ARTICLE

Glycoprotein V is a relevant immune target in patients with immune thrombocytopenia

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Richard Vollenberg,[1] Rabie Jouni,[2] Peter A. A. Norris,[3] Monika Burg-Roderfeld,[4] Nina Cooper,[1] Mathias J. Rummel,[5] Gregor Bein,[1] Irene Marini,[2] Behnaz Bayat,[1] Richard Burack,[6] Alan H. Lazarus,[3] Tamam Bakchoul[2*] and Ulrich J. Sachs[1,7*]

**TB and UJS contributed equally to this work*

[1]Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, Giessen, Germany; [2]Center for Clinical Transfusion Medicine, Medical Faculty of Tübingen, Eberhard Karls University, Tübingen, Germany; [3]The Canadian Blood Services & The Keenan Research Centre of St. Michael's Hospital, Toronto, ON, Canada; [4]Faculty for Chemistry and Biology, Fresenius University of Applied Sciences, Idstein, Germany; [5]IVth Department of Internal Medicine (Hematology/Oncology), Justus Liebig University, Giessen, Germany; [6]Department of Pathology and Laboratory Medicine, University of Rochester, NY, USA and [7]Center for Transfusion Medicine and Hemotherapy and Hemostasis Center, University Hospital Giessen and Marburg, Marburg, Germany

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ABSTRACT

Platelet autoantibody-induced platelet clearance represents a major pathomechanism in immune thrombocytopenia (ITP). There is growing evidence for clinical differences between anti-glycoprotein IIb/IIIa and anti-glycoprotein Ib/IX mediated ITP. Glycoprotein V is a well characterized target antigen in Varicella-associated and drug-induced thrombocytopenia. We conducted a systematic study assessing the prevalence and functional capacity of autoantibodies against glycoprotein V. A total of 1140 patients were included. In one-third of patients, platelet-bound autoantibodies against glycoproteins Ib/IX, IIb/IIIa, or V were detected in a monoclonal antibody immobilization of platelet antigen assay; platelet-bound autoanti-glycoprotein V was present in the majority of samples (222 out of 343, 64.7%). Investigation of patient sera revealed the presence of free autoantibodies against glycoprotein V in 13.5% of these patients by an indirect monoclonal antibody immobilization of platelet antigen assay, but in 39.6% by surface plasmon resonance technology. These antibodies showed significantly lower avidity (association/dissociation ratio 0.32±0.13 *vs.* 0.73±0.14; $P<0.001$). High- and low-avidity antibodies induced comparable amounts of platelet uptake in a phagocytosis assay using $CD14^+$ positively-selected human macrophages [mean phagocytic index, 6.81 (range, 4.75-9.86) *vs.* 6.01 (range, 5.00-6.98); $P=0.954$]. In a NOD/SCID mouse model, IgG prepared from both types of anti-glycoprotein V autoantibodies eliminated human platelets with no detectable difference between the groups from the murine circulation [mean platelet survival at 300 minutes, 40% (range, 27-55) *vs.* 35% (16-46); $P=0.025$]. Our data establish glycoprotein V as a relevant immune target in immune thrombocytopenia. We would suggest that further studies including glycoprotein V will be required before ITP treatment can be tailored according to platelet autoantibody specificity.

Introduction

Immune thrombocytopenia (ITP) is an acquired hemorrhagic autoimmune disease characterized by isolated thrombocytopenia.[1,2] Autoantibodies against platelet membrane glycoproteins cause platelet destruction and insufficient compensatory platelet production in the bone marrow (BM).[3-5] Cytotoxic effects of T cells have also been described.[6] Phagocytosis of antibody-decorated platelets *via* Fc-receptors or, following complement activation, *via* complement receptors were long-accepted concepts for the understanding of platelet destruction.[6,7] Recent studies have pro-

Correspondence:

ULRICH J. SACHS
ulrich.sachs@med.uni-giessen.de

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vided some evidence that autoantibodies may also trigger more complex processes, such as platelet activation, platelet desialylation, or platelet apoptosis, all of which could lead to Fc-independent platelet clearance.[8-11] More recently, there has also been evidence that the glycoprotein specificity of the autoantibodies could be important; for example, in a study by Li *et al.*,[10] desialylation occurred in the presence of anti-GPIbα, but not in the presence of anti-GP IIb/IIIa antibodies.

