Poly (I:C) downregulates platelet production and function through type I interferon

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Summary

Thrombocytopaenia is a frequent complication of viral infections; the underlying mechanisms appear to depend on the identity of the virus involved. Previous research, including reports from our group, indicates that as well as having antiviral activity type I interferons (IFN I) selectively downregulate platelet production. In this study we extended understanding of the role of endogenous IFN I in megakaryo/thrombopoiesis by evaluating platelet and megakaryocyte physiology in mice treated with polyinosinic:polycytidylic acid [poly (I:C)], a synthetic analogue of double-stranded RNA, Toll-like receptor-3 ligand and strong IFNβ inducer. Mice-treated with poly (I:C) showed thrombocytopaenia, an increase in mean platelet volume and abnormal haemostatic and inflammatory platelet-mediated functionality, indicated by decreased fibrinogen binding and platelet adhesion, prolonged tail bleeding times and impaired P-Selectin externalisation, RANTES release and thrombin-induced platelet-neutrophil aggregate formation. These changes were associated with an increase in size and an abnormal distribution of bone marrow megakaryocytes within the vascular niche and were directly correlated with the plasmatic and bone marrow IFNβ levels. All these effects were absent in genetically modified mice lacking the IFN I receptor. Our results suggest that IFN I is the central mediator of poly (I:C)-induced thrombocytopaenia and platelet dysfunction and indicate that these abnormalities are due to changes in the last stages of megakaryocyte development. These data provide new evidence for the role of IFN I in megakaryocyte distribution in the bone marrow niches and its influence on thrombopoiesis and haemostasis.

Keywords

Platelets, poly (I:C), thrombocytopaenia, virus, interferon

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Introduction

Thrombocytopenia is a frequent complication of viral infections. The exact mechanism appears to depend on the nature of the viruses involved; various mechanisms have been proposed including immunological platelet destruction, inappropriate platelet activation and consumption and impaired megakaryopoiesis (1).

Type I interferons (IFN I) produced during viral infections are critical because, in addition to their well-known antiviral activity they modulate both the innate immune response and the subsequent development of adaptive immunity to viruses (2–4). There are seven classes of IFN I: IFNβ, IFNα, IFNω, IFNτ, IFNκ, IFNδ, IFNε. There is only one human IFNβ and one functional IFNω, but humans and many other species have multiple IFNα variants (5). All IFNs bind to the same cell-surface receptor, the interferonα/β receptor (IFNAR), which comprises two transmembrane subunits, IFNAR1 and IFNAR2 (6). IFN I can be produced in response to stimulation of different pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), protein kinase R (PKR), or RNA helicases (7). A pioneering study by Iannacone et al. showed that mice infected with the prototypical arenavirus lymphocytic choriomeningitis virus (LCMV) exhibit an IFN I-dependent alteration in ADP-induced aggregation, together with a reduction of almost 80% in platelet numbers. This IFN I-mediated-platelet dysfunction caused lethal haemorrhage in mice with extreme thrombocytopenia (8). We demonstrated that infection of haematopoietic progenitors or megakaryocytes with Junin virus (the arenavirus that cause Argentine Haemorrhagic Fever) or treatment with polyinosinic: polycytidylic acid (poly I: C), a synthetic double-stranded RNA, TLR3 ligand that mimics viral replication (9), selectively impairs proplatelet formation and platelet release via effects on IFN I production, without affecting cell survival or megakaryocyte generation. Megakaryo/thrombopoiesis seemed to be linked to IFN I levels; high levels of these cytokines markedly decreased megakaryocyte growth and pla-

telet biogenesis, whereas low IFN I levels only impaired platelet production (10). Interestingly, both phenomena were independently demonstrated using recombinant IFNα. This cytokine was shown to hinder mouse megakaryopoiesis by altering proliferation and ploidy (11), but it also decreases platelet production without altering megakaryocyte growth (12). We also demonstrated that early progenitors and mature megakaryocytes, but not platelets, synthesise and release IFNβ, as well as express functional IFNAR, suggesting that IFN I plays a role in megakaryo/thrombopoiesis and raising the possibility that megakaryocytes are involved in the antiviral host response (13).

To improve understanding of the novel role of IFN I in platelet production we studied megakaryo/thrombopoiesis and platelet function in mice treated with poly (I: C), which predominantly stimulates activation of NF-κB and synthesis of IFNβ (9). Interestingly, due to the severe adverse effects of IFN I when administered in antitumour therapy (14), several agonists of TLRs that trigger physiological concentrations of IFN I, including poly (I: C) are being or have been tested in oncology trials (15). Because of its modulatory and optimisation antigen-specific immune response, poly (I: C) is also increasingly used as an adjuvant in human vaccination (16). A better understanding of the role of endogenous IFN I in megakaryo/thrombopiesis is necessary to ensure that clinical usage of these drugs is safe and evidence-based.

Our results provide new evidence that IFNβ-triggered by poly (I: C) causes thrombocytopenia and platelet dysfunction as a consequence of the inability of megakaryocytes, once formed, to reach the appropriate vascular niche and produce sufficient functional platelets. The novel observations presented here open up new avenues for research into the mechanisms controlling platelet biogenesis and could represent a convergent pathway for the effects of many viral infections on platelet production and function.

Materials and methods

Reagents

FITC-conjugated anti-mouse CD62P, FITC-conjugated anti-mouse CD61, PE-conjugated anti-mouse Ly6G and irrelevant FITC and PE monoclonal antibodies (Ab) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). PE-conjugated anti-mouse Ly6G was purchased from Biolegend (San Diego, CA, USA).α-thrombin was purchased from Enzyme Research Labs (Swansea, UK). Prostacyclin (PGI2) was purchased from Cayman (Ann Arbor, MI, USA). Alexa 488-conjugated fibrinogen was obtained from Invitrogen (Eugene, OR, USA). CD61 antibody was purchased from SantaCruz biotechnology (Santa Cruz, CA, USA). The synthetic analogue of dsRNA polyinosinic-polycytidylic acid, poly (I: C), was obtained from InvivoGen (San Diego, CA, USA). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Mice treatment and blood sample collection

Eight-to-ten-week-old C57BL/6J female mice and IFNAR$^{-/-}$ mice with a C57BL/6J background and their control littermates, originally obtained from Jackson Lab (Bar Harbor, ME, USA),were housed and bred in the animal facility of the Institute of Experimental Medicine. All experiments were performed in accordance with the American Physiological Society's Guiding Principles for the Care and Use of Animals in Research (American Physiological Society) and federal law on the use of experimental animals (Animal Welfare Act) and were approved by the local authorities.

Avertin-anesthetised mice were inoculated intravenously with poly (I: C) each 24 h and after different time points blood samples were collected by puncture of the retro-orbital plexus.

Blood cell count

Blood samples were drawn into plastic tubes containing EDTA. Haematological counts were performed using a veterinary haematological analyser (Abacus Jr Vet, Vienna, Austria).

Mouse platelet preparation

Orbital blood from Avertin-anesthetised mice was drawn into plastic tubes containing 10% by volume 3.8% citrate. The anticoagulated blood was diluted 1: 1 in PBS/citrate 0.38% and platelet-rich plasma (PRP) was obtained by centrifugation (5 minutes [min] at 100*g* at room temperature [RT]). Washed platelets (WP) were obtained by centrifuging PRP (5 min at 200*g* at RT) in the presence of PGI_2 (75 ng/ml) and EDTA (2 mM) to avoid platelet activation. After washing, WP were re-suspended in RPMI, adjusted to 3×10^4 platelet/μl and maintained at RT for 30 min prior to experimentation.

