A novel splice-specific GFI1B mutation in a family with recessive macrothrombocytopenia

H. Schulze¹, A. Schlagenhaus², G. Manukjan¹, O. Andres¹, E. Klopcoki⁵, S. Panzer³, C. Beham-Schmid², K. Althau², A. Greinacher⁴, T. Bakchoul⁴, M. G. Seidel² (¹Würzburg, Germany; ²Graz, Austria; ³Vienna, Austria; ⁴Greifswald, Germany)

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Objective: Growth Factor Independent 1B (GFI1B) is a transcription factor, essential for the erythroid and megakaryocytic lineage. Alternative splicing leads to a shorter p32 isoform which lacks the two out of 6 zinc fingers. Germline mutations have been reported to cause autosomal-dominant macrothrombocytopenia with a grey-platelet syndrome phenotype. We report on a Chechen family whose affected family members present with severe, life-threatening bleedings. The index patient had recurrent hematoma and multiple petechiae since childhood. Both her children (age 9 and 7) have platelet counts less than 45/nL and a similar cutaneous bleeding pattern. The brother also had low platelet counts and died with age 33 due to a spontaneous cerebral hemorrhage. The index patient's husband, her parents and the children of the deceased brother were clinically unaffected.

Methods: We analyzed blood smears with May-Grünwald-Giemsa and immunofluorescence microscopy. Platelet function was tested by aggregometry and flow cytometry. DNA was subjected to targeted next generation sequencing to screen for variants in 59 genes reported to be relevant for platelet formation or function.

Results: Platelets showed reduced staining for alpha-granule markers. White and red blood parameters were unaffected. Aggregometry with ADP, TRAP-6, or arachidonic acid was impaired. We found a novel homozygous single nucleotide insertion in GFI1B (NM_004188.5; c.551insG), expected to cause a premature stop-codon and which segregated with the phenotype. The unaffected mother, the husband and two unaffected nephews were heterozygous, confirming an autosomal-recessive trait. Dysplastic micromegakaryocytes and peripheral platelets were CD34-positive. Quantitative PCR of platelet RNA showed residual homozygous c.551_G insertion in the p37 transcript and an unexpected expression of p32. The p37 transcript was markedly reduced, with an increased p32/p37 ratio compared to controls.

Conclusion: Our findings imply that the first two zinc fingers of GFI1B are dispensable for human erythropoiesis, but essential for normal megakaryopoiesis and functional platelets. While previous mutations affect both isoforms, this insertion variant results in a premature stop-codon and affects only the p37 isoform. We conclude that the transcriptional regulation of erythropoiesis is uncoupled from that of megakaryopoiesis through alternative splicing of GFI1B.
Antibody-mediated platelet-desialylation: a potential role in platelet destruction in autoimmune thrombocytopenia

R. Jouni1, J. Alex2, I. Marini2, L. Janzen2, A. Greinacher2, T. Bakchoul1 (1Tuebingen, Germany, 2Greifswald, Germany)

Objective: Immune thrombocytopenia (ITP) is a bleeding disorder caused by autoantibodies (AAbs) directed against platelet (PLT) glycoproteins (GP). Recently, Fc-independent PLT clearance via Ashwell-Morell receptors (AMRs) has been suggested as a novel mechanism of antibody-mediated PLT destruction in mice. In this study we analyzed the impact of AAbs from ITP patients on the glycan pattern of human PLTs and the subsequent effect on their survival in vivo.

Methods: Sera from ITP patients and healthy donors were analyzed using monoclonal platelet antigen capture assay (MAIPA) and lectin binding assay (LBA). In LBA, sera were incubated with PLTs from healthy donors, and the change in glycan pattern was analyzed by flow cytometry using lectins; Ricinus communis agglutinin (RCA), Erythrina cristagalli lectin (ECL) and Peanut agglutinin (PNA) that bind to galactose, N-acetyllactosamine and N-acetylgalactosamine residues, respectively. The impact of different glycan patterns on the survival of human PLTs was investigated using the NOD/SCID mouse model.

Results: 37 sera from ITP patients and 25 sera from healthy donors were investigated in this study. Different patterns of glycan modification were observed after incubation with AAbs in the LBA. 17/37 sera induced a significant increase in PNA binding compared to healthy donors: (median fold increase (FI): 1.21, range: 1.08 – 1.40). 9/37 sera caused higher ECL binding (median FI: 1.02, range: 1.08 – 1.15). In contrast, 8/37 sera showed strong decrease in RCA binding (median FI: 0.52, range: 0.50 – 0.59). Interestingly, not only GP-Ib/IX AAbs but also GPIIb/IIIa AAbs were able to modify glycan pattern. No significant change was induced by sera from healthy donors. The injection of AAbs resulted in accelerated clearance of human PLTs from the circulation of the NOD/SCID mice. The destruction of human PLTs by ITP-AAbs was reduced but not completely inhibited by a specific neuraminidase inhibitor that prevents glycan changes on PLT surface (survival of human PLTs after 5h: 29%, range 22-40% vs. 48%, range 41-53%, p=0.014, respectively).

