Abstract Number - OC02

Single-cell RNA sequencing identifies a critical role of G6b-B in regulating megakaryocyte maturation

Z. Nagy[1], M. Englert[1], P. Arampatzi[2], P. Khoueiry[3], B. Nieswandt[1]

[1]Institute of Experimental Biomedicine, University Hospital Würzburg, Würzburg, Germany., [2]Core Unit Systems Medicine, University Hospital Würzburg, Würzburg, Germany., [3]Helmholtz Institute for RNA-based Infection Research (HIRI), Würzburg, Germany.

Background:
Platelets are produced by megakaryocytes (MKs), which differentiate from hematopoietic stem cells in the bone marrow. Although thrombopoietin receptor signalling constitutes a key pathway regulating MK differentiation, it remains unclear how other receptors regulate this process. In the absence of the MK lineage-specific receptor G6b-B (encoded by Mpig6b), human patients and transgenic mice develop severe macrothrombocytopenia and myelofibrosis. Using bulk RNA-sequencing on MKs from Mpig6b^{mut} mice, we recently found a reduction in mRNA levels suggesting that G6b-B is required for MK maturation (Becker, Nagy et al, Blood Adv. 2022), however the underlying molecular mechanisms remain unknown.
Aim:
To determine how G6b-B regulates the transcriptional program of MKs.
Methods:
Native bone marrow MKs of wild-type and Mpig6b^{mut} mice were enriched using magnetic cell separation and processed for single-cell RNA sequencing (scRNA-seq) utilizing the 10x Genomics Chromium system. Data analysis was performed using the Seurat package. Differences in MK subsets were assessed using immunofluorescence microscopy.
Results:
Our data show that the loss of G6b-B leads to an increased number of MK progenitors and a striking expansion of the immune-modulatory MK subset. These changes, combined with the upregulation of genes involved in regulating the extracellular matrix in stromal cells, could contribute to the rapid development of myelofibrosis in these mice. Additionally, we observed a decrease in the proportion of platelet-generating mature MKs, which showed reduced expression of several MK-specific transcripts, potentially contributing to the severe macrothrombocytopenia.
Conclusion:
Our results highlight G6b-B as a central receptor in MK development and provide novel insights into its role in coordinating the transcriptional program of maturing MKs. Loss of G6b-B results in aberrant differentiation of MK subsets, which could provide a potential explanation for both the macrothrombocytopenia and myelofibrosis phenotypes.

Abstract Number - OC03

Real-time assessment of inter-platelet communication and regulation of thrombus structure by connexin gap junctions

Kirk A Taylor[1], Joanne L Dunster[1], Alexander P Bye[2], Joanne L Mitchell[1], Tanya Sage[1], Sabina Ray[1], Jonathan M Gibbins[1]

[1]Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, UK, [2]School of Pharmacy, University of Reading, Reading, UK

Background:
Platelets respond to vascular injury in a highly coordinated manner to prevent blood loss and promote wound repair. Mechanisms regulating inter-platelet communication and coordination of these responses are incompletely understood and may involve connexin gap junctions. Inhibition of connexins reduces thrombus formation in vivo but the inter-cellular signals conveyed via platelet gap junctions are unknown.
Aims:
To evaluate the role of platelet connexins in coordination of intercellular signalling and regulation of thrombus structure.
Methods:
Real-time transfer of calcein dye was used to evaluate gap junction function during thrombus formation. Calcium spikes were recorded from platelets spread on fibrinogen-coated coverslips and coordination of signals was modelled in Matlab and R. Labelled dextrans (3kDa and 70kDa) were used to assess the structure of thrombi treated with gap junction inhibitors or from platelet-specific GJA10 knockout mice.
Results:
Thrombi were formed under arteriolar shear and calcein movement between the cytosol of adjacent platelets was tracked. To evaluate the role for connexins in this process, we used the pan-connexin inhibitor carbenoxolone (Cbx), the connexin 37 (Cx37)-selective peptide inhibitor 37,43Gap27 or a peptide cocktail targeting Cx37, Cx40 and Cx62. 37,43Gap27 reduced calcein transfer by 30%, whilst Cbx or the peptide cocktail reduced transfer by >60%, suggesting a role for multiple connexins. We next evaluated TRAP6-evoked calcium signals in single platelets and calculated the time interval between calcium spikes in cells that were physically interacting (paired). The lag time increased following treatment with Cbx. Finally, we evaluated the impact of connexin inhibitors on thrombus structure using labelled dextrans. We observed an increase in thrombus porosity when blood was pre-incubated with Cbx or peptide cocktail, suggesting a reduced packing density. A similar phenotype was observed in platelet-specific GJA10 knockout mice. Gap junction blockers also reduced the time to initiation of thrombolysis in a halo clot lysis assay.
Conclusions:
Connexins form gap junctions during thrombus formation that facilitate transfer of intercellular signalling molecules (e.g. cytosolic calcium or IP3). Targeting connexins also increased thrombus porosity and reduced the time to initiation of thrombolysis. Connexins may therefore represent a target to enhance thrombolytic therapies.

Abstract Number - OC04

Neutrophil extracellular trap components differentially induce S100A8/A9 expression on platelets associated with a procoagulant phenotype

Gina Perrella[1], Poppy Gant[1], Beata Grygielska[1], Benjamin Fisher[2], Mark Thomas[1], Rahul Madiha[2], Asif J. Iqbal[1], Julie Rayes[1]

[1]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Vincent Drive, B15 2TT, Birmingham, U.K., [2]Institute of Inflammation and Ageing, University of Birmingham, Birmingham, U.K.

Background:
Neutrophil Extracellular Traps (NETs) are observed in patients with COVID-19 and sepsis and markers of NETosis correlate with disease severity and thrombosis. S100A8/A9, a damage associated molecular pattern released during NETosis, supports the formation of procoagulant platelets through GPIbα. S100A8/A9 was also detected in platelets from patients with COVID-19 and sepsis but the mechanism driving its expression and its functional relevance in platelet function and inflammation are unknown.
Aim:
We investigated S100A8/A9 expression on platelet microvesicles from patients with sepsis and COVID-19 and its correlation with inflammatory and thrombotic markers. We further investigated the mechanism(s) driving S100A8/A9 expression in platelets and its relationship with platelet phenotype.
Methods:
Inflammatory and thrombotic markers in healthy donors, patients with COVID-19 and sepsis were measured by Luminex (ICAM-1, VCAM-1, CD40L, P-selectin, S100A12, lipocalin-2, CCL5) and ELISA (VWF, S100A8/A9). The expression of S100A8/A9, phosphatidylserine (PS) and P-selectin on platelet microvesicles and platelets was measured by flow cytometry.
Results:
Soluble (s) ICAM-1, sVCAM-1, sP-selectin and VWF were increased in patients with COVID-19 and sepsis. sCD40L and CCL5 were significantly higher in ICU patients with COVID-19. Neutrophil activation markers S100A8/A9, S100A12, Lipocalin-2 were increased in both cohorts, but significantly higher in ICU patients with COVID-19 and correlated with the presence of S100A8/A9- and phosphatidylserine-double positive platelet microvesicles. S100A8/A9 was absent in unstimulated platelets isolated from healthy donors as assessed by western blot and flow cytometry. NET components but not classical platelet agonists, differentially upregulated S100A8/A9 expression on the platelet surface. Anti-GPIbα antibody SZ2, which inhibits S100A8/A9-induced phosphatidylserine expression, drastically decreased S100A8/A9 expression on platelets. These S100A8/A9-positive platelets display a procoagulant and proinflammatory phenotype.
Conclusion:
Our results show that high levels of NETosis markers correlate with the presence of S100A8/A-positive platelet microvesicles. NET-components differentially induce the expression of S100A8/A9 on the platelet surface, driving in an autocrine way phosphatidylserine exposure in a GPIbα-dependent manner. These data suggest a new mechanism by which NETs induce inflammatory and procoagulant platelets.

