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

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

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

MI and JP contributed equally to this work

 doi:10.3324/haematol.2014.115634
Manuscript received on August 20, 2014. Manuscript accepted on February 2, 2015.
Correspondence: j.perdomo@unsw.edu.au

Materials

Antibodies: FITC Mouse anti-Human CD41a, PE Mouse anti-Human CD42a, APC Mouse anti-Human CD42b and Ig Isotype Control cocktail-C were purchased from BD Biosciences, (San Diego, CA). V450-Mouse anti-Human CD41a and V450-Mouse IgG1, κ Isotype control were from BD Biosciences (San Diego, CA). Mouse anti-Human CD41a-Alexa Fluor 647, Mouse anti-Human CD42a-Alexa Fluor 647 and Mouse Negative Control-Alexa Fluor 647 were from AbD Serotec (Kidlington, Oxford, UK). Simultest control γ1/γ1 (IgG1/IgG1) was from BD Biosciences (San Jose, CA). Anti-human IgM (μ-Chain specific) FITC conjugate and anti-human IgA (α-Chain specific) FITC conjugate were from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 488 goat anti-human IgG (H+L) antibody was from Molecular Probes, Life Technologies (Eugene, Oregon). Reagents and cells: Trypan Blue (0.4%), EDTA disodium salt solution for molecular biology 0.5M, Propium iodide and Stemline II media were from Sigma-Aldrich (St. Louis, MO, USA). DMEM media, phosphate buffered saline (PBS) and 0.5% Trypsin-EDTA (10X) were from Gibco (Invitrogen). Recombinant Human TPO, Research grade and CD34 MicroBead kit were from Miltenyi Biotec (Germany). CountBright Absolute Counting Beads were from Molecular Probes, Life Technologies (Eugene, Oregon). Protein G-Agarose Kit was from Roche diagnostics GmbH (Mannheim, Germany). PF4 ELISA kit was from Abcam (Cambridge, UK). TGF-β1, sFAS and sTRAIL ELISA kits were from R&D Systems, Inc (Minneapolis, USA). Nplate (romiplostim) was from AMGEN (Thousand Oaks, CA, USA). Chinese Hamster Ovary (CHO) cells were from ATCC. GPIbIX-CHO cells and GPIIb/IIIa-CHO cells were generated in our laboratory.

Supplemental Methods

ITP serum autoantibodies adsorption

Washed platelets were obtained from whole blood as previously described [1, 2]. ITP serum (400 μl) was mixed with 400 × 10^9 washed platelets for 1 h at 4°C. After centrifugation for 5 min at 3000 x *g*, the supernatant was mixed for a second time with the same number of fresh, washed platelets and incubated for 1.5 h at 4°C. The autoantibody-depleted serum was then analysed for the presence of autoantibodies by flow cytometry as described below.

ITP serum binding to platelets

To evaluate the presence of antiplatelet autoantibodies of different classes (IgG, IgM and IgA) in ITP patients' sera, we used expired group-O platelets from the Blood Bank (St George Hospital, Kogarah, NSW, Australia). The platelets were washed twice with buffer (PBS/10mM EDTA) and gently resuspended to 5 x 10^8 cells/ml. Equal volumes of suspended platelets and ITP or normal sera were incubated for 30 min at room temperature. The cells were then washed twice with the same buffer, and then incubated with Alexa Fluor 488-anti-human IgG (1:100 in the same buffer containing 1%BSA) for 30 min at 4ºC. Finally, the cells were washed twice and analysed by flow cytometry. For IgM and IgA detection, the same procedure was followed in separate experiments, except that FITC-anti-human IgM (1:16) and FITC-anti-human IgA (1:32) were added after the first incubation step, respectively.

ITP serum binding to human GPIIb/IIIa and GPIbIX expressed on CHO cells

CHO cells expressing human GPIIb/IIIa or GPIbIX were used to determine the specificity of IgG antiplatelet autoantibodies in ITP sera. The cells were dislodged from DMEM culture plates with 0.1%Trypsin-EDTA, resuspended in PBS/1%BSA

and 1 x 10^5 cells were incubated with each ITP or normal serum at different dilutions (1:10, 1:25, 1:50 and 1:100) for 30 min at room temperature. The cells were then washed with PBS and Alexa Fluor 488-anti-human IgG was added (1:100 in 1%BSA/PBS buffer) for 30 min at 4ºC. Finally, the cells were washed and analysed by flow cytometry.

