Autoantibodies in immune thrombocytopenia affect the physiological interaction between megakaryocytes and bone marrow extracellular matrix proteins

Immune thrombocytopenia (ITP) is characterized by its heterogeneity among patients with regard to both clinical features and response to treatment. Although there is still much to investigate, this heterogeneity could be the reflection of multiple mechanisms contributing to thrombocytopenia, affecting individual patients in variable proportions. These mechanisms include increased platelet clearance by autoantibodies targeting main platelet glycoproteins (GPs) (IIbIIIa, IbIX and IaIIa) and impaired megakaryopoiesis (McMillan *et al*, 2004). Recently, we reported that ITP plasma samples inhibit proplatelet (PP) formation by normal megakaryocytes (MKs) (Lev *et al*, 2014), demonstrating that thrombopoiesis, the key step of platelet production, is altered, a finding further confirmed by others (Iraqi *et al*, 2015). In agreement with these experimental data, studies on platelet kinetics show inadequate platelet production according to the degree of thrombocytopenia (Ballem *et al*, 1987).

Interaction between these GP receptors of the megakaryocytic lineage and their corresponding extracellular matrix ligands is essential for MK development and platelet production in the bone marrow (BM). Proof of the relevance of this interaction include the abnormalities in platelet generation found in several inherited conditions: (i) patients with *MYH9*-related thrombocytopenia (Pecci *et al*, 2009) carrying mutations in myosin IIA (MYH9), downstream of collagen type I receptor, GPIaIIa; (ii) macrothrombocytopenia due to mutations that partially activate fibrinogen receptor, GPIIbIIIa (Kunishima *et al*, 2011); and (iii) mutations in GPIbIXV, the von Willebrand factor (VWF) receptor, leading to Bernard-Soulier syndrome (Strassel *et al*, 2009).

Given that the main ITP autoantibodies target GPIIbIIIa, GPIbIX and GPIaIIa, we hypothesized that their binding could impair GP function, altering MK interaction with extracellular matrix proteins and thus, megakaryocytic behaviour within the BM environment. In order to test this assumption, we studied the effect of ITP plasma on normal MK adhesion, spreading, activation of downstream signalling pathways and thrombopoiesis.

After approval was given by the institutional Ethics Committees and written informed consent was obtained, blood samples from 14 ITP patients (Table SI) and controls were obtained and used to prepare recalcified plasma and IgG fractions (Lev *et al*, 2014). Autoantibodies were detected using the PAKAUTO kit (GTI Diagnostics Inc., Waukesha, WI, USA). Normal mature MKs were obtained after 13-day culture of human cord blood-derived $CD34^+$ cells, as described (Balduini *et al*, 2008), and incubated with recalcified plasma or purified IgG on type I collagen, fibrinogen and VWF-coated surfaces. Then, adhesion, spreading and activation of downstream signalling pathways were assessed. Details are provided in the Supporting Information.

The percentage of MK adhesion to type I collagen in the presence of anti-GPIaIIa ITP samples ($n = 2$) was below the normal reference range. These values were also lower than those obtained for ITP plasmas bearing anti-GPIIbIIIa ($n = 4$), or negative for autoantibodies ($n = 4$), which behaved normally (Fig 1A). Similar results were obtained when MKs were incubated with the corresponding purified IgG fractions, indicating that autoantibodies are responsible for this inhibitory effect (Fig 1B). MK spreading followed the same pattern (Fig 1C), demonstrating that autoantibodies also affect the ability of MKs to actively attach to this substrate (Fig 1D). To test GPIaIIa intracellular signalling after collagen engagement, we evaluated myosin light chain 2 (MLC_2) phosphorylation, a downstream mediator of GPIaIIa and a critical regulator of several aspects of MK physiology, including proper myosin contractility during spreading (Malara *et al*, 2011) and modulation of PP production (Chang *et al*, 2007). MKs showed decreased phospho-MLC_2 levels in the presence of anti-GPIaIIa-bearing plasmas (Fig 1E).

According to the classical haematopoiesis model, MKs begin their development in the osteoblast niche, a type I collagen-enriched environment. MK binding to this extracellular protein through GPIaIIa inhibits thrombopoiesis (Sabri *et al*, 2004), precluding premature platelet release within this BM compartment. In previous studies, we observed the absence of this physiological inhibition in the presence of anti-GPIaIIa ITP autoantibodies (Lev *et al*, 2014). Our current results indicate that this lack of inhibition relies on the fact that anti-GPIaIIa autoantibodies block MK-type I collagen interaction and interfere with MLC_2 phosphorylation, which is a key downstream effector mediating GPIaIIa-induced negative regulation of thrombopoiesis.