Abstract Number - OC05

Platelet-released mitochondria promote neutrophil activation and the formation of neutrophil extracellular traps

Harriet E Allan[1], Nicola Dark[1], Plinio M F Ferreira[2], Marilena Crescente[3], Paul C Armstrong[1], Timothy D Warner[1]

[1]Blizard Institute, Queen Mary University of London, [2]National Heart and Lung Institute, Imperial College London, [3]Centre for Bioscience, Manchester Metropolitan University.

Background:
Activated platelets produce a heterogenous population of extracellular vesicles (EVs) packaged with pro-inflammatory and pro-thrombotic molecules. A small subset of these vesicles contains functional mitochondria that actively respire and consume oxygen. Interactions between platelet vesicles and cells within the circulation have been described, however to date, mitochondria containing vesicles have not been defined as a separate population. Notably, mitochondria are damaged associated molecular patterns (DAMPs) which raises the question whether mitochondria vesicles will interact with and affect immune cells.
Aim:
To investigate if platelet EVs containing mitochondria interact with and change neutrophil phenotype
Methods:
Platelet EVs stained with MitoTracker Orange were sorted (BD FACS Aria IIIu) into two subpopulations, mitochondria negative (PMV) and positive (mitoPMV) EVs. Neutrophils incubated with sorted vesicles (45 mins, 37˚C) were analysed for activation marker expression by flow cytometry. Neutrophils incubated with PMVs and mitoPMVs were stimulated to form neutrophils extracellular traps (LPS 50ng/ml, 90mins, 37˚C), stained with DAPI and imaged using a confocal microscope. Data were analysed using Image J, FlowJo v.10 and GraphPad Prism.
Results:
MitoPMVs accounted for 19±1% of the vesicle population and interacted with neutrophils. Neutrophils incubated with mitoPMVs, but not with PMVs, showed increases in expression of CD66b (1.5±0.2-fold; n=6, $p<0.05$) and CD11b (1.3±0.1-fold; n=6, $p<0.05$) with concurrent reductions in CXCR2 expression (0.7±0.07-fold; n=6, $p<0.05$). NET formation in response to LPS was enhanced in the presence of mitoPMVs (LPS-stimulated DNA Area, 107±21μm2 vs. 238±6μm2; neutrophils vs. neutrophils + mitoPMVs; n=4, $p<0.05$).
Conclusion:
Mitochondrial transfer has been demonstrated in numerous cells highlighting a mechanism through which mitochondrial function may be improved. Here we show that mitochondria encapsulated within platelet EVs interact with neutrophils causing an increase in activation and phagocytosis markers. Further, platelet-derived mitochondria enhance NETosis, consistent with their capacity to act as DAMPs. Further work is required to determine the influence of platelet-derived mitochondria on neutrophils, but data presented here indicate they enhance neutrophil activation and inflammatory function.

Abstract Number - OC06

Endothelial inflammation affects platelet activation via surface receptors and mediator secretion

Claudia Schönichen[1,2], Isabella Provenzale[1,3], Annekathrin S. Nedwed[4], Fiorella A. Solari[5], Federico Marini[4], Audrey Cleuren[6], Andreas Daiber[7,8], Kerstin Jurk[2], Monika Stoll[1], Albert Sickmann[5], Marijke J.E. Kuijpers[1], Johan W.M. Heemskerk[1]

[1]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, The Netherlands, [2]Center for Thrombosis and Hemostasis, University Medical Center of the Johannes Gutenberg-University of Mainz, Germany, [3]Institute for Cardiovascular and Metabolic Research (ICMR), School of Biological Sciences, University of Reading, Reading (UK), [4]Institute of Medical Biostatistics, Epidemiology and Informatics (IMBEI), University Medical Center of the Johannes Gutenberg-University, Mainz, Germany, [5]Leibniz-Institut for analytical sciences – ISAS – e.V. Dortmund, Germany, [6]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA, [7]Department of Cardiology, University Medical Center Mainz, Johannes Gutenberg University, Mainz, Germany, [8]German Center for Cardiovascular Research (DZHK), Partnersite Rhine-Main, Mainz, Germany.

Background:
Endothelial cells regulate platelet activation to prevent adhesion and thrombus formation, via the release of cAMP- and cGMP-elevating agents. Inflammatory changes in endothelial cells can result in impaired platelet inhibition by mechanisms that are not well understood.
Aim:
Identify biological pathways underlying the endothelial regulation of platelet activation in inflammatory settings, using a vessel-on-a-chip model and multi-omics approaches.
Methods:
Platelet activation and thrombus formation were assessed in whole blood perfused over resting and inflamed human umbilical vein endothelial cells (HUVEC) cultured on collagen. Inflammation of the cells was induced by tumor necrosis factor (TNF)α or lipopolysaccharide (LPS). Inflammatory HUVEC were assessed by RNA-Seq. Releasates of HUVEC were screened for nitric oxide, prostacyclin and secreted proteins. Platelet reactivity after exposure to resting or inflamed HUVEC was assessed with flow cytometry and aggregation. Phosphorylation changes in the HUVEC-exposed platelets were investigated using isobaric label phospho-proteome analysis.
Results:
The potent inhibitory effect of HUVEC on platelet adhesion and coagulation activation was strongly reversed after pre-treatment of the cells with TNFα and to a lower extent with LPS. Transcriptome analysis showed that TNFα (LPS) treated endothelial cells over-expressed ~2800 (~250) mRNAs. These included >150 (23) transcripts of surface receptors and 23 (12) transcripts of endothelial release products, i.e. chemokines and other platelet-coagulation modulators. Pathway analysis confirmed an increased inflammatory state of the cells. Markedly, inflammatory treatment increased the prostacyclin production by 2-fold, while the release of nitric oxide was not affected. After exposure to the TNFα (but not LPS) treated HUVEC, washed platelets were considerably less inhibited in terms of aggregation and integrin αIIbβ3 activation. Phospho-proteome analysis revealed an altered pattern of activities of protein kinases and phosphatases in platelets after exposure to inflamed endothelial cells. The (inflamed) endothelial cells and releasates are currently examined for novel modulators of platelet function.
Conclusions:
Inflammatory stimulation of HUVEC with TNFα>LPS induces the surface expression and secretion of platelet-activating mediators, resulting in altered thrombogenicity via a panel of endothelial products. This may provide interesting therapeutic targets.

Abstract Number - OC07

Platelet-derived Integrin and Tetraspanin-enriched Tethers (PITTs) orchestrate neutrophil recruitment and drive pulmonary thrombo-inflammation

Timo Vögtle[1], Denise Johnson[1], Charly Kusch[1], Philipp Burkard[1], Paquita Nurden[2], Carina Gross[1], Lukas J. Weiss[1], Dirk Weismann[3], Wolfgang Kastenmüller[4], Harald Schulze[1], Katrin G. Heinze[5], Markus Sauer[5,6], David Stegner[1], Alan Nurden[2], Bernhard Nieswandt[1]

[1]Institute of Experimental Biomedicine I, University Hospital and Rudolf Virchow Center for Integrative and Translational Bioimaging, University of Würzburg, Germany, [2]Institut Hospitalo-Universitaire LIRYC, Hôpital Xavier Arnozan, Pessac, France, [3]Department of Internal Medicine I, Intensive Care Unit, University Hospital Würzburg, Germany, [4]Würzburg Institute of Systems Immunology, Max Planck Research Group, University of Würzburg, Germany, [5]Rudolf Virchow Center for Integrative and Translational Biomaging University of Würzburg, Germany, [6]Department of Biotechnology and Biophysics, Biocenter, University of Würzburg, Germany.

