Role of platelet factor 4 (PF4) and anti-PF4/polyanion IgG in human platelet FcγRIIA mediated anti-bacterial activity

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Objective: Platelets contribute to innate immunity by interacting directly and indirectly with bacteria. Here we addressed the role of the chemokine platelet factor 4 (PF4), anti-PF4/Heparin (H) IgG and human platelet FcγRIIA in bacterial recognition and killing.

Methods: Planar and biomimetic bacteria-like micropatterns functionalized with IgG, heat aggregated IgG (HAG) and PF4+anti-PF4/H IgG immune complexes were prepared using lithography. E. coli wild-type BW30270 and mutants KPM53 (ΔwaaC)/KPM121 (ΔwaaA) expressing truncated lipopolysaccharide (LPS) were micropatterned as live bacteria arrays. These E.coli show PF4-binding activity (WT<ΔwaaC<ΔwaaA).

Platelet adhesion, morphodynamics and activation on ligand functionalized micropatterns and on bacterial killing on micropatterns were assessed in the presence or absence of FcγRIIA, αIIbβ3 blockers and cytoskeletal inhibitors. Calcium mobilization, quantitative fluorescence microscopy and platelet-bacteria co-culture experiments were performed.

Results: Platelets adhered on planar micropatterns functionalized with IgG [% area covered 60.23 % ±13.1 mean (SD)], HAG [83.41 % ±11.36] and PF4 + anti-PF4/H IgG [74.41 % ±10.2]. FcγRIIA blocking mAb IV.3 reduced (P < 0.0001) platelet adhesion on IgG [3.39% ±3.35], HAG [11.41%±3.96] and PF4 + anti-PF4/H IgG [5 % ± 2.89]. Similarly, blocking of αIIbβ3 or the use of cytochalasin D and blebbistatin reduced platelet adhesion and spreading on these micropatterns. Similar results were obtained on functionalized bacteria-like microbead arrays. In the presence of anti-PF4/H IgG, platelets were able to kill E.coli strains within two hours, directly dependent on the bacterial PF4 binding capacity (up to 65.4 ± 6.3% killing rate), which was inhibited by mAb IV.3 blocking FcγRIIA (P < 0.005).

Conclusion: The chemokine PF4 not only binds to polyanion such as heparin but it can also bind to polyanions on bacteria, thereby enabling opsonization by anti-PF4/H IgG, which in turn mediates killing of E. coli via platelet FcγRIIA. Because PF4 binds to both Gram-negative and Gram-positive bacteria, preformed anti-PF4/polyanion IgG can recognize bacterial pathogens the host may not have encountered before. Moreover, the role of PF4 and anti-PF4/Polyanion IgG in FcγRIIA mediated platelet anti-bacterial activity shows bridging of innate and adaptive immune system by platelets and may be relevant in controlling bacterial infections.
Platelet activation and aggregation provoked by *Staphylococcus aureus* secreted proteins

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Objective: *Staphylococcus aureus* is an opportunistic human pathogen provoking a wide range of severe community-acquired and nosocomial infections amongst others infective endocarditis (IE) and disseminated intravascular coagulopathy (DIC). Both clinical manifestations constitute an uncontrolled activation of both platelets and coagulation cascade resulting in thrombocytopenia. *S. aureus*-platelet interactions occur either directly or indirectly via recruitment of serum components. Bacterial factors inducing platelet activation or aggregation are mostly secreted proteins possessing ECM binding activity.

Methods: Fifty-six recombinant secreted or surface-localized staphylococcal proteins were screened for their capacity to activate platelets, measured by the activation markers P-selectin and αIIbβ3 conformation, using whole blood, platelet-rich-plasma (PRP), and washed platelets in buffer from a constant set of donors. Micropattern protein array (MiPA) chips were functionalized with His6-tagged staphylococcal proteins to assess the interactions on a single cell level. Real-time calcium mobilization assay, single platelet imaging and P-selectin expression were used to detect platelet activation.

Results: This study confirmed the potential of the SERAM (secretable expanded repertoire adhesive molecules) protein Eap to induce platelet activation and aggregation. In addition, this study further identified the chemotaxis inhibitory protein CHIPS, the formyl peptide receptor-like 1 inhibitory protein FLIPr, all involved in immune evasion, as well as the major autolysin Atl as potent platelet activators. Furthermore, the domains of Atl and the extracellular adherence protein (Eap), responsible for platelet activation could be narrowed down. MiPa chips enabled to follow platelet activation by the candidates at single platelet level. Likewise, platelet aggregation activity of these candidates in whole blood could be determined.