Expression of MK markers on $CD34^+$ cells cultured with rhTPO

Cultured $CD34^+$ cells were collected at different time points and stained for MK markers GPIIb (CD41) and GPIX (CD42a) using FITC-anti-human CD41 and Alexa Fluor 647-anti-human CD42a, respectively. FITC-conjugated and Alexa Fluor 647-conjugated isotype antibodies were used as controls. After incubation for 30 min at 4ºC, the cells were washed and analysed by flow cytometry (FACSCalibur or FACSCantoII, BD Biosciences, San Diego, CA).

MK numbers, cell size and marker expression

At day 12 or 13 of culture, the cells were collected and stained for MK markers GPIIb, GPIX and GPIbα (CD42b) using V450-anti-human CD41, PE-anti-human CD42a and APC-anti-Human CD42b, respectively. V450-conjugated isotype antibody and PE- and APC-conjugated isotype antibody were used as controls. After incubation for 15 min at 4ºC, the cells were washed and analysed by flow cytometry. The percentage of cells expressing CD41, CD42a, CD42b and $CD41^+/CD42a^-$ as well as the percentage of double positive mature MK (i.e., $CD41^+/CD42a^+$) were determined. For cell size analysis, the cells were cytospun and subjected to Wright staining. Cells were imaged using a Zeiss Axio Observer.A1. Images were taken from random areas and the cell size was automatically calculated with Image-Pro Premier 9.1 software (Media Cybernetics, Inc. Rockville, MD, USA). Cells were also

collected and counted using Trypan blue exclusion staining and the total number of viable cells determined (Countess Automated Cell Counter, Invitrogen). To determine the total number of MK/well, the following formula was used:

$$The\,total\,MK\,number = Total\,cell\,number \times CD41\%$$

Ploidy pattern of MK

At day 12 or 13 of culture, MKs were collected for ploidy determination. The cells were stained with FITC-anti-human CD41 (30 min at 4^{o}C), washed once with binding buffer (PBS/0.5%BSA/2mM EDTA, pH 7.2), and resuspended in hypotonic citrate buffer (1.25mM sodium citrate, 2.5mM sodium chloride, and 3.5mM dextrose) containing 20 µg/ml of propidium iodide (PI) and 0.05% Triton-X 100 for 15 min at 4^{o}C in the dark. RNase was then added to 20 µg/ml, and the cell suspension was incubated for 30 min at 4^{o}C. Finally, the intensity of DNA staining by PI was determined by collecting 30,000 to 50,000 events in the $CD41^{+}$ population using a flow cytometer (FACSCalibur).

Platelet release in culture

At day 14 or 15 of culture, the number of platelet released in culture was enumerated. The cells (100µl) were collected and stained with Alexa Fluor 647-anti-human CD41 for 30 min at 4ºC. Then, 150µl of buffer (PBS/0.5%BSA/2mM EDTA, pH 7.2) and 50µl of CountBright counting beads were added and the cells were analysed immediately by flow cytometry. Normal blood platelets were treated in a similar way and were used for setting the analytical gate for the cultured platelet. The number of platelet/well was determined based on the CountBright counting beads manufacturer's instructions.

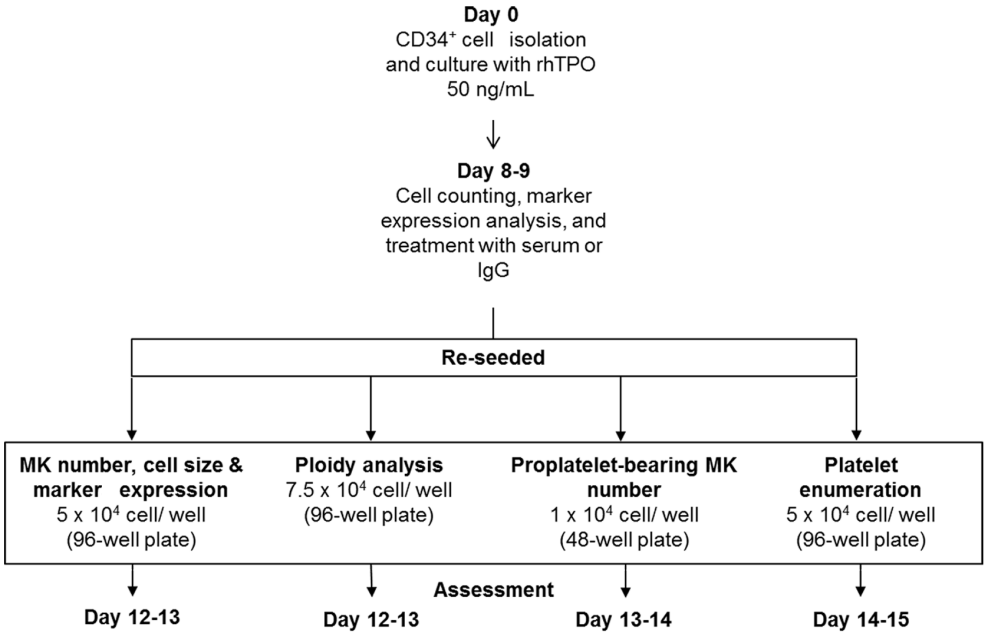
PF4, TGF-β1, sFAS and sTRAIL concentration in serum

