bjh research paper

Impaired proplatelet formation in immune thrombocytopenia: a novel mechanism contributing to decreased platelet count

Paola R. Lev,[1]* Matías Grodzielski,[1]* Nora P. Goette,[1] Ana C. Glembotsky,[1] Yesica R. Espasandin,[1] Marta S. Pierdominici,[2] Geraldine Contrufo,[1] Verónica S. Montero,[3] Luciana Ferrari,[1] Felisa C. Molinas,[1] Paula G. Heller[1] and Rosana F. Marta[1]

[1]Departamento de Hematología Investigación, Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), [2]Departamento de Hematología, Hospital Ramos Mejía, and [3]Departamento de Análisis Clínicos, Centro de Educación Médica e Investigación Clínica "Norberto Quirno" (CEMIC), Buenos Aires,Argentina

Received 12 December 2013; accepted for publication 5 February 2014
Correspondence: Rosana F. Marta, Departamento de Hematología Investigación, Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires, CONICET, Combatientes de Malvinas 3150, Buenos Aires C1427ARO, Argentina.
E-mail: rfmarta2005@gmail.com
*PRL and MG equally contributed to this study.

Summary

The pathophysiological mechanisms contributing to the decreased platelet count in immune thrombocytopenia (ITP) are not entirely understood. Here, we investigated the key step of proplatelet formation (PPF) by studying the effect of ITP plasma in thrombopoiesis. Normal cord blood-derived mature megakaryocytes were cultured in the presence of recalcified plasma from ITP patients, and PPF was evaluated by microscopic analysis. Patient samples induced a dose-dependent inhibition in PPF, as well as decreased complexity of proplatelet architecture. Although slightly increased, plasma-induced megakaryocyte apoptosis was not related to PPF impairment. Purified IgG reproduced the inhibitory effect, while platelet-adsorbed plasma induced its reversion, suggesting the involvement of auto-antibodies in the inhibition of thrombopoiesis. Impaired PPF, induced by ITP plasmas bearing anti-GPIIb-IIIa antibodies, was related to their ability to interfere with the normal function of this integrin, as assessed by megakaryocyte PAC-1 binding and β3 integrin phosphorylation while the presence of anti-glycoprotein Ia-IIa auto-antibodies was associated with loss of normal inhibition of PPF induced by type I collagen. In conclusion, abnormal thrombopoiesis comprising decreased PPF and morphological changes in proplatelet structure are induced by patient samples, unveiling new mechanisms contributing to decreased platelet count in ITP.

Keywords: auto-antibodies, immune thrombocytopenia, thrombopoiesis, proplatelets, platelets.

Immune thrombocytopenia (ITP) is an acquired autoimmune disorder characterized by isolated low platelet count. The currently identified underlying mechanisms leading to the decreased number of circulating platelets are peripheral destruction due to auto-antibody binding to platelet antigens and suppression of megakaryopoiesis (McMillan, 2009). The main antigenic targets include the fibrinogen receptor glycoprotein (GP) IIb-IIIa, von Willebrand receptor GPIb-IX and the collagen receptor GPIa-IIa. It has been shown that auto-antibodies present in plasma from some patients with ITP inhibit *in vitro* megakaryocyte (MK) production (McMillan *et al*, 2004), suggesting that a similar effect may occur *in vivo*.

The final step in which mature MKs give raise to platelets represents a unique cellular transformation event characterized by the formation of cytoplasmic extensions bearing periodic platelet-sized swellings connected by thin shafts, called proplatelets (PP). These PP elongate, branch and release platelet-like structures from their distal tips, finally consuming the MK cytoplasm. During this process, integrins and adhesion molecules, which play key roles in platelet activation, display different functions modulating platelet birth (Larson & Watson, 2006; Kanaji *et al*, 2012). The involvement of GPIIb-IIIa as well as GPIb-IX in platelet formation is evidenced by the deep abnormalities observed during thrombopoiesis in patients with macrothrombocytopenia due to GPIIb-IIIa mutations inducing partial activation of the integrin (Ghevaert *et al*, 2008; Kunishima *et al*, 2011; Bury *et al*, 2012) and in Bernard–Soulier syndrome (Balduini *et al*, 2009; Strassel *et al*, 2009) respectively.

Megakaryocyte behavior within the bone marrow relies, at least partially, on matrix composition. Type I collagen plays an essential role in inhibiting megakaryocyte PP formation through its binding to GPIa-IIa and the subsequent activation of the Rho-ROCK pathway (Sabri *et al*, 2004), thus, preventing

  doi:10.1111/bjh.12832

premature platelet release in the osteoblastic niche. This physiological regulation is lost in *MYH9*-related disease, supporting the notion that the failure of this mechanism contributes to the pathogenesis of this disorder (Pecci *et al*, 2009). Thus, it is not surprising that monoclonal antibodies directed against specific megakaryocytic antigens influence proplatelet formation (PPF) (Takahashi *et al*, 1999).

Dysregulation of MK apoptosis has been proposed as a mechanism contributing to decreased platelet production in ITP, although opposite theories were raised. Houwerzijl *et al* (2004) found ultrastructural abnormalities compatible with (para-) apoptosis in bone marrow MKs, implying that MK damage could reduce platelet production in ITP. On the other hand, Yang *et al* (2010) described lower expression of tumour necrosis factor-related apoptosis-inducing ligand, higher expression of BCL2L1 (Bcl-xL) and lower platelet release in normal MKs incubated with ITP plasma, pointing to decreased apoptosis as a contributing factor to dysmegakaryopoiesis and reduced platelet production. Concerning the specific process of platelet generation, Josefsson *et al* (2011) demonstrated that normal mature MKs must restrain the intrinsic apoptotic pathway to survive and progress safely through PPF and platelet shedding.

The present study was designed to investigate proplatelet formation by mature MKs in ITP. We hypothesized that the key step of thrombopoiesis could be altered by the presence of auto-antibodies in this disorder. In addition, we evaluated the influence of ITP plasma on normal mature MK survival. Our results showed quantitative as well as qualitative abnormalities in PP production in the presence of ITP plasma, unveiling new mechanisms contributing to the development of thrombocytopenia in ITP.

Materials and methods

Patients and blood samples

Twenty-one patients with chronic ITP (median age, 42 years, range 21–80) diagnosed according to current criteria (Rodeghiero *et al*, 2009) were included. The study was approved by the Ethics Committee from Instituto de Investigaciones Médicas Alfredo Lanari. Clinical and laboratory data are presented in Table I.

Blood samples were drawn after written informed consent was obtained in accordance with the Declaration of Helsinki as follows: 10 ml were collected into 342 mmol/l EDTA and 10 ml into 129 mmol/l sodium citrate. Cord blood was obtained following normal pregnancies and deliveries at Hospital Materno-Infantil Dr Gianantonio upon written informed consent of the parents.

