Immune thrombocytopenia: antiplatelet autoantibodies inhibit proplatelet formation by megakaryocytes and impair platelet production *in vitro*

Muna Iraqi,[1,2]* Jose Perdomo,[1,2]* Feng Yan,[1,2,3] Philip Y-I Choi,[1,2] and Beng H. Chong[1,2,3]

[1]Department of Medicine, St George and Sutherland Clinical School, University of New South Wales; [2]Centre for Vascular Research, University of New South Wales; and [3]Haematology Department, St George and Sutherland Hospitals, Sydney, Australia

MI and JP contributed equally to this work

ABSTRACT

Primary immune thrombocytopenia is an autoimmune disease mediated by antiplatelet autoantibodies that cause platelet destruction and suppression of platelet production. *In vitro* effects of autoantibodies on megakaryocyte production and maturation have been reported recently. However, the impact of these autoantibodies on crucial megakaryocyte functions, proplatelet formation and subsequent platelet release, has not been evaluated. We examined the effects of serum and IgG from 19 patients with immune thrombocytopenia using day 8 or 9 megakaryocytes (66.3 ± 10.6% $CD41^+$), derived from cord blood hematopoietic stem cells ($CD34^+$). The number of proplatelet-bearing megakaryocytes, the number of platelets released in the culture, total megakaryocyte numbers, ploidy pattern and caspase activation were measured at various times after treatment. After 5 days of treatment the number of proplatelet-bearing megakaryocytes was significantly decreased by 13 immune thrombocytopenia autoantibodies relative to the control group ($P<0.0001$) and this decrease was accompanied by a corresponding reduction of platelet release. Other features, including total megakaryocyte numbers, maturation and apoptosis, were not affected by immune thrombocytopenia antibodies. Treating the megakaryocytes with the thrombopoietin receptor agonists romiplostim and eltrombopag reversed the effect of the autoantibodies on megakaryocytes by restoring their capacity to form proplatelets. We conclude that antiplatelet antibodies in immune thrombocytopenia inhibit proplatelet formation by megakaryocytes and hence the ability of the megakaryocytes to release platelets. Treatment with either romiplostim or eltrombopag regenerates proplatelet formation from the megakaryocytes.

Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune disease mediated by antiplatelet autoantibodies that cause increased platelet clearance.[1] Most of these antibodies are directed against platelet membrane glycoprotein (GP) complexes, GPIIb/IIIa (CD41/CD61) or GPIbIX (CD42b and CD42a).[2,3] In some ITP patients platelet production is normal but in others it is decreased.[4,5] As megakaryocytes (MK) also express CD41/CD61 and CD42b/CD42a on their surfaces,[6,7] it has been proposed that antiplatelet antibodies might bind MK and cause their destruction, impair their function or delay their maturation and consequently interfere with platelet production.[8] *In vitro* studies showed that plasma from patients with ITP suppressed MK growth and/or maturation.[8,9] On the other hand, Yang and colleagues[10] reported that ITP plasma stimulated the production of MK, but impaired their differentiation and the production of platelets. *In vitro*, the capacity of MK to form proplatelets can be inhibited by monoclonal antibodies against CD41 and CD42b.[11] In addition, we have observed that quinine-induced thrombocytopenia sera containing anti-CD42a antibodies decreased the number of MK and suppressed their capacity to form proplatelets.[12]

On examination of bone marrow from ITP patients, Barsam *et al.* found that both responders and non-responders to treatment with eltrombopag, a thrombopoietin receptor (TPO-R) agonist, showed a boost in MK proliferation without, however, the expected increase in platelet production in the non-responders.[13] These observations may be explained by failure of eltrombopag to counter the antibody-induced defective proplatelet production in non-responding patients, suggesting that antiplatelet autoantibodies can have a direct, deleterious effect not only on MK production and maturation, but also on their crucial capacity to form proplatelets and consequently on platelet production.

Some critical aspects have not been addressed: the effect of ITP antibodies on terminal differentiation, i.e. proplatelet formation and platelet release, the effects of patients' IgG or other serum components, and the impact of TPO-R agonists on proplatelet production in the presence of ITP antibodies are yet to be investigated. We have explored these issues. MK cultures derived from human $CD34^+$ cells were used to examine the effect of ITP sera and IgG on proplatelet formation, platelet production and on several related megakaryocytic features such as viability, ploidy pattern and apoptosis. We found that a large proportion of ITP antibodies markedly

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Correspondence: j.perdomo@unsw.edu.au

decreased the number of proplatelet-bearing MK and hence the number of platelets released in culture, without altering MK proliferation, differentiation or apoptosis. A small subset of sera decreased MK numbers, inhibited maturation and enhanced caspase activation, but the corresponding patients' IgG did not recapitulate these effects. Notably, TPO-R agonists were able to overcome the inhibitory effect of several ITP antibodies on MK by enhancing their capacity to form proplatelets.

Methods

Patients and controls

Whole blood samples were collected with informed consent from 19 randomly selected patients with chronic ITP treated at St. George Hospital (Kogarah, NSW, Australia) and from nine healthy individuals (control group). The diagnosis of ITP was based on previously described criteria:[14] exclusion of other causes of thrombocytopenia, isolated thrombocytopenia and absence of hepatosplenomegaly and lymphadenopathy. The patients, nine females and ten males, were aged from 19.7 to 85.7 years (median, 53.9 years). Their details are shown in Table 1. This study was approved by the Institutional Human Ethics Committee and was conducted in compliance with the Declaration of Helsinki.

Serum preparation

Serum was obtained from coagulated whole blood by centrifugation at 1800 x *g* for 15 min. The serum was heat-inactivated at 56°C for 30 min and stored in aliquots at -80°C until required for analysis.

Purification of total IgG

The total IgG fraction was purified from ITP and normal sera using protein-G agarose beads (Roche, Germany) according to the manufacturer's instructions. The final IgG fractions were dialyzed overnight with 1 x phosphate-buffered saline at 4°C, concentrated to 10 mg/mL (within the normal range of IgG concentration in serum, which is 7-16 mg/mL)[15] and stored in aliquots at -20°C until required for analysis.