In general, antibody identification (or the use of monoclonal antibodies in animal models) in these studies was restricted to two types of autoantibody specificities: anti-GP IIb/IIIa and anti-GP Ib/IX. This was because these two glycoproteins are currently considered to be the most important autoimmune targets in ITP.[12,13] However, glycoprotein V (GP V) is a major protein on the platelet membrane, with approximately 10,000 copies per platelet.[14] More than 30 years ago, GP V was first thought to be the immune target of quinidine-related platelet antibodies by Stricker and Shulman,[15] and Garner *et al.*[16] described GP V as the antigen in a gold-triggered autoimmune response in patients with rheumatoid arthritis. GP V was also described as the target protein in pediatric varicella-associated thrombocytopenia.[17] Some evidence for a potential role of GP V in ITP came from preliminary studies in patients with different types of thrombocytopenia.[18,19] A valuable systematic study on GP V in patients with ITP was recently published,[20] but whether or not anti-GP V autoantibodies contribute to thrombocytopenia in ITP remains unknown.

Here, we investigated the potential of anti-GP V autoantibodies in patients with ITP. Our work shows that autoantibodies to GP V are found in a majority of patients with ITP and can potentially cause platelet clearance mechanisms. This new information helps fill in some of the missing pathophysiological events in ITP.

Methods

Adult patients with a suspected diagnosis of ITP were identified, as previously described.[7] In brief, standardized questionnaires covering relevant criteria to refute or confirm a diagnosis of ITP according to the British guidelines were used.[21] Patients with relevant other diagnoses that could explain thrombocytopenia, such as aplastic anemia, leukemia, lymphoma, myelodysplastic syndrome, solid tumors, liver cirrhosis, recent cardiac surgery, BM/blood stem cell transplantation, sepsis, and drug-induced thrombocytopenia, were not included. Platelet-bound and free anti-platelet autoantibodies of the IgG type were detected by the monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay, as described by Kiefel *et al.*[22] Assay sensitivity was controlled by the use of the anti-HPA-1a World Health Organization (WHO) standard (NIBSC, Potters Bar, UK).

Leftover material was used for the additional experiments performed. All anti-GP V sera used in these experiments were negative for the presence of anti-GP IIb/IIIa, anti-GP Ib/IX, anti-GP Ia/IIa, and anti-GP IV by MAIPA. Immunoglobulin G (IgG) fractions were isolated using a commercial purification kit (MelonTM-Gel IgG Spin Purification Kit, Thermo Fisher Scientific, Waltham, MA, US).

Surface plasmon resonance (SPR) analysis allows label-free, real-time investigation of antigen-antibody interactions. This was performed on a protein interaction array system (ProteOn XPR36, Bio-Rad, Munich, Germany). Recombinant His-tagged GP V as the target protein and GP IV as an irrelevant control (R&D Systems; Life Technologies, Carlsbad, CA, USA) were immobilized onto flow cells of an HTE sensor chip. Phosphate buffered saline-tween (PBS-T) was used as running buffer for all steps. The SPR signal originates from changes in the refractive index at the chip's surface. For antigen-antibody interactions, changes in the refractive index are linear to the number of antibodies bound. Data were acquired with the computer software (ProteOn Manager Software, BioRad). Interaction curves were referenced by interspot, second flow cell with immobilized GP IV and monoclonal anti-GP V (MAB42, R&D Systems, 6 μg/mL) as standard. The R700/R350 ratio was used to differentiate high-avidity (>0.5) and low-avidity (<0.5) antibody binding.[23]

A phagocytosis assay was performed using $CD14^+$ positively-selected macrophages (autoMACS Pro Separator; Miltenyi Biotec, Germany) from cryogenically stored human spleen specimens obtained from ITP patients. Healthy donor platelets were fluorescently labeled with CellTracker Green 5-chloromethylfluorescein diacetate (Thermo Fisher Scientific, MA, USA), washed, then opsonized with the ITP serum samples and added to the splenic macrophages for phagocytosis. Macrophages were observed by spinning-disc confocal microscopy under 63x objective oil immersion with differential interference contrast (DIC) and laser fluorescence (488, 647 excitation) on a Quorum multi-modal imaging system (Quorum Technologies, ON, Canada) equipped with a 50 micrometer pinhole spinning disc and an ORCA-Flash 4.0 V2 PLUS sCMOS camera. Four images were taken at the center of each well with Z-stacking every 0.33 μm with >30 stacks. Images were reconstructed in 3D for analysis using Imaris 8.0.2 (Bitplate, UK) and phagocytic index was calculated as (total engulfed platelets / splenic macrophages counted) x 100.