The level of platelet factor 4 (PF4), transforming growth factor-β1 (TGF-β1), soluble FAS (sFAS) and soluble tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) in ITP and normal sera were measured by enzyme-linked immunosorbent assay (ELISA) kits mentioned in materials and methods section above according to manufacturer's instruction.

1. Asvadi P, Ahmadi Z, Chong BH. Drug-induced thrombocytopenia: Localization of the binding site of GPIX-specific quinine-dependent antibodies. *Blood*. 2003;102(5):1670-1677.

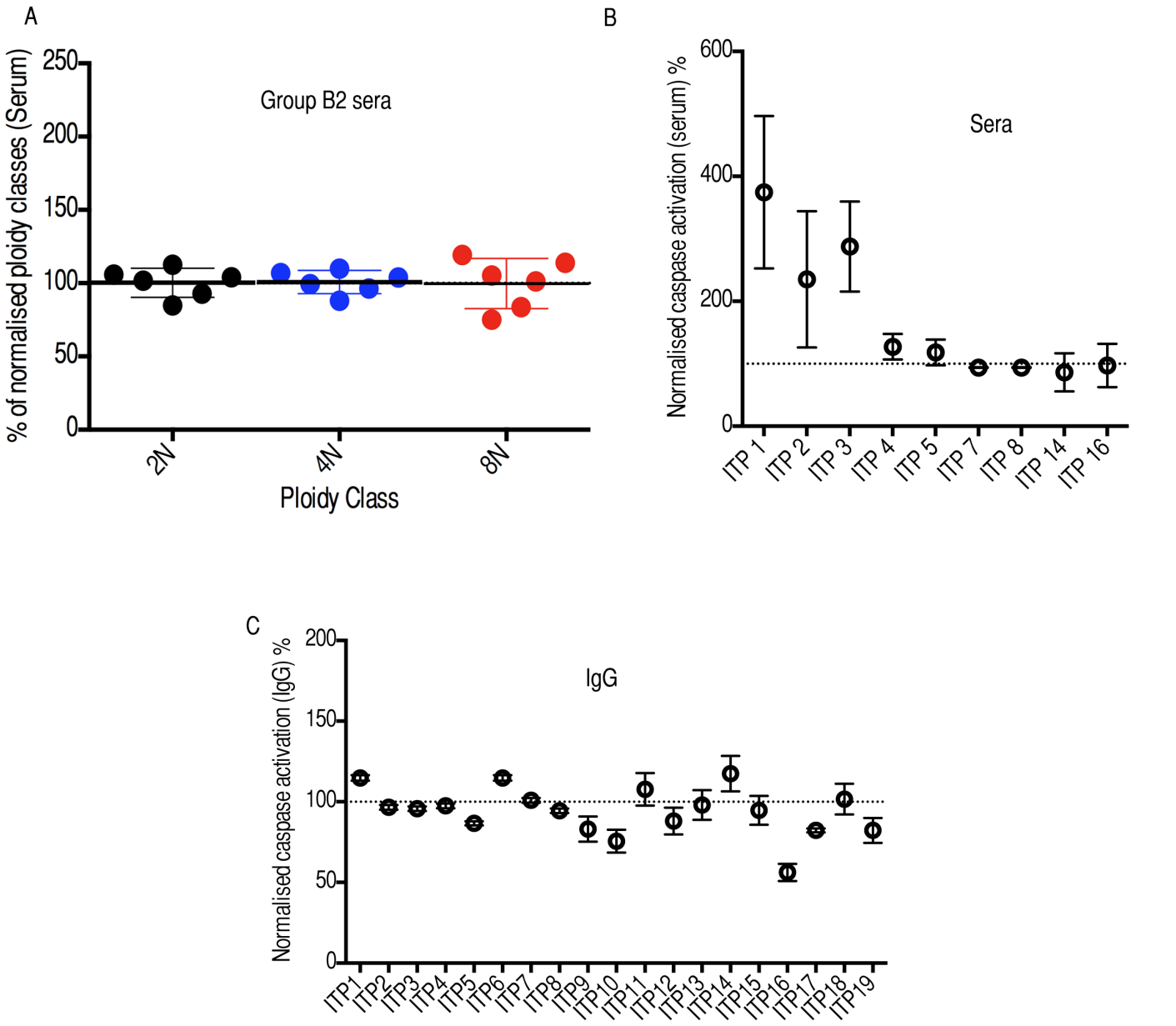
2. Burgess JK, Lopez JA, Gaudry LE, *et al*. Rifampicin-dependent antibodies bind a similar or identical epitope to glycoprotein IX-specific quinine-dependent antibodies. *Blood*. 2000;95(6):1988-1992.

