterized with regard to plasma immunoglobulin concentrations and anti-platelet autoantibody isotype. Five patients

presented with pure anti-platelet autoantibodies of the isotype IgM (i.e. IgM-mediated AITP) and six patients with pure anti-platelet autoantibodies of the isotype IgG (i.e. IgG-mediated AITP). Anti-HLA antibodies were detected in the plasma in none of the patients. The eluates used in the assay contained also pure anti-platelet antibody populations (four IgG and four IgM). Healthy individuals did not exhibit anti-platelet or anti-HLA antibodies.

Flow cytometric monocyte-based phagocytosis assay. Monocytes and platelets. The phagocytosis assay was performed similar to Lim *et al.* [22]. The monocytes and platelets used in each assay were obtained from two different healthy donors with blood type 0 (one female and one male donor). The monocytes were collected by apheresis (Cobe Spectra LRS; Gambro BCT Inc., Lakewood, CO, USA) in the blood donation centre by standard procedures. The apheresis product was diluted with PBS buffer. The mononuclear cells (MNCs) were isolated by density gradient centrifugation (density 1.077, Biocoll Separating Solution; Biochrom AG, Berlin, Germany). MNCs were washed twice with RPMI (RPMI 1640; Bio-Whittaker Europe, Verviers, Belgium) and resuspended in RPMI with 5% FCS to 5×10^6 white blood cells per mililitre. We used the adhesion method to enrich the monocytes as followed: 2 ml of the resuspended MNCs was poured in each well of eight well multi-plates (Nunc, Wiesbaden Biebrich, Germany). After incubation for 1 h at 37 °C/5% CO_2 and after washing the well with warm RPMI solution to discharge non-adherent cells, the monocyte layer was checked by light microscopy.

Platelets. The platelets were obtained by peripheral blood collection from the same donor as the MNCs. The blood was anticoagulated with EDTA. The platelet-rich plasma was washed three times and resuspended in 0.5% EDTA–PBS buffer to a final concentration of 5×10^8/ml.

All cell concentrations were determined using an automated cell counter (Sysmex, K1000; Toa Medical Electronics, Kobe, Japan).

Labelling of platelets. For each assay, monocytes and platelets were obtained from the same donor. Platelet-rich plasma was obtained from EDTA whole blood. Platelets were labelled with CellTracker Green CMFDA (Molecular Probes, Eugene, OR, USA). CMFDA was used as described by Baker *et al.* [23]. We used a concentration of 5 μM CMFDA to label the platelets. After 45 min of incubation, the platelets were centrifuged at 2000 *g* for 10 min, resuspended with 0.5% EDTA–PBS and washed again. The labelled platelet solution was adjusted to a concentration of 5×10^8 platelets/ml.

Sensitization of CMFDA-labelled platelets. One millilitre of the CMFDA-labelled platelet solution was incubated with patients' plasma or eluates from platelet membrane of AITP patients. Different antibody isotypes were adjusted to same (weight dependent) concentrations with respect to the number of antigen-binding sites. In case of plasma 300 μg immunoglobulin and in case of eluates 150 ng immunoglobulin were used. The incubation was performed in the absence or presence of 5 μl rabbit complement (Biotest, Dreieich, Germany). Plasma from AITP patients with strong IgG anti-GPIIb/IIIa reactivity and plasma from healthy blood donors served as positive and negative controls.

Monocyte–platelet incubation. Two millilitre of the RPMI/FCS medium and 250 μl of the sensitized platelet solution were added to wells coated with a monocyte monolayer. The plates were gently moved to mix the solutions. Each assay was performed in triplets, and each assay was repeated the following day. Positive and negative controls were performed in each assay. The plates were incubated in a CO_2 (5%) incubator for 90 min at 37 °C. To remove non-adherent cells, each well was washed twice with 2 ml of warm RPMI/FCS medium. Adherent monocytes were mobilized with 1.5 ml cold PBS and the help of cell scraper (Falcon, Becton Dickinson Labware, Franklin Lakes, NY, USA). The collected monocyte cell suspensions were centrifuged at 1200 *g* for 10 min and resuspended with 100 μl PBS.

Flow cytometric analysis of the platelet phagocytosis. After phagocytosis, monocytes were incubated with 3 μl PE-conjugated anti-human CD14 mouse antibody (Caltag Laboratories, Burlingame, CA, USA) and 15 μl PerCP-conjugated anti-human CD61 mouse antibody (Becton Dickinson, San Jose, CA, USA) for 15 min at room temperature. Monocytes were washed twice with PBS and were analysed by flow cytometry (FACS Calibur; Becton Dickinson, Mountain View, CA, USA).