A NOD/SCID mouse model was used to investigate the elimination of human platelets by anti-GPV autoantibodies in ITP patients.[24] In brief, NOD/SCID mice (NOD.CB17-Prkdcscid/J; Stock No. complexes, 001303) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) *via* Charles River, Research Models and Services (Sulzfeld, Germany). Sex- and age-matched (8-16-week old) animals were used in this study. Human platelets (200 μL, $2x10^9$/mL) were injected into the lateral mouse tail vein. After 30 minutes (min) a blood sample was collected by tail vein puncture to determine the baseline of circulating human platelets (100%). Subsequently, IgG fractions isolated from human sera containing anti-GPV antibodies or control sera from healthy donors were injected into the other lateral tail vein (2 mg/g body weight). The survival of human platelets in the mouse circulation was analyzed over time using flow cytometry (Cytomics FC 500; Beckman Coulter) after staining platelets with anti-human CD41-PE-Cy5 (Beckman Coulter) and anti-mouse CD41-FITC (BD Biosciences, San Diego, CA, USA). Animal experiments were performed with the approval of the local authorities in Tuebingen, Germany. The study was conducted in accordance with the Declaration of Helsinki, and the use of human material was approved by the local ethics committees in Giessen, Germany and Toronto, ON, Canada.

Results

Prevalence of platelet-bound autoantibodies against GP V

A total of 1645 patients with no alternative reason for a low platelet count were included. The amount of autologous platelets was sufficient for a complete direct test (including all 3 glycoprotein specificities) in 1140 patients (69.3% of n=1645 patients with a clinical suspicion of

Table 1. Summary of autoantibody specificities detected in 343 of 1140 immune thrombocytopenia patients, either on the surface of the patient's platelets (platelet-bound) or free in patient serum.

Glycoprotein specificity	Platelet-bound autoantibodies		Free autoantibodies	
	N. of positive samples	%	N. of positive samples	%
GP IIb/IIIa only	71	20.7	6	13.3
GP Ib/IX only	30	8.8	6	13.3
GP V only	10	2.9	6	13.3
GP IIb/IIIa plus GP Ib/IX	20	5.8	3	6.7
GP IIb/IIIa plus GP V	10	2.9	3	6.7
GP Ib/IX plus GP V	61	17.8	9	20.0
GP IIb/IIIa plus GP Ib/IX plus GP V	141	41.1	12	26.6
Total	343	100.0	45	99.9*

*Rounding error.

ITP). This group was further assessed in order to ensure comparability of data. Results are summarized in Table 1. For patients with a positive test result for at least one glycoprotein, the frequency of immunization against GP V was similar to the other glycoproteins: 242 out of 343 (70.6%) patients were positive for anti-GP IIb/IIIa, 232 out of 343 (67.6%) patients were positive for anti-GP Ib/IX, and 222 out of 343 (64.7%) patients were positive for anti-GP V (Kruskal-Wallis test; P=0.67) (Table 1).

Interestingly, there was also no difference in the amount of antibodies attached to GP V (antibody load), as determined by the optical density of the MAIPA assay between glycoproteins: mean values were 1.86 [95% confidence interval (CI): 1.49-2.23] for anti-GP IIb/IIIa, 1.63 (1.27-1.99) for anti-GP Ib/IX, and 1.82 (1.37-2.26) for anti-GP V; Kruskal-Wallis test, P=0.77.

Prevalence and binding properties of free autoantibodies against GP V

Sera from patients with any positive result in the direct MAIPA test (n=343) were further assessed by indirect MAIPA for the presence of free autoantibodies against these platelet glycoproteins. Results are summarized in Table 1. Free autoantibodies were detected in 45 out of 343 (13.1%) patient samples. The glycoprotein-specific distribution was GP IIb/IIIa (25 out of 45, 55.5%), GP Ib/IX (30 out of 45, 66.6%), and GP V (29 out of 45, 64.4%). Identified free autoantibody specificities matched the platelet-bound specificities from the same patient throughout. Addition of recombinant GP V to sera prior to testing completely blocked the detection of anti-GP V autoantibodies, but did not interfere with the detection of anti-GP IIb/IIIa or anti-GP Ib/IX autoantibodies (*data not shown*).