Supplemental Figures

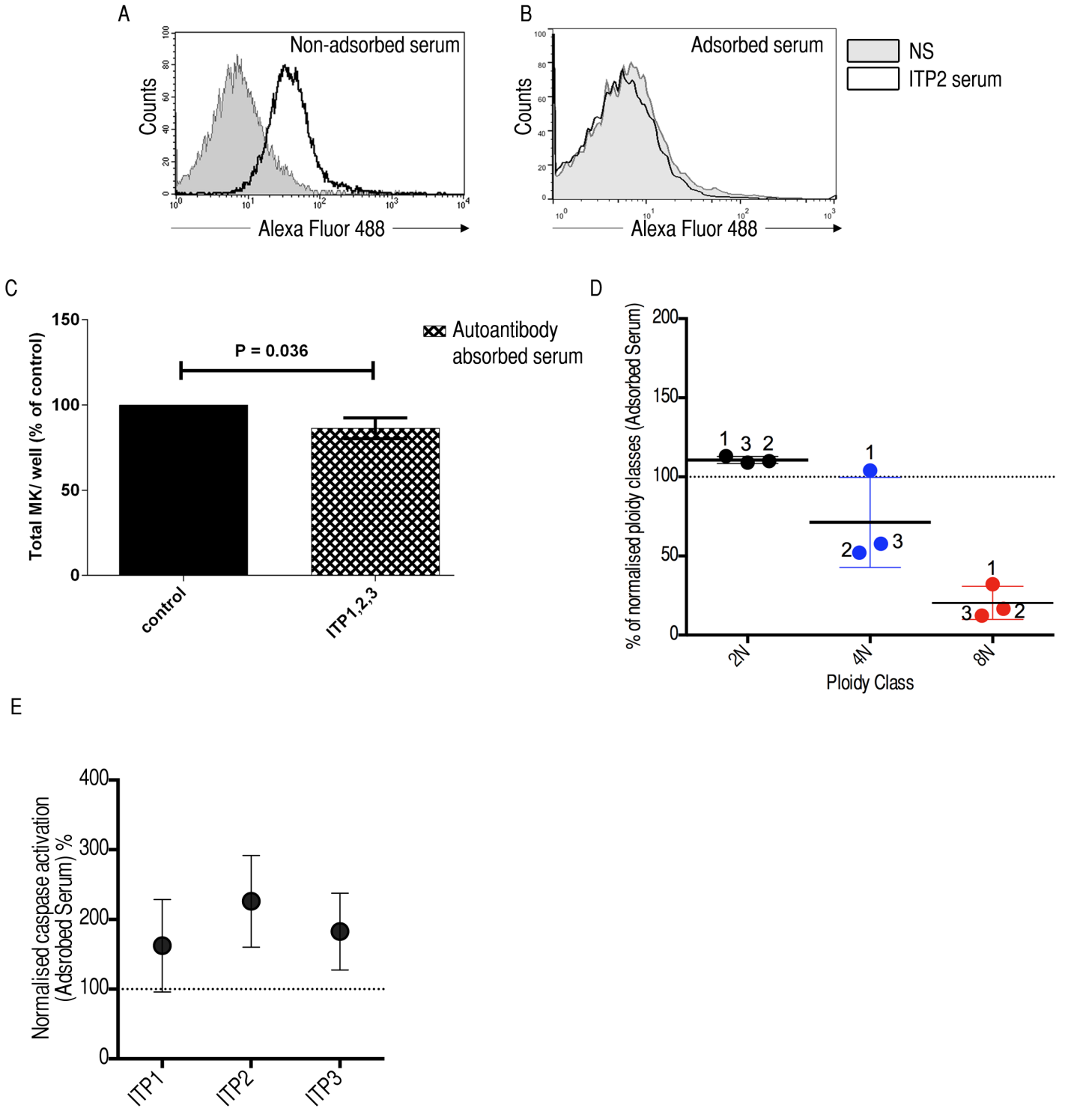


Supplemental Figure 1. Schematic representation of the MK treatment protocol. CD34+ cells were isolated from umbilical cord blood and cultured with rhTPO (50 ng/ml). After 8-9 days, the cells were counted, stained for MK markers and re-seeded at different densities with ITP and control sera or total IgG at 1:10 dilution.
The assessment of MK numbers, cell size, markers' expression and ploidy was done 4 days after treatment (12-13 days from the start of the culture). The number of proplatelet-bearing MKs was assessed 5 days after treatment and the number of platelets released in culture was analysed 6 days after treatment.