The platelets were internally labelled with CMFDA. CMFDA shows properties similar to FITC and was detected in fluorescence channel 1. The monocytes were detected by PE-conjugated anti-human CD14 mouse antibody in fluorescence channel 2. The adherent and non-internalized platelets were identified by PerCP-conjugated anti-human CD61 mouse antibody in fluorescence channel 3 (see Fig. 1).

The data were analysed by software (CellQuest; Becton Dickinson). Phagocytosed platelets were detected as a CMFDA-positive population in an ellipsoid $CD14^+$ monocyte gate. Additionally, platelet adherence was measured in an ellipsoid $CD14^+$ monocyte gate by the quantification of CD61-PerCP.

Two populations of the monocytes could be identified, regarding the quantity of CMFDA fluorescence (see Fig. 1). The population with the higher intensity ($CMFDA^+$) was the source for the quantification of phagocytosis. We used a modified index, originally described by Rinder *et al.* [24, 25], to quantify the index of phagocytosis that we defined as the product of percentage of CMFDA-positive monocytes ($CMFDA^+$ and $CD14^+$ cells of all cell events) and mean fluorescence

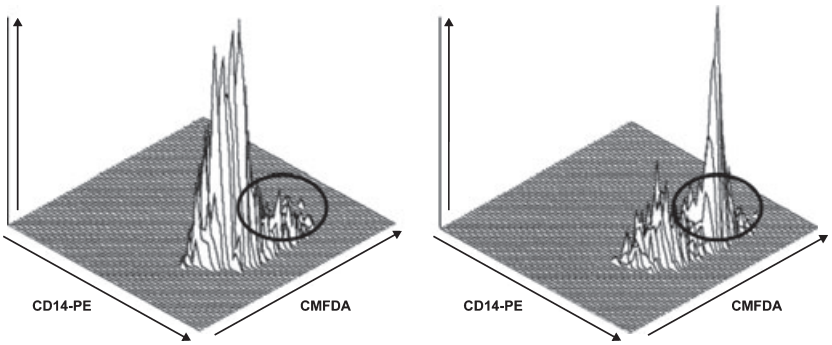

Figure 1 Three-dimensional graphics show two results of the monocyte-based phagocytosis assay. The monocyte population ($CD14^+$) is depicted in dependence of the fluorescence intensity of 5-chloromethyl fluorescein diacetate. Left: negative control (healthy control); right: autoimmune thrombocytopenic purpura patient.

intensity (MFI) of CMFDA. This index is referred to as phagocytic index (PI) and is given as mean ± SD.

Statistical analysis. The results were analysed using the Mann–Whitney *U*-test. All statistics were performed by software (SPSS 16.0; SPSS Inc., Chicago, IL, USA).

Results

Phagocytosis of platelets sensitized with antibodies from plasma of AITP patients

Autoantibody-coated platelets were differentially *in vitro* phagocytosed by monocytes depending upon immunoglobulin isotype and complement activation. As shown in Fig. 2 and supplemental Table S1 in more detail, the platelets either sensitized with IgG antibodies from AITP patients showed in the absence of complement a phagocytic index (PI) of 330 ± 24 (n = 6) or sensitized with IgM antibodies a PI of 250 ± 29 (n = 5), whereas platelets incubated with healthy control plasma resulted in a PI of 202 ± 35 (n = 5). Only the phagocytosis of platelets with autoantibodies of the IgG isotype achieved significance when compared with the IgM isotype and control group [Mann–Whitney *U*-test (P < 0.01 for both)]. The monocyte-based phagocytosis assay was performed additionally in the presence of complement. As expected, the controls showed a slight increase in the PI (260 ± 55), indicating a near equal 'background' level including spontaneous phagocytosis. In contrast to the above-mentioned experiments without complement supplementation, the phagocytic indices were now significantly increased for both groups of antibody-sensitized platelets (IgG isotype 796 ± 158 (n = 6), IgM isotype 539 ± 78 (n = 5), P < 0.01, respectively). Furthermore, donor platelets sensitized with IgG autoantibodies of AITP patients were phagocytosed to significant higher amounts when compared to those with IgM autoantibodies (P < 0.05).

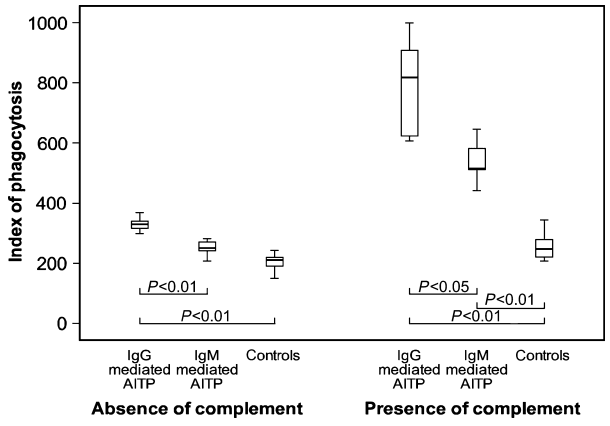

Figure 2 Index of phagocytosis of antibody-sensitized platelets depending upon the antibody isotype (IgG or IgM) with or without complement supplementation.