Serum IgG fractions from all 222 patients with platelet-bound anti-GP V were further analyzed by SPR (Figure 1); 88 out of 222 (39.6%) patients showed specific binding to the GP V flow cell. These 88 sera included all 29 identified as containing anti-GP V by indirect MAIPA. Further analysis demonstrated that these 29 sera with autoantibodies detected both in the indirect MAIPA and in SPR were of higher avidity (R_{700}/R_{350}=0.73±0.14; Wilcoxon rank test, P<0.001) (Figure 1B, top panel) than the 59 sera that gave positive signals in SPR, but were negative in the indirect MAIPA assay (R_{700}/R_{350}=0.32±0.13) (Figure 1B, bottom panel). These results indicate that SPR has better sensitivity compared to the gold standard MAIPA assay in detecting anti-GP V autoantibodies.

Autoantibody-triggered phagocytosis and *in vivo* platelet clearance

Anti-GP V autoantibodies were grouped according to their SPR binding profiles into a "high avidity" and a "low avidity" group. IgG fractions prepared from two high-avidity and two low-avidity anti-GP V antibody-containing ITP sera were tested in a phagocytosis assay using CD14 positively-selected human macrophages from ITP spleens (Figure 2). One high- and one low-avidity GP V sera induced significant platelet uptake relative to normal human serum controls (P=0.003 and P=0.026, respectively). Of those positive, high- and low-avidity antibodies induced similar amounts of platelet uptake [mean phagocytic index, 6.81 (range, 4.75-9.86) *vs.* 6.01 (range, 5.00-6.98), respectively; P=0.954]. To further assess the biological effect of anti-GP V autoantibodies on platelet destruction, the NOD/SCID mouse model was used. First, moab SW16 against human GP V was injected at two concentrations and the results verified against a murine monoclonal antibody (SZ21) specific for GPIIb/IIIa known to cause thrombocytopenia. SW16 induced similar clearance of human platelets from the murine circulation as SZ21 (mean platelet survival after 300 min, 16±5% *vs.* 8±8%; P=0.140) (Figure 3A). Platelet elimination was slower when SW16 was injected at a lower concentration (27±4%; P=0.018) (Figure 3A). Next, we analyzed IgG fractions isolated from ITP sera which contained anti-GP V autoantibodies only. Unexpectedly, anti-GP V reduced the survival of human platelets compared to control IgG regardless of their binding properties [median platelet survival after 300 min, "high avidity", 35% (range, 16-46%; P=0.029) and "low avidity": 40% (range, 27-55%; P=0.025), respectively] (Figure 3B). After 24 h, only a few injected human platelet circulated in the presence of anti-GP V antibodies [median platelet survival after 1440 min, "high avidity", 22% (range, 11-23%; P=0.0286) and "low avidity", 20% (range, 13-24%; P=0.029) *vs.* 46% (range, 43-76%)] (Figure 3B). No difference in platelet elimination was observed between the two groups (P=0.229 and P=0.441, after 300 min and 1440 min, respectively). As expected, autoantibodies were generally less effective in