Background:
In thrombo-inflammatory disease settings, platelets orchestrate immune cell recruitment and regulate vascular barrier function, but the underlying mechanisms are not fully understood. The platelet integrin αIIbβ3 associates with the tetraspanin CD9 and is required for haemostasis and thrombosis, but its role in thrombo-inflammation is less well defined.
Aims:
In this study, we characterized a novel pathway of αIIbβ3 regulation in platelets that drives thrombo-inflammation.
Methods:
Mice received the anti-αIIbβ3 mAbs (MWReg30, JON mAbs, 2µg/g body weight) intravenously and platelets were monitored ex vivo and in vivo. To induce pulmonary inflammation, lipopolysaccharide, S. pneumoniae or S. aureus were instilled intranasally into C57BL/6 or vWF-/- mice. Lungs and blood were taken for further analysis.
Results:
Application of anti-αIIbβ3 mAb induced platelet recruitment to the liver with flow-dependent binding of the opsonized platelets to FcγRIIB on endothelial cells. αIIbβ3/CD9 clustered at the platelet 'rear edge' and segregated within tethers (termed Platelet-derived Integrin and Tetraspanin-enriched Tethers) that separated and left attached to the endothelial cells. The flow-dependent release of PITTs was reproduced in vitro employing surface-immobilized antibodies but also on vWF-coated surfaces. Notably, neutrophils adhered to PITTs formed on vWF, indicating a role for PITTs in immune cell recruitment. In support of this hypothesis, PITT formation was detected in blood smears obtained from patients with Covid-19 and their circulating platelets showed a partial loss of αIIbβ3/CD9. Strikingly, PITT formation was detected in lungs of mice challenged in models of pulmonary infection/inflammation, but not in control mice. These PITTs were deposited on endothelial cells and interacted with neutrophils. PITT formation/deposition in lungs was abolished in vWF-/- mice that also showed reduced LPS-induced pulmonary neutrophil recruitment.
Conclusion:
This study reports a previously unrecognized, inflammation-triggered mechanism of αIIbβ3 regulation, which facilitates the recruitment of neutrophils and fuels thrombo-inflammation.
This work was supported by the DFG (SFB 1525 – project A06 and NI556/13-1).

Abstract Number - OC08

Antiplatelet drugs do not protect from platelet-leukocyte aggregation in Coronary Artery Disease

Christian Schulte[1], Luise Pieper[1], Maike Frye[2], Christoph Waldeyer[1], Johannes T Neumann[1], Fabian J Brunner[1], Giordano Pula[3]

[1]Department of Cardiology, University Heart & Vascular Center Hamburg, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, [2]Institute for Clinical Chemistry and Laboratory Medicine, University Medical Center Eppendorf, Hamburg, Germany, [3]Centre for Biomedicine, Hull and York Medical School (HYMS), Hull, UK.

Background:
Despite the advances in cardiovascular medicine, coronary artery disease (CAD) remains a leading cause of mortality. Among the pathophysiological features of this condition, platelet-leukocyte aggregates (PLAs) require further attention, either as diagnostic/prognostic disease markers or as potential interventional targets.
Aims:
In this study, we characterised PLAs in CAD patients. Primarily, we investigated the association of PLA levels with CAD diagnosis. In addition, the basal levels of platelet activation and degranulation were also assessed in CAD patients and controls, and their correlation with PLA levels was analysed. Finally, the effect of antiplatelet treatments on circulating PLA numbers, basal platelet activation and degranulation was studied in CAD patients.
Methods:
Participants were recruited at the Department of Cardiology of the University Heart and Vascular Centre Hamburg Eppendorf. Amongst patients admitted with severe chest pain, the diagnosis of CAD was made angiographically and patients without CAD were used as controls. PLAs, platelet activation and platelet degranulation were assessed by flow cytometry.
Results:
Circulating PLAs and basal platelet degranulation levels were significantly higher in CAD patients compared to controls. Surprisingly, there was no significant correlation between PLA levels and platelet degranulation (or any other measured parameter). In addition, CAD patients on antiplatelet therapy did not display lower PLA or platelet degranulation levels compared to controls.
Conclusions:
Overall, these data suggest a mechanism of PLA formation that is independent of platelet activation or degranulation and highlights the inefficiency of current antiplatelet treatments for the prevention of basal platelet degranulation and PLA formation.

Abstract Number - OC09

Single platelet function in thrombocytopenia patients measured with droplet microfluidics

Maaike Jongen[1], Petter Höglund[1]

[1]Karolinska Institutet, Sweden.

Background:
Platelet functional heterogeneity has been a topic of interest in several studies. However, only a droplet-based method can study pure intrinsic heterogeneity without the influence of adjacent platelets. This method has previously shown to detect novel subtypes of differently responsive platelets that contribute to the total response of platelets as a collective.
Aims:
To adapt and apply the single platelet droplet microfluidic assay to detect subtypes of platelets in different patient groups suffering from thrombocytopenia.
Methods:
The innovative droplet microfluidic protocol involves compartmentalising platelets in monodisperse droplets with agonists while excluding paracrine signalling. High frequency encapsulation (250 Hz) is coupled with flow cytometry for high throughput quantification of platelet responses to multiple agonists. The ideal intake of platelets is in platelet rich plasma at a concentration of $5x10^9$/L and consumption is 60 µL/hr, so for mildly thrombocytopenic patients no adaptation is required and for severely thrombocytopenic patients a slow centrifugation of the platelet rich plasma can increase the concentration as required.
Results:
Platelets were individually encapsulated in water-in-oil droplets with a mean diameter of 25 µm. In healthy individuals, it is shown that there exist hyperresponsive platelet subpopulations visible after addition of low concentration agonist that are not detectable outside droplets and drive the collective response. Furthermore, at high concentrations of agonists we can detect hyposensitive platelets that don't respond with submaximal agonist activation. We hypothesize that these are increased in leukaemia patients where there is a functional defect in addition to the low counts and that the hyperresponsive population is enhanced in immune thrombocytopenic patients where a compensatory mechanism prevents bleeding even at very low counts.
Conclusions:
This study demonstrates the value of a high throughput droplet microfluidics and flow cytometry workflow for measuring platelet heterogeneity and its application to thrombocytopenic patients of different origins.

Abstract Number - OC10

Supercharged platelets as a novel therapy for reducing blood loss post-cardiac surgery

Alexi Crosby[1], Souradip Mookerjee[1], Amie K Waller[1], Holly R Foster[1], Annett Mueller[1], Lily M Du[2], David A Wilcox[2], Cedric Gheveart[1]

[1]Department of Haematology, Cambridge Univeristy, UK, [2]Department of Pediatrics, Medical College of WI, USA 53226, [7]Department of Pediatrics, Medical College of WI, USA 53226.

Background:
Cardiovascular surgery is one of the surgical disciplines that requires the most blood components for transfusion. In addition to surgical techniques, the transfusion of blood components and pharmacological interventions, measures to restore haemostasis in actively bleeding patients include the administration of concentrated coagulation factors including recombinant FVIIa (rFVIIa). However, this has huge cost implications given the very short half-life of clotting factors and an inherent risk of adverse thrombotic events, resulting in increases in hospital, morbidity and mortality.
Aims:
We sought to determine whether loading donor MK's and subsequently MK's generated in vitro from iPSC's with FVIIa, would enhance the haemostatic ability of their platelet progeny, with a view to translating this to the clinic.
Methods:
Immunofluorescence and flow cytometry were used to determine whether donor platelets endocytose extracellular rFVIIa. Using the bleeding time NRG mouse model, made profoundly thrombocytopenic through antibody depletion, we intravenously administered donor-derived platelets loaded with rFVIIa.
Using viral transduction we over-expressed 3 specific transcription factors (FLI1, TAL1 and GATA1) (FoP) to generate MKs from iPSCs and virally transduced these MKs with FVIIa. PBMKs were generated from cord blood.
Results:
We have shown that donor platelets endocytose rFVIIa and target it to their alpha granules. We have also shown a reduction in early haemorrhage in the NRG mouse administered donor platelets loaded with FVIIa, compared with those administered donor platelets or PBS. We have also shown that we can generate MKs from iPSCs and that they express FVIIa. Preliminary data shows that peripheral blood megakaryocytes (PBMKs) virally transduced with FVIIa have an increase in thrombin production and a decrease in thrombin lag time, compared with platelets from untransduced MKs.
Conclusion:
We have demonstrated that donor platelets 'supercharged' with rFVIIa have an enhanced haemostatic ability and that we can 'over-express' FVIIa in our FoP platelets. We are currently determining whether platelets from PBMKs, supercharged with FVIIa, exhibit a greater haemostatic ability than untransduced platelets. This will enable the clinic to have a more effective, safe, continuous and universal supply of platelets.