Conclusion: Taken together, this study identified two members of the SERAM family (Eap and Atl) and two additionally secreted proteins of *S. aureus* as platelet activators and aggregators. These results emphasize the importance and diversity of *S. aureus*-platelet interactions. Interestingly, Atl and Eap were also shown to interact with the platelet-derived protein thrombospondin-1 (Kohler et al., 2014, Hussain et al., 2008).
Altered platelet lipidomic profile influences thrombotic disposition: modulation by the CXCL12-CXCR4-CXCR7 Axis


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Objective: CXCR7 agonist ameliorates atherosclerosis in Apoe-/- mice. Since CXCR7 surface expression on platelets and plasma CXCL12 levels are enhanced in acute coronary syndrome (ACS), both with significant prognostic impact, this study explores the atherothrombotic influence of CXCL12-CXCR4/CXCR7 axis on platelet lipid associations and characterizes the platelet lipidome in coronary artery disease (CAD) patients.

Methods: Flow cytometry, immunofluorescence confocal microscopy, impedance aggregometry, untargeted lipidomics analysis (UHPLC-ESI-QTOF-MS/MS), live imaging of single platelets by scanning ion conductance microscopy, calibrated automated thrombinoscopy, ex vivo flow chamber assay, in vivo occlusive thrombosis model.

Results: LDL/oxLDL enhanced reactive oxygen species (ROS), mitochondrial superoxide generation, whereby platelets serve as intracellular compartment for (per)oxidative lipid modification, counteracted by SOD2-mimetic MnTMPyP. Lipidomics revealed enhanced intraplatelet oxidized phospholipids, cholesteryl esters, sphingomyelin, lysopC, di-,mono-acylglycerols, decreased ceramides levels in CAD patients suggesting a dynamic interaction between plasma and platelet lipids. Enhanced platelet-oxLDL in CAD patients, correlated with platelet CXCR7 surface expression, plasma tryglycerides, HDL, while inversely with CXCR4, plasma LDL. Platelet-oxLDL was elevated in ACS patients with angiographic evidence of intracoronary thrombi. Ex vivo analysis of intracoronary thrombi sections revealed oxLDL deposition in platelet-enriched areas. LDL/oxLDL induced degranulation, αIIbβ3-integrin activation, aggregation, apoptosis, thrombin generation and dynamic shape change. Further, LDL/oxLDL enhanced thrombus formation ex vivo and in vivo in mice (FeCl3 induced carotid injury). LDL-oxLDL enhanced platelet CXCL12 release, differentially regulated CXCR4-CXCR7 surface exposure decreasing CXCR4 while enhancing CXCR7 expression. CXCL12-CXCR4-CXCR7 prompted LDL/oxLDL uptake by upregulating surface availability of the scavenger receptors CXCL16-SR/PS-OX and ApoER2, also synergistically augmented the LDL/oxLDL-induced pro-oxidative and thrombogenic platelet functions.

Conclusion: Pro-oxidative platelet lipidome might propagate thrombotic disposition, a mechanism potentially modulated by CXCL12-CXCR4-CXCR7 axis to influence disease progression and prognosis in CAD.
Metformin alters the epigenetic regulation of tissue factor in diabetes

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Objective: Diabetes mellitus is characterized by chronic vascular inflammation and a main risk factor for cardiovascular mortality. In particular, elevated levels of circulating tissue factor (TF) result in increased diabetes-related thrombogenicity.

Metformin remains the first-line therapy for an oral anti-diabetic treatment. Beyond its glucose-lowering effects, metformin was shown to reduce vascular inflammation and levels of circulating coagulation factors, including TF. Recently, metformin has been reported to alter the expression of endothelial microRNA(miR)s, such as miR-19a and miR-126. In the present study we sought to elucidate the role of miR-depending TF expression and procoagulability in patients with or without metformin therapy.

Methods: Plasma samples of 40 patients with known diabetes were assessed for the expression of miR-19a, TF protein, TF activity, and markers for vascular inflammation. Human microvascular endothelial cells (HMEC-1) and monocytes (THP-1) were transfected with a miR-mimic or a control-miR.

Results: In those patients on metformin we observed increased expression of miR-19a as compared to the patients without metformin. We found plasma miR-19a to strongly correlate with miR-126 (r= 0.92, p<0.0001). Metformin treatment was associated with reduced TF protein and TF-mediated procoagulability, while the expression of vascular adhesion molecule-1 was not altered. In HMEC-1 and THP-1 miR-19a transfection led to suppression of TF mRNA, TF protein, and TF activity. miR-19a and miR-126 in concert exhibited additive inhibition of TF expression and activity of a luciferase reporter construct containing the F3 3’UTR.

