Materials and Methods

Antibodies and peptides
Monoclonal antibody (moab) Gi5 against αIIbβ3 complex was characterized in our laboratory.[1] Moab 23C6 against αvβ3 complex[2] and moab P2W7 against αv[3] were purchased from Milipore (Temecula, CA, USA) and Calbiochem (La Jolla, CA, USA). Moab AP3 against the common β3 subunit[4] was produced by hybridoma AP3 cell line. RGD peptide was from Bachem, Bubendorf, Switzerland. Moab SZ21 specific against the HPA-1a alloform of β3 was from Beckman Coulter (Sinsheim, Germany). A soluble recombinant αIIbβ3 protein was produced in High Five cells.[5]

Serum samples
Maternal serum samples were selected from cases diagnosed with FNAIT in the Giessen laboratory based on the availability of adequate amounts of serum leftovers. For all selected samples, mothers were HPA-1a negative and had anti-HPA-1a antibodies, but no other HPA antibodies. Clinical data entries included neonatal brain ultrasound results, based on which the samples were assigned to the +ICH cohort (presence of ICH, n=18) or the –ICH cohort (absence of ICH, n=18). Demographic parameters (age, race) and clinical parameters as given in tables 1 and 2 including, neonatal platelet counts, were not statistically different between the +ICH and the –ICH cohort. From each cohort, 9 sera were chosen by list randomizer (http://www.random.org) for use in subsequent experiments. Serum samples from healthy blood donors were used as controls throughout the study. IgG fractions were purified using Melon Gel IgG Purification Kit as recommended by the manufacturer (Pierce Biotechnology, Rockford, IL, USA). IgG concentration was measured by Nano Drop (Thermo Scientific, Wilmington, DE, USA) and adjusted to a concentration of 20 µg/ml. Purified IgG was used immediately. Investigators in the laboratory were unaware of the clinical background of samples.

Endothelial cells
Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical vein as described[6] and maintained in endothelial basal medium-2 (EBM2; Lonza, Bazel, Switzerland). All experiments were performed with primary, secondary or tertiary post confluent monolayers of HPA-1aa or HPA-1bb genotyped HUVECs. HPA typing of HUVECs was performed by real-time PCR using VIC and FAM reporter probes (ABI Applied Bioscience, Washington, DC, USA) specific for HPA-1a and HPA-1b, respectively.

Stable cell lines expressing human αIIbβ3 and αvβ3 integrins
Chinese Hamster Ovary (CHO) cells expressing αIIbβ3 were generated as described.[7] CHO cells expressing the HPA-1a form of the αvβ3 integrin were a generous gift from Dr. Mark Ginsberg (University of California, La Jolla, CA, USA) and were cultured in DMEM medium (PAN) supplemented with 1% non-essential amino acids (Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 10% FCS (PAN).

Characterization of HPA-1a alloantibodies by antigen capture assay
Aliquots of 30 µl platelets ($20x10^6$ cells) or HUVEC ($5x10^5$) were incubated with 20 µl serum and 10 µl moabs (concentration 20 µg/ml). Binding of human antibodies was analysed by MAIPA as described.[8] Cut-offs were calculated by the use of control sera. All experiments were run in triplicates, and results were given as arithmetic means of optical density (OD).

Production of αIIbβ3 coated beads
In brief, 2 ml amino-link resin beads (Thermo Scientific, Rockford, IL, USA) were coupled with 2 ml αIIbβ3 recombinant protein (0.35 mg/ml) in 200 µl coupling buffer overnight at 4°C.

Concentration of αIIbβ3 bound to the beads was measured by bicinchoninic acid assay (BCA; Thermo Scientific). To verify the specificity and absorption capacity of αIIbβ3 coated beads, 100 µl moabs specific for αIIbβ3 (clone Gi5), β3 (clone AP3), and αvβ3 (clone P2W7) (concentration 20 µg/ml) were absorbed with 100 µl αIIbβ3 beads (6.8 µg αIIbβ3/ml) overnight at 4°C. Absorbates were tested with αvβ3 and αIIbβ3 transfected CHO cells by flow cytometry (FACS Canto, Heidelberg, Germany) for the presence of remaining antibodies.