Conclusion: Our data demonstrate that AAbs from ITP patients are able to induce cleavage of glycan moieties on the PLT surface in distinct ways. Although, AAb-mediated glycan-modification seems to contribute to PLT destruction in ITP, the impact of GP-specificity needs further investigations.
Reelin is important for PLCgamma2 phosphorylation upon platelet activation and integrin outside-in signaling

I. Krueger, M. Klier, N. S. Gowert, M. Elvers (Duesseldorf, Germany)

Objective: Reelin is an important mediator for cell migration in brain development. Recent studies provide evidence that Reelin is found in plasma and platelet-alpha-granules and plays an important role in platelet activation. Furthermore, Reelin expression was also reported to increase upon inflammatory processes. This and the findings in reeler mice showing reduced thrombus formation under high shear conditions ex vivo and protection against arterial thrombosis in vivo prompted further investigation regarding the exact Reelin-mediated signaling pathways, especially regarding the cytoskeletal reorganization following platelet activation and adhesion.

Methods: In vitro and in vivo analysis of mutant Reelin deficient mice to investigate Reelin mediated signaling cascades in platelets.

Results: Reelin activated small GTPases of the Rho family and is important for the phosphorylation of Rho target proteins such as PAK1/2 supporting lamellipodia formation upon spreading on fibrinogen. Moreover, adhesion and cytoskeletal reorganization of reeler platelets on a collagen-related peptide (CRP) matrix was reduced. In line with this result, platelet activation in response to CRP was strongly reduced in Reelin deficient platelets. Engagement of glycoprotein (GP)VI induced robust phosphorylation of PLCgamma2 and Syk while significantly reduced phosphorylation was detected using platelets from reeler mice. To analyze integrin outside-in signaling in further detail, clot retraction experiments were performed showing almost absent clot formation of Reelin deficient platelets. Thus, we found Reelin effecting GPVI dependent signaling and integrin outside-in signaling suggesting that Reelin modulates PLCgamma2 activation.

Conclusion: This study identifies Reelin as an important mediator in hemostasis and arterial thrombosis showing great promise for a therapeutic target in antithrombotic treatments.
Platelets are a previously unrecognized source of immune-modulatory CXCL14

A. Witte, J. Lu, M. Gawaz (Tuebingen, Germany)

**Platelets**
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**Objective:** Platelets are the source of a variety of chemokines which are crucial for immune responses due to their immune-modulatory or antimicrobial activity. The relatively newly discovered antimicrobial chemokine CXCL14 is widely expressed in different kinds of tissue and immune cells. The objective of this study was to investigate whether platelets contain CXCL14 and, like other platelet-derived chemokines, whether CXCL14 is released upon activation. Furthermore a possible immune-modulatory function of platelet derived CXCL14 was investigated.

**Methods:** Flow cytometry, western blot analysis, Boyden Chamber migration assays, scratch assay (HUVECs)

**Results:** Western Blot analyses with isolated platelets show that human as well as murine platelets express the chemokine CXCL14, also known as BRAK, which was unknown until now. Upon activation of human platelets in platelet-rich-plasma (PRP) with different agonists, CXCL14 cell surface expression was significantly enhanced indicating that CXCL14 is released and can bind back to the platelet membrane. Furthermore, activation of platelets with increasing concentrations of Collagen Related Peptide (CRP) correlated with CXCL14 surface expression. Since CXCL14 is known to be chemotactic for monocytes and NK cells, we investigated the chemotactic effect of platelet-derived CXCL14 on isolated human monocytes. Treatment of activated platelet supernatant with a neutralizing antibody against CXCL14 led to a decreased number of migrated monocytes towards the platelet supernatant, indicating that platelet-derived CXCL14 is involved in chemotactic responses as an immune modulatory chemokine. Blocking the chemokine receptor CXCR4 reduced the number of monocytes migrated towards recombinant CXCL14 in contrast to blocking of CXCR7, which indicates an involvement of CXCR4 in the CXCL14 mediated migration of monocytes.

In addition, a scratch assay with HUVECs indicates that platelet-derived CXCL14 counteracts the angiogenic effect of VEGF on these endothelial cells, which might be of importance during wound healing and angiogenesis.

**Conclusion:** We show that platelets are a source of CXCL14 and that CXCL14 is released upon activation. Platelet derived CXCL14 induces monocyte migration and counteracts the angiogenic effect of VEGF on HUVECs.
Specific inhibition of extracellular CyPA affects platelet and monocyte functions in vitro and in vivo

S. von Ungern-Sternberg¹, B. Walker-Allgaier¹, S. Vogel¹, E. Kremmer², O. Borst³, T. Billar³, A. May¹, M. Gawaz¹, P. Seizer¹ (¹Tubingen, Germany, ²Munich, Germany, ³Pittsburgh, USA)

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Objective: Cyclophilin A (CyPA) is an ubiquitously expressed intracellular chaperon protein and, under inflammatory conditions, CyPA is secreted and binds to its receptor EMMPRIN (CD147). Due to the binding between CyPA and EMMPRIN platelets get activated. CyPA is involved in the pathophysiology of several inflammatory diseases, such as myocarditis, myocardial infarction, and atherosclerosis. At present, there is no specific inhibitor targeting extracellular CyPA without affecting other extracellular cyclophilins or the intracellular CyPA. In this study, we developed an antibody-based inhibitor of CyPA that specifically neutralizes extracellular CyPA in vitro and in vivo.