Hematopoietic stem ($CD34^+$) cell isolation and culture

Umbilical cord blood obtained from healthy donors was provided by the Sydney Cord Blood Bank (Sydney, NSW, Australia) in accordance with institutional human ethics approval. $CD34^+$ cells were isolated from cord blood mononuclear cells using a CD34 MicroBead kit (Miltenyi Biotec, Australia) according to the manufacturer's instructions. Isolated cells were cultured in Stemline II media supplemented with 50 ng/mL recombinant human thrombopoietin (rhTPO) to stimulate MK differentiation, unless otherwise stated.

Treatment of cultured cells with immune thrombocytopenia serum or IgG

After 8 or 9 days of culture, the cells were collected and counted using trypan blue exclusion staining. The cells were re-seeded at different densities to assess various aspects of MK (*Online Supplementary Figure S1*). ITP and control serum or IgG were added

Table 1. Details of ITP patients.

Patient	Antiplatelet antibodies IgG	IgM	IgA	IgG specificity	Splenectomy	Platelet count x 10^9/L	Recent medications
Group A							
ITP1	+	-	-	GPIIb/IIIa	Yes	133	None
ITP2	+	-	-	GPIIb/IIIa	Yes	1	IVIG, Prednisone
ITP3	+	-	-	GPIIb/IIIa	No	12	None
ITP4	+	-	-	GPIIb/IIIa & GPIbIX	No	30	NA
ITP5	+	+	+	GPIIb/IIIa & GPIbIX	No	80	None
ITP6*	+	-	+	GPIIb/IIIa & GPIbIX	Yes	115	NA
ITP7	+	+	+	GPIIb/IIIa	No	16	None
ITP8*	+	-	+	GPIIb/IIIa & GPIbIX	No	55	IVIG
Group B1							
ITP9	+	-	-	NA	No	11	None
ITP10	+	-	+	GPIIb/IIIa & GPIbIX	No	43	None
ITP11	+	+	+	GPIIb/IIIa & GPIbIX	Yes	28	NA
ITP12	+	+	+	GPIIb/IIIa	No	60	None
ITP13	+	+	-	GPIIb/IIIa & GPIbIX	NA	NA	NA
Group B2							
ITP14	+	-	-	GPIIb/IIIa	Yes	355	None
ITP15	-	-	-	-	No	9	None
ITP16	+	-	+	GPIIb/IIIa & GPIbIX	No	45	Danazol
ITP17	-	-	-	-	No	133	None
ITP18	-	-	-	-	No	41	None
ITP19	+	-	-	GPIIb/IIIa	No	160	None

*The presence of antiplatelet autoantibodies (IgG, IgM and IgA) was evaluated by flow cytometry using blood group O platelets. The IgG specificity was further determined with Chinese hamster ovary (CHO) cells expressing human GPIIb/IIIa (CD41/CD61) or GPIbIX (CD42b/CD42a). Fluorescence mean of the binding of each ITP serum three standard deviations (3SD) above the mean of a panel of normal sera was considered positive for the presence of antiplatelet antibodies. *Binding was done using CHO cells only; NA: not available. IVIG: intravenous immunoglobulin.*