Separation of anti-HPA-1a subtypes from human sera

Aliquots of 100 µl αIIbβ3 beads were incubated with 100 µl serum overnight at 4°C. After centrifugation (1 min at 10,000g), supernatant was collected, and beads were washed twice with 500 µl isotonic saline. Antibodies bound to αIIbβ3 beads were eluted with 40 µl acid buffer (saline containing 1.5% BSA, and 1.0% acetic acid, pH 2.8) for 10 min at RT. Eluted antibodies were neutralized with 3 µl 2.5M Tris buffer, pH 7.2. IgG was purified as described IgG and was adjusted to a concentration of 20 µg/ml before use.

Analysis of different anti-HPA-1a subtypes by immunoprecipitation

CHO cells expressing αIIbβ3, αvβ3 or non-transfected CHO cells were surface labelled with 2 ml NHS-LC-Biotin (5 mmol/L; Pierce) as previously described.[24] Labelled cell lysates (100 µl) were incubated with 35 µl adsorbate or eluate (20 µg/ml) overnight at 4°C in the presence of 50 µl protein G beads (Gerbu Biotechnik, Heidelberg, Germany). After washings with immunoprecipitation buffer (50 mm Tris, 150 mm NaCl, 1% Triton X-100), bound proteins were eluted by adding SDS buffer for 5 min at 100 °C. Eluates were analyzed on 7.5% SDS-PAGE under reducing conditions. Separated proteins were transferred onto polyvinylidenfluorid (PVDF) membranes and developed with peroxidase-labeled streptavidin using chemiluminescence system (Immobilon Western Substrat, Millipore).

Apoptosis assay

Cell apoptosis was measured by the Caspase-Glo 3/7 assay (Promega, Madison, WI, USA). 100 µl vitronectin (2 µg/mL; Athens Research & Technology, Athens, GA, USA) were coated on 96 white well plates (Corning Incorporated, Corning, NY, USA) for 8 hours. Aliquots of 450 µl HUVEC (1-2x10^6 cells in EBM-2 medium) were incubated with cRGD (final concentration 40 µg/ml) or purified human IgG (final concentration 40 µg/ml) and seeded onto vitronectin coated wells for 16 hours at 37°C, 5% CO_2. 100 µl of Caspase-Glo 3/7 reagents were then added at RT and luminescence was measured using a fluorescence microtiter reader (FLX800, Biotek Instrument Winooski, VT, USA). In some experiments, 5 µl of 30 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma) were added for 30 min at 37°C prior to addition of IgG.

Morphological assessment of apoptosis

Aliquots of 10^5 HUVEC were seeded onto µ-slide well (Ibidi, Martinsried, Germany) coated with vitronectin (see above) together with purified human IgG (final concentration 40 µg/ml) or cRGD (final concentration 40 µg/ml) for 16 - 18 hours at 37°C, 5% CO_2. After washings with 300 µl ice-cold PBS pH 7.4 (PAN, Aidenbach, Germany), cells were fixed with 300 µl 4% paraformaldehyde (PFA; Sigma) for 15 min and incubated with 1µg/ml blue fluorescing dye (Hoechst 33342; Thermo Scientific, Rockford, IL, USA) for 3 min at RT. Stained chromatin DNA was analysed using confocal microscopy with 60x magnification (Nikon Eclipse TE2000-E, Tokyo, Japan).

ROS detection assay
ROS produced in HUVEC was measured with 2',7'-dichlorofluorescein diacetate (DCFDA; Abcam, Cambridge, UK). In brief, 10^5 HUVEC were seeded in 6-wells plates for 6 hours and cultured overnight in EBM-2 medium containing 0.5% FCS. After removal of the medium, 1.5 mL medium containing DCFDA (final concentration, 10 mmol/L) was added. Subsequently, cells were incubated with mab against 23C6 or mouse IgG (5 µg/mL), cRGD (5 µg/mL), and anti-HPA-1a or control IgG (50 µg/mL) for 1 hour at 37°C. Cells were detached with accutase (Sigma), fixed by adding 250 µl CELLFIX (1:10 dilution; Becton Dickinson, Heidelberg, Germany), and measured by flow cytometry as described above. In some experiments prior to stimulation with antibodies, HUVEC were treated with AEBSF for 1 hour at 37°C.