Flow cytometry

WP were stimulated for 10 min with different concentrations of thrombin in the presence of FITC-CD62P (anti-P-Selectin), an equivalent amount of isotype-matched control mAb or Alexa-488 fibrinogen, and then washed and fixed in 1% PFA. Samples were analysed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) and using CellQuest and WinMDI software; results were expressed as mean fluorescence intensity (MFI).

Mixed aggregates were tested in whole blood that was lysed with NH_4CL, washed and then stimulated with thrombin or not, according to treatment group, for 15 min at RT in the presence of FITC-CD61 and PE-Ly6G Abs or the corresponding isotypes. Samples were analysed using flow cytometry and the results were expressed as the percentage of the $CD61^+$ cells in the $Ly6G^+$ population.

Determination of RANTES release from platelets

Washed platelets were stimulated with 0.5U/ml of thrombin for 30 min at RT. The reaction was halted by the addition of 75 nM of PGI2 and supernatants were stored at –80 °C until assayed. Release of RANTES was measured by using a commercial kit (Peprotech, Rocky Hill, NJ, USA) according to the manufacturer's instruction.

Bleeding times

The tail bleeding assay was performed as described previously (17). Briefly, mice were anaesthetised with Avertin before the terminal tail segment tail was cut off with a sharp sterile blade then immediately immersed in saline solution at 37 °C. Tail bleeding time was defined as the time required for bleeding to stop. In some mice the bleeding had not stopped after 600 seconds (sec), in these mice bleeding time was recorded as >600sec.

Platelet adhesion assay

A 96-well plate was coated with fibrinogen (100 µg/ml), and left overnight at 4 °C. The plate was then washed and blocked with

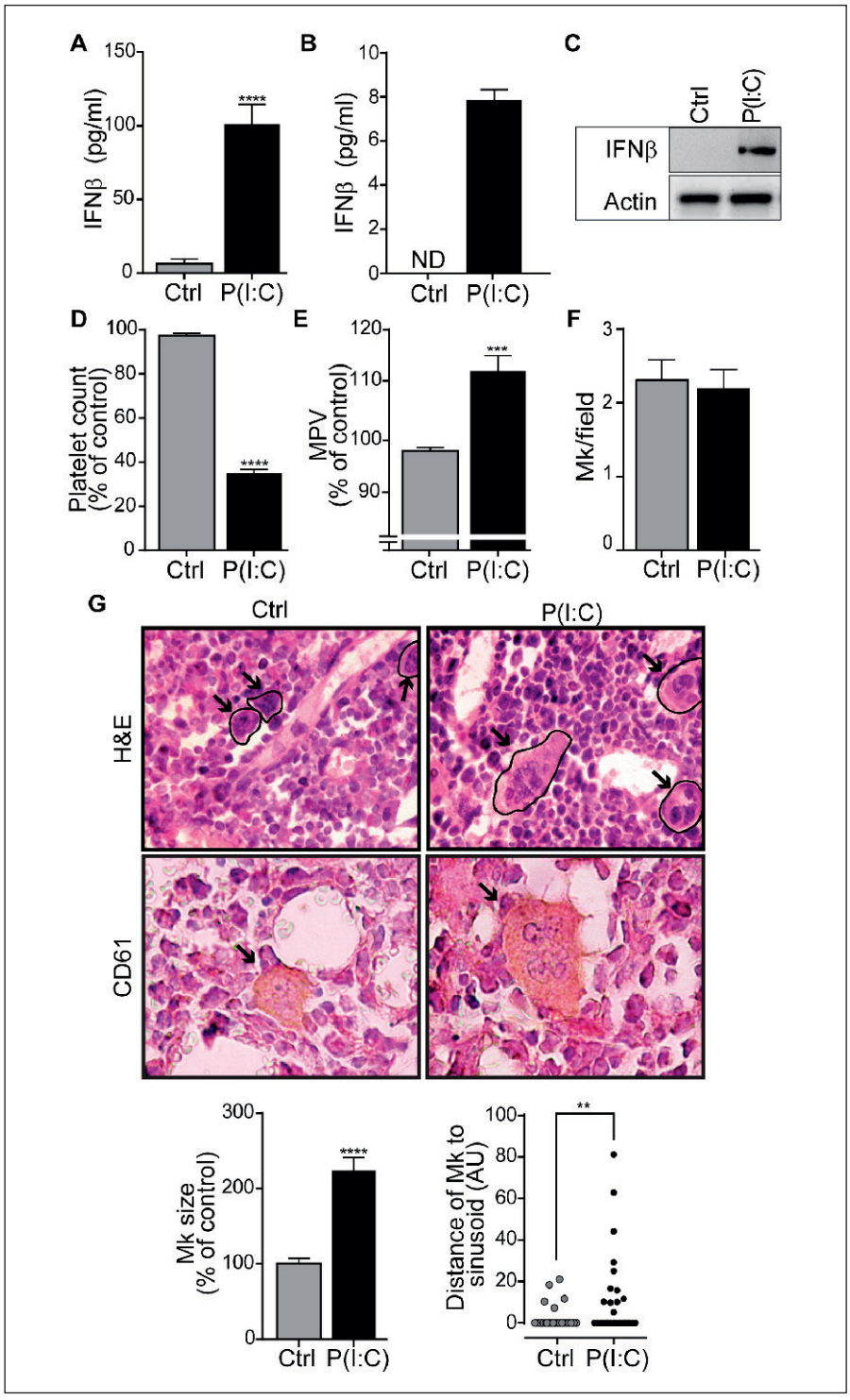


Figure 1: Poly (I:C) effects on platelets and megakaryocytes. Mice were inoculated daily with poly (I:C) [P(I:C), (25 µg/mouse/day)] for three days, blood samples were taken at 72 h. Plasmatic (A) and bone marrow (B) IFNββ levels were determined by ELISA. C) RT-PCR were performed in bone marrow cells. Platelet count (D) and mean platelet volume (MPV) (E) were analysed in whole blood using a haematological analyser, the results are expressed as percentage of control values. Mice were euthanised and femur samples were removed, fixed and stained with H&E and CD61 to determine megakaryocyte (Mk) number (F), size and distance to medullar sinusoids (G). The Mk have been indicated with black arrows. (n=6, comparison with control: **p <0.01, ***p <0.001, ****p <0.0001, ND: Non Detectable).