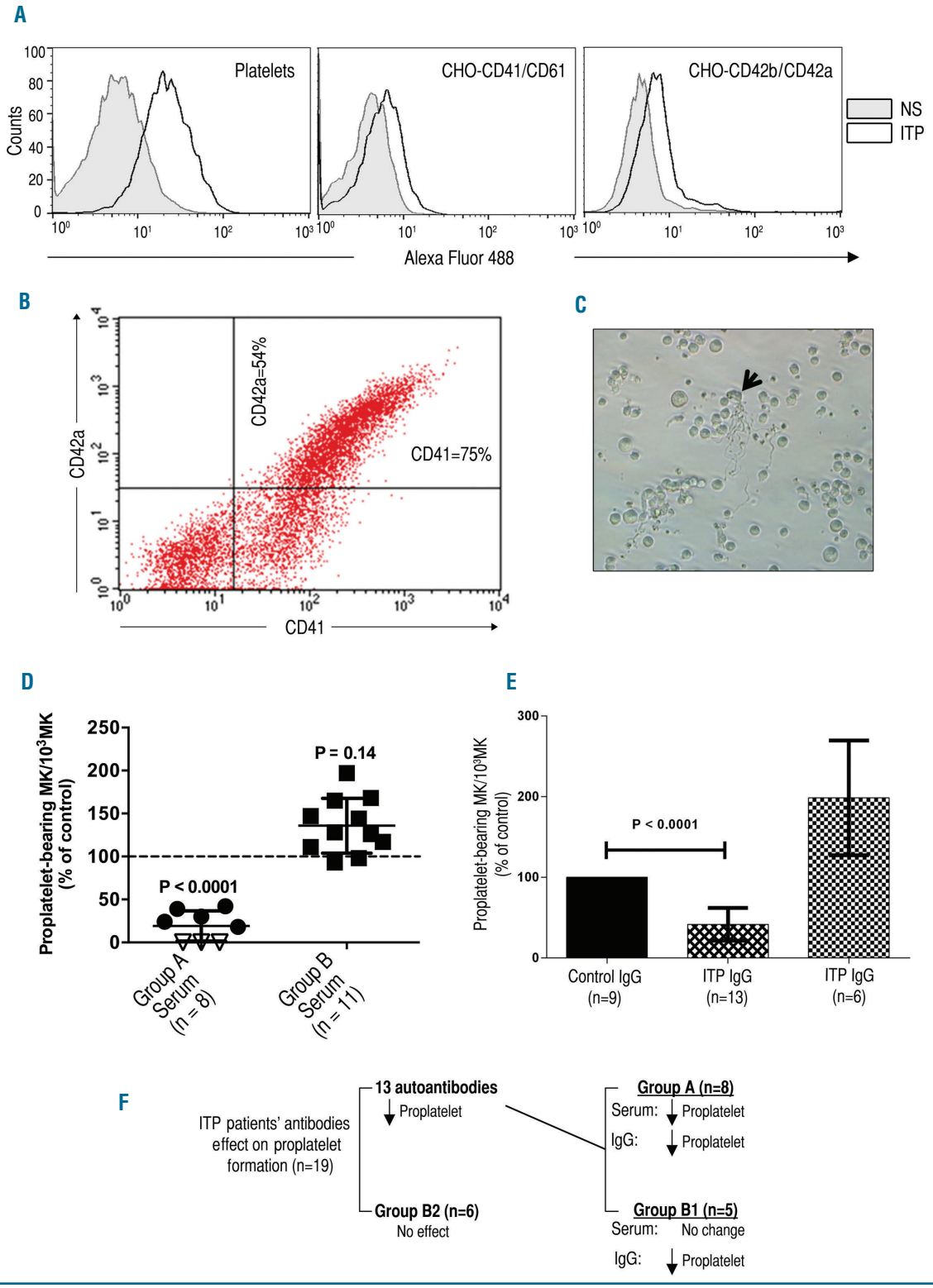

Figure 1. Groups of ITP patients based on the effect of the patients' autoantibodies on the proplatelet formation capacity of MK. (A) Representative histograms of ITP serum binding to platelets, CHO-CD61/CD41 cells and CHO-CD42b/CD42a cells. ITP sera with a fluorescence mean above 3SD that of a panel of normal sera was considered positive for the presence of antiplatelet antibodies. (B) Flow cytometry profile of day 8 cultured MK, stained with anti-human CD41-FITC and anti-human CD42a-Alexa Fluor 647. Dots in the upper right quadrant represent mature MK ($CD41^+/CD42a^+$ cells) (representation of many determinations). (C) Morphological characteristics of proplatelet-bearing MK (arrow) in culture, taken by a Leica DMIRB inverted microscope, 20X objective, using a Leica DC200 camera (representative image of many determinations). (D) Relative to the control group, eight out of 19 ITP sera reduced the number of proplatelet-bearing MK (group A, $P<0.0001$), while the remaining sera did not (group B, $P=0.14$). (E) The number of proplatelet-bearing MK was significantly reduced in MK cultures treated with IgG from 13 samples ($P<0.0001$). (F) Based on the correlation of serum and IgG results the samples were classified as follows: group A: both sera and IgG affected proplatelet formation; group B1 the effect was restricted to purified IgG only; group B2, no effect. NS: normal serum.

to each well at 1:10 dilution and the experiments were repeated three to five times for all samples to ensure the reproducibility of the findings.

Megakaryocyte proplatelet formation

At day 13 or 14 of culture, the proplatelet-bearing MK (i.e., cells with one or more cytoplasmic projections) were counted using a Leica DMIRB inverted light microscope (Leica Microsystems, Australia) at 20X magnification. In addition, the number of GPIIb$^+$ (CD41$^+$) cells was determined by flow cytometry as described in the *Online Supplementary Methods*. The number of proplatelet-bearing MK was determined per 10^3 total MK:

$$The\ number\ of\ proplatelet-bearing\ MK/10^3\ MK = \frac{proplatelet-bearing\ MK/well}{total\ GPIIb^+cells/well} \times 1000$$

To evaluate the number of proplatelet-bearing MK in the presence of TPO-R agonists, 100 ng/mL romiplostim (according to previously published data[16]) or 1 μM of eltrombopag[17] (in DMSO) were added to ITP-IgG cultures. For normal IgG without eltrombopag, DMSO was added to the culture media.

Statistical analysis

To account for variations caused by the use of different CD34$^+$ cell preparations and any other assay variations, three or four normal samples were treated identically to the ITP samples in each experiment. For data presentation, the mean of three to five trials of each ITP sample was compared to the mean of control samples used in the same experiment and expressed as percentage increase or decrease relative to control (mean ITP culture/mean control culture × 100). In the text, the results are expressed as the mean ± SD for the ITP and control groups. The probability of differences between the two groups being statistically significant was determined by the unpaired Student t-test. For ploidy analysis, the Welch t-test was applied. Statistical calculations were performed using GraphPad Prism6 software (GraphPad Software Incorporation, La Jolla, CA, USA). *P* values <0.05 were considered statistically significant.

Results

Immune thrombocytopenia serum and IgG affect megakaryocyte proplatelet formation and platelet production

The presence of antiplatelet antibodies in ITP serum was determined by flow cytometry (Figure 1A; Table 1) and their specificities were consistent with our previous findings.[2] Beside IgG, IgA and IgM antibodies were detected in eight and five patients, respectively (Table 1). The presence of HLA class-I antibodies was also analyzed (FlowPRA Class-I screening test) and the results are shown in *Online Supplementary Table S4*. Cord blood CD34$^+$ cells provide a good source for the production of functional MK when cultured with TPO in serum-free conditions.[18] The differentiation kinetics of CD34$^+$ cells cultured with rhTPO was monitored at several time points by evaluating the expression of specific MK markers (CD41 and CD42a). We observed that the level of expression of CD41 increased significantly after 8 to 9 days of culture (% CD41$^+$ cells; mean ± SD, 66.3±10.6%). We, therefore, considered these days as suitable for treating the MK with ITP sera or IgG (Figure 1B).

To determine the effects of ITP serum/IgG on proplatelet formation (Figure 1C), cultured MK were treated with either serum or purified IgG from 19 ITP patients and nine normal controls and were evaluated 5 days later. Based on the effect of their sera on the proplatelet production of MK, the ITP patients could be divided into two groups (Figure 1D): group A, comprising eight patients whose sera significantly reduced the number of proplatelet-bearing MK (P<0.0001), and group B, consisting of 11 patients whose sera did not affect the number of proplatelet-bearing MK (P=0.14). We next analyzed the correlation between serum and IgG results in terms of proplatelet formation. Unexpectedly, IgG from 13 ITP patients reduced the number of proplatelet-bearing MK (P<0.0001), while the rest had no effect (Figure 1E). The patients' samples were, therefore, finally categorized as shown in Figure 1F.