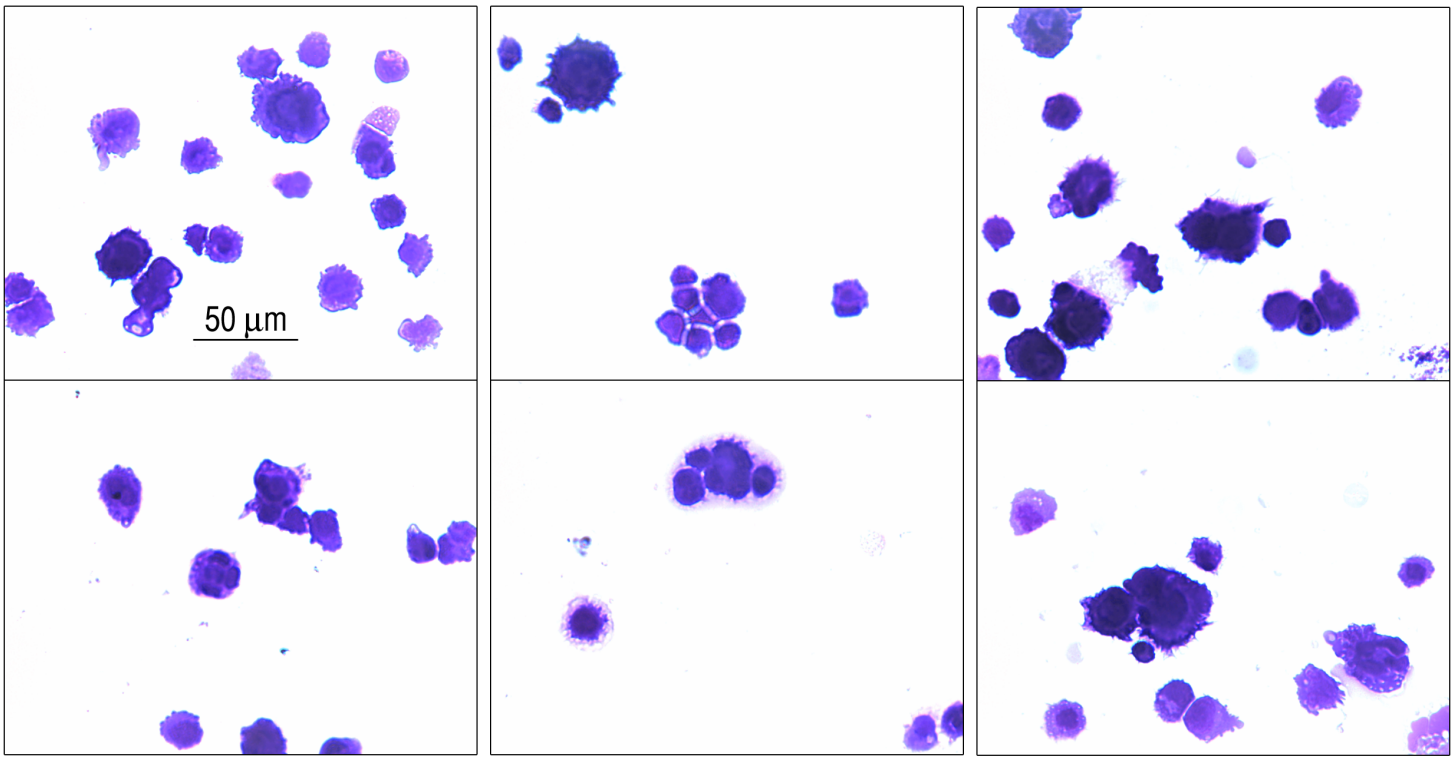
Figure S1



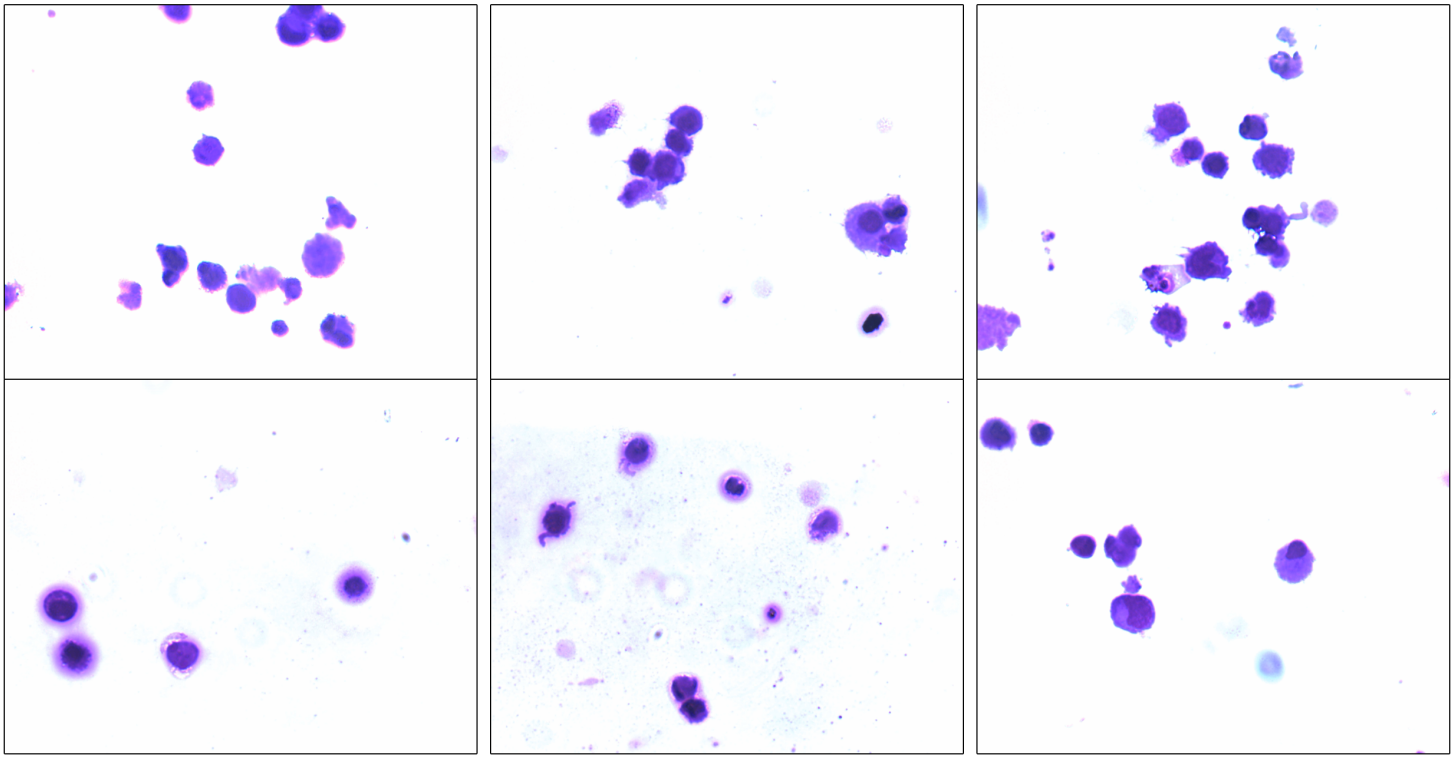
Supplemental Figure 2. Ploidy distribution of group B sera and total caspase activation. (A) Ploidy distribution of group B2 ITP sera treated samples nomalized to control sera. Each dot represents the mean of three independent experiments. No significant differences were observed relative to control samples. (B) Total caspase activation was evaluated by flow cytometry in the CD41+ population four days after treatment. Caspase activation was normalized to control samples (%) (mean ± SEM is shown). (C) Normalized caspase activation using purified total IgG (mean ± SEM is shown).