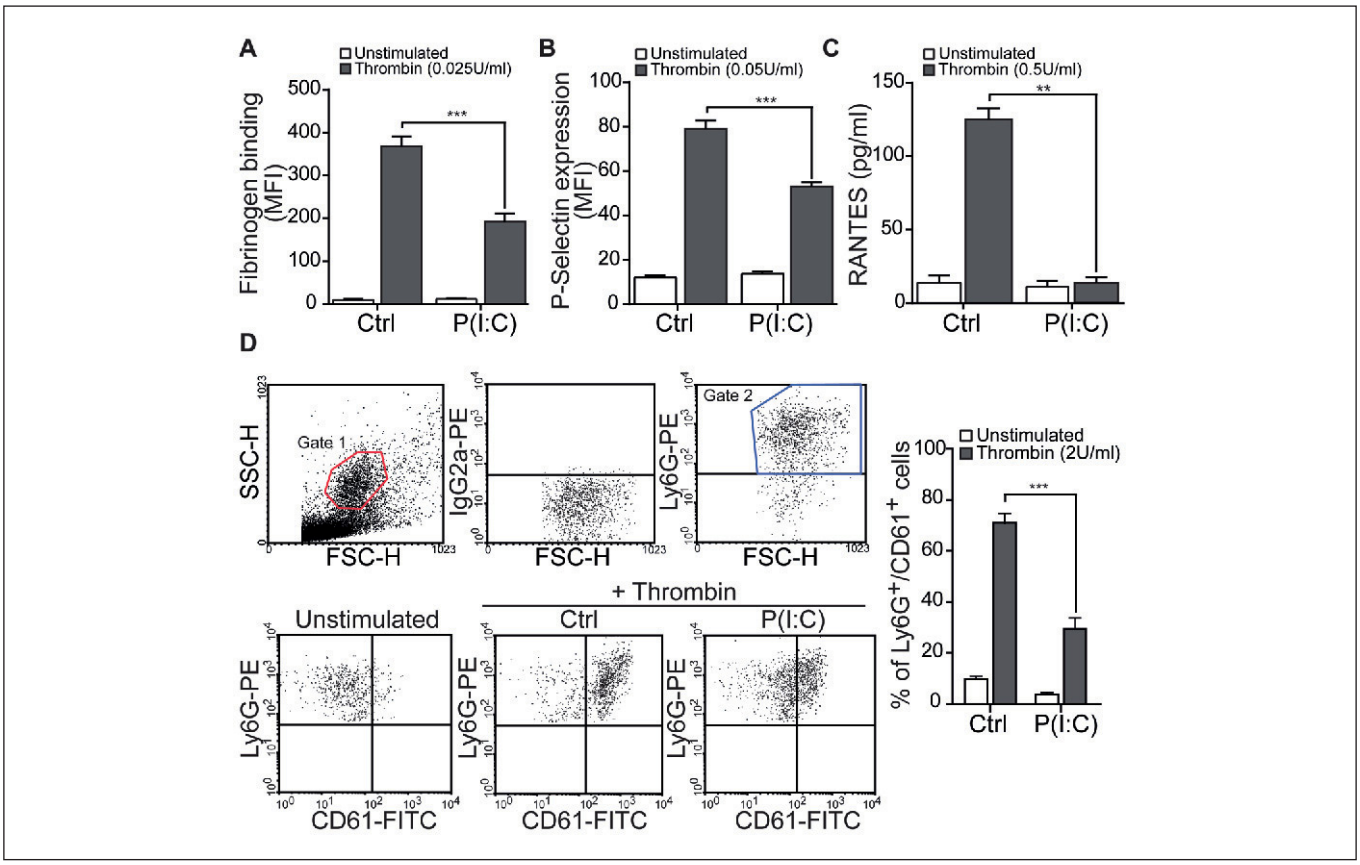


Figure 2: Poly (I:C) dampened platelet function. Washed platelets (WP) (3×10^4/ µl) from mice that were treated for 72 h with poly (I:C) [P(I:C), 25 µg/mouse/day] were stimulated with thrombin at the indicated concentration to assess fibrinogen binding (A) or P-Selectin expression (B) by flow cytometry and RANTES release by ELISA (C) (n=6, **p<0.01, ***p<0.001 vs control -WP stimulated with the same thrombin concentration-). D) Mixed aggregates were tested in whole blood that was lysed with NH_4Cl, washed and then stimulated with thrombin, or not, in the presence of FITC-CD61 and PE-Ly-6G Abs or the corresponding isotypes. The figure depicts the analysis strategy. Cells were gated for FSC and SSC (Gate 1, red) and then for Ly6G+ cells (Gate 2, blue). An analogical gate was used to include both conditions and then analysed. Results are expressed as % of CD61 cells in the Ly6G+ population. Flow cytometry assay were analysed on a FACSCalibur flow cytometer using CELLQUEST software and the results are expressed as mean fluorescence intensity; MFI. (n=6, ***p<0.001 vs control blood stimulated with the same thrombin concentration).

2 % heat-inactivated BSA and aliquots of WP were added to each well and incubated at RT for 1 hour (h). After washing, a solution of 5 mM p-nitrophenyl phosphate in 0.1 M citrate buffer containing 0.1 % Triton X-100, pH 5.4 was added and incubated for 1 h at 37°C. The reaction was stopped by adding 2N NaOH and absorbance at 405 nm was measured with a microplate reader (Dynatech MR 5000; Dynatech Laboratories, Munich, Germany).

Platelet spreading

Coverslips were coated with fibrinogen (100 µg/ml) and left overnight at 4°C then blocked with 2 % BSA for 2 h. Following this WP (3×10^4 platelet/µl) were added and incubated at RT for 20 min. Adhered platelets were fixed in 4 % PFA, permeabilised with 0.1 % Triton X-100 then stained with TRITC-Phalloidin and visualised with confocal microscopy (Olympus FV-1000; Olympus, Tokyo, Japan). Images were analysed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Clot retraction assay

Clot retraction was measured by mixing 200 µl of PRP from control or poly (I: C)-treated mice with 300 µl of Tyrode's buffer, 10 µl of erythrocytes (to enhance clot contrast for photography), 2 mM of calcium and 2 U/ml of thrombin. As infusion of poly (I: C) induced thrombocytopenia, in order to obtain the same final platelet concentration (3×10^4 platelet/µl) the PRP from control samples was adjusted with platelet-poor plasma from the same animal. A rod was placed in each glass test tube and incubated at RT. Clot formation and subsequent retraction was photographically recorded at various time intervals up to 180 min.

Determination of von Willebrand factor (vWF).

Plasma vWF levels were determined by ELISA as previously described (18). The results are expressed in ng/ml and were extrapolated from serial dilutions of normal pooled plasma, assuming a 7 µg/ml vWF concentration.

Histology

Femur samples were removed from mice and immersed in 10% buffered formalin. Thin transversal sections of the whole bone were cut and morphological features were evaluated in standard haematoxylin-and-eosin (H&E) slides using a Zeiss Axiolab microscope. In selected experiments, CD61 was immunostained using a monoclonal antibody anti CD61 to confirm megakarycytes morfology. After washing, the primary antibody was detected using a kit (Zymed, San Francisco, CA, USA) according to the manufacturer's instructions. Megakaryocyte numbers in bone marrow were estimated by counting numbers in thirty 400X magnification fields and expressed in terms of megakaryocytes/field. Sinusoidal blood vessels were identified by the visibly clear lumen in direct contact (sinusoidal) with the parenchymal cells and the characteristically flattened endothelial lining around the lumen. Megakaryocytes were identified by typical morphological characteristics. The perimeter or area of each megakaryocyte was determined using the ImageJ software.

RNA isolation and RT-PCR

Bone marrow cells were obtained by flushing the murine femurs with RPMI (19). After red cell lysis and washing, total RNA was isolated using TriReagent (Biodynamics, Buenos Aires, Argentina). RT-PCR was performed as previously described (19). Briefly, cDNA was synthesised from 20 ng of total RNA using 15 mM of random hexamers (Biodynamics) and Moloney murine leukaemia virus reverse transcriptase (Promega, Buenos Aires, Argentina). The PCR was conducted at an annealing temperature of 55°C. Specific primer for murine actin and IFNβ were used (Actin fwd: AACCCCAAGGCCAACCGCGAGAAGATGACC, Actin rev: GGTGATGACCTGGCCGTCAGGCAGCTCGTA, IFNβ fwd: CACACCCTCTCCATCAACT, IFNβ rev: TCTTGAAGTCCG CCCTGTAG).