Conclusion: We conclude that metformin increases the expression of miR-19a and miR-126 in patients with diabetes and thereby epigenetically controls the post-transcriptional TF expression. This mechanism may in part explain the metformin-dependent reduction in cardiovascular mortality.
D-Dimer, but not the Khorana score predicts venous thromboembolism in lung cancer patients

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Objective: Patients with lung cancer frequently suffer from venous thromboembolism (VTE). The Khorana-Score was introduced in 2008 to assess the risk of VTE in cancer patients and to identify patients who may benefit from primary prophylaxis with anticoagulation. Previous studies have shown that elevated D-dimer levels are associated with an increased risk of VTE in cancer patients.

Methods: In this prospective observational study we investigated, if the Khorana Score can predict VTE in lung cancer patients. We also examined, if a dichotomized D-dimer can predict VTE in newly diagnosed lung cancer patients. The 75th percentile (1.77 µg/mL) of D-dimer in the lung cancer population was defined as the cut-off. The Khorana Score is calculated based on the type of cancer, the body mass index (BMI), and the complete blood count. The endpoints of the study were objectively confirmed VTE with diagnostic imaging and adjudicated by an independent committee. Competing-risks regression according to Fine and Gray was used to calculate the risk and distinguish between high- and low risk of VTE.

Results: From Oct. 2003 to Apr. 2014, 320 lung cancer patients (60.3% male; mean age: 61 years) were included in the study. 28 (8.8%) patients developed VTE within 2 years. The median observation time was 370 days, and 198 (61.9%) patients died during the observation time. The Khorana-Score did not predict VTE in lung cancer patients, the subdistribution hazard ratio (SHR) was 0.3 (95% Confidence Interval [CI]: 0.1 – 1.1) in patients with a Khorana Score of two, and 0.7 (95% CI: 0.2 – 2.5) in patients with a score of three or higher. D-dimer was significantly associated with the occurrence of VTE in lung cancer patients; the SHR was 2.82 (95% CI: 1.32 – 6.03) after adjusting for age and sex. The cumulative probabilities of VTE occurrence were 12.5% and 16.3% in patients with D-Dimer above the 75th percentile, respectively after 180 and 365 days, and 2.92% and 5.4% in those below the 75th percentile (Figure 1).

Conclusion: D-dimer independently predicts VTE in lung cancer patients. The Khorana-Score does not distinguish between lung cancer patients with high- and low risk for VTE.
Randomized, double-blind, placebo-controlled trial of recombinant human C1 inhibitor for prophylaxis of hereditary angioedema attacks

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Objective: Recombinant human C1 inhibitor (rhC1INH) is an approved, on-demand treatment for hereditary angioedema (HAE) attacks. Open-label data suggest that rhC1INH may prevent HAE attacks.

Methods: Patients ≥13 years of age with functional C1INH levels <50% of normal and history of ≥4 HAE attacks during the preceding 3 months were included in this phase 2, double-blind, 3-period crossover study. Patients received intravenous rhC1INH 50 IU/kg (max, 4200 IU) once and twice weekly and placebo in three 4-week treatment periods that were separated by 1 week. With the crossover design, all patients received each of the dosing regimens. Attack symptoms were recorded daily. The number of HAE attacks per 4-week treatment phase (primary endpoint) and percentage of patients with clinical response (≥50% reduction in number of attacks from treatment with placebo to treatment with rhC1INH; secondary endpoint) were determined. Adverse events (AEs) and immunogenicity were also assessed.

Results: Thirty-two patients were randomized to treatment. Mean number of HAE attacks was significantly reduced with rhC1INH twice weekly (P < 0.0001) and once weekly (P = 0.0004) versus placebo (Figure). Most patients (74.2%; 95% CI, 57-86) who received rhC1INH twice weekly and 41.9% (95% CI, 26-59) who received rhC1INH once weekly had ≥50% reduction in number of HAE attacks. No patients withdrew because of AEs and no thrombotic or thromboembolic events, drug hypersensitivity or anaphylactic reactions, or neutralizing antibodies were reported.

Conclusion: rhC1INH provided clinically relevant reductions in HAE attack frequency and was well-tolerated. Data support the continued development of rhC1INH for HAE prophylaxis.