Driven by a chemoattractant SDF-1 (also termed CXCL12) gradient, MKs migrate to the vascular niche, a

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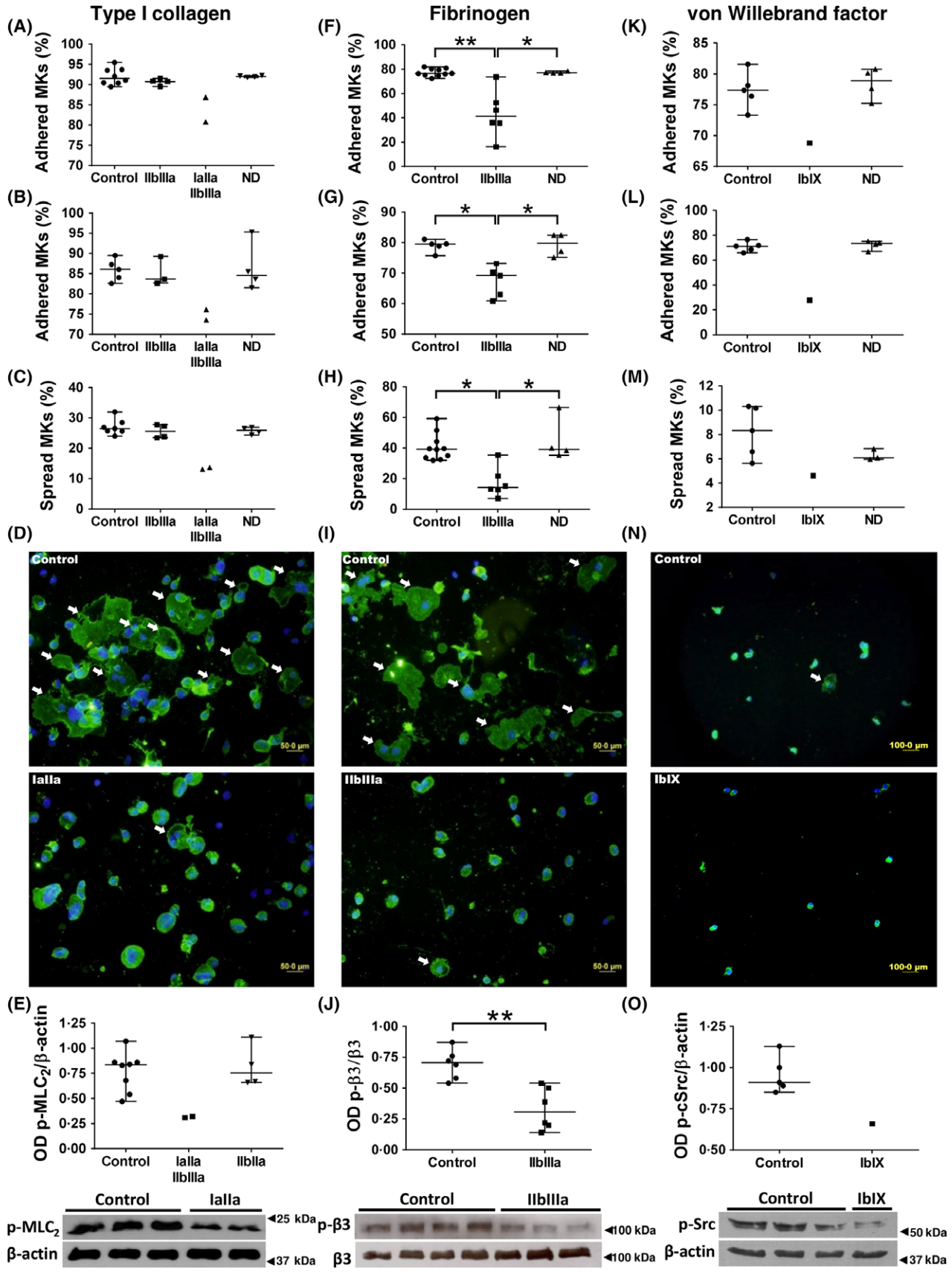