Sera from group A patients (n=8) caused significant reductions in the number of proplatelet-bearing MK/10^3MK compared to sera from the control group (mean ± SD: 2.5±2.3 *versus* 12.9±1.7; P<0.0001) (Figure 2A), while group B1 and group B2 sera did not significantly alter the number of proplatelet-bearing MK/10^3MK compared to sera from the control group (mean ± SD: 14.3±8.2 *versus* 8.9±3.5; P=0.22 and mean ± SD: 8.1±2.4 *versus* 6.7±1.7; P=0.27, respectively) (Figure 2B,C). Interestingly, three sera from group A (called ITP1, ITP2 and ITP3) completely abolished proplatelet formation (Figure 2A, open triangles). We then analyzed the potential role of the antiplatelet antibody fraction from ITP serum in the inhibition of proplatelet formation. Both serum and IgG from group A patients reduced proplatelet formation (Figure 2A) (mean ± SD: 2.6±1.9 *versus* 7.1±2.5 control IgG; P=0.0012). Although group B1 serum did not affect proplatelet formation, the purified total IgG from this group was able to reduce the number of proplatelet-bearing MK (Figure 2B) (mean ± SD: 2.7±0.99 *versus* 6.2±1.6 control IgG; P=0.003). As expected, IgG from group B2 did not significantly change the number of proplatelet-bearing MK/10^3MK (mean ± SD: 5.3±2.6 *versus* 2.7±1.4 control IgG; P=0.059), (Figure 2C). Together, these results show that purified IgG from most ITP patients leads to a marked decrease in proplatelet formation capacity of MK. To determine whether the impairment of proplatelet formation from cultured MK attenuates their ability to release platelets, we treated MK with ITP or control sera and the platelets released in culture were counted 6 days later (Figure 3A). As group A sera inhibited proplatelet formation, the number of platelets was also significantly reduced (Figure 3B) (mean ± SD: 208 ± 94×10^3 *versus* 338 ± 80 × 103; P=0.01). On the other hand, the number of platelets was not altered in cultures treated with group B sera (mean ± SD: 346±152×10^3 *versus* 262±97×10^3; P=0.14) (Figure 3B). These results are consistent with the findings on proplatelet formation.

Most immune thrombocytopenia sera and IgG have no effect on total megakaryocyte numbers and maturation

The observed decrease in proplatelet formation and platelet release could be a consequence of reduced MK proliferation and maturation in the presence of ITP autoantibodies or a direct antibody effect on proplatelet formation. After 4 days of treatment, the total number of MK and the percentage of mature (CD41$^+$/CD42a$^+$) MK were calculated.

We observed that three sera from group A (ITP1, ITP2, ITP3, which were previously found to completely inhibit proplatelet production), markedly decreased the total MK number ($CD41^+$) relative to control sera (mean ± SD: $66\pm13\times10^3$ *versus* $288\pm34\times10^3$; P=0.0005) (Figure 3C). These sera also caused a reduction in the percentage of mature MK ($CD41^+/CD42a^+$) (mean ± SD: 57.5%±1.1% *versus* 78.7%±1.2%; P<0.0001) (Figure 3D). In contrast, no significant differences in MK numbers or maturation were detected in cultures incubated with sera from all other patients (Figure 3C,D). Despite the marked effects on MK numbers and maturation exerted by ITP1, ITP2 and ITP3 sera, no significant differences were observed in cultures treated with purified IgG from these patients (Figure 3E), indicating that the effects of the sera on these two parameters were due to factors other than the autoantibodies in the serum. Consistent with the serum effects (Figure 3C,D), total IgG from all other patients had no effect on total MK numbers or maturation (*data not shown*).

Most immune thrombocytopenia sera and IgG have no effect on megakaryocyte ploidy distribution, cell size or caspase activation

The effect of treatment with both ITP serum and purified IgG on MK ploidy distribution was assessed in all patients by comparing ploidy levels in the $CD41^+$ population after 4 days of treatment in the control and treated populations. Consistent with the observations presented above for serum-treated MK, ploidy distribution was not affected significantly by most ITP sera, except for ITP1, ITP2 and ITP3, which caused significant increases in the 2N population and corresponding decreases in the percentages of higher ploidy cells (Figure 4A; *Online Supplementary Table S1*). Moreover, when the cell area was determined for selected samples, only ITP1, ITP2 and ITP3 demonstrated a significantly reduced average cell size (Figure 4B and *Online Supplementary Figure S4*), suggesting the presence of more immature cells in these cultures. Sera from group B2 patients had no effect on ploidy distribution (*Online Supplementary Figure S2A*). Unlike the shift towards lower ploidy classes observed in MK treated with ITP1, ITP2 and ITP3 sera, incubation with purified IgG from these and all other samples did not affect ploidy distribution (except for a shift towards 2N observed with ITP13) (Figure 4C; *Online Supplementary Table S1*).

Caspase activation is a feature of MK development and changes in the apoptotic status of MK have been shown to have an impact on proplatelet formation.[19] Even though caspase-9 is dispensable for proplatelet formation in mice, intrinsic apoptosis is involved in MK survival[20] and alterations of this system are, therefore, likely to affect MK function. Analysis of total caspase activation in the $CD41^+$ population revealed strong caspase activation only in cultures treated with ITP1, ITP2 and ITP3 sera (*Online Supplementary Figure S2B*). However, incubation with IgG from these and the remaining ITP patients did not alter caspase activation significantly (*Online Supplementary Figure S2C*). Collectively, the data presented here indicate that ITP IgG can affect proplatelet formation independently of changes to ploidy distribution, cell size and total caspase activation.