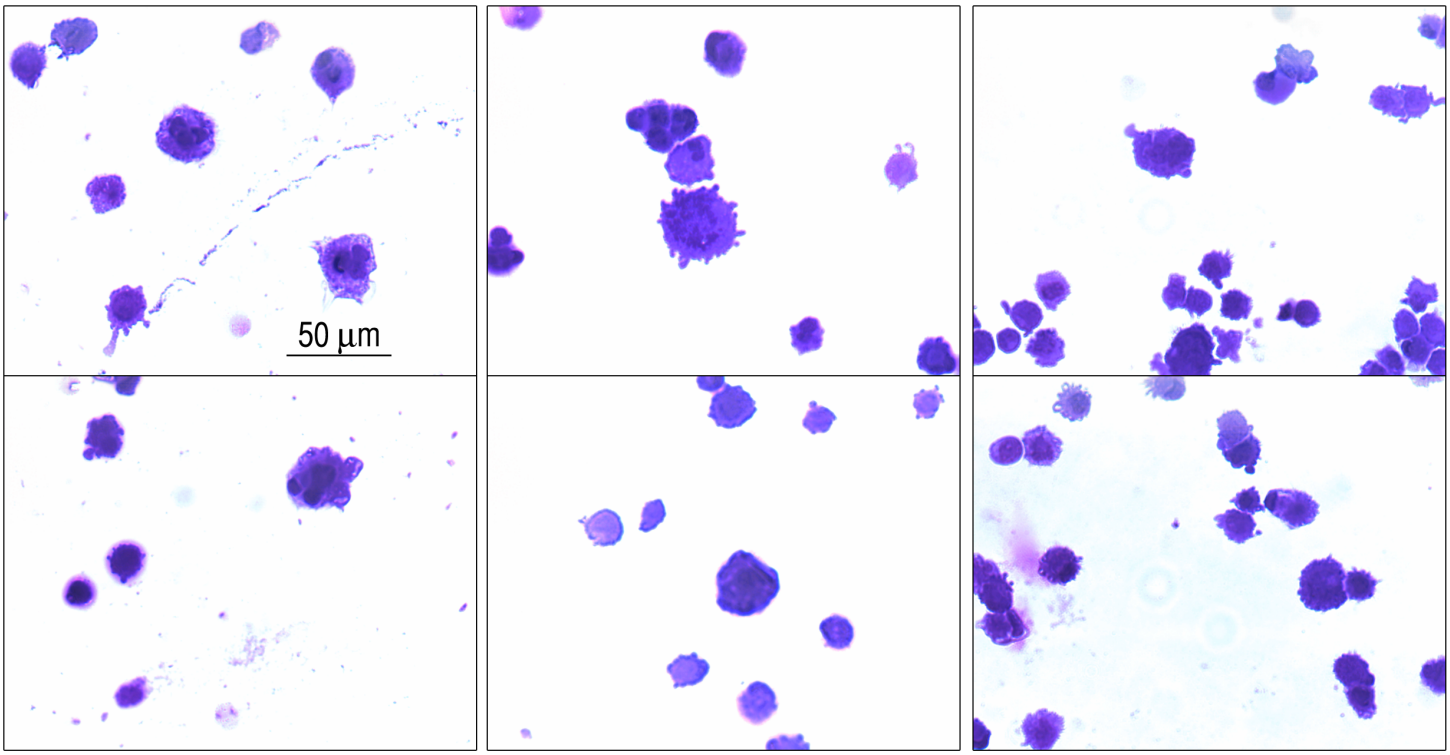
Supplemental Figure 3. Adsorbed ITP1, ITP2 and ITP3 sera affect cultured MKs. ITP1, ITP2 and ITP3 sera were adsorbed on washed platelets to remove antiplatelet autoantibodies. The grey histogram represents normal serum (NS) and the open histogram depicts ITP serum (A) ITP2 serum binds to platelets before adsorption. (B) Removal of autoantibodies after adsorption abolished platelet binding. (C) Normalized MK numbers. Incubation of MKs with adsorbed sera caused significant reduction in MK numbers for ITP1, ITP2 and ITP3 samples (Mean ± SD). (D) Normalized ploidy distribution of MKs treated with adsorbed sera. Significant changes in 2N and 8N ploidy classes were observed after treatment.
2N distribution for ITP1, ITP2 and ITP3 (P= 0.01, 0.05 and 0.03), respectively. For 8N (P= 0.02, 0.01, 0.01), respectively. Only ITP2 showed a significant change in 4N distribution (P= 0.04). (E) Relative to control, treatment with ITP1, ITP2 and ITP3 adsorbed sera caused and increase in total caspase activation (mean plus SD).