Phagocytosis of platelets sensitized with antibodies from platelet eluates of AITP patients

In addition, comparable experiments were carried out by using autoantibodies initially bound on the surface of AITP platelets and subsequently eluated by acid elution technique. Because there was a shortage of eluate, these assays were exclusively performed without complement. The mean indices of phagocytosis of IgG-sensitized platelets were 212 ± 26 (n = 4) and of IgM-sensitized platelets 118 ± 12 (n = 4). The indices of phagocytosis of IgG-sensitized platelets compared with IgM-sensitized platelets could be discriminated significantly using Mann–Whitney *U*-test (P < 0.05).

Platelet adherence to monocytes

The fluorescence level of anti-CD61-PerCP was measured with respect to the sensitizing antibody isotype or control, see Fig. 3. Adherent platelets 'sensitized' with control plasma without any known antibody-mediated platelet reactivity showed a MFI of 2621 ± 71. The MFI of adherent platelets sensitized with plasma-soluble IgG antibodies was 2872 ± 120 (n = 4) and with eluated IgG antibodies 2007 ± 41 (n = 4). Platelets sensitized with IgM antibodies achieved MFI of 2857 ± 64 (n = 4) and 2025 ± 30 (n = 4), respectively, without complement

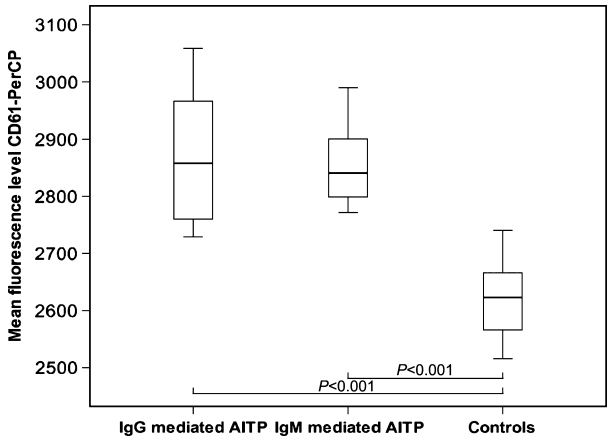

Figure 3 Mean fluorescence intensity of anti-CD61-PerCP of $CD14^+$ monocytes showing the properties of adherence depending upon autoimmune thrombocytopenic purpura-mediating antibody isotype.

supplementation. The control group could be discriminated from the AITP patients group independent upon the immunoglobulin isotype (IgG or IgM) by the level of anti-CD61-PerCP fluorescence ($P < 0.001$), whereas there was no statistical significance between these groups of sensitizing antibody isotypes ($P = 0.862$).

Discussion

Early *in vitro* studies have shown that the thrombocytopenia in AITP patients based primarily upon the destruction of platelets by the antibody-induced phagocytosis [26, 27]. However, to this end, no further investigations concerning autoantibody isotype and phagocytosis capacity were made yet, although specific inhibitors of phagocyte-mediated consumption of platelets are currently investigated for new treatment options of AITP and Fc-FcR interactions are thought to be brilliant targets for immunomodulatory therapies [13].

Phagocytosis in AITP

Antigen processing is thought to be a key determinant of the quality and quantity of a $CD4^+$ T cell activation [28], and so it is postulated that presentation of antigen and activation of antigen-presenting cells (APC) play a crucial role for the understanding of tolerance and autoimmunity [29]. Over the last 10 years, abnormal T cell activation patterns could be demonstrated in the setting of AITP [7, 8, 30], and autoreactive T cells are believed to drive autoantibody production in patients with AITP [31]. Yet, it is unknown how the T cells are activated, but probably, APCs such as monocytes and dendritic cells may be involved [9, 32]. Antigens are usually internalized by APCs and processed into smaller antigenic fragments by proteolytic degradation. These antigen fragments are attached to major histocompatibility complex class II molecules and presented on the cell surface to be the target for antigen-specific T-helper cells [33]. During senescence, phagocytosis occurs physiologically in the manner that platelets are taken up by macrophages and presumably destroyed within lysosomes. Under certain inflammatory stimuli, the monocyte/macrophage system might be activated, which may alter the physiological way to process autoantigens and lead to altered T cell and APC interactions that may force antigen-primed B cells to secrete autoreactive antigen-specific antibodies [9].