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Fig 1. Effect of immune thrombocytopenia (ITP) autoantibodies in normal megakaryocyte (MK) interaction with extracellular bone marrow matrix proteins. $CD34^+$ cells from normal cord blood were cultured for 13 days at 37°C in 5% CO_2 to obtain mature MKs. 1×10^5 cells were seeded on 25 μg/ml type I collagen (A–E), 100 μg/ml fibrinogen (F–J) or 10 μg/ml von Willebrand factor (VWF) (K–O) -coated coverslips and incubated in the presence of 10% recalcified plasma from ITP samples bearing anti-GPIIbIIIa and GPIaIIa autoantibodies (IaIIa–IIbIIIa, $n = 2$), anti-GPIIbIIIa autoantibodies (IIbIIIa, $n = 4$) or anti-GPIbIX autoantibodies (IbIX, $n = 1$). To evaluate the anti-GPIbIX sample, MKs were incubated with 1 mmol/l RGDS peptide to block GPIIbIIIa binding to VWF. MKs were also incubated with ITP plasma samples with no detectable autoantibodies (ND, $n = 4$) and control samples ($n = 5$–10). After 16 h, cells in the supernatants were counted and the percentage of adherent cells was calculated (A, F, K). Adhesion studies were also performed in the presence of the corresponding purified IgG fractions (B, G, L). Adhered MKs were fixed with 4% paraformaldehyde and immunostained with mouse anti-CD61, FITC-conjugated chicken anti-mouse secondary antibody and Hoechst 33258. Photographs of randomly chosen fields were captured in an epifluorescent microscope (Carl Zeiss, Oberkochen, Germany) equipped with a digital camera (Canon Power Shot G6, Tokyo, Japan) and used to calculate the percentage of spread cells (C, H, M). Images from representative fields of MKs adhered to type I collagen (D), fibrinogen (I) or VWF (N) in the presence of control plasma (top) or ITP anti-GPIaIIa, GPIIbIIIa or GPIbIX, respectively (bottom), are shown. Arrows indicate spread MKs. For GP downstream signalling analysis, 1×10^6 mature MKs were incubated with 10% of plasma for 30 min, after which the cells were seeded on the corresponding matrix proteins for 1 min in the presence of ITP or control plasma samples. Cells were lysed, resolved on 12% one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto nitrocellulose (E, J, O). Membranes were probed with anti-phospho-MLC_2 (Ser19), anti-phospho-β3 (Tyr773), anti-β3 and anti-phospho-Src (Tyr418). The ratio between the optical density of p-β3 and β3 was calculated. Phosphorylation of MLC_2 and Src was normalized to β-actin. Immunoreactive bands were detected using peroxidase-conjugated secondary antibodies and chemiluminescence detection. Representative examples of Western blots are shown. Each sample was evaluated three times in independent experiments and the average values are plotted. Median and range values are shown. For analysis of adhesion and spreading on fibrinogen, Kruskal–Wallis test, followed by Dunn's multiple comparisons, was applied. To evaluate β3 phosphorylation, data were analysed by Mann–Whitney test. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. Due to the low number of anti-GPIaIIa/IIbIIIa and anti-GPIbIX plasma samples, these results were compared to the range of the control group. [Colour figure can be viewed at wileyonlinelibrary.com]