Determination of IFNβ levels

Plasma samples were obtained by centrifugation of anticoagulated whole blood (10 min at 800g at RT). Bone marrow samples were obtained by flushing the murine femurs of three

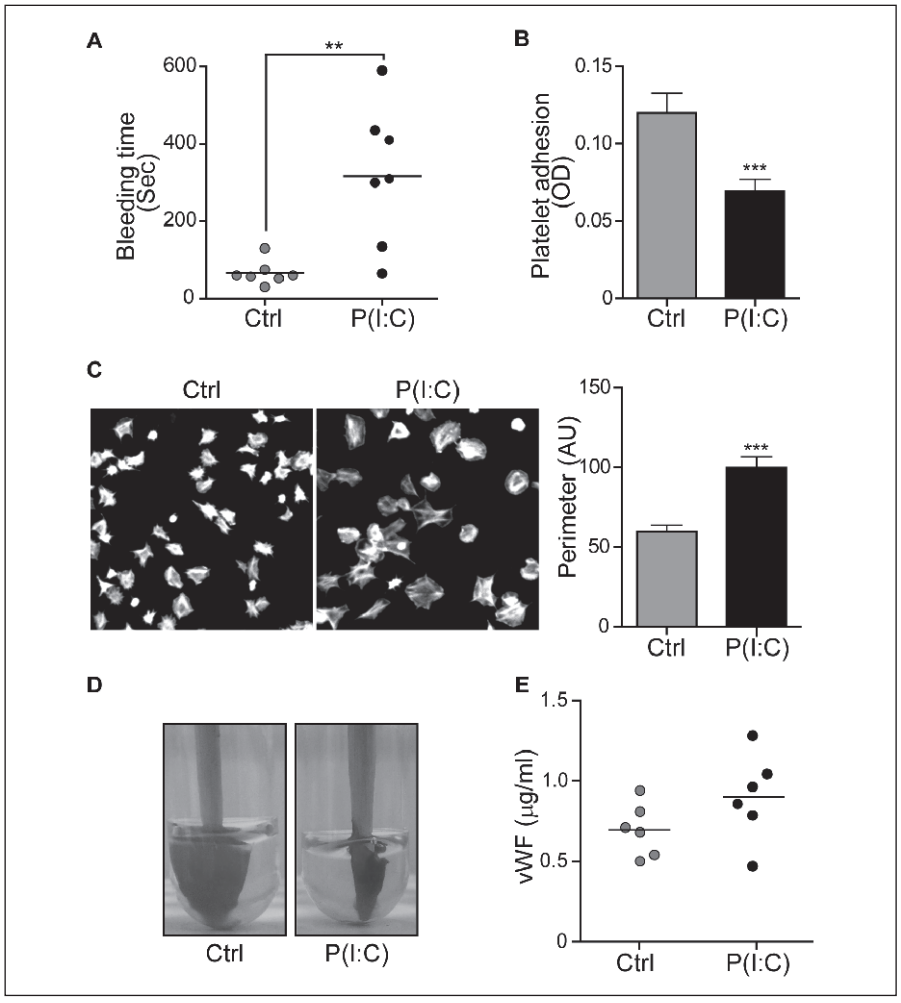
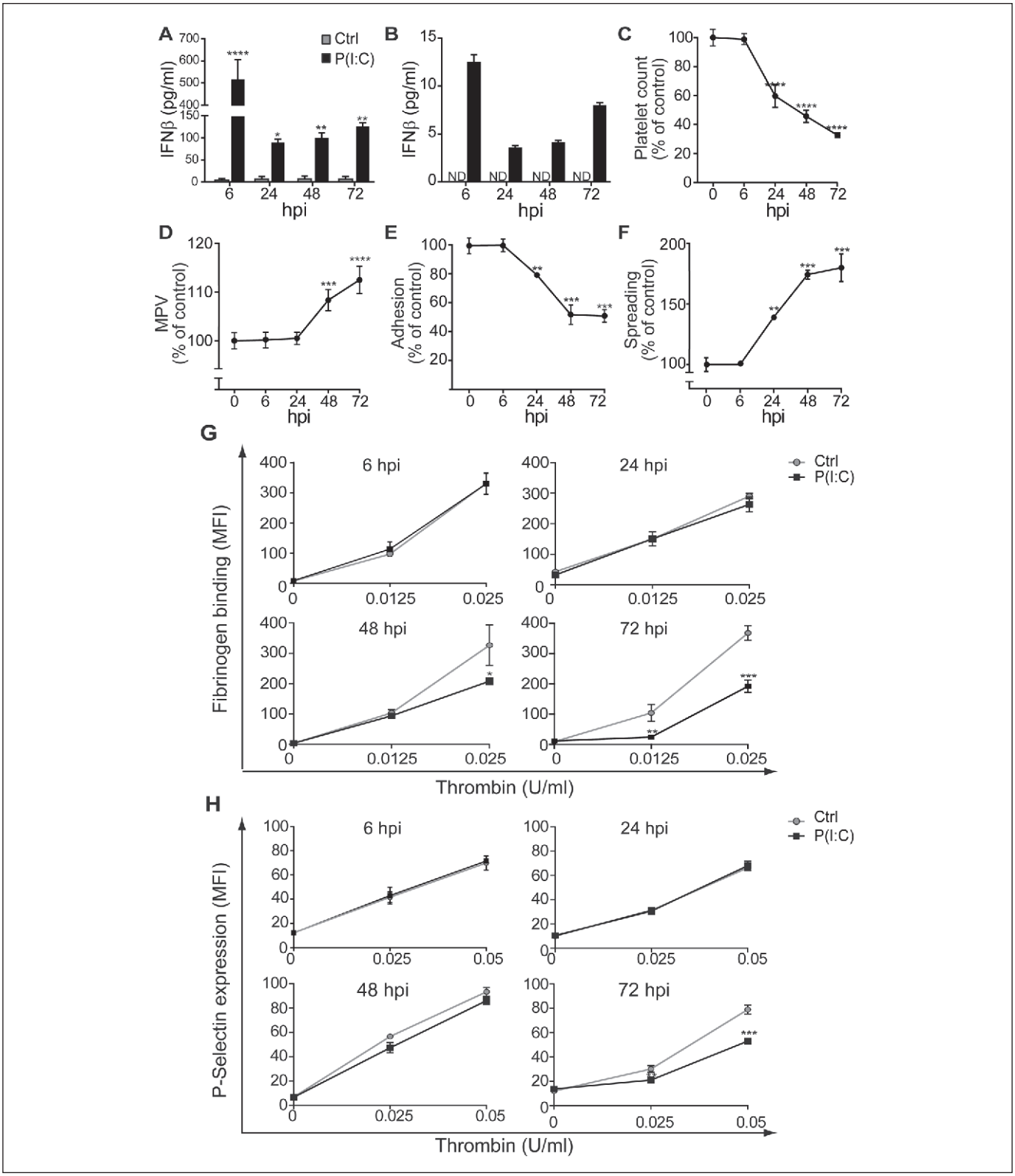


Figure 3: Primary haemostasis is altered in poly (I:C) treated mice. Mice were inoculated daily with poly (I:C) [P(I:C), 25 μg/mouse/day] for three days. A) The terminal 3 mm of the tail was cut off and immediately immersed in saline solution at 37°C. Tail bleeding time was defined as the time taken for bleeding to cease. B) Washed platelets (WP) (3×10^4/ μl) were seeded on fibrinogen-coated wells and platelet adhesion was assessed using an acid phosphatase activity assay. The results were expressed in terms of optical density (OD). C) WP (3×10^4/ μl) were plated on fibrinogen-coated wells for 20 min. Platelets were fixed and stained with TRITC-phalloidin. Platelet spreading was visualised using confocal microscopy (Olympus FV1000). D) Clot retraction was determined using PRP supplemented with red cells in the presence of thrombin (2 U/ml) and monitored for 180 min. E) Plasma vWF levels were determined by ELISA. (n=7, comparison with control **p<0.01, ***p<0.001).

mice and resuspended in 400 µl of RPMI. IFNβ levels were determined using a mouse High Sensitivity IFNβ ELISA Kit (PBL assay science, Piscataway, NJ, USA) according to the manufacturer's instructions.