Abstract Number - OC11

Dual anti-platelet therapies are ineffective at preventing blockage of the coronary microcirculation following ischaemia reperfusion injury in mice.

Joanne L Mitchell[1], Juma El awaisi[1], Dean P J Kavanagh[1], Neena Kalia[1]

[1]University of Birmingham.

Background:
Ischaemia-reperfusion injury (IRI) is caused by inadequate perfusion within the cardiac microcirculation due to thrombo-inflammation resulting in larger infarcts and heart failure (HF). Myocardial infarction (MI) treatments include dual anti-platelet therapies (DAPTs) which are effective at decreasing recurrent MI, however many DAPT patients still develop HF. Due to limitations of clinical imaging, effectiveness of DAPTs in the coronary microcirculation, which services large parts of the myocardium, is poorly understood.
Aim:
Determine the effectiveness of DAPTs in limiting thrombo-inflammation and re-establishing blood flow in the murine coronary microcirculation post IRI.
Methods:
Mice were pre-treated with vehicle control (VC), aspirin alone or combined with either ticagrelor, clopidogrel or prasugrel. Myocardial IRI on anaesthetised mice was performed using a reversible ligation of the left anterior descending artery for 45m, followed by 2h reperfusion. Intravital imaging of the coronary microcirculation in the beating mouse heart was used to visualise fluorescently labelled platelets and neutrophils during reperfusion. Laser speckle contrast imaging (LSCI) on the surface of the beating heart measured tissue perfusion. Flow cytometry detected P-selectin on mouse platelets following IRI in response to ex vivo agonist stimulation.
Results:
DAPTs were effective and reduced platelet activation ex vivo in response to CRP and ADP compared to VC. Intravital imaging showed no reduction in % area covered by platelet microthrombi in the coronary microcirculation in response to aspirin alone (1.79%), or combined with ticagrelor (1.08%), clopidogrel (1.05%) or prasugrel (0.69%) compared to VC (1.79%). Interestingly, neutrophil recruitment was increased compared to VC (2.08%) upon treatment with aspirin (6.22%; P=0.002) and aspirin and clopidogrel (6.35%; P=0.0001) but was unaffected by aspirin with ticagrelor (2.84%) or prasugrel (3.33%), suggesting certain DAPTs may enhance neutrophil infiltration to the coronary microcirculation causing further blockages. LSCI during reperfusion showed no DAPTs were able to restore left ventricle perfusion back to normal (sham) levels.
Conclusion:
DAPTs do not prevent blockage of the coronary microcirculation by platelet microthrombi following IRI and in some cases increase neutrophil infiltration. New targets are needed for MI treatment to allow adequate reperfusion of the coronary microcirculation and prevent HF.

Abstract Number - OC12

Novel Prostacyclin Analogue Produces Potent and Selective Platelet Inhibition

Megan F Allen[1], Shahida Mallah[1], James L Hutchinson[1], Georgia Tier[1], Changcheng Jing[2], Isabel Perez-Powell[2], Varinder K Aggarwal[2], Stuart J Mundell[1], Ingeborg Hers[1]

[1]School of Physiology, Pharmacology & Neuroscience, University of Bristol, UK., [2]School of Chemistry, University of Bristol, UK.

Background:
Prostacyclin (PGI2) is a potent inhibitor of platelet activation through the IP receptor. However, due to its short half-life (3-5 minutes) it is limited for use as an anti-thrombotic. We have synthesised a novel PGI2 analogue with superior stability compared to other analogues e.g. iloprost. We aimed to show that our compound, ligand 9, shows improved potency compared to iloprost, ralinepag and selexipag and is selective.
Methods:
PRESTO-Tango was performed to determine receptor selectivity through β-arrestin2 recruitment in transfected HTLA cells in the presence of different prostanoid receptors in a concentration-dependent manner. Flow cytometry for vasodilator-stimulated phosphoprotein (VASP) phosphorylation was performed on washed human platelets in a concentration-dependent manner. Light transmission aggregation and plate aggregation were performed using human platelet rich plasma to determine if ligand 9 can reverse U46619-induced aggregation and the concentration-dependent manner of this respectively.
Results:
Using PRESTO-Tango, Ligand 9 induced β-arrestin2 recruitment through the IP receptor and is 333-fold more potent than the synthetic IP-agonist AFP-07. However, ligand 9 may also activate the EP4 receptor. Ligand 9 was confirmed to induce VASP phosphorylation and is 4-fold more potent than AFP-07 and iloprost. Interestingly, this only occurred through the IP receptor and showed no activity at the EP4 receptor. Ligand 9 was capable of reversing U46619-induced aggregation with an Emax of 100 nM selectively through the IP receptor. It was also more potent than other IP receptor agonists in a concentration-dependent manner and interestingly reduced absorption below baseline levels.
Conclusion:
Our novel PGI2 analogue ligand 9 is more potent than current clinically available IP agonists and is selective for the IP receptor in platelets, highlighting promise as superior anti-thrombotic agent. Ligand 9 also reveals an interesting effect on platelet aggregation which requires further study.

Abstract Number - OC13

Role of MicroRNA-223-3p in regulating platelet-supported thrombin generation

Julia Charlon[1], Sylvie Dunoyer-Geindre[1], Séverine Nolli[1], Paulina Ciepla[1], Jean-Luc Reny[2], Pierre Fontana[3]

[1]Geneva Platelet Group, Faculty of Medicine, University of Geneva, Geneva, Switzerland, [2]Division of General Internal Medicine, Geneva University Hospitals, Geneva, Switzerland, [3]Division of Angiology and Haemostasis, Geneva University Hospitals, Geneva, Switzerland.

Background:
Platelets are anucleate cells mostly involved in hemostasis through their aggregating properties and their ability to promote thrombin generation at their activated surface. Platelet reactivity is variable among individuals and microRNAs (miRNAs) may regulate platelet function. Among platelet-derived miRNAs, miR-223-3p is the most highly expressed. Furthermore, several studies have reported an association between miR-223-3p level and platelet reactivity or recurrence of cardiovascular events, but the impact of miR-223-3p on platelet function is poorly understood.
Aim:
To investigate the role of miR-223-3p on platelet reactivity in platelet-like structures (PLS) derived from human hematopoietic stem cells (CD34+).
Methods:
MiR-223-3p upregulation and downregulation were carried out by transfecting CD34+-derived megakaryocytes with miR-223-3p mimic or CRISPR/Cas9 complexes, respectively. PLS were then timely collected for functional tests. Phosphatidylserine (PS) exposure was performed using flow cytometry. PLS-supported thrombin generation in human plasma was quantified with the Calibrated Automated Thrombogram using the velocity index. Finally, quantitative polymerase chain reaction was used to quantify the expression of miRNAs and selected mRNAs.
Results:
Increased levels of miR-223-3p induced a 30±5% decrease of PS exposure after activation (n=7, p=0.001), and a 10±3% decrease in PLS-supported thrombin generation (n=8, p=0.008), compared to mock condition. These findings were associated with a 47±10% decrease in TMEM16F mRNA expression (n=4, p=0.022), a major contributor in PS exposure. Transfection with CRISPR/Cas9 complexes led to a 59±8% decrease in the expression of miR-223-3p (n=4, p=0.0027).
Conclusion:
MiR-223-3p upregulation is associated with a decrease of PS exposure, PLS-supported thrombin generation, and TMEM16F mRNA expression. This observation suggests that miR-223-3p could regulate PLS procoagulant activity through the regulation of TMEM16F. CRISPR/Cas9 editing tool allowed to significantly decrease miR-223-3p and phenotypic experiments are underway.