Autoantibody-depleted sera affect megakaryocyte function

The effects on MK numbers, differentiation, ploidy and

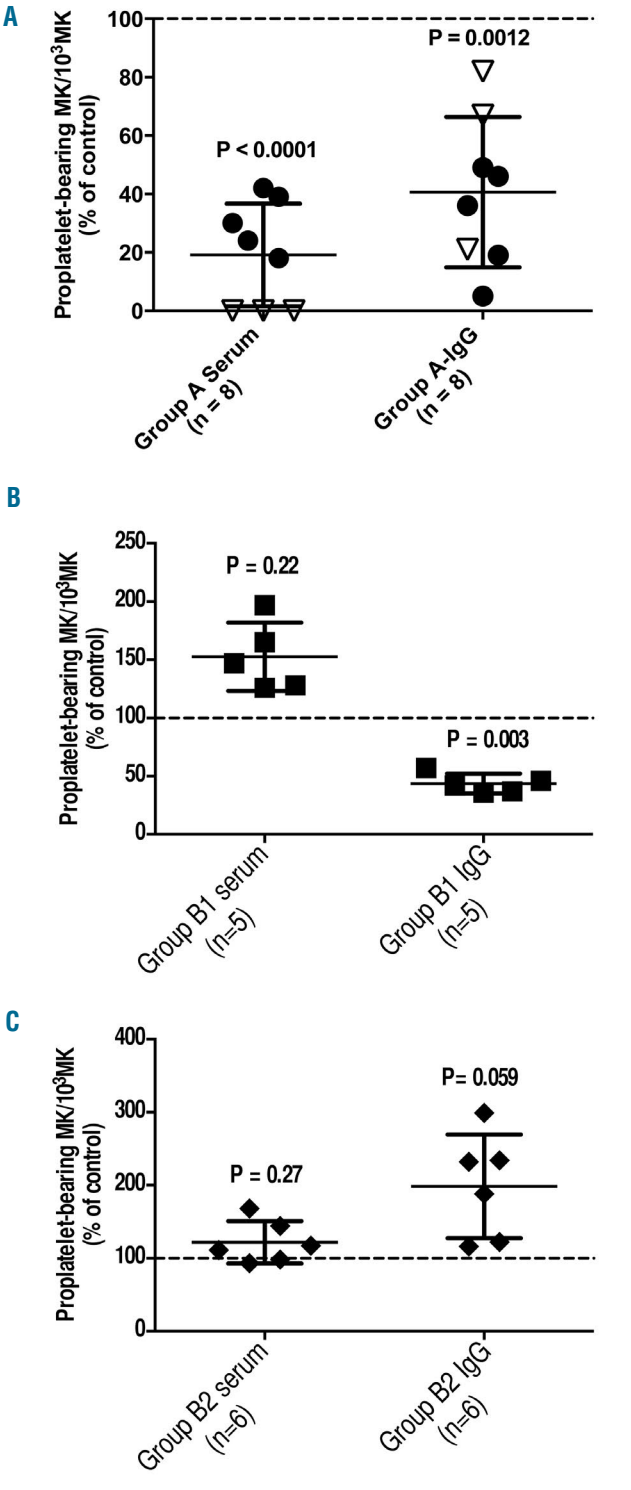

Figure 2. The effect of ITP serum and IgG on the number of proplatelet-bearing MK. (A) Compared to the control group, the number of proplatelet-bearing MK was significantly reduced in MK cultures treated with serum and IgG from group A samples (P<0.0001 and P=0.0012, respectively). Open triangles represent ITP1, ITP2 and ITP3. (B) Group B1 serum did not alter the number of proplatelets per MK (P=0.22), however proplatelet formation was inhibited by the respective total IgG (P=0.003). (C) Proplatelet formation was not decreased by either serum or IgG from group B2 samples (P=0.27 and P=0.059, respectively). % of control (proplatelet-bearing $MK/10^3MK$) = number of proplatelet-bearing $MK/10^3MK$ (ITP culture)/number of proplatelet-bearing $MK/10^3MK$ (control culture) × 100.

apoptosis exerted by ITP1, ITP2 and ITP3 sera are most likely due to factors other than specific antiplatelet IgG. To evaluate the effect of other components of these sera, ITP autoantibodies were removed from the samples by adsorption on washed platelets. The absence of antiplatelet autoantibodies in adsorbed sera was confirmed by flow cytometry (*Online Supplementary Figure S3A,B*). The autoantibody-depleted serum was used to treat MK to assess its potential effects. The depleted sera induced effects comparable to ITP serum before adsorption on megakaryocytic viability, differentiation and apoptosis (*Online Supplementary Figure S3C,E*). The detrimental impact of these sera on MK is not, therefore, due to the presence of antiplatelet autoantibodies but to other factors present in the samples.

Since several factors are known to inhibit MK development *in vitro*, particularly platelet factor 4 (PF4)[21] and transforming growth factor-β1 (TGF-β1),[22,23] we investigated whether the presence of these cytokines/chemokines in ITP1, ITP2 and ITP3 sera was different from that in control sera. PF4 levels in these ITP sera were variable but not higher than in control sera or other ITP sera (*Online Supplementary Table S2*). However, the levels of TGF-β1 were very high in ITP1 serum (*Online Supplementary Table S3A*). TGF-β1 is a negative regulator of MK proliferation[24] and it could be one of the factors affecting the MK treated with ITP1 serum.

ITP1, ITP2 and ITP3 sera enhanced MK caspase activation, and because sFas and TRAIL are apoptosis-inducing factors known to affect MK apoptosis; we measured sFas