Tube formation assay
Aliquots of 50 µl ice-cold matrix gel (Biovision, Milpitas, CA, USA) were plated onto microtiter wells (Greiner, Frickenhausen, Germany) for 30-60 min at 37°C. 100 µl HUVEC (1-5 x 10^5 cells in EBM2 medium supplemented with 2.5% FCS) were seeded carefully onto the gel for 45 min at 37°C. Thereafter, thrombin (1 U; Sigma, Steinheim, Germany) or IgG (40 µg/ml) were added. Cells were allowed to grow for 20 hours at 37°C. Data were analysed using a F-view monochrome fluorescence microscope (Olympus, Tokyo, Japan) with 10x magnification. For quantification, data were imported as TIFF files into ImageJ (http://imagej.nih.gov/ij/) using the stage micrometer as calibrator. In some experiments, 5 µl AEBSF was added for 30 min at 37 °C prior to addition of the antibodies.

Adhesion assay
Microtiter wells (Greiner Bio-one, Frickenhausen, Germany) were coated with 2 µg vitronectin (Athens Reasearch and Technology, Athens, GA, USA) or BSA (Serva, Heidelberg, Germany) in HBS buffer (119 mM NaCl, 4 mM KCl, 11 mM Glucose in 20 mM Hepes buffer) overnight at 4°C. After washings with HBS buffer, wells were blocked with 100 µl 3% BSA. Aliquots of washed HUVEC (1-4 x10^6) were added together with purified IgG or cRGD (final concentration 5 µg/ml) for 1 hour at 37°C. Wells were washed, adherent cells were stained with crystal violet (Sigma, Steinheim, Germany) and measured by a microtiter reader at 592nm (Sunrise™, Tecan, Maennedorf, Germany).

Structural analysis
The crystal structure of integrin αIIbβ3 extracellular domain (PDB code 3FCS) (ref: PMID: 19111664) was superimposed on the crystal structure of αvβ3 extracellular domain (PDB code 4G1M) (ref: PMID: 23106217) with PyMOL based on β3 PSI domain or β3 hybrid domain.

Statistical analysis
Statistical comparisons were made using an unpaired, 2-tailed Student's t test or 1-way ANOVA followed by Bonferroni's post-hoc test, as appropriate. A p-value <0.05 was assumed to represent statistical significance.

Study approval
The use of all human material was approved by the Ethics Committee of the Medical Faculty, Justus Liebig University, Giessen, Germany.

References:

1. Santoso S, Zimmermann U, Neppert J, Mueller-Eckhardt C. Receptor patching and capping of platelet membranes induced by monoclonal antibodies. *Blood.* 1986; 67(2):343-349.
2. Horton M A, Lewis, D, McNulty K, Pringle JA, Chambers TJ. Monoclonal antibodies to osteoclastomas (giant cell bone tumors): definition of osteoclast-specific cellular antigens. *Cancer research*. 1985; *45*(11 Part 2), 5663-5669.

3. Kamata T, Handa M, Takakuwa S, Sato Y, Kawai Y, Ikeda Y, Aiso S. Epitope Mapping for Monoclonal Antibody Reveals the Activation Mechanism for aVb3 Integrin. PLoS ONE. 2013; 8(6): e66096.
4. Newmann PJ, Allen RW, Kahn RA, Kunicki TJ.1985. Quantitation of membrane glycoprotein IIIa on intact human platelets using the monoclonal antibody, AP3. *Blood.* 65:227–232.
5. Peterson JA, Visentin GP, Newman PJ, Aster RH. A recombinant soluble form of the integrin alphaIIbbeta 3 (GPIIb-IIIa) assumes an active, ligand-binding conformation and is recognized by GPIIb-IIIa-specific monoclonal, allo-, auto-, and drug-dependent platelet antibodies. Blood. 1998;92(6):2053-63.
6. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphological and immnulogic criteria. *J Clin Invest.* 1973;52(11):2645-2656.
7. Santoso S, Kiefel V, Richter IG, Sachs UJ, Rahman A, Carl B, Kroll H. A functional platelet fibrinogen receptor with a deletion in the cysteine-rich repeat region of the beta(3) integrin: the Oe(a) alloantigen in neonatal alloimmune thrombocytopenia. *Blood.* 2002;99(4):1205-1214.
8. Kiefel V, Santoso S, Weisheit M, Mueller-Eckhardt C. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. *Blood.* 1987;70(6):1722-1726.