Abstract Number - OC14

c-Mpl is a platelet receptor for platelet factor 4

Richard J Buka[1], Samantha J Montague[1,2], Luis A Moran[1], Eleyna M Martin[1], Alexandre Slater[1], Stephen P Watson[1,2], Phillip LR Nicolson[1,3]

[1]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham UK, [2]Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, The Midlands, UK, [3]Department of Clinical Haematology, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK.

Background:
Platelet factor 4 (PF4) is an abundant chemokine, released from platelet α-granules upon activation and has a central role in vaccine-induced immune thrombocytopenia and thrombosis (VITT). In VITT, PF4 and anti-PF4 antibodies form immune complexes which activate platelets through FcγRIIA. The addition of PF4 to VITT serum enhances platelet activation in vitro which is thought to be due to enhanced immune complex formation. However, the effect of PF4 alone on platelet activation has not been extensively investigated.
Aim:
To characterise the effect of PF4 on platelet function and identify the platelet receptor for PF4.
Methods:
Platelet activation by PF4 was assessed by light transmission aggregometry using washed platelets. Signalling pathways were investigated by mass spectrometry and western blot using phosphospecific antibodies or immunoprecipitation (IP). PF4-c-Mpl binding was quantified by surface plasmon resonance (SPR).
Results:
PF4 induced platelet aggregation at doses between 10-100 µg/mL (1.28-12.8 µM) in a variable, but dose-dependent manner (n=13), with the aggregation pattern characterised by a slow initial phase, followed by a faster second phase after >10 minutes. Tyrosine phosphorylation after PF4 stimulation revealed two prominent bands at ~95 kDa. Phosphorylated signal transducer and activator of transcription (STAT)5a/b was detected by mass spectrometry and confirmed by western blot. Phosphorylated STAT3 was confirmed by western blot and Janus kinase 2 (JAK2) phosphorylation by IP. The JAK2 inhibitor ruxolitinib (100 nM) blocked aggregation (n=8, $p<0.001$) and phosphorylation (n=3). Thrombopoietin (TPO) induces JAK2-STAT3/5 phosphorylation so we hypothesised that c-Mpl was the receptor for PF4. This was confirmed using a phosphospecific antibody to c-Mpl (Tyr626) to show increased c-Mpl phosphorylation after PF4 stimulation (n=3). SPR gave an estimated affinity constant (KD) of 744±19 nM for PF4-cMpl binding. Finally, we found that ruxolitinib inhibited platelet aggregation to VITT serum (n=7, $p=0.014$).
Conclusion:
These data support a model in which PF4, as well as its role in immune complex formation, directly contributes to platelet activation. Furthermore, binding of PF4 to c-Mpl likely increases the avidity of the immune complex binding to FcγRIIA, enhancing platelet activation.

This work was supported by funding from the National Institute of Health Research and the British Heart Foundation.

Abstract Number - OC15

Development of a fully in vitro approach to generation of megakaryocytes and platelets from mouse embryonic stem cells

Jordan Vautrinot[1], Moyra Lawrence[2], Samantha Mason[2], Xiaojuan Zhao[1], Cedric Ghevaert[2], Alastair W. Poole[1]

[1]University of Bristol, [2]University of Cambridge.

Background:
Present understanding of platelet function derives substantially from genetic mouse models and pharmacological targeting of signalling pathways, each having their own drawbacks. The field is limited by the technology currently in place: gene-specific studies are limited to either gene knockouts in animals or in rare human cohorts. Therefore, there is a need to generate large numbers of specific genetically modifiable platelets. Here we present data showing the ability to generate megakaryocytes (MK) and platelets from a genetically modifiable source in murine embryonic stem cells.
Aims:
To generate megakaryocytes from ES-R1 embryonic mouse stem cells and subsequently generate large numbers of functional platelets.
Methods:
Mouse embryonic stem cells (ES-R1) were transformed into megakaryocytes using an established forward programming method. Post day 10 cells were recovered, and ploidy, granularity and surface markers were assessed by FACS, confocal and TEM imaging. Assessment of platelet generation capability was demonstrated in a novel ex vivo heart lung system (https://www.biorxiv.org/content/10.1101/2021.11.01.466743v1), generated platelets function was assessed by FACS for P-selectin expression and activation of integrin aIIbb3 induced by agonists.
Results:
FACS analysis of generated megakaryocytes showed cells became CD41a and CD42d positive as well increased ploidy comparable to that of bone marrow-derived controls. TEM and confocal imaging showed cells to be granular and polyploid. Following passage through a mouse heart-lung preparation, forward programmed MKs were able to generate platelets comparable to bone marrow-derived Generated platelets were also functional, showing agonist-stimulated P-selectin expression and activation of integrin aIIbb3.
Conclusion:
We conclude that it is possible to generate functional megakaryocytes from mouse embryonic stem cells, that not only have comparable features to those derived from bone marrow but are also capable of producing large amounts of functional platelets. The genetic tractability of ES cells establishes a process for generating large numbers of genetically modified platelets in future work.

Abstract Number - OC16

Platelets-tumor cells interaction: a shield against chemotherapy

Jorge Cerón-Hernández[1], Abel García-Díaz[2], Gonzalo Martínez-Navajas[1], Iris Simón-Sáez[3], Alba Rodríguez-Martínez[4], Inés Aznar[2], María Pilar Molina[2], Pedro J Real[1], María J Serrano[2]

[1]GENYO - Centro Pfizer- Universidad de Granada- Junta de Andalucía de Genómica e Investigación Oncológica, Biochemistry and Molecular Biology I, Granada, Spain., [2]GENYO - Centro Pfizer-Universidad de Granada- Junta de Andalucía de Genómica e Investigación Oncológica, Liquid Biopsy and Cancer Interception Lab, Granada, Spain., [3]Mayo Clinic, Mayo Clinic, Rochester-Minnesota, United States., [4]Hospital Costa del Sol, Hospital Costa del Sol, Málaga, Spain.

Background:
Platelets are active players in tumorogenesis, although the exact interactive mechanisms and their direct impact on tumor cells remain largely unknown. Preliminary studies in our laboratory showed that coculturing lung and other cancer cell lines with platelets enhances proliferation of the former and induces phenotype changes.One of the many functions proposed of this interaction between platelets and tumor cells is that platelets protect tumor cells against cell death and apoptosis and they could be protecting against treatments with different drugs.
Aim:
In order to demonstrate this hypothesis, in vitro tests were carried out with three different lung cancer cell lines (A549, H1975 and H1299) and platelets from a healthy donor in coculture treated or not with CIS-platin, Docetaxel or Erlotinib.
Methods:
Platelets were obtained from peripheral blood samples from a healthy donor. Platelets were added at concentration of 100,000 plt/µL. The tests were carried out with different treatments (CIS-platin, Docetaxel or Erlotinib) at previously calculated IC50 concentration with or without platelets. The effect on cell death and apoptosis was evaluated at 24 h after treatment by flow cytometry using annexin V / 7AAD kit. We also carried out xCelligence assays analyzing cell proliferation and cytotoxicity.
Results:
The IC50 concentration of all drugs increased in all tumour lines when cells had 24 h of contact with platelets. Apoptosis in the A549 cell line co-cultured with platelets and treated with CIS-platin, Docetaxel or Erlotinib showed a statistically significant downward at 24h versus cells without platelets treated with the same drug ($P<0.05$). In the coculture of H1975 cell line with platelets treated with CIS-platin apoptosis and cell death were decreased but not significant. Regarding the xCelligence proliferation assays, significant differences were found between the condition of treated co-culture and treated cells without platelets for both cell lines at 24 and 48 h after treatment ($P<0.05$). No significant differences were found in the migration assay, but there was a strong trend that at 24 h the cells in contact with the platelets were able to move and they survived more.
Conclusions:
These results suggest that tumor cells might be protected by platelets. Platelets are able to interact with tumor cells and prevent apoptosis. Furthermore, platelets promote growth of tumor cells even these cells are treated with different drugs.

Abstract Number - OC17

Understanding the structure/function relationship of PLCγ2 downstream of platelet (hem)ITAM receptors

Safa Gayes[1], Selorm P.Segbefia[1], Alice Pollitt[1], Craig Hughes[1]

[1]University of Reading.