Statistical analysis

All results are expressed as mean + SEM. According to the experiments, results were analysed using Student's paired t-test or one-



and two-way analyses of variance (ANOVAs) followed by post-hoc multiple comparisons using the Bonferroni procedure to assess differences between means. All statistical analyses were performed with Prism 6 software (GraphPad, San Diego, CA, USA). The significance threshold was set at $p < 0.05$.

Results

Poly (I: C) induces alterations in platelets and megakaryocytes

We assessed the role played by endogenous IFN I in the regulation of megakaryo/thrombopoiesis in an *in vivo* model by inoculating mice daily with poly (I: C) (25 µg/mouse/day) for three days, then evaluating platelet and megakaryocyte numbers and morphology. As expected, injection of poly (I: C) triggered the production of IFNβ. Increased levels of IFNβ were detected in plasma as well as in bone marrow samples. (►Figure 1A-C). There was also a significant decrease in platelet numbers (►Figure 1D) which was accompanied by an increase in mean platelet volume (MPV, ►Figure 1E). Given that an increase in MPV is a classical physiological response to thrombocytopenia induced by stimulation of megakaryopoiesis, we decided to examine the bone marrow megakaryocyte population in more detail. Bone marrow analysis showed that although numbers were not affected (►Figure 1F), megakaryocytes were significantly larger in poly (I: C) treated animals than control mice (►Figure 1G). There was also a shift in their bone marrow location; in treated-animals megakaryocytes were located further from bone marrow sinusoids than in control animals (►Figure 1G).

Poly (I: C) down-regulated platelet haemostatic and proinflammatory responses

Platelet activation modulates not only haemostasis but also inflammation, the latter mainly due to the expression of P-Selectin and the release of several proinflammatory molecules from alpha granules (20). To analyse the effect of poly (I: C) in these platelet responses in the next experiments, we assayed fibrinogen binding, P-Selectin expression and the release of RANTES, a CCL5 chemokine, induced by thrombin. We found that all activation responses (►Figure 2A-C) were markedly decreased in mice treated with poly (I: C). In line with the down-regulation of P-Selectin expression, thrombin-induced formation of heterotypic aggregates between platelets and neutrophils was also inhibited in treated animals (►Figure 2D).

Poly (I: C) alters primary haemostasis

Having demonstrated that poly (I: C) treatment reduced platelet count and impaired platelet function, we then evaluated the functional relevance of these abnormalities by investigating tail bleeding times. We found that bleeding time was significantly prolonged in poly (I: C)-treated animals (►Figure 3A).

When exposed to extracellular matrix platelets adhere, and as a result of integrin αIIbβ3 outside-in signalling, they develop filopodia and spread. We found that adhesion of platelets to fibrinogen-coated wells was impaired (►Figure 3B), a result in line with observation of prolonged bleeding times. Surprisingly, however, spreading on fibrinogen coated wells was increased (►Figure 3C). Furthermore, the time for clot retraction, another platelet response regulated by outside-in signalling (21), was shorter in mice treated with poly (I: C) than in control animals (►Figure 3D). Bleeding time is influenced not only by platelet numbers and functionality, but also by vWF levels and release of platelet endothelium-derived inhibitors such as prostacyclin (PGI2) and nitric oxide (NO). We therefore measured plasma levels of vWF and PGI2 to gain a better understanding of the mechanisms underlying the prolongation of bleeding in poly (I: C)-treated animals. The increase in vWF levels in poly (I: C)-treated mice was not significant (►Figure 3E). PGI2 levels were similar in poly (I: C)-treated mice and controls (data not shown).

Alterations of platelets mediated by poly (I: C) are not associated to a direct effect

To determine whether poly (I: C) acted directly on platelets or on the parent megakaryocytes mice were injected with 25µg of poly (I: C) every 24 hours (h) and plasma IFNβ production and haematological features were evaluated at 6, 24, 48 and 72 h post-injection (hpi). Plasmatic and bone marrow IFNβ levels in poly (I: C)-treated animals followed a similar kinetic pattern; they peaked at 6 hpi, then decreased but remained higher than in the control animals for all time intervals (►Figure 4A and B). Platelet count, MPV, adhesion to fibrinogen, spreading, fibrinogen binding and the P-Selectin expression were unaffected at 6 hpi (►Figure 4C-H). Platelet count was significantly lower than in non-treated mice from 24 hpi (►Figure 4C); but the effect on MPV only became evident at 48 hpi (►Figure 4D). Platelet dysfunction was observed at 24 hpi for adhesion and spreading, 48 hpi for fibrinogen binding and 72 hpi for P-Selectin (►Figure 4E-H) suggesting that the effect of poly (I: C) was associated with an alteration of the

Figure 4: Platelets alterations mediated by poly (I:C) were not associated with an acute effect. Mice were inoculated with poly (I:C) [P(I:C), 25 µg/mouse/day] and blood samples were collected by puncture of the retro orbital plexus at the indicated time. Plasmatic (A) and bone marrow (B) IFNββ levels were determined by ELISA. Platelet count (C) and mean platelet volume (MPV) (D) were analysed in whole blood with a haematological analyser. E) Washed platelets (WP) (3×10^4/ µl) were seeded on fibrinogen-coated wells and platelet adhesion was assessed using an acid phosphatase activity assay. The results were expressed in terms of optical density (OD). F) WP (3×10^4/ µl) were plated on fibrinogen-coated wells for 20 min. Platelets were fixed and stained with TRITC-phalloidin. Platelet spreading was visualised using confocal microscopy (Olympus FV1000). (n=6, comparison with control *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). G) WP (3×10^4/ µl) were stimulated with thrombin and fibrinogen binding or P-Selectin expression were analysed by flow cytometry on a FACSCalibur flow cytometer using WinMDI software and the results are expressed in terms of mean fluorescence intensity; MFI. (n=6, ***$p<0.001$ vs control WP stimulated with the same thrombin concentration).

megakaryocyte rather than a direct effect on platelets. Moreover, the *in vitro* treatment of murine platelets with the same concentrations of poly (I: C) did not influence either basal or thrombin-induced fibrinogen binding or P-Selectin expression (data not shown).

Poly (I: C)-induced megakaryo/thrombopoiesis alterations correlate with IFNβ levels

In a previous study using an *in vitro* model we observed that IFNβ differentially alters megakaryo/thrombopoiesis in a concentration-dependent manner (10). In the next set of experiments we sought to replicate this finding *in vivo*. We observed concentration-dependent suppression of platelet numbers, increase in MPV and loss of platelet functionality (fibrinogen binding and P-Selectin expression) (►Figure 5A-E). Tail bleeding time also increased with poly (I: C) dose; at higher poly (I: C) doses tail bleeding lasted longer than 600s, our selected measurement end point (►Figure 5F). Bone marrow megakaryocytes were unaffected by treatment with poly (I: C) (data not shown) but their size increased in proportion to poly (I: C) concentration (►Figure 6A). Notably, at high poly (I: C) concentrations (50–200 μg), bone marrow sinusoids were markedly dilated (►Figure 6A-C). All the quantitative and qualitative alterations in the platelets and megakaryocytes apart from vascular dilation and bleeding time were linearly correlated with IFNβ level (►Figure 6D).