Autoantibodies in AITP

Anti-platelet autoantibodies in AITP mainly belong to the IgG class. The most common antibody subclass in chronic AITP patient was IgG1 (77%), either alone or with other IgG subclass antibodies [34]. Observations were made that patients with acute AITP show predominantly IgM autoantibodies [35]. He *et al.* evaluated sera from 47 chronic AITP patients for antibodies of IgG, IgA and IgM classes. In 85%, the sera contained one or more classes of Abs. IgG and IgA occurred together in 51% of sera, in 17% IgA alone. IgM antibodies were found in 15% in combination with IgG or IgA [36]. Porcelijn and von dem Borne [37] mentioned an occurrence of anti-platelet autoantibodies of the IgM isotype in up to 26% of cases. This observation was previously confirmed by our group, indicating that AITP is mediated partially by antibodies of both isotypes, IgM and IgG [38]. In some clinical settings, the detection of IgG autoantibodies might not be sufficient. The autoantibody assays in AITP should be broadened with autoantibody isotypes like IgM and IgA. However, it remains unclear whether autoantibodies in AITP follow the rules of isotype switch.

In AITP, autoantibody-dependent phagocytosis is considered to be the primary mechanism of peripheral platelet destruction [1, 6, 39]. However, there might be additional components such as a decreased T cell-mediated platelet production involved in the pathophysiology of AITP [3, 40, 41]. Apart from this, we used an *in vitro* monocyte-based phagocytosis assay to investigate the link between FcR-mediated phagocytosis and different autoantibody classes.

We could show that autoantibody-coated platelets were differentially *in vitro* phagocytosed by monocytes depending upon immunoglobulin isotype. With respect to the index of phagocytosis used in our study, IgG antibodies led to the highest quantity of phagocytosis, compared with IgM and controls. The presence of a complement source increases the phagocytosis capacity of monocytes for each group or makes antibody-sensitized platelets more 'mouth-watering' for phagocytosis comparable to the process of opsonization. As shown by our own results, bound autoantibodies of IgM isotype could only be discriminated from healthy controls in the presence of complement. In early

studies, increased levels of platelet-associated C3, C4 and C9 have been demonstrated on AITP platelets [42, 43], and our results underline that complement activation may be important in some AITP patients and this is probably dependent on autoantibody isotype.

In our experiments, the actual internalization of platelets was controlled by the simultaneous measurement of merely adherent platelets by anti-CD61-PerCP. There were no differences of non-internalized and adherent platelets depending upon the IgM or IgG isotype. It can be assumed that the monocyte surface might be saturated with antibody/platelet bindings. As expected, healthy controls showed a significant lower level of antibody-mediated adherence of platelets on monocytes.

In conclusion, we were able to confirm our hypothesis that the individual quantity of phagocytosis of antibody-sensitized platelets depends first on the immunoglobulin isotype of the respective AITP initiating autoantibody and second on complement activation. The observed differences might be mediated by distinct Fc receptor classes, specific for different antibody isotypes. A human Fcα receptor (also known as CD89) with IgA-binding specificity has been characterized [44]. Fcα receptor is involved in renal autoimmune disease [45]. More recently, the Fcα/μ receptor (Fcα/μR) could be identified on B cells and macrophages and seems to be specific for IgA and IgM antibodies [46]. Fcα/μR is thought to be involved in the protection of bacterial infections by mediating phagocytosis of IgM-coated organisms, facilitating subsequent antigen processing and presentation to helper T lymphocytes [47]. It is well known that IgG antibodies bind to Fcγ receptor on macrophages and neutrophils, mediating phagocytosis of opsonized micro-organisms and secretion of proinflammatory cytokines. In AITP, inhibitory FcγRIIB receptors and its balance with activating receptor subtypes (e.g. FcγRIIA) have highly been investigated and are now targets for immunomodulatory therapies [4]. Low-affinity receptors FcRIIA and FcRIIIA are thought to be primarily responsible for removal of opsonized platelets [6].

Taken all findings together, in AITP, little is known about specific platelet-associated signalling pathways that follow the specific Fc receptor/ligand interaction, but it has to be postulated that diverse isotype-dependent pathways follow different Fc receptor classes and might be crucial for tolerance induction and autoimmunity. Therefore, the immunoglobulin isotype of the underlying autoantibodies should be carefully considered in diagnostic procedures, novel treatment options and the pathophysiology of AITP.

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Conflict of interest

The authors do not have any conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article

Table S1 Index of phagocytosis of antibody-sensitized platelets (PI) depending upon the antibody isotype (IgG or IgM) with or without complement supplementation.

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