Background:
Phospholipase C gamma 2 (PLCγ2) is a key signalling hub, playing a role in a number of platelet signalling pathways. These include the collagen receptor GPVI which activates via an ITAM (immunoreceptor tyrosine-based activatory motif), and the podoplanin receptor CLEC-2, which activates through a similar mechanism known as a hemITAM. PLCγ2 is a large, multi-domain protein. The regulation of its function is through molecular interactions with a number of signalling proteins, although this is not fully understood.
PLCγ2 plays a similar role downstream of ITAM-linked receptors in other cells, namely B-cells. PLCγ2 mutations have been identified in 80% of patients who develop resistance to ibrutinib therapy. Some of these PLCγ2 mutations have been shown to cause a gain-of-function, but the effect of the others is unknown. It is also unknown whether these, or other mutations may arise in
Aim:
To understand the structure/function relationship of PLCγ2 downstream of (hem)ITAM receptors, using pharmacological inhibition and genetic manipulation in vivo and in vitro.
Methods:
CRISPR/Cas9 gene editing was used to generate PLCγ2 knock-in and knock-out mutations in DT40 cells. PLCγ2 mutations were also generated using a gene overexpression system. To characterise the functional effect of the mutations, calcium assays, NFAT assays, and tyrosine phosphorylation were used. Zebrafish were used for in vivo assessment of PLCγ2 function using pharmacological
Results:
Calcium responses and NFAT signalling in response to GPVI and CLEC-2 activation were abolished in PLCγ2 knock-out DT40 cells. Similar results were seen with a PLCγ2 inhibitor in both DT40 cells and platelets. Experiments are in progress to assess the effect of the PLCγ2 knock-in mutations and their susceptibility to the inhibitor in both cells and zebrafish.
Conclusion:
These results confirm the essential role for PLCγ2 downstream of platelet (hem)ITAM receptor signalling. Characterisation of PLCγ2 knock-in point mutations will identify whether mutations result in a gain or loss of function, and where there is a gain, whether PLCγ2 pharmacological inhibition is still possible.

Abstract Number - OC18

Platelet p110β mediates platelet-leukocyte interaction and curtails bacterial dissemination in pneumococcal pneumonia

Waltraud C. Schrottmaier[1], Julia B. Kral-Pointner[1], Manuel Salzmann[1], Marion Mussbacher[1], Anna Schmuckenschlager[1], Anita Pirabe[1], Laura Brunnthaler[1], Yasemin Ekici[1], Ulrike Heber[2], Cecilia Söpderberg-Nauclér[3], Bernard Payrastre[4], Marie-Pierre Gratacap[4], Sylvia Knapp[5], Gernot Schabbauer[1], Alice Assinger[1]

[1]Department of Vascular Biology and Thrombosis Research, Centre for Physiology and Pharmacology, Medical University of Vienna, Austria, [2]Department of Pathology and Comprehensive Cancer Centre, Medical University of Vienna, Austria, [3]Department of Medicine, Solna, Centre for Molecular Medicine, Microbial Pathogenesis Unit, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden, 4Inserm UMR1297, Institut des Maladies Métaboliques et Cardiovasculaires (I2MC), Université Toulouse III Paul Sabatier, France, [5]Department of Medicine I, Research Division of Infection Biology, Medical University Vienna, Austria.

Background:
Class IA phosphatidylinositol 3-kinase catalytic subunit p110β promotes tumour cell proliferation, but p110β is also central for platelet activation to maintain haemostasis. However, it remains unclear if p110β also regulates platelet-mediated immune responses, which could have consequences for immune modulation upon treatment with p110β inhibitors, which are currently under investigation in clinical trials as anti-cancer treatment.
Aim:
We aimed to elucidate the role of platelet p110β for platelet-leukocyte interplay and the subsequent impact on inflammation and infection.
Methods:
Mice with platelet-specific p110β deficiency (p110βΔPLT) or wildtype mice receiving the p110β-specific inhibitor TGX-221 were challenged with Streptococcus pneumonia-induced pneumonia. Leukocyte infiltration, pulmonary damage, bacterial burden and survival were evaluated to assess inflammation and disease severity. The cell-specific contribution of platelet p110β for individual immune responses were confirmed by investigating neutrophil phagocytosis in vitro and bacterial clearance in vivo as well as leukocyte recruitment in a murine model of thioglycollate-induced sterile peritonitis.
Results:
Using a mouse model of S. pneumoniae-induced pneumonia and bacteraemia, we found that pneumococcal infection rapidly induced platelet-monocyte and platelet-neutrophil aggregate formation. Both platelet-specific p110β deficiency and pharmacologic inhibition of p110β with TGX-221 prevented platelet-leukocyte interactions and diminished pulmonary leukocyte infiltration. This enhanced bacterial burden and led to exacerbated disease pathogenesis. Platelet p110β mediated neutrophil phagocytosis of S. pneumoniae in vitro and curtailed bacteraemia in vivo. Further, genetic deficiency or inhibition of platelet p110β also impaired macrophage recruitment in an independent model of sterile peritonitis.
Conclusion:
Our results demonstrate that platelet p110β dysfunction exacerbates pulmonary infection by impeding leukocyte recruitment and pathogen clearance. Thereby, our findings provide important insights into the immunomodulatory potential of PI3K inhibitors in bacterial infection.

Abstract Number - OC19

Super-resolution microscopy of α-granule secretion in spreading platelets

Smita Patil[1], Ingmar Schoen[1]

[1]Irish Centre for Vascular Biology, Royal College of Surgeons in Ireland (RCSI) Dublin, Ireland.

Background:
α-granule secretion is a key element of platelet function in haemostasis, thrombosis and inflammation. In suspended platelets, agonist-induced α-granule secretion requires dynamic changes of the actin cytoskeleton. Since the actin cytoskeleton also changes dramatically during platelet adhesion and spreading, the question arises if and how α-granule secretion and platelet spreading influence each other through the shared actin cytoskeleton.
Aim:
To understand the spatiotemporal coordination of α-granule secretion in adherent platelets.
Methods:
Washed platelets from healthy human donors were seeded onto fibrinogen (Fg) or collagen I (Col) coated glass coverslips without or in the presence of different agonists. Platelets were fixed after 2…60 minutes, fluorescently labelled for f-actin and α-granule components P-selectin (Psel), Thombospondin-1 (TSP-1), or von Willebrand Factor (vWF). Stimulated Emission Depletion (STED) microscopy or 3D direct stochastic optical reconstruction microscopy (dSTORM) images were analysed using custom ImageJ and MATLAB scripts.
Results:
The super-resolution microscopy techniques ambiguously distinguished single α-granules from dispersed/secreted staining, membrane-residing from internal granule components, and upwards from downwards secretion. Our data show that in many α-granules of resting platelets, Psel was not homogeneously distributed but excluded from sub-regions where vWF occupied the area beneath the membrane. Granule secretion on Col was faster compared than on Fg, while platelets retained more and longer filopodia on Col vs Fg during spreading. Soluble agonists accelerated platelet spreading and granule secretion on Fg depending on agonist strength (Thrombin>CRP-XL>ADP). Especially PAR-agonists (TRAP-6 and PAR-4 AP) induced excessive filopodia formation. In all cases, α-granules remained located centrally and were secreted upwards as well as downwards but were never observed in peripheral lamellipodia nor in filopodia. Downwards secreted thrombospondin was contained below platelets, sealed off by the lamellipodium.
Conclusion:
The cross-talk between major actin cytoskeletal structures and α-granule secretion in adherent platelets is limited to a mutual excluded volume effect. The compartmentalised organisation of Psel on α-granules as revealed here for the first time might have functional implications for granule-membrane docking and differential secretion.