Preparation of recalcified plasma

Platelet-poor plasma was obtained from sodium citrate-anticoagulated blood by centrifugation at 700 ***g*** for 20 min and a second centrifugation at 10000 ***g*** for 10 min at 4°C. Then, plasma was recalcified with 25 mmol/l $CaCl_2$ for 2 h at 37°C. The clot was removed and samples were centrifuged at 10000 ***g*** for 10 min at 4°C. Supernatants were stored at −70°C until use.

Auto-antibody evaluation

The specificity of the auto-antibodies was evaluated on EDTA-anticoagulated plasma processed using PAKAUTO kit (GTI Diagnostics Inc., Waukesha, WI, USA), according to the manufacturer's instructions. This methodology allows the detection of auto-antibodies (IgG, IgM or IgA subclasses), identifying those directed against GPIIb-IIIa, GPIb-IX and GPIa-IIa.

Adsorption of auto-antibodies from ITP plasma

Recalcified ITP plasma (0·5 ml) was incubated with washed control platelets (2×10^9/ml) at room temperature (RT) for 1 h. After centrifugation at 2200 ***g*** for 10 min, the supernatant plasma was incubated with fresh platelets under the same conditions. Platelet-adsorbed plasma was then analysed for the presence of auto-antibodies as described above and stored at −70°C until use. Normal control plasma was processed simultaneously under the same conditions.

IgG purification

IgG was purified from patient and control plasma by affinity chromatography, using protein G Sepharose (GE Healthcare Bio-Science Corp. Piscataway, NJ, USA) and further washed and concentrated using ultrafiltration centrifugal devices (Thermo Scientific, Rockford, IL, USA). IgG fractions were resuspended in the initial volume with phosphate-buffered saline (PBS), measured at 280 nm and stored at −70°C until use.

Cell culture

CD34-positive cells were obtained from umbilical cord blood and cultured for 13 d to obtain mature MKs, as described previously (Balduini *et al*, 2008). Cultures with at least 90% MK, as established by fluorescein isothiocyanate (FITC)-conjugated anti-CD61 percentage of positive cells (BD Bioscience, San José, CA, USA), were used.

Proplatelet count. To evaluate the effect of recalcified and platelet-adsorbed ITP plasma, as well as IgG fractions, on thrombopoiesis, 1×10^4 mature MKs were cultured with the addition of variable amounts of either ITP or control samples. After 24 or 48 h, the total number of MK producing proplatelets was counted under an inverted microscope (Axiovert 25; Carl Zeiss GmbH, Göttingen, Germany) using a 32× objective, and the percentage of proplatelet-bearing MKs was calculated on the basis of the viable cell count and

Table I. Clinical and laboratory data from ITP patients.

Patient no.	Platelet count ($\times 10^9$/l)	Type of auto-antibody			Treatment
		Anti-GPIIb-IIIa	Anti-GPIb-IX	Anti-GPIa-IIa	
1	46	−	−	−	None/Spl.
2	30	+	−	−	None
3	38	−	−	−	None
4	30	−	−	−	Corticosteroids
5	52	−	−	+	None/Spl.
6	85	−	−	−	Danazol
7	6	+	−	−	None
8	30	−	−	−	Corticosteroids
9	10	−	−	−	None/Spl.
10	16	−	+	−	Corticosteroids/Spl.
11	29	−	−	−	Corticosteroids/Spl.
12	30	+	−	−	Azathioprine/Spl.
13	40	+	−	+	Corticosteroids
14	16	−	−	−	None
15	48	−	−	−	Corticosteroids
16	50	+	−	−	Corticosteroids
17	30	−	−	−	None
18	22	−	−	−	Corticosteroids/Spl.
19	18	−	−	−	Anti-D/IVIg
20	36	−	−	−	None
21	48	+	−	+	None/Spl.

Platelet count and treatment at the time of study. Spl: splenectomy; IVIg: intravenous immunoglobulin.

the purity of CD61+ cells in the culture. Although PPF inhibition by ITP samples was observed as early as 24 h, further studies were performed at 48 h because of the higher percentage of PP observed. For some experiments, patient plasma was diluted with normal plasma, and the effect of serial ITP plasma dilutions in PPF was evaluated. All samples were processed in duplicate and at least in two separate experiments using different cord blood samples. MK maturation after a 48-h incubation with recalcified plasma was assessed by flow cytometry using anti-CD61-FITC and phycoerythrin (PE)-conjugated monoclonal antibody against GPIb (CD42b) (BD Biosciences).

PPF inhibition on type I collagen. In order to test inhibition of PPF by type I collagen, mature MKs were seeded on cell culture wells previously coated with 25 μg/ml type I collagen (kindy provided by Prof. Tira and Dr Gruppi, University of Pavia or by BD Bioscience) as described (Balduini *et al*, 2008). Ten percent recalcified plasma or 2% IgG from ITP patients bearing anti GPIa-IIa auto-antibodies and from normal controls was added and incubated for 48 h, after which PP count was performed. Results on type I collagen were expressed as fold-change compared to that of MK seeded on uncovered wells.

Morphological analysis of MKs and proplatelets

To assess PP morphology, PP count on fibrinogen matrices and analysis of MK maturation stages, mature MKs were seeded at a concentration of 1×10^5 cells/well on glass coverslips previously coated with 100 μg/ml fibrinogen (Sigma-Aldrich, St. Louis, MO, USA) (Balduini *et al*, 2008). Cells were incubated for 48 h with 10% recalcified plasma from either ITP patients or normal controls. Then, cells were fixed with 3% paraformaldehyde for 20 min at RT, permeabilized with 0·2% Triton X-100 for 6 min, and stained with 62·5 ng/ml anti-CD61-FITC in PBS for 1 h. In order to evaluate MK apoptosis, samples were simultaneously labelled with rabbit anti-active-caspase 3 antibody, (Cell Signaling Technologies Inc., Danvers, MA, USA) followed by incubation with rhodamine-conjugated goat anti-rabbit secondary antibody (Thermo Scientific, Rockwood, TN, USA). Nuclear staining was performed with 100 ng/ml Hoechst 33258 (Sigma-Aldrich) for 4 min at RT. Samples were mounted in ProLong gold Antifade Reagent (Molecular Probes Inc, Eugene, OR, USA) and analysed under an epifluorescent microscope (Carl Zeiss GmbH) using a 40× or a 100× oil-immersion objective. Microphotographs were obtained using a digital camera (Canon Power Shot G6, Tokyo, Japan). MKs were categorized into maturation stages according to standard morphological criteria (Williams & Levine, 1982). For PP analysis, the number of PP extensions, branching points, swellings and platelet-like structures (tips) on each proplatelet-bearing megakaryocyte was counted. PP measurements comprising length and maximum shaft thickness were performed with the VideoTesT-Master Morphology image analysis software (St. Petersburg, Russia). For PP morphology, at least 40 MKs producing PPs were analysed in each

sample; for evaluation of chromatin condensation, at least 100 MKs were evaluated and for PP count on fibrinogen, at least 1000 MKs were counted in each sample.