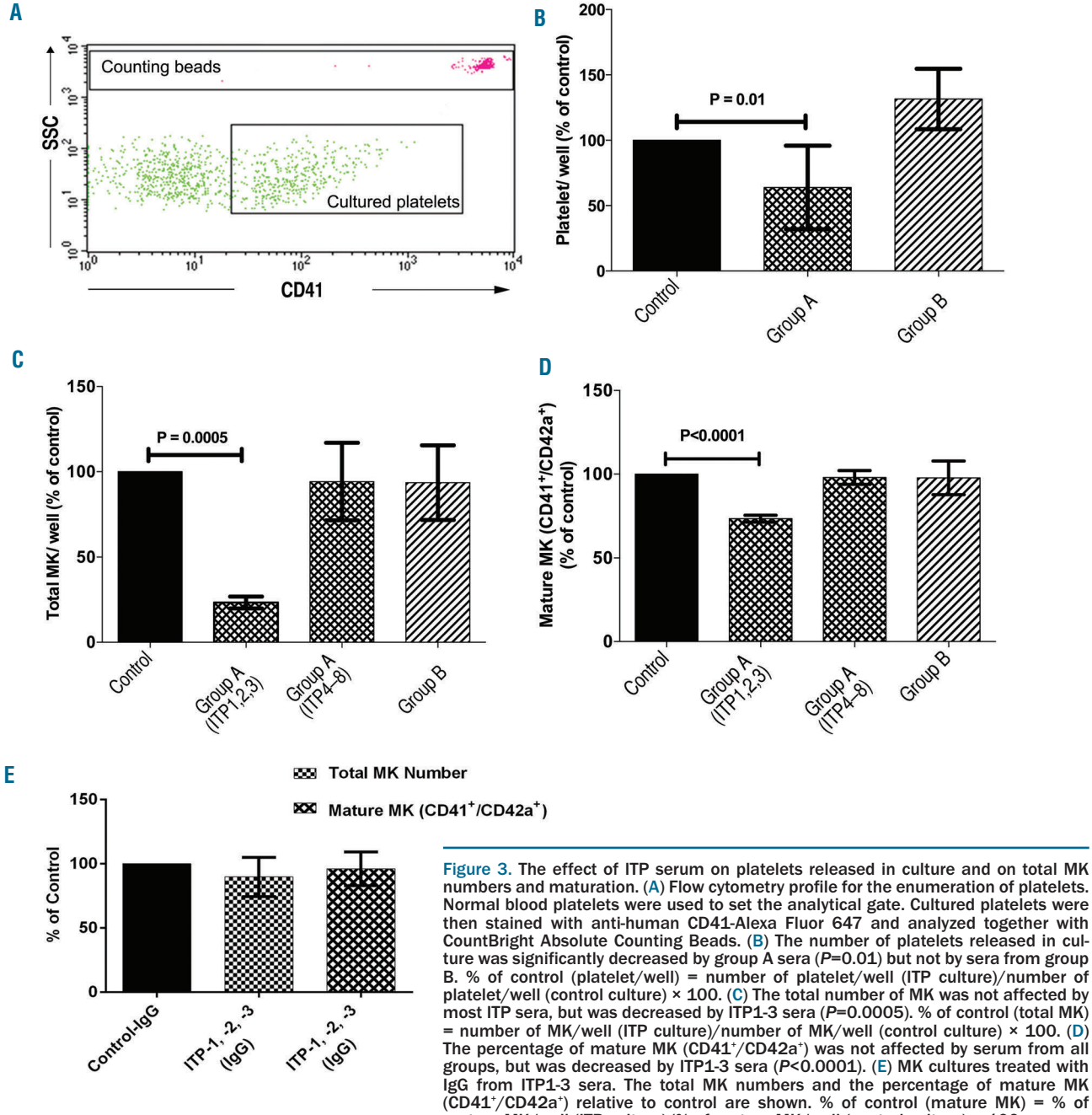

Figure 3. The effect of ITP serum on platelets released in culture and on total MK numbers and maturation. (**A**) Flow cytometry profile for the enumeration of platelets. Normal blood platelets were used to set the analytical gate. Cultured platelets were then stained with anti-human CD41-Alexa Fluor 647 and analyzed together with CountBright Absolute Counting Beads. (**B**) The number of platelets released in culture was significantly decreased by group A sera (P=0.01) but not by sera from group B. % of control (platelet/well) = number of platelet/well (ITP culture)/number of platelet/well (control culture) × 100. (**C**) The total number of MK was not affected by most ITP sera, but was decreased by ITP1-3 sera (P=0.0005). % of control (total MK) = number of MK/well (ITP culture)/number of MK/well (control culture) × 100. (**D**) The percentage of mature MK ($CD41^+/CD42a^+$) was not affected by serum from all groups, but was decreased by ITP1-3 sera (P<0.0001). (**E**) MK cultures treated with IgG from ITP1-3 sera. The total MK numbers and the percentage of mature MK ($CD41^+/CD42a^+$) relative to control are shown. % of control (mature MK) = % of mature MK/well (ITP culture)/% of mature MK/well (control culture) × 100.

and TRAIL levels in these samples. We found that the concentration of TRAIL in ITP1, ITP2 and ITP3 was not increased relative to that in normal serum although it was higher than in other ITP sera examined (*Online Supplementary Table S3B*). The sFas concentration was higher in ITP3 serum relative to normal sera, suggesting a possible role for sFas in this sample (*Online Supplementary Table S3C*). Collectively, these observations indicate that TGF-β1 and sFas may have influenced MK cultured with ITP1 and ITP3, respectively.

Thrombopoietin receptor agonist treatment counters the effect of immune thrombocytopenia IgG on proplatelet formation by megakaryocytes

The TPO-R agonists romiplostim and eltrombopag have recently been approved for the treatment of ITP.[25] Romiplostim is a peptide that binds the extracellular domain of the TPO-R, while eltrombopag is a non-peptide drug that activates the receptor by interacting with the transmembrane domain.[25] To test their activity on cultured MK, day 8-9 cells were transferred to media containing 100 ng/mL romiplostim or 1 μM eltrombopag plus either 10% ITP or normal IgG. Wells containing normal IgG without TPO-R agonists served as controls. As expected, treatment with TPO-R agonists increased the number of proplatelet-bearing MK in cultures containing normal IgG (Figure 5). Importantly, TPO-R agonists also increased the number of proplatelet-bearing MK in the presence of most of the ITP IgG samples examined (Figure 5). The evidence from these experiments suggests that TPO-R agonists promote proplatelet formation in mature MK and can overcome the deleterious effects of ITP autoantibodies.