Abstract Number - OC20

New role of platelets on liver metabolism in early NASH

Maxime Borret[1], Marie Bellio[1], Alicia Vauclard[1], Emma Lefrancais[2], Pierre-Damien Denechaud[1], Anne Abot[3], Claude Knauf[3], François Briand[4], Thierry Sulpice[4], Laurence Payrastre[5], Philippe Valet[6], Hervé Guillou[5], Jean-Philippe Pradère[6], Bernard Payrastre[1], Sonia Severin[1]

[1]Inserm U1297 and Université Paul Sabatier, Institut des Maladies Métaboliques et Cardiovasculaires, Toulouse, France, [2]CNRS and Université Paul Sabatier, Institut de Pharmacologie et de Biologie Structurale, Toulouse, France, [3]Enterosys, Labège, France, [4]Physiogenex SAS, Escalquens, France, [5]Toxalim (Research Center in Food Toxicology), INRAE, ENVT, INP- PURPAN, UMR 1331, UPS, Université de Toulouse, Toulouse, France, [6]RESTORE Research Center, University of Toulouse, INSERM 1301, CNRS 5070, EFS, ENVT, Toulouse, France.

Background:
Non-alcoholic fatty liver diseases (NAFLD) are a major public health issue on a global scale. The World Health Organization categorizes them as the number one cause of liver disease in Western countries, with hundreds of thousands of people affected in France. NAFLD begins with a high accumulation of lipids in the liver, called hepatic steatosis, and progresses to an inflammatory and fibrotic state called non-alcoholic steatohepatitis (NASH). These two reversible stages can remain asymptomatic or progress to cirrhosis and liver cancer. Due to its multifactorial etiology, this pathology is still poorly understood, and current therapeutic strategies are still limited. The recently obtained results from the laboratory show that induction of NASH in mice is responsible for an abnormal recruitment of platelets and thrombi formation.
Aim:
This project aims at characterizing the role of platelets in liver metabolism under NASH conditions.
Methods:
We use pharmologically induced thrombocytopenia or thrombocytosis mice before submitting them to a NASH diet. Experiments are performed on total liver tissue, blood and freshly isolated liver sinusoidal endothelial cells (LSEC) or hepatocytes.
Results:
We show that in vivo, platelets have a preventive effect on NASH development by decreasing hepatic lipid accumulation, inflammation and fibrosis development. We also show that platelets have a rescuing effect with on an installed NASH on lipid storage and liver damage. LSEC are a crossroad between blood and liver functional cells. Interestingly, we observe that platelets modify LSEC phenotype by modulating their fenestration with an impact on vessel permeability in vivo. In vitro experiment on freshly isolated LSEC show that platelet secretome is responsible of this fenestration modulation effect. We are currently investigating if platelet secretome also has a direct effect on hepatocyte metabolism.
Conclusion:
To conclude, these promising results give new insights on the benefic role of platelets in NAFLD development. Further investigation will aim at deciphering molecular mechanisms involving platelets in early metabolic liver diseases.

Abstract Number - OC21

GPIb-alpha – VWF interaction mediates platelet accumulation in a new vessel-on-a-chip model of deep vein thrombosis

Alexander Brill[1], Hosam A Baksamawi[2], Alessio Alexiadis[2], Daniele Vigolo[2]

[1]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom, [2]School of Chemical Engineering, University of Birmingham, Birmingham, United Kingdom.

Background:
Deep vein thrombosis (DVT) is a hectic medical problem taking lives of millions of people around the world. DVT designated together with its main complication, pulmonary embolism (PE), as venous thromboembolism (VTE), has an average prevalence of ~1.6 per 1000 and dramatically increases with age.
Aim:
Given both technical and ethical issues of using animals in research, we aimed at developing an appropriate in vitro model that would recapitulate the conditions of thrombus development in the vein.
Methods:
We have developed a novel microfluidics chamber with moving valve leaflets to mimic the hydrodynamics of a large vein. The chamber was coated with human umbilical vein endothelial cell (HUVEC) monolayer under constant flow of the medium. The monolayer was stable and did not denude under flow. A “back and forth” flow pattern, typical for the veins, was used in experiments.
Results:
Passage of whole blood through the chamber did not induce platelet activation. Unstimulated platelets demonstrated moderate accumulation at the leaflet tips, which directly correlated with leaflet flexibility. Platelet activation by thrombin induced robust platelet accrual over the luminal side of the leaflets, but the conglomerate was unstable and gradually destroyed during 12-min experiment. Inhibition of glycoprotein (GP) IIb-IIIa by eptifibatide did not affect platelet accumulation. In contrast, blockade of the interaction between GPIb-alpha with A1 domain of von Willebrand factor (VWF) completely abolished deposition of both resting and thrombin-activated platelets. Stimulation of the endothelium with histamine, a known secretagogue of Weibel-Palade bodies, promoted platelet accrual at the basal side of leaflets in the valve pockets, where human thrombi are usually observed.
Conclusion:
Blood flow hydrodynamics plays a pivotal role in DVT initiation. Accumulation of activated platelets at the valve leaflets is mediated by GPIb-alpha-VWF interaction and occurs at the leaflets tips, whereas endothelial activation results in platelet accrual in the valve pockets.

Abstract Number - OC22

Characterising architecture of platelet aggregations using expansion microscopy (ExM).

Emma L Faulkner[1], Natalie S Poulter[1], Steven G Thomas[1]

[1]Institute of Cardiovascular Sciences , College of Medical and Dental Sciences , University of Birmingham, Birmingham, B15 2TT, UK.

Background:
Platelets play critical roles in haemostasis and thrombosis. Platelet aggregation is a multi-step process, driven by activation of G-protein coupled and glycoprotein receptors, rearrangement of cytoskeletal components and secretion of intracellular granules. Intravital microscopy revealed haemostatic clots adopt a core-and-shell architecture with highly activated and densely packed platelets at a core, which is overlaid by a shell of less activated platelets. This structure serves to both stabilise and limit overall thrombus growth. Thrombosis is an undesirable form of haemostasis, often associated with disease, occurring as a result of systemic or local pathology and producing thrombi with differing compositions and architecture. Platelet aggregation has been considered from an agonist centric viewpoint, yet this view is minimalistic and fails to account for the impact of the architecture of thrombi.
Aim:
In this work we apply expansion microscopy (ExM) to platelet aggregations to allow description of the three-dimensional architecture with sub-cellular resolution. Modelling platelet aggregate structure will improve understanding of how this affects clinical outcomes and treatment efficacy.
Methods:
Platelet aggregates were formed in parallel plate flow chambers under arterial shear. Platelet aggregates were fixed and prepared via a 4-fold ExM preparation. The net effect of this is spreading out features of interest in the sample, allowing for 60-70 nm lateral resolution. ExM-prepared samples are optically clear, increasing achievable imaging volume and depth. ExM therefore overcomes key difficulties in imaging thrombi; their small size and dense packing of platelets.
Results:
This work incorporates >1500 platelet aggregations to allow detailed qualitative and quantitative characterisation of structural heterogeneity in the presence and absence of actin inhibitors. We developed an analysis workflow to allow us to describe relative spatial organisation of features e.g. actin, membranes.
Conclusion:
This study shows ExM is a powerful, relatively high-throughput approach to describing platelet aggregations. With our now optimised experimental and analysis workflow, we can apply this in a number of ways, for example, determining structural impact of anti-thrombotic drug regiments on pathological thrombi. We are now working on techniques to improve resolution and to investigate additional platelet features e.g. receptor clustering.

Abstract Number - OC23

Role of platelet GARP in post-myocardial infarction inflammation and fibrosis

Julie Bodart[1], Cécile Dufeys[1], Yotis A Senis[2], Zoltan Nagy[3], Luc Bertrand[1], Christophe Beauloye[4], Sophie Lucas[5], Sandrine Horman[1]

[1]Université catholique de Louvain (UCLouvain), Institut de Recherche Expérimentale et Clinique (IREC), Pôle de Recherche Cardiovasculaire (CARD), Brussels, Belgium, [2]Université de Strasbourg, Institut National de la Santé et de la Recherche Médicale (INSERM), Etablissement Français du Sang Grand Est, Unité Mixte de Recherche (UMR)-S 1255, Fédération de Médecine Translationnelle de Strasbourg, Strasbourg, France., [3]Institute of Experimental Biomedicine, University Hospital and Rudolf Virchow Center, University of Würzburg, Würzburg, Germany, [4]Cliniques Universitaires Saint-Luc, Division of Cardiology, Brussels, Belgium., [5]Université catholique de Louvain (UCLouvain), Institut De Duve, Pôle de Recherche génétique et cellulaire (GECE), Brussels, Belgium.