Activated caspase-3 and -7 detection by fluorochrome-labelled inhibitors of caspases (FLICA)

In order to quantitatively evaluate active caspase-3 and -7, MK previously incubated with recalcified normal or ITP plasma for 48 h, were tested with the CaspaTagTM caspase-3/7 In situ assay kit (Chemicon International, Millipore, Billerica, MA) according to the manufacturer's instructions. MKs stimulated with 24 μmol/l A23187 (Sigma-Aldrich) for 1 h were processed as positive controls.

GPIIb-IIIa functional studies

PAC-1 binding. Normal mature MKs were incubated with 10% recalcified plasma for 24 h at 37°C in 5% CO_2. After incubation, MKs were washed and stimulated with 20 μmol/l ADP/20 μmol/l epinephrine, in the presence of FITC-conjugated PAC-1 (antibody recognizing the active site of the glycoprotein) (BD Bioscience) and anti-CD42b-PE, for 5 min at RT. Cells were then fixed in 1% paraformaldehyde, diluted in PBS and acquired in a flow cytometer (BD Biosciences). The MK population was selected as anti-CD42b-PE positive cells. Irrelevant staining was determined using FITC-conjugated mouse IgM (BD Biosciences). Basal GPIIb-IIIa activation was assessed in unstimulated MK processed as described above.

Detection of β3 integrin phosphorylation by western blot. 10^6 mature MKs were stimulated with 8 μmol/l ADP, 32 μmol/l epinephrine and 3 μmol/l TRAP, in the presence of 100 μg/ml fibrinogen and 10% recalcified either control or Patient 2 plasma for 15 or 30 min at 37°C in 5% CO_2. Cells were then washed, lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mmol/l Tris–HCl, 150 mmol/l NaCl, 1% NonidetP-40, 0·5% sodium deoxycholate, 0·1% sodium dodecyl sulphate [SDS]), resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose. Membranes were tested with rabbit polyclonal anti-phospho-β3 (Y773) (Abcam, Cambridge, UK) or mouse anti-β actin (Sigma Aldrich), and immunoreactive bands were detected using anti-rabbit (Cell Signaling Technology) or anti-mouse horseradish peroxidase-conjugated antibodies (Sigma Aldrich), respectively, followed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Buckinghamshire, UK). Protein bands were quantified by densitometry.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Differences between data obtained in the presence of ITP samples and normal controls were assessed using unpaired *t*-test or Mann–Whitney–Wilcoxon test. Reference values were taken as the mean value of normal population ±2SD. Relationship between two sets of variables was determined by Pearson or Spearman correlation. P values <0·05 were considered statistically significant.

Results

Plasma from ITP patients inhibit normal proplatelet formation

To investigate whether abnormal thrombopoiesis represents an additional mechanism contributing to thrombocytopenia in ITP, 10% of either ITP or normal recalcified plasma was added to normal mature MKs obtained at day 13 of culture and PP count was carried out after 48 h, during peak PPF. ITP plasma induced inhibition of PPF compared to control plasma, 2·57 ± 1·53% vs. 4·93 ± 1·03%, $P < 0·0001$, unpaired *t*-test (Fig 1A). In particular, PP counts in the presence of 10 of 21 patient samples were below the reference range, as established by mean ± 2SD PP counts obtained after incubation with normal plasma. Representative images of cultured cells in the presence of 10% patient and control plasma are shown in Fig 1B and C respectively, and time-course inhibition in PPF is depicted in Fig 1D, showing that ITP plasma-induced decrease in PPF is evident as early as 24 h. Percentage of PPF obtained on a fibrinogen-coated surface (not shown) correlated to that observed in cell suspension cultures ($P = 0·0004$, Spearman correlation), indicating that abnormalities induced by ITP plasma may be reproduced when MKs are plated on this extracellular matrix protein.

Proplatelets production observed in the presence of plasma from untreated patients did not differ between that produced with plasma from patients who were under different treatments, 3·33 ± 1·47% vs. 2·47 ± 1·63%, $P = 0·221$, unpaired *t*-test (see Table I for individual platelet count and treatment) and no relationship was found between PP counts in the presence of ITP plasma and patient peripheral platelet counts ($P = 0·62$, Pearson correlation). Of note, all but one patient included in this study had platelet counts below 50×10^9/l.

As ITP plasma has been previously shown to affect MK maturation when added at the beginning and throughout the culture period (McMillan *et al*, 2004), we assessed whether ITP plasma added to day 13-MK and incubated for a further 48 h induced similar effects. CD61 and CD42b expression analysis showed no difference between MK cultured with patient and control samples (CD61 patients, 89·4 ± 5·2%, controls, 90·5 ± 2·4%; CD42b patients, 72·9 ± 4·5%, controls, 74·5 ± 9·0%; $P = 0·43$ and $P = 0·51$ respectively, Mann–Whitney–Wilcoxon), which was confirmed by evaluation of MK maturation stages by immunofluorescent microscopy (not shown). These results indicate that inhibition in thrombopoiesis under these experimental conditions is not due to decreased MK maturation but, rather, to a direct effect of ITP plasma on PPF.