Type I IFN is the mediator involved in poly (I: C)-induced alterations in megakaryo/thrombopoiesis

To confirm that poly (I: C)-mediated impairment in megakaryo/thrombopoiesis is a consequence of IFNβ production we explored similar platelet responses in mice deficient in the IFN I receptor ($IFNAR^{-/-}$). As shown in ►Figure 7A-H although infusion of poly (I: C) increased plasma and bone marrow levels of IFNβ in these mice, this failed to produce thrombocytopenia or alterations in platelet function or MPV as there were no alterations in megakaryocyte size or localisation of megakaryocytes in the marrow sinusoids.

Discussion

In this study we demonstrated in mice that poly (I: C) decreased platelet production, impaired platelet function, affected primary

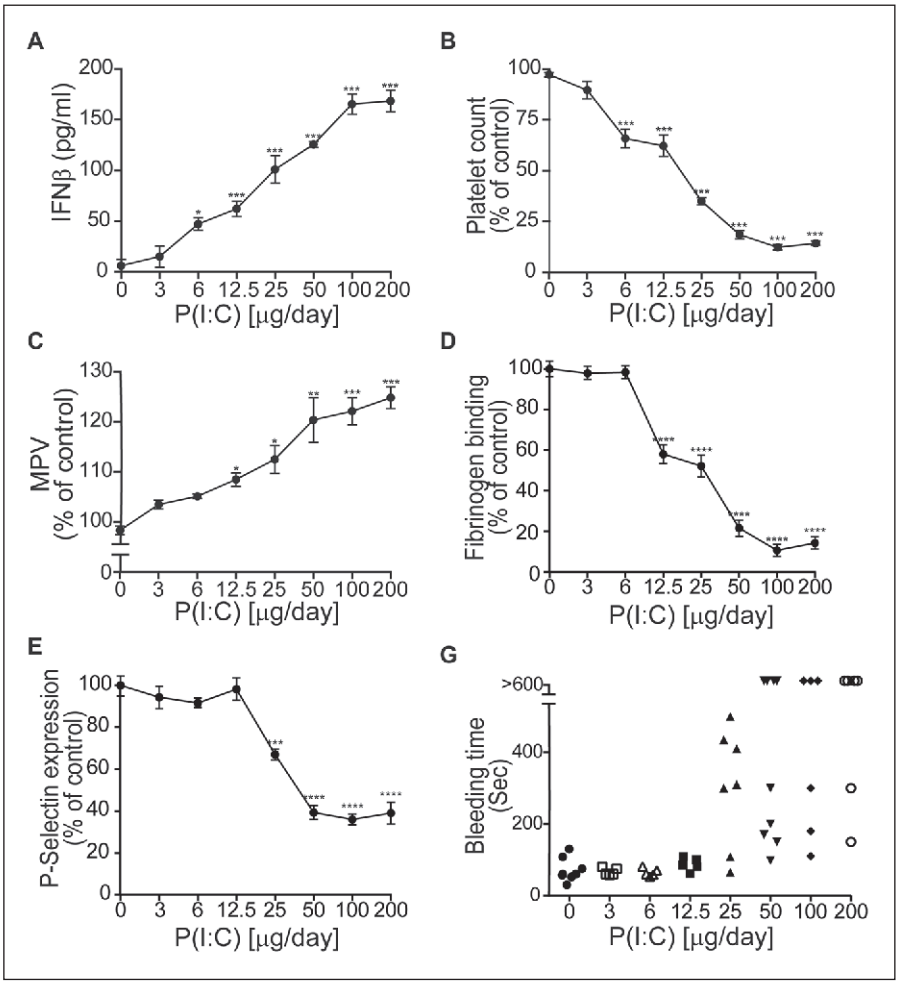


Figure 5: Poly (I:C) dampened megakaryo/thrombopoiesis and platelet function in a concentration dependent manner. Mice were inoculated daily with various concentrations of poly (I:C) for three days; blood samples were taken at 72 h and plasma IFNββ levels (A), platelet count (B) , (C) mean platelet volume (MPV), (D) fibrinogen binding, (E) P-Selectin expression and (F) bleeding time were determined. Results are expressed as percentage of control values (n=6, comparisons with control *p< 0.05, ***p< 0.01, ***p< 0.001, ****p< 0.0001).