Background:
After myocardial infarction (MI), necrotic cardiomyocytes induce an inflammatory phase, which involves infiltration of immune cells and up-regulation of inflammatory genes leading to fibrosis. Although transforming growth factor β1 (TGFβ1) is a master regulator of inflammation and fibrosis, mechanisms that trigger its activation after cardiac injury remain poorly understood. Interestingly, platelets have been shown to early infiltrate the infarcted myocardium, producing large amounts of molecules. Specifically, platelets were shown to contain 40-100 times more TGFβ1 than other cell types therefore contributing to 40-50% of the systemic TGFβ1. Moreover, platelets are also involved in TGFβ1 activation via the Glycoprotein A Repetitions Predominant (GARP) present on their surface. The present study sought to determine the contribution of platelet GARP in TGFβ1 activation, inflammation and fibrosis after MI.
Methods:
We generated a Cre transgenic mouse strain allowing megakaryocyte/platelet specific invalidation of GARP (GpIba-CrexGARPfl/fl). Platelet function and production of active TGFβ1 were assessed by FACS and ELISA/western blot, respectively. Platelet specific GARP KO (pKO) and WT mice were subjected to permanent ligation of a coronary artery, and cardiac function was assessed by echocardiography. Cardiac inflammation and fibrosis were evaluated by immunohistochemistry, FACS, western blot and RT-qPCR.
Results:
GARP exposure at platelet surface increases upon agonist stimulation. Interestingly, GARP deficiency does not impair platelet function but dramatically reduces active TGFβ1 in the serum and platelet releasates. We show that platelets infiltrate the myocardium between 1-3 days after MI, similarly in both genotypes. However, we find a drastic reduction of active TGFβ1 in the myocardium and the plasma of pKO mice. While no difference in inflammatory cells infiltration is observed, we reveal a significant increase in proinflammatory cytokines expression in pKO infarcts, compared to WT. Furthermore, pKO infarcted hearts present a disturbed MMP/TIMP/collagens expression. Finally, echocardiographic data highlight the presence of aneurysm and increased left ventricular dilation in the pKO infarcted hearts.
Conclusion: Our data demonstrate that platelet GARP is crucial for TGFβ1 activation. Moreover, GARP invalidation in mouse platelets is associated with a modulation of the inflammatory response which impacts the healing process after MI.

Abstract Number - OC24

The subtilisin-like protease furin regulates hemin-dependent ectodomain shedding of glycoprotein VI (GPVI)

Anne-Katrin Rohlfing[1], Annalena Fink[1], David Jonah Schaale[1], Valerie Dicenta-Baunach[1], Zoi Laspa[1], Andreas Pelzer[1], Tatsiana Castor[1], Xiaoqing Fu[2], Michael Lämmerhofer[2], Meinrad Paul Gawaz[1]

[1]Department of Cardiology and Angiology, University Hospital Tübingen, Eberhard Karls University Tübingen, Tübingen, Germany, [2]University of Tübingen, Department of Pharmaceutical and Medicinal Chemistry, Institute of Pharmaceutical Sciences, Tübingen, Germany.

Background:
Free hemoglobin and hemin are the result of severe hemolysis of erythrocytes, which can be caused by various conditions diseases such as unstable sickle cell disease, b-thalassemia, or paroxysmal nocturnal hemoglobinuria. The liberated hemin can cause platelet activation and is able to trigger thrombosis.
Aim:
Analyze the hemin induced receptor shedding.
Methods:
The effect of hemin on the platelet function and surface expression off the glycoprotein VI (GPVI) was evaluated. Isolated platelets were stimulated with increasing concentrations of hemin and the effect analyzed by stand methods such as flow cytometry, aggregometry, flow chamber experiments and immunoblotting.
Results:
Platelet activation, aggregation and aggregate formation on immobilized collagen under flow was strongly enhanced in our experiments. In addition, GPVI surface expression was significantly reduced upon hemin stimulation in a concentration-dependent manner. Immunoblotting experiments showed, that the hemin-induced reduced GPVI surface expression was caused by an enhanced shedding of the GPVI ectodomain. Further inhibitor studies revealed that the GPVI shedding is independent of the GPVI or CLEC-2 mediated ITAM signaling pathway. Previously, ADAM10 and ADAM17 have been described to regulate GPVI shedding, but the hemin-induced GPVI shedding was independent of these metalloproteases. Surprisingly, inhibitor studies using the specific furin inhibitor SSM3 revealed, that the subtilisin-like proprotein convertase furin controls hemin-dependent GPVI shedding. Hemin associated GPVI degradation was substantially reduced in the presence of SSM3. Further, SSM3 inhibited hemin-induced but not CRP-induced platelet aggregation, indicating that furin controls specifically hemin associated platelet functions.
Conclusions:
In summary, we describe a novel furin-mediated mechanism of hemin-dependent GPVI shedding and thereby modulated platelet function.

Abstract Number - OC25

Protein kinase A regulates platelet phosphodiesterase 3A through an A-kinase anchoring protein dependent manner

Jawad S Khalil[1], Bethany Williams[1], Robert Law[1], Bethany Webb[1], Khalid M Naseem[1]

[1]Leeds Institute of Cardiovascular & Metabolic Medicine, UK.

Background:
Endogenous platelet inhibitory mechanisms are mediated by prostacyclin (PGI2) stimulated cAMP signalling, which is propagated and terminated by protein kinase A (PKA) and phosphodiesterase 3A (PDE3A), respectively. However, the tight spatiotemporal regulation of this signalling pathway that allows it to facilitate haemostasis while inhibiting thrombosis is unclear.
Aims:
To examine the molecular mechanism by which cAMP signalling controls PDE3A activity in platelets
Results:
We first examined the localisation of PDE3A in platelets by subcellular fractionation. We found platelets express three isoforms of PDE3A; PDE3A1, PDE3A2 and PDE3A3. Consistent with cardiomyocytes, PDE3A1 and A3 are in the membrane fraction and PDE3A2 in the soluble fraction. Treatment of platelet with thrombin or PGI2 led serine and threonine phosphorylation of multiple sites in a PKC and PKA dependent manner respectively. Focussing on cAMP signalling we confirmed that PGI2 induced a dose and time-dependent increase in phosphorylation and of PDE3A at serine312 in a PKA-dependent manner that was associated with an increase in phosphodiesterase enzymatic activity. PKA dependent PDE3A phosphorylation and activation was prevent by the generic A-kinase anchoring peptide disruptor, Ht31, suggesting a supramolecular complex controls PDE£A activity. In vitro experiments have shown that PDE3A may associated with several known AKAPs including Gravin, AKAP7, AKAP79 and mAKAP. Using a combination of immunoprecipitation and proximity ligation assays we found AKAP7 was associated with a PDE3A isoform.
To confirm the composition of the molecular complex that regulated PDE3A activity we performed a series of co-immunoprecipitation studies. Immunoprecipitation of total PDE3A revealed the presence of AKAP7, PKAcat and PKARII, but not PKA-RI. Immunoprecipitation of AKAP7 showed associated with PKAcat, PKARII and primarily PDE3A2 rather than other isoforms of the enzyme. Importantly, the AKAP-7 immunoprecipitates possessed milrinone sensitive PDE activity. Finally, we immunoprecipitated PKA-RII, where we found PDE3A2, PKAcat and AKAP7, and milrinone
Conclusions:
These data suggest a novel AKAP signalling complex that integrates propagation and termination of cAMP signalling through coupling of PKA and PDE3A, and the first evidence of selective compartmentalisation of PDE activity in platelets.

This work was funded by the British Heart Foundation (RG/F/22/110067)