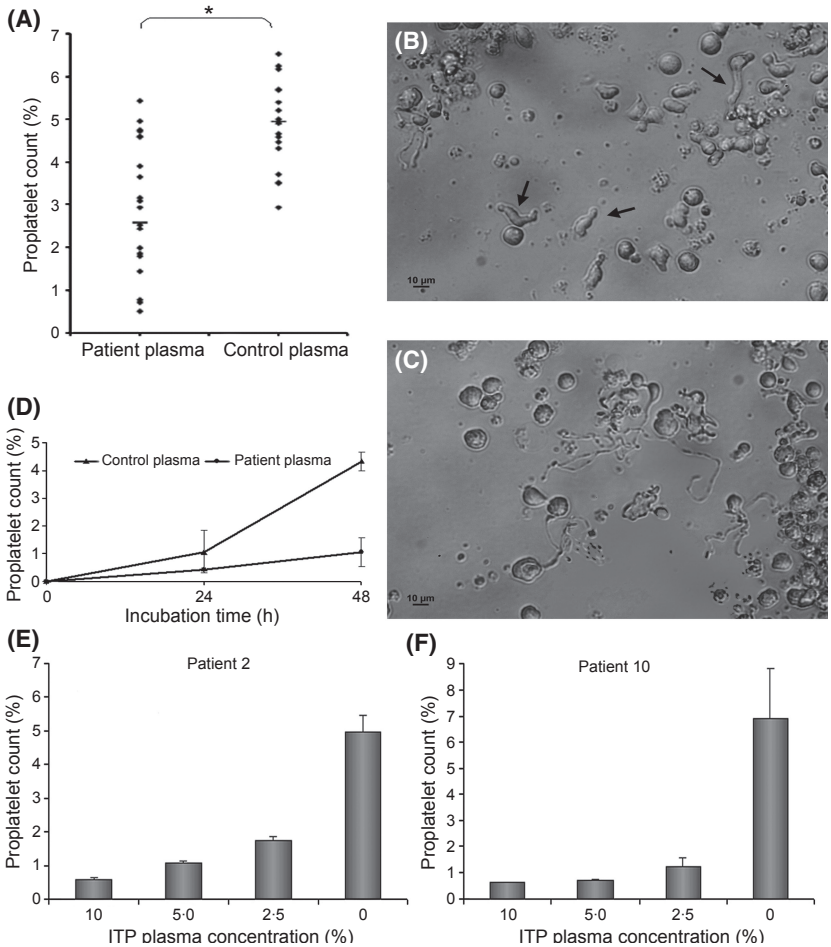

Fig 1. Proplatelet count in the presence of ITP plasma. CD34+ cells from normal cord blood were cultured for 13 d as described, to obtain mature megakaryocytes (MKs). Then, 10% of either immune thrombocytopenia (ITP; $n = 21$) or normal ($n = 18$) recalcified plasma was added and proplatelet (PP) count was carried out by microscopic observation under an inverted microscope (Carl Zeiss). (A) Percentage of proplatelet formations (PPF) after 48 h of culture was calculated on the basis of CD61+ (flow cytometry) living cells (excluding Trypan blue stain), and plotted (*$P < 0{\cdot}0001$, unpaired *t*-test). Dots represent mean value of at least two separate experiments using different cord blood samples performed in duplicate. (B) Representative pictures of MKs cultured in the presence of recalcified ITP plasma or (C) normal control plasma. Images were captured with a 32× objective, photographed with an Olympus DP70 CCD camera (Olympus) and acquired through Lumina Vision software (Mitani, Fukui, Japan); Arrows in B indicate atypical PP features observed in the presence of patient plasma. (D) Representative example of time-course PPF in the presence of control and patient plasma. (E and F) Dose-dependent PP inhibition induced by plasma from two ITP patients (ITP plasma dilutions were performed with control plasma). Results represent mean ± standard deviation of at least two independent experiments performed in duplicate.

In order to establish whether PP inhibition was dependent on plasma concentration, PPF from normal MKs was evaluated in the presence of serial plasma dilutions from two patients who had both shown a strong inhibition in PPF when previously tested at 10%. Inhibition of PP count was dose-dependently attenuated at increasing plasma dilutions by both samples, and was evident even when ITP plasma was tested at 2·5% (Fig 1E,F).

Proplatelet morphology from normal megakaryocytes in the presence of ITP plasma

With the aim of studying if plasma from ITP patients also induced qualititative abnormalities in thrombopoiesis, mature MKs cultured from day 13 to 15 on a fibrinogen matrix and in the presence of recalcified either ITP or control plasma, were stained with anti-CD61-FITC, and PP architecture was analysed. Proplatelet-bearing MKs obtained after culture with ITP plasma ($n = 12$) revealed atypical structural features characterized by decreased PP length and increased shaft thickness, poorly delimited swellings along the PP length, decreased branching, lower number and poorly delimited PP tips and, overall, a lower complexity of PP processes. Examples and numerical data are shown in Fig 2A–D and E–G, respectively. Statistical differences between patients and controls were observed for all these parameters. These atypical features can be observed also in pictures taken in cell suspension culture (Fig 1B). Overall, these results suggest that abnormal thrombopoiesis comprises not only a lower number of MKs capable of producing PP, but also structural changes in PP architecture impairing platelet production.

Evaluation of normal mature megakaryocyte apoptosis induced by ITP plasma

To investigate if abnormal platelet production in ITP is related to apoptosis of mature MKs, chromatin condensation and caspase activation were evaluated in normal MKs in the presence of ITP plasma. A barely significant difference was seen in the percentage of MK with chromatin condensation incubated with ITP compared to control plasma ($n = 14$), 13·03 ± 4·66% vs. 9·17 ± 2·35%, $P = 0{\cdot}031$, (Mann–Whitney–Wilcoxon test), while no difference in the percentage of MKs displaying active caspase 3/7 was observed in cultures incubated with patient or control plasma as measured by