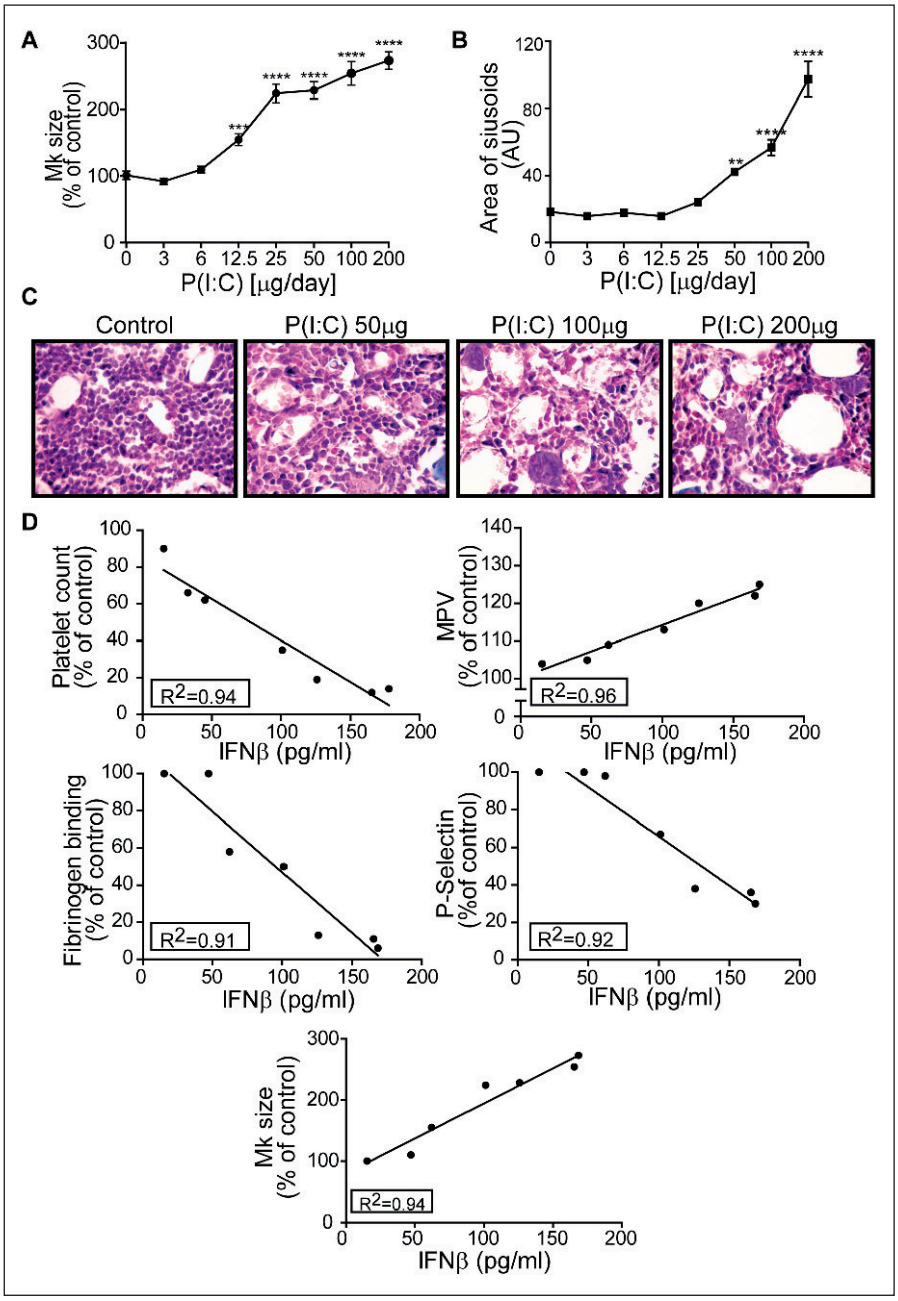


Figure 6: Direct correlation between platelets and megakaryocytes alterations and plasmatic IFNββ levels. Mice were inoculated daily with various concentrations of poly (I:C) [P(I:C)], for three days. At 72 h mice were euthanised and femur samples were removed, fixed and stained with H&E to determine megakaryocyte (Mk) size (A) and sinusoids area (B) (n=6, comparison with control **p<0.01, ***p<0.001, ****p<0.0001). C) Images of sinusoid size are representative of six mice. D) Alterations in platelet count, mean platelet volume (MPV), fibrinogen binding, P-selectin expression and Mk size were correlated with IFNββ levels.

haemostasis and altered the distribution of bone marrow megakaryocytes in the vascular niche; these changes were correlated with an increase in plasma and bone marrow IFNβ levels.

Administration of a daily dose of poly (I: C) over a three-day period significantly increased IFNβ levels, decreased platelet numbers and increased MPV. MPV is generally higher in the context of platelet destruction or inherited macrothrombocytopenia (22, 23) and abnormally low MPV values are more likely to be correlated with thrombocytopenia when it is due to impaired bone marrow production (24). Despite the increase in MPV, the fact that thrombocytopenia was observed 24 h after poly (I: C) treatment started and peaked 48 h later and bearing in mind that murine platelets have a life span of 3–4 days (25), our results suggested that the low platelet count was not related to acute platelet destruction. Moreover, we found that the increase in MPV was independent of the level of thrombocytopenia, occurring only when bone marrow megakaryocyte size was affected. Previous research, including studies by our group, showed that demarcation of the membrane system and platelet territories are absent in IFNα- or β-treated human megakaryocytes (10, 12). Thus, the increase in platelet size could have been associated with alterations in megakaryocyte cytoskeleton. Collectively our data indicated that the reduction in platelet count was due to altered megakaryo/thrombopoiesis in the bone marrow.

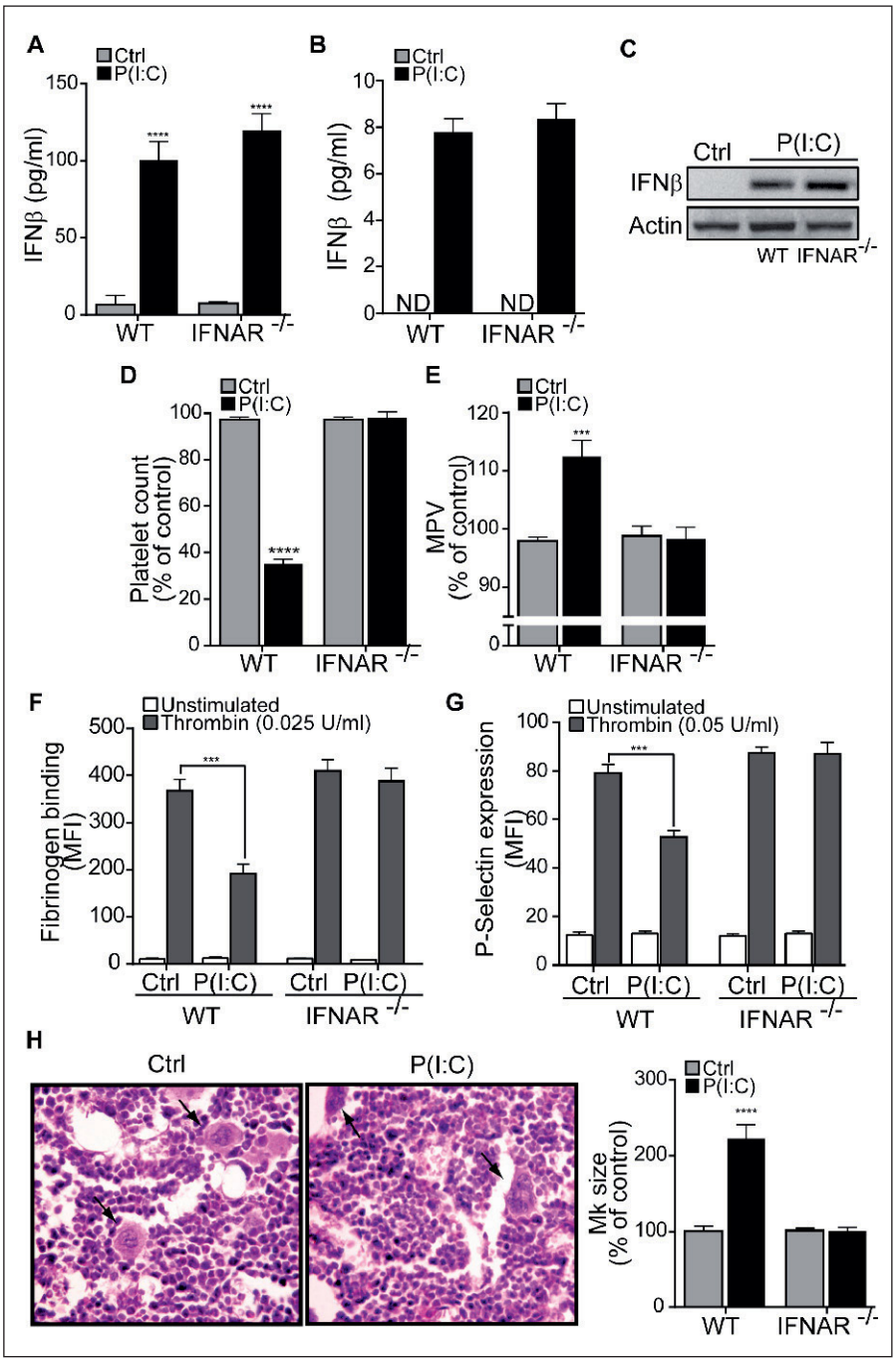


Figure 7: Poly (I:C)-induced alterations were absent in IFNAR$^{-/-}$ mice. IFNAR$^{-/-}$ and control littermates (WT) mice were inoculated daily with poly (I:C) [P(I:C), 25 µg/mouse/day] for three days and blood samples were taken at 72 h. Plasmatic (A) and bone marrow (B) IFNββ levels were determined by ELISA. C) RT-PCR were performed in bone marrow cells. Platelet count (D) and mean platelet volume (MPV) (E) were analysed in whole blood with a haematological analyser (n=6, comparison with control ***p< 0.001, ****p< 0.001). F and G) Washed platelets (3×10^4/ µl) were stimulated with thrombin and fibrinogen binding or P-Selectin expression were analysed by flow cytometry on a on a FACSCalibur flow cytometer using CELLQUEST software and the results were expressed in terms of mean fluorescence intensity; MFI. (n=6, ***p< 0.001 vs WP stimulated with the same thrombin concentration). H) Mice were euthanised and femur samples were removed, fixed and stained with H&E to determine megakaryocyte (Mk) size. The Mk were indicated with black arrows. The images are representative of six mice. (n=6, ****p< 0.0001).