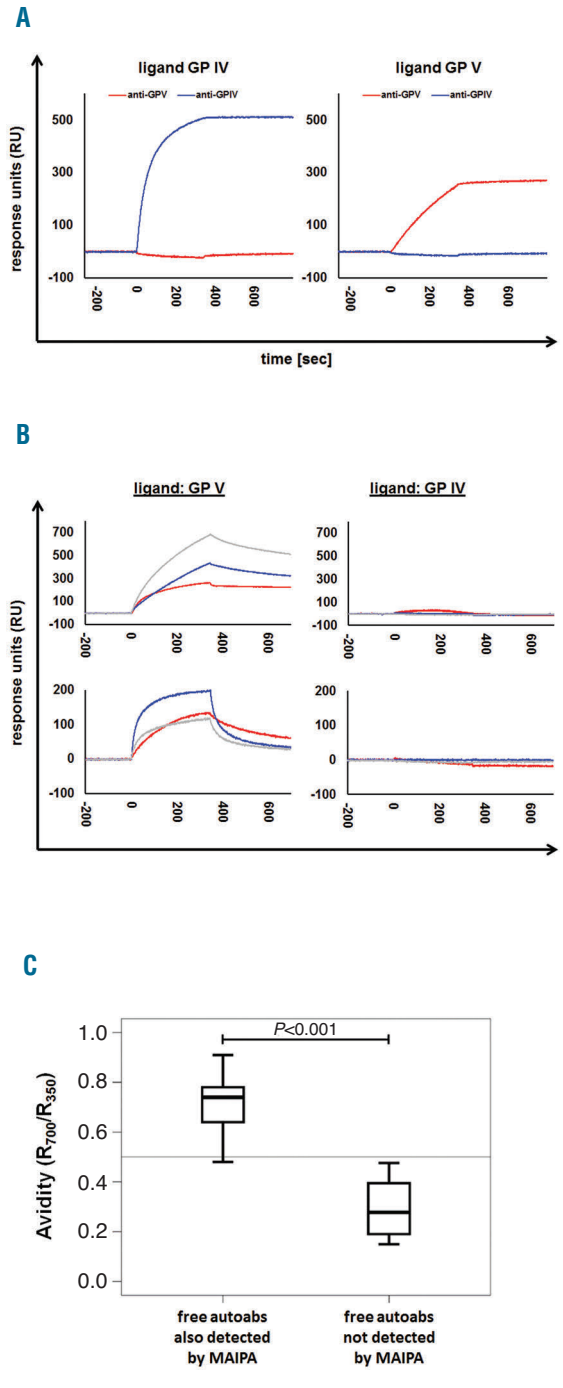

Figure 1. Detection of anti-GPV autoantibodies (autoabs) by surface plasmon resonance (SPR). (A) SPR analysis was performed on a protein interaction array system. Recombinant histidine (His)-tagged GP V and GP IV (CD36) were immobilized onto HTE sensor chips. Representative curves for the interaction of monoclonal antibodies with the respective proteins are shown. (B) Representative response curves from n=6 different IgG fractions obtained from immune thrombocytopenia (ITP) patients. (Top) Reactivity of IgG fractions from n=3 sera from patients with free autoabs that were also detectable by standard serology (monoclonal antibody immobilization of platelet antigens, MAIPA). (Bottom) Reactivity of IgG fractions from n=3 sera without detectable anti-GP V by standard serology (MAIPA). Note the difference in the maximum response units (y-axis) and the different behavior of antibodies with regard to association and dissociation characteristics. (C) Comparison of the avidity of MAIPA positive (n=29) *versus* MAIPA negative (n=59) ITP sera detected by SPR in a box-and-whisker plot with median, interquartile range, and highest/lowest value per group. Avidity was calculated as the R700/R350 rate, where R350 indicates the maximum anti-GP V antibody binding after 350 seconds (s) of association, and R700 indicates the remaining antibody binding after additional 350 s of dissociation.

removing platelets from the murine circulation than human alloantibodies (anti-HPA-1a as present in the WHO standard). These data demonstrate that anti-GP V antibodies of high or low avidity are capable of removing circulating platelets and thus represent a functionally relevant specificity of autoantibodies in ITP. To further substantiate the hypothesis that the observed effects are mediated by anti-GP V IgG, we directly compared the median human platelet survival after injection of IgG fractions prepared from one ITP serum containing anti-GP V autoantibodies only (Figure 3C), either after the absorption with recombinant glycoprotein V (rGPV; dashed line) or without (full line). The median platelet survival at t=1440 min after absorption was 48.5% (range, 44-53%) *versus* 18% (range, 11-20%) without absorption (P=0.028). This experiment supports our conclusion that anti-GP V IgG is capable of removing human platelets from the murine circulation.

Discussion

In this study, we demonstrate that GP V is a frequent immune target in ITP patients. Anti-GP V autoantibodies are detectable with the same frequency as those against GP IIb/IIIa and GP Ib/IX. Anti-GP V autoantibodies have the ability to induce a modest level of phagocytosis and to eliminate human platelets in a murine model.