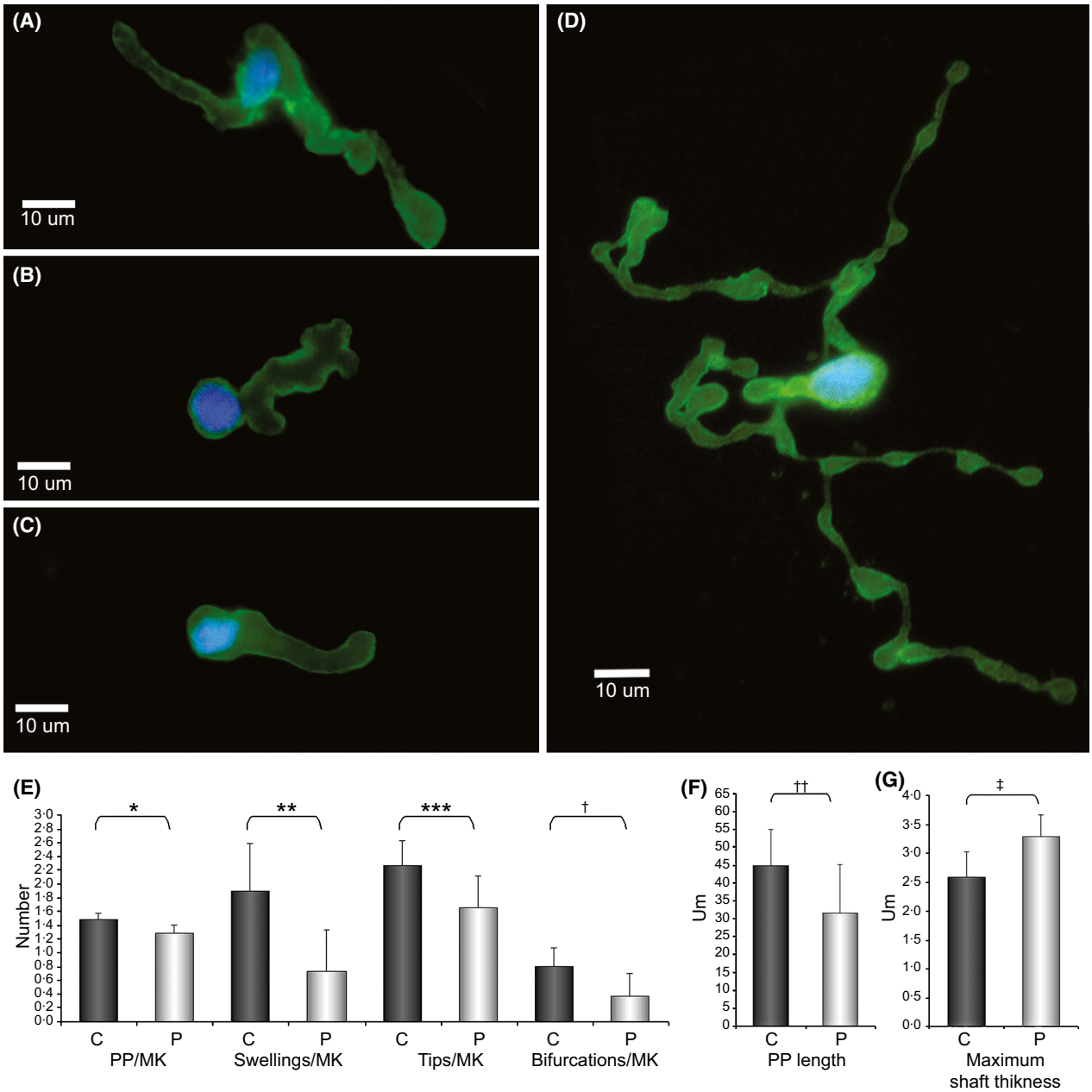

Fig 2. Analysis of proplatelet morphology in normal MKs in the presence of ITP plasma. Mature megakaryocytes (MKs) were seeded on fibrinogen and 10% of the final culture volume was replaced by patient or normal recalcified plasma. After 48 h, staining was performed using CD61-FITC/Hoechst. Representative pictures of proplatelet-bearing MKs in the presence of (A–C) ITP plasma and (D) control plasma. Images were acquired using a 100× oil immersion objective. (Carl Zeiss GmbH). (E) Evaluation of proplatelet (PP) morphological features: number of PP extensions (*P = 0·0004), swellings (**P = 0·0002), tips (***P = 0·015) and bifurcations (†P = 0·002) per MK in the presence of immune thrombocytopenia (ITP) patient (P, n = 12) and control (C, n = 12) plasma were plotted. (F) PP length (††P = 0·012) and (G) maximum shaft thickness (‡P = 0·0003) measured in μm, using cell analysis software (VideoTesT-Master Morphology image analysis software). Results represent mean ± standard deviation values of at least 40 MKs producing PPs in each sample. In order to avoid morphological variations due to intrinsec features among MK derived from different cord blood samples, all plasma samples were evaluated using MK derived from the same cord blood.

FLICA, 18·17 ± 2·31 vs. 17·08 ± 2·46, P = 0·12, (Mann–Whitney–Wilcoxon test) (Fig 3A) and by immunofluorescent staining using a specific anti-active caspase 3 antibody (data not shown).

No correlation was found between PP count performed on immunofluorescent-stained samples and the percentage of MK with chromatin condensation induced by ITP plasma (P = 0·14, Spearman correlation), suggesting that apoptosis of mature MK and PPF inhibition are independent events.

Megakaryocytes producing PP did not display overt caspase 3 activation and did not show chromatin condensation either in the presence of normal or ITP plasma

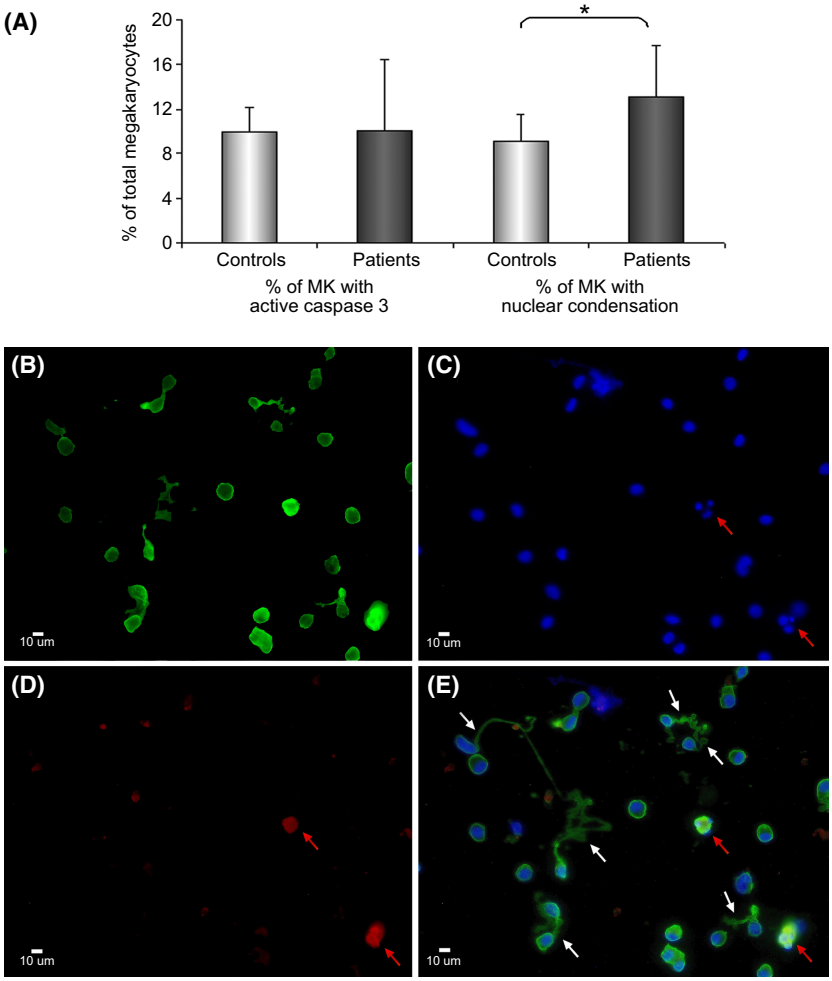

Fig 3. Effect of ITP plasma on normal MK apoptosis. Mature megakaryocytes (MKs) incubated for 48 h with immune thrombocytopenia (ITP) or normal plasma were either assayed for active caspase 3 and 7, by fluorochrome-labelled inhibitors of caspases (FLICA) methodology or stained with anti-CD61-FITC/Hoechst/anti-active caspase 3 followed by rhodamine-conjugated secondary antibody, as described in Materials and methods. (A) Mean ± standard deviation percentage of MK showing highly condensed chromatin (right, * $P = 0{\cdot}031$, Mann–Whitney–Wilcoxon test) and expressing active caspase 3 and 7 (left, $P = 0{\cdot}12$), in the presence of plasma from ITP patients ($n = 20$) or normal controls ($n = 19$). Representative images showing MK incubated with normal recalcified plasma stained with: (B) anti-CD-61-FITC (green), (C) Hoechst dye (blue), (D) anti-active caspase 3 (red), (E) overlapping pictures (scale bar: 10 μm). White arrows indicate proplatelet-bearing MKs and red arrows indicate apoptotic MKs.

(Fig 3B–E). These findings are in agreement with recently published data (Josefsson *et al*, 2011), showing that MK apoptotic signals must be restrained in order to allow platelet production to take place.

Role of auto-antibodies in PPF inhibition

In order to identify the molecular target of auto-antibodies, a qualitative enzyme-immunoassay (ELISA) specific for antibodies against GPIIb-IIIa, GPIb-IX and GPIa-IIa was performed on ITP plasmas. Samples from eight patients (38%) displayed detectable auto-antibodies (Table I).