Discussion

During maturation, MK express CD41/CD61 and CD42b/CD42a on their surfaces,[6,7,26] so it is likely that the binding of antiplatelet autoantibodies could inhibit MK maturation[27-29] and interfere with platelet release. During endomitosis, the ploidy of diploid (2N) MK precursors increases[30] and each of these mature MK is able to release thousands of platelets[31] by extending proplatelets.[32-34] Platelet production can be affected by abnormalities at any stage of MK production.[30] The impact of ITP sera on MK production and differentiation from $CD34^+$ cells has been evaluated previously,[8-10] while other work has assessed the effect of antiplatelet monoclonal antibodies[11] and drug-dependent antiplatelet antibodies[12] on proplatelet formation. Previous work did not, however, fully examine the activity of antiplatelet IgG from ITP patients on terminal MK differentiation (proplatelet formation and platelet release). Recent observations suggest that increasing MK production in ITP patients does not always correlate with a recovery of platelet counts,[13] indicating that additional factors may prevent platelet release from MK. In this study we explored the following questions: (i) Do ITP antibodies affect terminal MK differentiation, i.e. proplatelet formation and platelet release? (ii) Are these effects due to IgG or to other serum components? (iii) What is the role of TPO-R agonists on proplatelet production in the presence of ITP antibodies: do they increase megakaryocyte production or do they enhance proplatelet formation? We show that the majority of ITP antiplatelet antibodies analyzed effectively prevented proplatelet formation in MK cultures, that the effect is due to IgG antibodies and that TPO-R agonists reverse the effect of some ITP antibodies and enhance proplatelet formation.

Group A sera decreased the number of proplatelet-forming MK, and this suppression was ascribed to the presence of specific antibodies as confirmed by cultures using IgG, whereas sera and IgG from group B2 patients had no impact on proplatelet formation. On the other hand, group B1 patients seemed paradoxical: their sera had no effect on proplatelet formation but significant inhibition

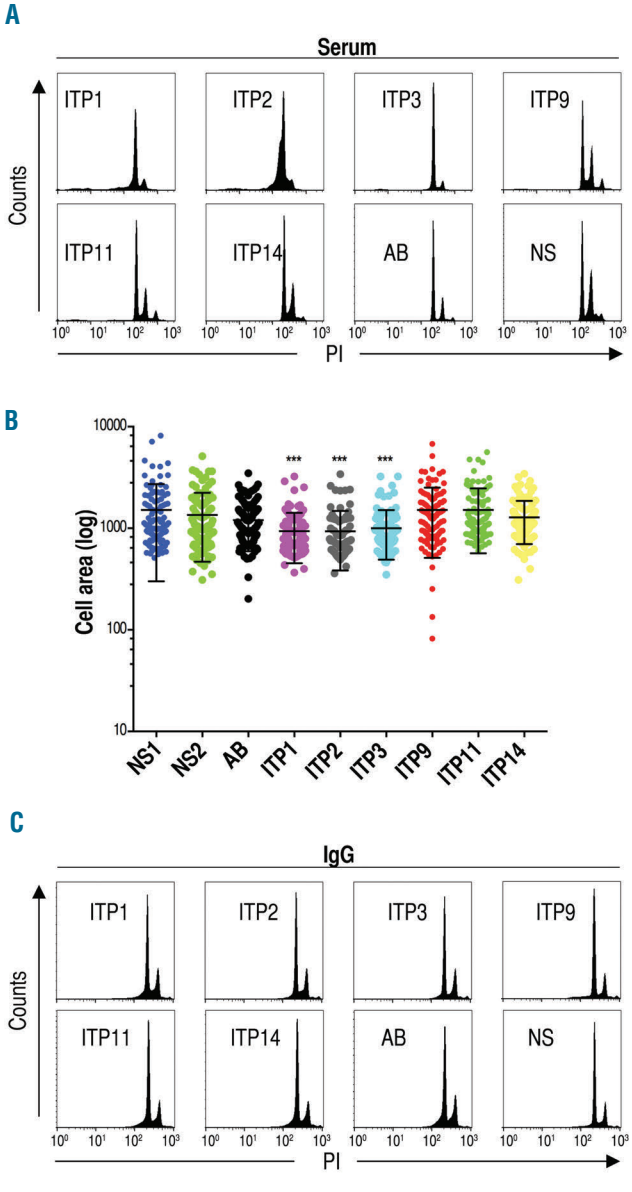

Figure 4. Effect of ITP serum and IgG on MK ploidy distribution and cell size. (A) Representative histograms showing the ploidy distribution of $CD41^+$ cells in populations treated with serum from ITP patients and controls (AB and NS). ITP1, ITP2 and ITP3 show an increase in the 2N population and a corresponding decrease in higher ploidy classes. (B) Treated cells were cytospun on slides, stained with Wright stain, imaged and the cell area calculated (arbitrary units, n=110, except for ITP2, n=88). ITP1, ITP2 and ITP3 had significantly decreased cell area relative to controls (NS1, NS2 and AB). (C) No obvious changes in ploidy distribution were observed after treatment with IgG from all groups. NS: normal serum; AB: normal pooled AB serum. *** $P<0.0001$.

was observed when MK were cultured with total IgG from these patients. These observations could be explained by the fact that: (i) the final dilution of patients' serum in the culture medium was 1 in 10 for all patients and hence the antibody concentration was effectively diluted ten times in the MK culture; (ii) in contrast, purified patients' IgG was added to the MK culture at a final concentration of 10 mg/mL which approximates the concentration of IgG in the plasma. Since the effects of an autoantibody may be dependent on its specificity, affinity and titer,[35] the antibody concentration/titer in group B1 sera might not have been sufficient to inhibit proplatelet formation. Alternatively, some cytokines that enhance megakaryopoiesis (e.g., TPO and interleukin-11)[36-38] might have induced increased proplatelet formation by group B1 sera, thus negating the effect of IgG.

There was a positive correlation between the number of proplatelet-bearing MK and the number of platelets released in culture. For instance, group A sera interfered with proplatelet formation by MK thus reducing the production of platelets (platelet release). This synchronicity between proplatelet and platelet findings supports the notion that antiplatelet autoantibodies that inhibit proplatelet formation are likely to impair platelet production.