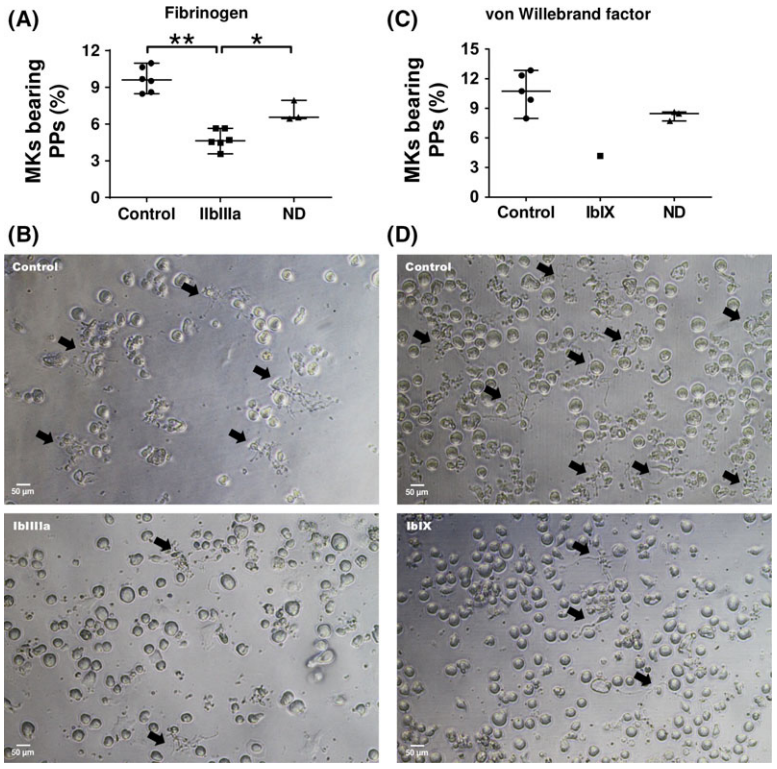

Fig 2. Effect of immune thrombocytopenia (ITP) autoantibodies in proplatelet formation on extracellular bone marrow matrix proteins. $CD34^+$ cells from normal cord blood were cultured for 13 days at 37°C in 5% CO_2 to obtain mature megakaryocytes (MKs). Then, 1×10^4 cells were incubated on fibrinogen (A, B) or von Willebrand factor (VWF) (C, D) -coated surfaces in the presence of ITP samples bearing anti-GPIIbIIIa ($n = 6$) or anti-GPIbIX autoantibodies (IbIX, $n = 1$), respectively. In each case, MKs were also incubated with ITP plasma samples with no detectable autoantibodies (ND, $n = 3$) and control samples ($n = 5$–6). After 48 h, the percentage of MKs bearing proplatelets (PPs) were established by direct observation under an inverted microscope (Carl Zeiss) using a 32× objective. Representative fields of MKs forming PPs on fibrinogen (B) or VWF (D) in the presence of control plasma (top) or ITP anti-GPIIbIIIa or GPIbIX, respectively (bottom) are shown. Images were captured with a 32× objective, photographed with an Olympus DP70 CCD camera (Olympus, Tokyo, Japan) and acquired through Lumina Vision software (Mitani, Fukui, Japan). Arrows indicate MKs forming PPs. Each sample was evaluated twice on independent experiments and the average values are depicted. Median and range values are shown. For proplatelet formation analysis on fibrinogen, Kruskal–Wallis test, followed by Dunn's multiple comparisons, was applied. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. As only one anti-GPIbIX plasma sample was studied, its result was compared to the range of the control group. [Colour figure can be viewed at wileyonlinelibrary.com]

microenvironment permissive for thrombopoiesis, where fibrinogen and VWF binding to GPIIbIIIa and GPIbIXV, respectively, enhances platelet production (Balduini *et al*, 2008). ITP plasma samples bearing autoantibodies against GPIIbIIIa and GPIbIXV, and the purified IgG fractions, significantly reduced MK adhesion to their corresponding ligands (Fig 1F, G, K, L). MK spreading was also compromised in the presence of these samples (Fig 1H, M). Plasma from ITP patients with no detectable autoantibodies allowed normal MK adhesion and spreading on these matrices (Fig 1I, N).

Downstream signalling after fibrinogen-GPIIbIIIa interaction, evaluated by phosphorylation of β3 tyrosine residues (Tyr773 and Tyr785), was reduced in MKs incubated with anti-GPIIbIIIa autoantibodies (Fig 1J). Similar results were obtained when phosphorylation of c-Src (also termed CSK), a kinase that plays a major role in the GPIbIX pathway, was investigated after VWF-GPIbIX interaction in the presence of the ITP plasma bearing anti-GPIbIX autoantibodies (Fig 1O).

These results suggest that MK interaction with fibrinogen and VWF in the vascular niche could be impaired in ITP patients with anti-GPIIbIIIa and anti-GPIbIX autoantibodies, leading to functional abnormalities in mature MKs and interfering with PP production and platelet release. Indeed, PP formation evaluated in MKs cultured on surfaces coated with fibrinogen and VWF were reduced in the presence of anti-GPIIbIIIa and anti-GPIbIX ITP plasmas, respectively (Fig 2A, D). The slight decrease in PP formation observed in the presence of patient samples with no detectable autoantibodies could be due to other factors, including the presence of autoantibodies different from those tested in this study.

Considering the heterogeneous nature of ITP, the study of additional patients harbouring autoantibodies against these three main targets will be useful to determine the relevance of our findings in a larger ITP cohort.

Overall, our results indicate that ITP autoantibodies induce abnormal MK interaction with different components of the bone marrow extracellular microenvironment that affect MK physiological functions and thus contribute to defective platelet production in ITP. These novel findings unveil additional mechanisms by which ITP autoantibodies hinder MK biology, highlighting their multiple effects in platelets and their precursors.

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Author disclosure

All authors state that they have no conflict of interest.

Author contributions

M. Grodzielski designed and performed experiments, analysed data, wrote the manuscript and prepared figures. C.A. Di Buduo and P.M. Soprano performed experiments. N.P. Goette and P.R. Lev performed experiments and analysed data. P.G. Heller discussed the results and wrote the manuscript. A. Balduini designed experiments, analysed data and supervised the project. R.F. Marta designed and performed experiments, analysed data, wrote the manuscript and supervised the project. All authors edited the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. Patient features.

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