To determine if platelet auto-antibodies were responsible for PP inhibition, two different strategies were carried out using plasma from two patients previously shown to inhibit PPF: Patient 2, harbouring anti-GPIIb-IIIa antibodies, and Patient 10, who had anti-GPIb-IX antibodies:

1 Evaluation of PPF in the presence of platelet-adsorbed ITP plasma: Plasma samples were adsorbed with washed platelets from healthy donors, resulting in complete depletion of auto-antibodies, as assessed by ELISA measurement. Next, normal MKs were incubated with 10% platelet-adsorbed recalcified plasma and PP count was carried out 48 h later. Partial reversion of the inhibitory effect induced by recalcified plasma was observed in the presence of the corresponding platelet-adsorbed plasma (Fig 4A).
2 Purified IgG action on PPF: IgG fraction was purified from these plasma samples and tested for PPF at 2·5% final concentration. Both IgG samples displayed an inhibitory effect on PPF similarly to that induced by patients' plasma (Fig 4B). These results are in agreement with those observed in the presence of platelet-adsorbed plasma and suggest that the anti-GPIIb-IIIa and anti-GPIb-IX antibodies present in the IgG fraction of Patients 2 and 10 respectively, could be, at least in part, responsible for PPF reduction.

Auto-antibody specificity and its role in PPF

Auto-antibodies against GPIIb-IIIa were the most frequently observed in our population, being found in six of 21 ITP samples. Interestingly, PP inhibition was seen in the presence of four of these samples (one of them also carrying anti-GPIa-IIa auto-antibodies). In addition, one sample with

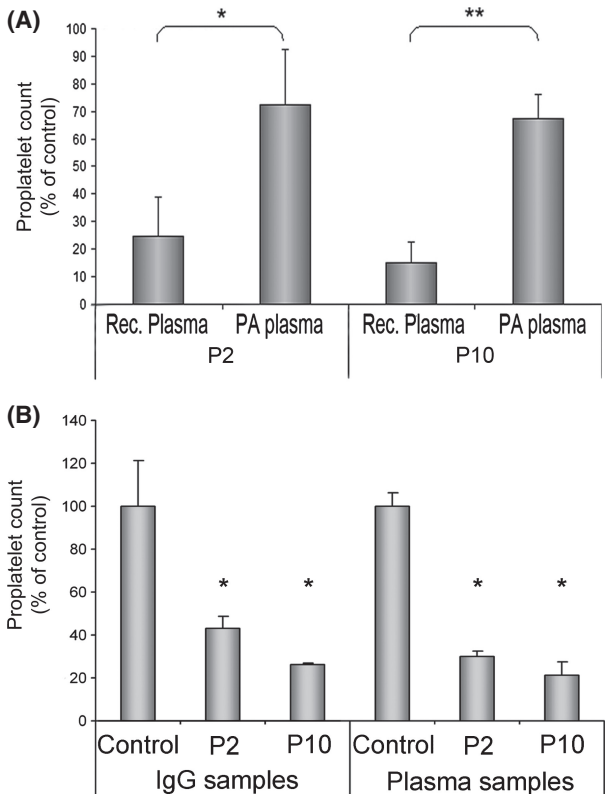

Fig 4. PPF in the presence of platelet-adsorbed plasma and purified IgG from immune thrombocytopenia patients. (A) Auto-antibody adsorption was carried out by incubation of recalcified plasma with normal platelets. Proplatelet formation (PPF) was assayed in the presence of 10% of recalcified (Rec.) and platelet-adsorbed (PA) plasma. Assays were performed in duplicate at least in two separate experiments using different cord blood samples. Results were expressed as mean ± standard deviation (SD) percentage of proplatelet (PP) counts obtained in the presence of recalcified and platelet-adsorbed control samples, respectively, set as 100%. *$P = 0{\cdot}008$; **$P = 0{\cdot}0001$, unpaired *t*-test. P2: Patient 2, harbouring auto-antibodies against GPIIb-IIIa; P10: Patient 10, with auto-antibodies against GPIb-IX. (B) PPF was evaluated in the presence of 2% purified IgG and expressed as percentage of that obtained in the presence of the corresponding control IgG (left). PPF in the presence of 2% of the corresponding plasma samples is also shown for comparison (right). Results represent the mean ± SD of two independent experiments.

anti-GPIb-IX and five in whom auto-antibody detection was negative also induced decreased PPF. In contrast, one patient carrying anti-GPIIb-IIIa, one with anti-GPIa-IIa, one with both anti-GPIIb-IIIa and GPIa-IIa, and eight in whom no auto-antibodies were detected, did not show quantitative inhibition in PPF.

Auto-antibodies against GPIIb-IIIa that interfere with integrin function impair PPF. After the finding that four of six ITP plasmas containing antibodies against GPIIb-IIIa inhibited PPF, we sought to investigate the mechanisms underlying this effect. To this end, GPIIb-IIIa function was assessed by two different strategies:

1 PAC-1 binding: Incubation of unstimulated MKs with ITP plasma did not increase PAC-1 binding compared to control plasma, $5{\cdot}98 \pm 2{\cdot}46\%$ vs. $8{\cdot}40 \pm 1{\cdot}67\%$, ($P = 0{\cdot}79$, Mann–Whitney–Wilcoxon test), indicating that anti-GPIIb-IIIa auto-antibodies do not induce the expression of the active site of the integrin by themselves. Furthermore, ITP samples bearing anti-GPIIb-IIIa antibodies, shown above to inhibit PPF, exerted an inhibitory effect on MK PAC-1 binding after stimulation with 20 μmol/l ADP/epinephrine compared to controls, $36{\cdot}31 \pm 11{\cdot}31\%$ vs. $51{\cdot}76 \pm 4{\cdot}50\%$ ($P = 0{\cdot}034$, Mann–Whitney–Wilcoxon test), while PAC-1 binding to MK incubated with both ITP plasma samples that induced normal PPF was similar to controls (Fig 5A). The fact that anti-GPIIb-IIIa-bearing plasmas were not related to a unique phenotype regarding PPF suggests that differences in the intrinsic auto-antibody features among these ITP patients (i.e. epitope specificity, affinity degree) could determine their ability to interfere with proplatelet production, as previously shown using monoclonal antibodies directed against different epitopes of the same glycoprotein complex (CD41 and CD61) (Takahashi *et al*, 1999).
2 β3 integrin phosphorylation: In order to further confirm that anti-GPIIb-IIIa auto-antibodies interfere with the normal function of GPIIb-IIIa, β3 phosphorylation was evaluated in MKs incubated for 15 and 30 min with recalcified plasma from Patient 2, either unstimulated or after stimulation with TRAP/ADP/epinephrine. Patient plasma bearing anti-GPIIb-IIIa auto-antibodies did not induce β3 phosphorylation by itself (Fig 5B). Moreover, and in agreement with results from PAC-1 binding, β3 phosphorylation was prevented in TRAP/ADP/epinephrine-stimulated MKs incubated with patient's plasma.