NS1 NS2 AB



ITP1 ITP2 ITP3



Supplemental Figure 4. Wright/Giemsa staining of treated Mks. Cultured Mks were centrifuged onto slides after treatment with the samples indicated under each panel. The cells were stained for morphology with Wright/Giemsa staining, and imaged with a Zeiss Axioskop microscope and AxioVision 3.1 software (20X objective using an Axiocam camera; Zeiss). The images were processed using Adobe Photoshop CS5.Two representative fields are shown in each case. The cell area was calculated and used for the graph shown in Figure 4B. Scale bar, 50 µm; NS, normal serum; AB, pooled AB serum.

Ploidy distribution of megakaryocytes treated with ITP serum or total IgG

	Ploidy distribution (%), ± SD					
	Serum			IgG		
Sample	**2N**	**4N**	**8N**	**2N**	**4N**	**8N**
Control	52.2±14.3	35.5±9.0	10.9±5.8	76.1±4.5	21.4±3.5	2.5±1.7
ITP1	84.9±2.5***	13.8±2.5***	1.2±0.3**	75.5±7.6	22.7±6.9	1.8±0.6
ITP2	74.0±16.5	22.1±11.4	3.0±3.8*	78.8±3.6	18.7±4.0	2.5±0.8
ITP3	87.1±2.9***	12.5±2.8***	0.4±0.2**	76.5±4.0	20.4±4.2	3.0±1.3
ITP5	63.9±18.8	29.2±13.4	6.9±5.5	77.3±4.7	20.9±4.1	2.1±0.2
ITP6	47.3±19.2	47.2±15.9	5.2±3.1	78.3±6.5	19.8±5.4	1.9±1.1
ITP13	63.02±7.7	31.2±6.0	6.0±2.0	82.3±0.7*	15.7±1.8*	2.2±1.1

* .01 < P < .05; ** 0.0001<P< .01; ***P<0.0001

Supplemental Table 1

PF4 Serum Concentration (ng/mL)					
NS-mean > 2000					
ITP1	2207	**ITP9**	636	**ITP14**	34
ITP2	11	**ITP10**	2114	**ITP16**	2146
ITP3	40	**ITP11**	779	**ITP18**	1910
ITP4	204	**ITP12**	2137		
ITP6	692	**ITP13**	296		
ITP7	1546				
ITP8	1716				

Supplemental Table 2

A

TGF-β1 Serum Concentration (pg/mL)	
NS (mean ± SD)	278 ± 214
ITP1	9089
ITP2	263
ITP3	379
ITP13	362

B

TRAIL Serum Concentration (pg/mL)	
NS (mean ± SD)	91 ± 60
ITP1	104
ITP2	69
ITP3	114
ITP6	15
ITP11	52
ITP13	7

C

sFAS Serum Concentration (pg/mL)	
NS (mean ± SD)	121 ± 42
ITP1	156
ITP2	98
ITP3	259
ITP11	77
ITP13	124

Supplemental Table 3

Sample	HLA Class-I PRA%	HLA Class-I Antibodies
Negative Control	2	-
Positive Control	100	+
AB Serum	2.16	-
Group A		
ITP1	6.59	-
ITP2	0.85	-
ITP3	94.2	+
ITP4	NA	NA
ITP5	36.7	+
ITP6	99.1	+
ITP7	88.3	+
ITP8	8.11	-
Group B1		
ITP9	10.5	-
ITP10	14	+
ITP11	2.87	-
ITP12	85.8	+
ITP13	1.55	-
Group B2		
ITP14	2.18	-
ITP15	3.27	-
ITP16	33.1	+
ITP17	6	-
ITP18	2.89	-
ITP19	0.27	-

Supplemental Table 4. Panel Reactive Antibodies. To screen for the antibodies against Human Leukocyte Antigen (HLA) in ITP sera, FlowPRA Class I Screening Test (One Lambda Inc., Canoga Park, CA, USA) was used according to the manufacturer's instructions. One positive control and one negative control serum were purchased from the same company for setting the cut-off point for the sensitisation against HLA class I antigens. The samples were analysed by flow cytometry gating 10000 class I beads per sample. The positive and negative cut-off point were set at the end of the peak of the FL1 histogram of the negative control serum. A single shifted peak or multiple peaks, or a second distinct peak or a shoulder within the positive region of the histogram were considered positive. The percentage of positive events is shown in the table.