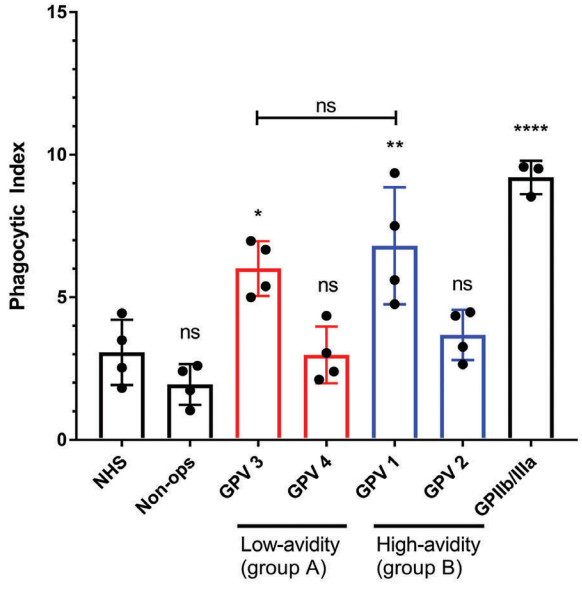

Figure 2. Phagocytosis of platelets by splenic macrophages. Healthy donor platelets were opsonized with human immune thrombocytopenia (ITP) sera containing low-avidity (group A, red bars) or high-avidity (group B, blue bars) anti-GP V autoantibodies from four different patients (n=4 each). Serum from one anti-GPIIb/IIIa-positive ITP patient was used as a positive control (n=3) and healthy donor sera [normal human sera (NHS), n=4] or phosphate buffered saline (PBS) [non-opsonized (non-ops), n=4] were used as negative controls. Human ITP splenic macrophages were isolated by CD14 positive selection from frozen adult ITP splenic single-cell suspensions and were incubated with opsonized human platelets for 40 minutes at 37°C. Phagocytosis was determined by spinning disc confocal microscopy and outside (non-phagocytosed) platelets were distinguished using an anti-GPIX antibody stain following macrophage fixation. Phagocytic index was calculated as (engulfed platelets counted / splenic macrophages counted) x 100. Error bars=Standard Deviation. Statistical significance was calculated by one-way ANOVA against NHS unless specified. NS: not significant; *P<0.05; **P<0.01; ****P<0.0001.

Autoantibodies against platelets are considered to be the major factors for platelet clearance in ITP.[25] GP IIb/IIIa and GP Ibα are generally reported as the most common antigenic targets.[26,27] We were able to analyze the amount of platelet-bound, glycoprotein-specific autoantibodies in 343 patients in parallel. Whereas anti-GP V antibodies as the sole autoantibody entity were less often detected (2.9%) than the other specificities (20.7% and 8.9%, respectively), two-thirds of all ITP patients reacted with GP V in combination with other specificities. Anti-GP V was more often seen in association with anti-GP Ib/IX (61 out of 91, 67%) than with anti-GP IIb/IIIa (10 out of 8, 12%), but all entities were clearly separable.

In contrast to platelet-bound autoantibodies, free autoantibodies in patient plasma are only rarely detectable.[28] Still, in our cohort, free anti-GP V was not less often detected than the other specificities, again, most frequently in association with other autoantibodies. Adding anti-GP V detection to the standard laboratory test would only mildly increase the overall test sensitivity (from 29.2% to 30.1% for platelet-bound glycoprotein specific autoantibodies and from 3.4% to 3.9% for free autoantibodies). In contrast to conventional testing by MAIPA, SPR technology significantly raised the test sensitivity, with no loss of specificity. Interestingly, we observed a clear difference in autoantibody avidity between those autoantibodies detected by standard serology plus SPR and those detected by SPR only. To our knowledge, autoantibodies against platelets have not been investigated for their avidity before. However, we previously demonstrated that anti-HPA-1a alloantibodies against platelets may be of low avidity and escape detection by MAIPA.[23] These antibodies had a comparable profile to the SPR-only autoantibodies detected in this study: a slow binding during the association and fast detachment during the dissociation phase. This suggests that these antibodies may become washed away in conventional test methods, whereas no-wash detection by SPR increases sensitivity. Low-avidity anti-GP V autoantibodies were able to induce platelet destruction *in vitro* and *in vivo*. This finding indicates that these antibodies, which are not detectable using conventional methods, are of clinical relevance. This observation demonstrates that low sensitivity could, in fact, be an important drawback of autoantibody testing in the laboratory. Further development of methods might be useful to increase the clinical utility of platelet autoantibody testing.[29]