Anti GPIa-IIa auto-antibodies interfere with the normal inhibition of PPF exerted by type I collagen. To evaluate the effect of anti-GPIa-IIa auto-antibodies present in ITP plasma in the normal inhibition of PPF exerted by type I collagen, PPF by MKs seeded on this matrix protein was evaluated and compared to that obtained in uncovered wells in the presence of plasma from Patients 5, 13 and 21, which carryied anti-GPIa-IIa antibodies. All these samples prevented the normal inhibition of type I collagen on proplatelet production, while, in the presence of plasma from Patient 7 (harbouring anti-GPIIb-IIIa antibodies) and five normal controls, the expected reduction in PP count could be seen. Results are expressed as fold change in the percentage of PPF produced on type I collagen versus uncovered wells (Fig 5C), (Fold change in PP production in the presence of plasma from patients bearing anti-GPIa-IIa auto-antibodies versus plasma from normal controls, $P = 0{\cdot}036$, Mann–Whitney–Wilcoxon test). This abnormal behavior was also observed when PPF was assayed in the presence of purified IgG from Patient 5 (not shown).

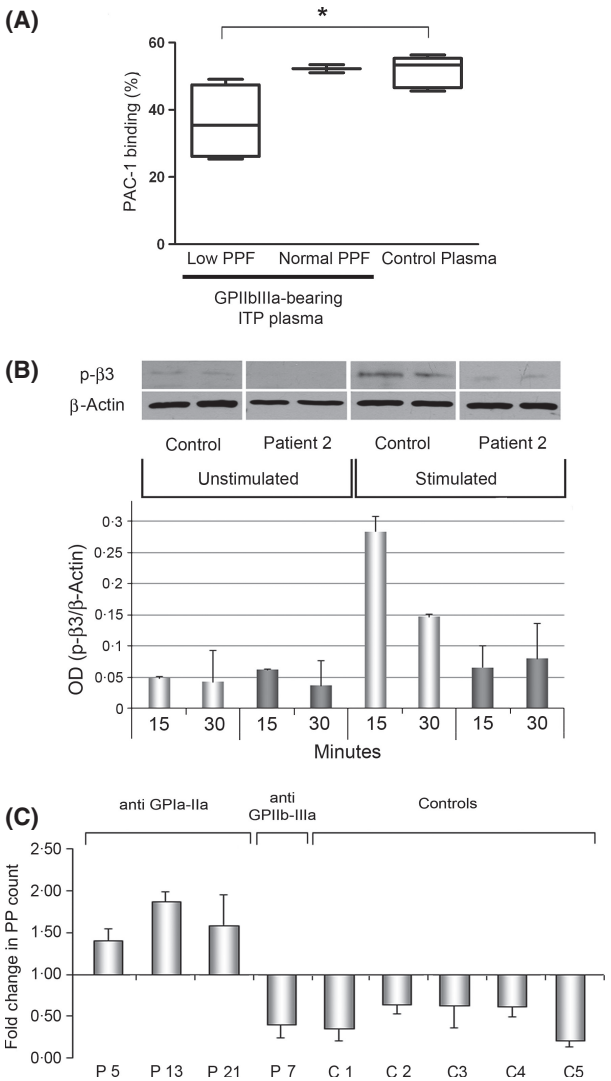

Fig 5. Anti-GPIIb-IIIa and GPIa-IIa auto-antibodies effect on glycoprotein function. (A) Mature megakaryocytes (MKs) incubated for 24 h with immune thrombocytopenia (ITP) samples bearing anti-GPIIb-IIIa auto-antobodies were evaluated for PAC-1 binding after ADP/epinephrine stimulation, by flow cytometry. Low PPF: plasma samples from ITP patients previously shown to inhibit proplatelet formation (PPF); Normal PPF: plasma samples from ITP patients that induced normal PPF. Box plots depict the average of two independent experiments. Statistical differences were seen between MK PAC-1 binding in the presence of anti-GPIIb-IIIa-bearing ITP samples that induced low PPF and normal controls, $*P = 0{\cdot}034$, Mann–Whitney–Wilcoxon test. (B) MKs were incubated for 15 and 30 min with 10% recalcified plasma from Patient 2 and a normal control, either with or without stimulation with TRAP/ADP/epinephrine, then washed, lysed, resolved by 8% sodium dodecyl sulphate polyacrylamide electrophoresis and transferred to nitrocellulose. Immunoblots were carried out using antibodies against p-β3 integrin and β-actin as a protein loading control. Results are expressed as mean ± standard deviation of the ratio between the optical density of p-β3 and β-actin, of two experiments. (C) Mature MKs were seeded on type I collagen in the presence of 10% recalcified plasma from three ITP patients bearing anti-GPIa-IIa auto-antibodies (Patient [P]5, P13 and P21), one ITP patient with anti-GPIIb-IIIa antibodies (P7) and five normal controls (C1–5), and proplatelet (PP) count was carried out as previously described. The fold change in PP count, calculated as PP count obtained on type I collagen and relative to that obtained on the corresponding uncovered well are plotted. Results represent mean ± standard error of the mean of at least two independent experiments. Statistical differences were seen between results obtained in the presence of anti-GPIa-IIa-bearing ITP plasmas and normal controls, $P = 0{\cdot}036$, Mann–Whitney–Wilcoxon test.

Discussion

Growing advances in the knowledge of the mechanisms of thrombopoiesis have been made during the last decade. Some of the evidence in this field has come from the study of patients with platelet disorders. Our results demonstrate an inhibitory effect of ITP plasma specifically on PPF. Extrapolation of these *in vitro* results to the ITP scenario would imply that, even in a bone marrow with normal or increased MKs, inhibition of platelet production could take place at the thrombopoietic step.

Morphological differences between PP produced in the presence of ITP and normal plasma were also observed in this study. Most of the plasma samples from ITP patients induced wider and shorter PP, bearing lower numbers of bifurcation points and tips, and overall, less complex PP than normal ones. Taking into account that MKs must elongate their PP through the bone marrow endothelial barrier in order to release platelets into the bloodstream, these morphological PP features could hinder thrombopoiesis. Altogether, these results could imply that, in addition to the lower number of MKs capable of producing PP in ITP, each proplatelet-bearing MK would be unable to produce appropriate numbers of platelets.

No differences were found in PPF between MKs incubated with samples from the group of patients who were not under treatment and those who were. However, both groups presented similar platelet counts, as in the latter, treatment was aimed just at achieving a safe platelet count (20–30 $\times$ 10^9/l). Further studies in a larger ITP population including patients with a wider spectrum of peripheral platelet counts, as well as longitudinal assessment of samples obtained before and during treatment, are in progress to broaden conclusions in this field and to ascertain whether ITP treatment is able to ameliorate the inhibition found in PPF.