Although bone marrow megakaryocyte numbers were unaffected by treatment with poly (I: C) we did observe an increase in their size. These data are consistent with previous studies which correlated reductions in platelet numbers with increases in megakaryocyte size as an attempt to improve the thrombopoietic potential of the marrow (26, 27). However in addition to the increased size, we also observed that the fraction of megakaryocytes associated with the bone marrow sinusoidal microvasculature was significantly lower in treated than in control animals. Megakaryocyte development in the bone marrow progresses from the endosteal niche, which promotes megakaryocyte progenitor proliferation, towards the vascular niche, where megakaryocytes transmigrate through the sinusoidal endothelium and release pro-platelets into the sinus (28). It is therefore possible that despite their increase in size, an impairment in megakaryocyte migration towards the sinusoids is one of the mechanisms underlying thrombocytopenia in poly (I: C)-treated animals. Interestingly, it has recently been shown that endogenous SDF-1 acutely regulates megakaryocyte location in the bone marrow. In a steady state SDF-1 directs megakaryocyte movement towards the vascular niche, resulting in

thrombopoiesis. Following radiation injury SDF-1 transcript numbers increase near the endosteum, directing megakaryocytes away from the vascular niche and reducing platelet production (29). Considering these data, it remains to be determined whether the impairment in the ability of megakaryocytes to reach the marrow sinusoids in poly (I: C)-treated animals is due to an IFN I-mediated regulatory effect on SDF-1 expression in marrow stroma.

It is notable that at high poly (I: C) concentrations we observed an increase in vascular bone marrow area due to vasodilation and re-association of megakaryocytes with the sinusoids. Although vascular dilation could be a physiological response instigated to bring megakaryocytes closer to the sinusoids in order to facilitate platelet release, we did not observe any corresponding increase in the number of circulating platelets. In line with our results, Niswander et al. postulated that thrombocytopenia induced by radiation injury promoted marrow vascular dilatation causing a reassociation with vasculature without functional consequences for platelet production (29).

Together with alterations in platelet number and megakaryocyte distribution in the bone marrow, we also found that thrombin-mediated fibrinogen binding and platelet adhesion were diminished in poly (I: C)-treated animals. Unexpectedly, platelet spreading and clot retraction were significantly increased. Although the reasons for these effects are not clear, a possible explanation could be that the enhancement of these haemostatic responses was a compensating mechanism for poly (I: C)-mediated alterations in platelet adhesion. However, the tail bleeding time, which is influenced by the quantitative and qualitative properties of platelets, was longer in poly (I: C)-treated mice than in controls. These results suggest that the increase in the outside-in platelet responses (spreading and clot-retraction) was not sufficient to prevent the effect of poly (I: C)-mediated thrombocytopenia and platelet function alterations.

Besides platelets, vWF levels and the release of platelet endothelium-derived inhibitors also regulates the bleeding time. Poly (I: C) has been shown to activate the endothelium (30) so the release of endothelial-derived platelet inhibitors may also have contributed to the prolongation of bleeding. We found that plasmatic vWF and PGI_2 levels were similar in controls and in treated animals precluding an alteration in the levels of these molecules as the cause of the prolonged bleeding in treated mice.

Platelets not only are key elements for haemostasis and thrombosis but also are relevant amplifiers of the inflammatory response. This feature is associated with the expression of P-Selectin on the surface membrane and the great variety of pro-inflammatory molecules that are released upon activation (20). Our data showing that the exposure of P-Selectin together with the formation of platelet-neutrophil mixed aggregates as well as the secretion of RANTES which were markedly decreased in poly (I: C)-treated mice pointed out that platelet-mediated inflammatory responses were also impaired by poly (I: C) treatment. Since we have recently reported that human platelets express TLR3 and its stimulation by poly (I: C) primed thrombin-mediated activation (31), it could be conceivable that poly (I: C) infusion in mice directly affected platelet function and promoted sequestration. However, our data showed that the platelet responses analysed were not modified until 24–48 or 72 h post poly (I: C) infusion. In addition, the *in vitro* experiments showed that poly (I: C) at the same concentrations used *in vivo*, neither induced platelet activation nor modified thrombin-induced platelet responses. Collectively these data suggest that the decreased platelet function appears to be associated with a direct effect on megakaryocytes rather than on platelets. Experiments using TLR3 KO mice would be helpful to elucidate this issue.

Best known as a robust inducer of IFN I, poly (I: C) is also associated with the induction of other proinflammatory cytokines such as interleukin (IL)-6, 10, 12, and tumour necrosis factor-α (32). We found that in spite of normal IFN I production, either plasmatic or in the bone marrow, none of these effects were observed in IFNAR-/- mice. These results together with our recent findings that TLR3 is expressed in human megakaryocytes and its activation impairs platelet production and promotes IFNβ release (31), strongly suggest that IFNβ produced by TLR3 stimulation either systemically or in a paracrine or autocrine manner in the bone marrow milieu has a major role in in the regulation of platelet biogenesis and functionality.

Our previous *in vitro* observation that high concentrations of IFNβ inhibit megakaryocyte growth and low concentrations selectively decrease platelet production (10) lead us to suggest that IFNβ levels could determine the fate of megakaryocytes, affecting either proliferation or their ability to produce platelets. The present *in vivo* data demonstrated that platelet production and func-

What is known about this topic?

- Lymphocytic choriomeningitis and Junin arenaviruses, and polyinosinic:polycytidylic acid (poly (I:C), a synthetic analogue of double-stranded RNA, Toll-like receptor-3 ligand, decrease platelet aggregation and platelet counts *in vitro* and *in vivo* due to type I interferon (IFN I) production.
- Human recombinant IFN alpha selectively diminishes platelet production without altering megakaryocyte number or endomitosis.
- Several clinical trials are currently investigating poly I:C as an adjuvant in human vaccination or cancer treatment.

What does this papers add?

- Endogenous production of IFN I triggered by the TLR3 ligand, poly (I:C), induces thrombocytopenia and also increases mean platelet volume; it dampens adhesion, the binding of fibrinogen, P-Selectin expression and RANTES release from platelets and prolongs the tail bleeding time in a concentration-dependent manner.
- Qualitative and quantitative platelet changes are not due to a direct effect on platelets or megakaryocyte numbers; rather they are associated with increased size and altered spatial distribution of megakaryocytes in the vascular bone marrow niche.
- These observations open up new avenues for research into the processes underlying platelet production pathophysiology in the context of arenavirus and other viral infections and should also be considered in the clinical use of poly I:C.

tion decreased with increasing IFNβ levels; however, although high poly (I: C) concentrations reduced platelet count almost to 15% of the mean control value and affected the megakaryocyte size and proximity to the sinusoids, megakaryocyte numbers were not markedly altered. The different *in vitro* and *in vivo* findings with respect to the effect of IFN I on megakaryocyte growth may be related to the use of different species or to the levels of IFN I attained in each condition. It should be noted that clinical studies have shown that the administration of IFNα to patients with chronic hepatitis, solid tumours or myeloproliferative disorders induce thrombocytopenia without affecting the number of megakaryocytes in bone marrow (33–35).

In conclusion, our results show that IFNβ downregulates platelet production and also hampers haemostatic and proinflammatory platelet responses, leading to impaired primary haemostasis. These alterations appear to be related to a direct effect on the late stages of megakaryocyte development and their spatial distribution in the bone marrow niches. Our data provide new evidence on the mechanisms involved in thrombocytopenia but may also be relevant to current clinical trials in which poly (I: C) is increasingly used as an adjuvant in human vaccination and cancer treatment (16).

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Conflicts of interest
None declared.

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