Anti-GP V autoantibodies were efficient in removing platelets, regardless of their avidity; indicating that platelets loaded with anti-GP V undergo the same fate as platelets loaded with other autoantibodies.[23] The presence of anti-GP V might affect the clinical picture of ITP patients in two ways: 1) by a more efficient platelet removal because of an increased overall IgG load; or 2) by

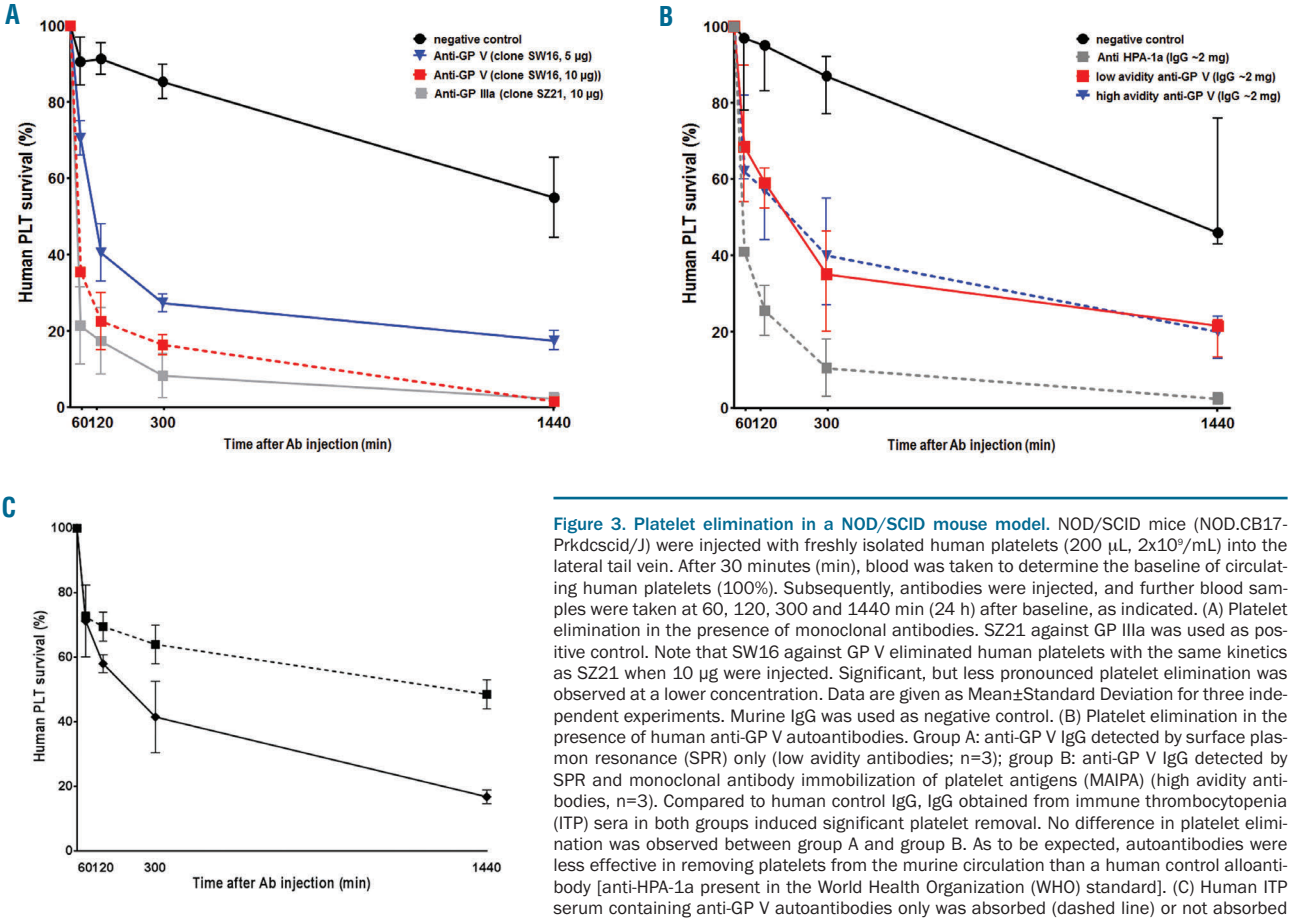

Figure 3. Platelet elimination in a NOD/SCID mouse model. NOD/SCID mice (NOD.CB17-Prkdcscid/J) were injected with freshly isolated human platelets (200 µL, 2x10^9/mL) into the lateral tail vein. After 30 minutes (min), blood was taken to determine the baseline of circulating human platelets (100%). Subsequently, antibodies were injected, and further blood samples were taken at 60, 120, 300 and 1440 min (24 h) after baseline, as indicated. (A) Platelet elimination in the presence of monoclonal antibodies. SZ21 against GP IIIa was used as positive control. Note that SW16 against GP V eliminated human platelets with the same kinetics as SZ21 when 10 µg were injected. Significant, but less pronounced platelet elimination was observed at a lower concentration. Data are given as Mean±Standard Deviation for three independent experiments. Murine IgG was used as negative control. (B) Platelet elimination in the presence of human anti-GP V autoantibodies. Group A: anti-GP V IgG detected by surface plasmon resonance (SPR) only (low avidity antibodies; n=3); group B: anti-GP V IgG detected by SPR and monoclonal antibody immobilization of platelet antigens (MAIPA) (high avidity antibodies, n=3). Compared to human control IgG, IgG obtained from immune thrombocytopenia (ITP) sera in both groups induced significant platelet removal. No difference in platelet elimination was observed between group A and group B. As to be expected, autoantibodies were less effective in removing platelets from the murine circulation than a human control alloantibody [anti-HPA-1a present in the World Health Organization (WHO) standard]. (C) Human ITP serum containing anti-GP V autoantibodies only was absorbed (dashed line) or not absorbed (full line) with recombinant GP V prior to IgG isolation. Platelet elimination was studied as outlined above. Data are given as Mean±Standard Deviation for three independent experiments.

functional effects of anti-GP V with subsequent changes in platelet reactivity. Since this was a laboratory-based study with one-stage clinical and laboratory data only, and no follow up, no definite conclusions can be drawn.