It is worth noting that group A and group B1 sera had antiplatelet autoantibodies against either CD41/CD61 and CD42b/CD42a or only CD41/CD61 and the effect on proplatelets was independent of the type of antibody. The presence of antibodies against CD41/CD61 in three group B2 sera with no measurable effects on proplatelet formation could be related to the recognition of certain epitopes or subunits within the same receptor, which do not result in deleterious effects on MK as inferred from the fact that monoclonal antibodies against individual glycoprotein subunits may affect proplatelet formation differently.[11] Anti-CD42b antibodies may act through different mechanisms and are more resistant to intravenous immunoglobulin or steroid treatment.[39-41] However the effect on proplatelet formation appears to be independent of the antibody specificity.

In cultures with ITP1, ITP2 and ITP3 sera, proplatelet formation was completely inhibited, while the corresponding IgG only partially inhibited proplatelet formation. It is likely that the presence of apoptotic cells in the serum cultures resulted in reduced MK viability and/ or maturation. This suggests a synergistic effect between certain factors present in serum and the antiplatelet antibodies, which results in total inhibition of proplatelet production. After showing that ITP1, ITP2 and ITP3 sera caused a decrease in total MK numbers, shifted ploidy patterns to lower N classes and increased caspase activation (even after removal of the antiplatelet antibodies), we sought to ascertain the presence of factors in serum that might influence MK development. PF4 and TGF-β1 are well-characterized negative regulators of megakaryopoiesis. Although no differences were found in PF4 levels among our ITP sera and control sera, TGF-β1 levels were higher in ITP1 serum, suggesting a possible impact on MK numbers and maturation. Kuter *et al.* showed that serum TGF-β1 is a potent inhibitor of MK proliferation and ploidy *in vitro*.[23] MK development and platelet release require cell proliferation and maturation and may also involve apoptosis.[42] TRAIL and sFas are apoptosis-inducing members of the tumor-necrosis factor family. TRAIL levels in ITP1, ITP2 and ITP3 sera were comparable to those in control sera while the concentration of sFas was increased in ITP3 serum. Thus, TGF-β1 and sFas may possibly have affected megakaryopoiesis in ITP1 and ITP3, respectively. The expression of Fas ligand on the MK surface stimulates programmed cell death after stimulation of certain pathways within MK.[43]

Treatment of MK with TPO-R agonists (romiplostim or eltrombopag) in the presence of ITP autoantibodies neutralized the antibody effect and enhanced MK proplatelet formation capacity, demonstrating an effect at the level of mature MK. TPO-R agonists engage the c-Mpl receptor, stimulate MK *in vitro*,[44] and increase platelet production *in vivo*.[25] However, it is unclear how these drugs increase platelet counts in patients with ITP. Is the increase in platelet numbers a reflection of enhanced MK differentiation? Or, is the same number of MK producing more platelets? There is evidence of enhanced MK production after romiplostim treatment,[45] while other observations suggest that increases in platelet production occur without a change in MK mass.[46] Our results provide experimental evidence that TPO-R agonists may increase platelet production in ITP by boosting the number of proplatelet-bearing MK within an existing MK population. Although most ITP samples tested responded similarly to both romiplostim and eltrombopag (either positively or negatively) (Figure 5) there was one, namely ITP7, which responded differently (responding to romiplostim but not to eltrombopag). This suggests that the response to TPO-R agonists may be determined by the nature/specificity of the autoantibody.

Examination of the bone marrow of ITP patients after eltrombopag treatment revealed an increment in MK proliferation in both responders and non-responders, implying that MK proliferation alone does not guarantee recovery of proplatelet and platelet production.[13] These observations suggest that in culture conditions ITP antibodies affect mainly the late stages of MK differentiation (proplatelet formation) rather than proliferation or early differ-

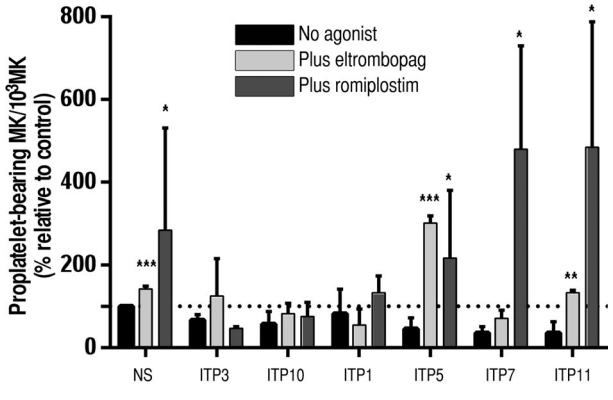

Figure 5. Romiplostim and eltrombopag treatment reverses the effect of ITP IgG on MK proplatelet formation capacity. Day 8-9 MK were cultured with 20 ng/mL rhTPO plus 100 ng/mL romiplostim or 1 μM eltrombopag and control or ITP IgG. Normal IgG without TPO-R agonists was used as the reference control. % of control (proplatelet-bearing MK/10³MK) = number of proplatelet-bearing MK/10³MK (ITP culture + TPO-R agonist)/number of proplatelet-bearing MK/10³MK (control culture without TPO-R agonists) × 100. NS, normal serum IgG. *0.01<*P*<0.05; ** 0.01<*P*<0.001; ****P*<0.0001.

entiation phases.

In agreement with our work, it was recently shown that ITP autoantibodies inhibit proplatelet formation without changes to apoptosis.[47] The differences between our and previous[8-10,47] reports regarding MK numbers and maturation may be related to the following factors: (i) our treatments with ITP sera and IgG were carried out in cultures containing already differentiating MK, i.e. cultures with over 50% $CD41^+$ cells; (ii) even though most of our patients had similar specificities of antiplatelet autoantiobodies to those previously reported, their target epitope/subunit, titer and avidity could be different; (iii) the subjects in the study by Chang *et al.* mostly had acute childhood ITP[9] and the pathophysiology of this condition may be somewhat different from that of chronic adulthood ITP.

In conclusion, autoantibodies from the majority of the ITP patients in this study inhibited *in vitro* proplatelet formation by MK and their subsequent ability to produce platelets, suggesting that an analogous mechanism may operate *in vivo*. We are now investigating the mechanisms by which antiplatelet autoantibodies inhibit proplatelet formation. These findings will contribute to a more complete understanding of the pathophysiological mechanisms of ITP.

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Authorship and Disclosures

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