Methods: Mice and rats were immunized with a peptide containing the EMMPRIN binding site and various antibody clones were selected and purified. At first, all antibodies were tested for their binding capacity on recombinant and cell-derived CyPA in Western Blot and their functional activity in a migration assay. The best antibody, 8H7-mAb, was chosen for further experiments. At first, 8H7-mAb was tested in CyPA-derived modified Boyden chamber migration assays in vitro and in vivo in a CyPA-induced peritonitis model. Then, the anti-thrombotic functions of 8H7-mAb were analyzed by measuring p-selectin expression on platelets by flow cytometry and thrombus formation in vitro and in vivo in a FeCl3 model. Next, we analyzed the functional effects of 8H7-mAb in a trauma/hemorrhagic shock mice model.

Results: According to the results of the Western Blot and migration assay, the clone 8H7-mAb was chosen for further experiments. In a binding assay, we could show that there is a specific binding of 8H7-mAb on recombinant CyPA compared to IgG control and that 8H7-mAb is only detecting CyPA in Western blot assay and not CyPB. We found that 8H7-mAb reduced the CyPA-induced migration of monocytes/macrophages in vitro and in vivo (CyPA-induced peritonitis). 8H7-mAb is able to reduce the thrombus formation on a collagen matrix in vitro and in a FeCl3 model in vivo. Interestingly, 8H7-mAb treated shock mice had significantly less platelet aggregation of circulating platelets and less platelet and monocyte infiltrates in the liver compared to IgG control treated mice.

Conclusion: Our study provides evidence that the novel CyPA-inhibitor 8H7-mAb affects CyPA-induced thrombosis and thrombo-inflammation in vitro and in vivo.
High shear induced platelet rolling velocity and adhesion onto von Willebrand factor are mediated by GpIb alpha clustering as demonstrated by FRET/FLIM and can be inhibited by alpha linolenic acid

S. Stivala¹², D. van den Heuvel³, S. De Meyer⁴, S. Gobbato¹, N. Bonetti¹, H. Deckmyn¹, G. G. Camici¹, T. F. Lüscher⁷, R. Urbanus³, H. Gerritsen¹, J. H. Beer¹² (¹Zurich, Switzerland, ²Baden, Switzerland, ³Utrecht, The Netherlands, ⁴Kortrijk, Belgium)

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Objective: We studied the shear-dependent GpIb alpha clustering and the structural and functional interference with this process by the C18 omega-3 fatty acid alpha linolenic acid (ALA).

Methods: Citrated- and EDTA-anticoagulated blood was incubated with vehicle (ethanol 0,1% v/v) or ALA at 7.5/15/30 μM final concentrations. Calcein-stained platelets were perfused on flow channels coated with human vWF at a shear flow of 100 dyne/cm² (shear force of 2500 s⁻¹). The platelet-covered area, platelet rolling velocity and distance travelled were calculated with the Bioflux software. GpIb clustering was analysed by Förster resonance energy transfer/ fluorescence lifetime imaging (FRET/FLIM). Platelet-rich plasma was pre-incubated with vehicle or ALA 30 uM for 1h, then subjected to 10'000 s⁻¹ shear in a cone and plate viscometer, and GpIb stained with the 6B4 Fab fragment conjugated with the donor and acceptor fluorophores 488 and 594, respectively. Fluorescence lifetime was analyzed on a CLSM C1 microscope equipped with a lifetime module with an objective of 60x/1.49 numerical aperture.

Results: Shear-induced platelet aggregation to vWF was dose-dependently reduced by ALA (platelet-positive area: 64,112 μm² in the vehicle versus 21,102 μm² ALA 30 uM, n=6, p=0.018). A reduction in GpIb-mediated platelet adhesion was also observed in EDTA-anticoagulated blood (platelet-positive area: 99,781 μm² vehicle versus 70,405 μm² ALA, n=6, p=0.016). Platelet velocity was double in ALA-treated samples compared to vehicle (velocity: 0.75 μm/sec vehicle versus 1.56 μm/sec ALA, n=10, p=0.023). Incubation with ALA did not change GpIb surface expression, as shown by flow cytometry (MFI: 3217 vehicle versus 3477 ALA, p=0.3). GpIb clustering (as measured by fluorescence lifetime) was increased by shear duration and partially inhibited by ALA as indicated by an increase in the donor fluorophore lifetime after shear in preliminary experiments (lifetime: 2.59±0.09 nsec vehicle versus 2.72±0.1 nsec ALA, n=3).

Conclusion: a) platelet adhesion and aggregation to vWF under high-shear flow can be inhibited by ALA, an n-3 fatty acid abundantly found in the Mediterranean diet; b) clustering of GpIb alpha on the platelet surface mediates the adhesion process under flow and ALA interferes with this process; c) this mechanism may contribute to explain the protective effects of the Mediterranean diet on platelet mediated vascular events.