The low level of phagocytosis induced by these autoantibodies may hint at a unique mechanism of thrombocytopenia, or could indicate that a co-factor found *in vivo* but not *in vitro* (complement components, C-reactive protein, or serum amyloid A) is required.[30] Alternatively, it is possible that the highest affinity antibodies remain bound to platelets and those in the sera have lower affinity and, therefore, trigger lower levels of phagocytosis.

Antibodies against GP V could exert different functional effects on platelets: GP V is cleaved by thrombin or, following platelet activation with collagen, by ADAM17/TACE.[31,32] GP V is thought to function as a negative modulator of thrombin-induced platelet activation.[33] *In vivo* studies in mice have demonstrated that the absence of GP V increases both platelet adhesion and aggregation; but also decreases thrombus stabilization.[34] Whether any of these physiological processes are affected by anti-GP V autoantibodies is currently not known. Since we have now established GP V as an important immune target in ITP, it will be important to study whether the presence (or absence) of anti-GP V antibodies also affects treatment efficacy, as previously reported for the two other autoantibody specificities.[35,36]

This study has some limitations. Only ITP patients in whom a complete direct MAIPA test could be performed qualified. This cohort may not be representative for all ITP patients. In addition, antibodies of the IgA or IgM type, which are rarely detected in ITP,[27,28,37] were not studied. We were also unable to characterize IgG subclasses in our cohort. Whereas others have shown that the majority of anti-GPIIb/IIIa autoantibodies are of the IgG1 subclass, some IgG2, 3 and 4 have been reported.[38] The IgG subclass distribution of anti-GP V may differ from anti-GPIIb/IIIa. Finally, any blood sample taken from an ITP patient may not reflect the *in vivo* situation, since platelets sensitized with high-avidity antibodies may have been cleared (together with these antibodies) from the circulation before the sample was taken.

Despite these restrictions, we have confirmed GP V as a frequent immune target in ITP and demonstrated that anti-GP V autoantibodies are of clinical relevance since they can remove platelets from the circulation. We have also, for the first time, demonstrated that low platelet autoantibody avidity might be the main reason why current serology does not detect platelet autoantibodies more often. We would suggest that studies including GP V as an immune target are required before ITP treatment can be tailored according to platelet autoantibody specificities.

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