Evidence that platelet production in ITP is suboptimal (Barsam *et al*, 2011) is supported by the success of thrombopoietin receptor agonists (TRAs) in the treatment of ITP patients. As shown for the first time in this study, reduced platelet production is caused, not only by autoimmune-mediated inhibition in megakaryopoiesis (McMillan *et al*, 2004), but also, by impaired PP formation from mature MKs. It remains to be established whether differences in the relative contribution of impaired PP and platelet formation versus increased platelet clearance might influence response to different ITP treatments, i.e. those aimed at enhancing platelet production or blocking platelet destruction. Further

work in this field may hopefully lead to improvement in ITP management and, eventually, to a tailored treatment approach for each patient.

As mentioned before, the role of MK apoptosis in ITP is controversial. Considering that, as recently shown, the apoptotic machinery is dispensable for PPF (Josefsson *et al*, 2011) and that its activation may rather impair this process, we aimed to assess the potential contribution of increased MK apoptosis to the reduction found in PPF. Incubation of mature MKs with ITP plasma in the same conditions under which PPF was assessed did not induce increased caspase activation. Moreover, although a slight increase in MKs showing nuclear pyknosis was found among cells exposed to patient samples, there was no correlation between this feature and reduced PP counts, further indicating that the inhibition in PPF was not due to a decrease in the number of viable MKs. Regarding the thrombopoietic process, proplatelet-bearing MKs incubated either with normal or ITP plasma did not display apoptotic features. Therefore, although MK damage might play a role in ITP pathogenesis in the *in vivo* setting, as demonstrated by morphological bone marrow features (Houwerzijl *et al*, 2004), our results suggest that neither the decrease in PPF nor the morphological PP abnormalities found in this study can be attributed to increased MK apoptosis, but rather to other factors, such as the direct effect of auto-antibodies on the MK surface.

The participation of platelet auto-antibodies in the inhibition of PPF is suggested by the partial correction of PP production in the presence of platelet-adsorbed ITP plasma and by the reproduction of the inhibitory effect by purified IgG. The unexpected finding that inhibition of thrombopoiesis was not restricted to the presence of a specific type of auto-antibody, suggests that even when the underlying mechanisms leading to reduction of platelet production may not be the same among ITP patients with auto-antibodies directed against different glycoproteins, abnormalities in thrombopoiesis could affect a wide spectrum of the ITP population.

In our cohort of ITP patients, only one presented auto-antibodies against GPIb-IX. Plasma from this patient induced a deep inhibition in PPF as well as morphological changes in PP from normal MK. Evidence for impaired PPF in the absence or decrease of GPIb-IX-V complex, [Bernard–Soulier syndrome (Strassel *et al*, 2009) and Bolzano mutation (Balduini *et al*, 2009), respectively] or filamin, a structural protein linking GPIb with the cytoskeleton (Jurak Begonja *et al*, 2011; Kanaji *et al*, 2012), has been reported. Taken together, these data suggest that the presence of anti-GPIb-IX auto-antibodies in ITP could interfere with the normal function of GPIb-IX-V complex, thus leading to impaired thrombopoiesis.

The role of GPIIb-IIIa in thrombopoiesis remains controversial. While patients with Glanzmann thrombasthenia do not show thrombocytopenia, monoclonal antibodies directed against GPIIb inhibit PPF *in vitro* (Takahashi *et al*, 1999) and αIIb −/− mice display a blunted platelet response to thrombopoietin administration, demonstrating that under certain conditions, this glycoprotein complex may play a role in platelet production (Larson & Watson, 2006). Moreover, activation of integrin signalling via SRC family kinases has been shown to have a positive role in thrombopoiesis (Mazharian *et al*, 2010, 2011). On the other hand, recent evidence revealed that partial activation of GPIIb-IIIa impairs thrombopoiesis, as shown by the presence of activating mutations in *ITGA2B* and *ITGB3* in patients with inherited macrothrombocytopenia (Ghevaert *et al*, 2008; Kunishima *et al*, 2011; Bury *et al*, 2012). The finding that anti-GPIIbIIIa-bearing ITP plasmas inhibit PPF, provides yet another setting in which abnormalities targeting GPIIb-IIIa lead to defects in thrombopoiesis. In this study, constitutive activation of GPIIb-IIIa, mimicking *ITGA2B/ITGB3*-related thrombocytopenia, could not be demonstrated. Rather, PP inhibition was associated with impaired GPIIb-IIIa function, as assessed by PAC-1 binding and β3 phosphorylation in MKs incubated with anti-GPIIb-IIIa bearing samples. Further studies on GPIIb-IIIa downstream signalling pathways during normal and pathological thrombopoiesis may provide additional insight into the effect of GPIIb-IIIa abnormalities in PPF.

Type I collagen plays a critical role in the bone marrow osteoblastic niche environment, preventing platelet formation through its binding to GPIa-IIa and activation of Rho-ROCK pathway. Plasma from ITP patients bearing anti-GPIa-IIa auto-antibodies interfered with normal inhibition of type I collagen on proplatelet production, suggesting that the presence of these auto-antibodies could induce precocious PPF and platelet release within the osteoblastic niche instead of the microvessel lumen in these patients.

Overall, these data suggest that differential mechanisms leading to impairment of PP production may depend on the specific target of the auto-antibodies and could contribute to explain the heterogeneous pathophysiology of ITP. Interestingly, inhibition of thrombopoiesis was not restricted to auto-antibody-positive ITP plasmas. Other factors present in these ITP samples, including auto-antibodies other than those tested in this study, could interfere with PP generation. Regarding ITP samples that did not induce reduction in PP count, differences in auto-antibody features, such as epitope specificity, affinity degree and/or concentration, could determine their ability to alter PP production.

In conclusion, quantitative and qualitative abnormalities in the key step of thrombopoiesis, are new mechanisms contributing to decreased platelet production and thrombocytopenia in ITP. Insight into the mechanisms underlying PPF inhibition reveal that these may be diverse among ITP patients, highlightening the heterogeneous nature of this disorder. These novel findings add to our understanding of the pathophysiology of this complex disease.

Acknowledgements

We are deeply grateful to the Departamento de Obstetricia from Hospital Materno-Infantil Dr Gianantonio and

especially to Sara Labat for collection of cord blood, to Dr. Judith Sarano, María Gargiulo, Lorena V. Suarez, Dr. Graciela N. Gomez and Dr. María V. Collado from Departamento de Inmunología, Instituto Lanari, for helpful discussion of the results and technical assistance. This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (Grants PICT32075 and PICT1860).

Author contributions

RFM, PGH performed the study design. PRL, MG, NPG, ACG, YE, GC, LF, FCM, PGH, RFM performed experiments and/or analysed data. FCM, PGH, MSP, VSM provided patient samples and clinical data. RFM, PGH wrote the manuscript. All authors are involved in manuscript editing and final approval.

Conflict of interest

The authors have no competing interests.

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