Novel platelet glycoprotein VI and CLEC-2 targeting strategies

IMPACT

In conjunction with the coagulation cascade, platelets are key regulators of physiological hemostasis. However, at site of a ruptured or eroded atherosclerotic plaque, uncontrolled platelet activation can lead to the generation of vaso-occlusive thrombi, and to subsequent to life-threatening conditions such as heart attack and stroke. The gold standard for secondary arterial thrombosis prevention is a prophylactic treatment with antiplatelet ADP receptor antagonists and aspirin.[1-3] However, this treatment carries an inherent increase in the risk of bleeding.[4] To overcome this side effect, there is ongoing research to discover new therapeutic targets and antiplatelet drugs, which will allow better treatment of cardiovascular diseases with reduced side effects. In addition, platelet targeting may also be beneficial for the treatment of other pathological conditions such as deep vein thrombosis, inflammatory diseases (*e.g.*, acute respiratory distress syndrome, cancers and sepsis).[5-8]

Glycoprotein VI (GPVI) is the main collagen receptor on the platelet surface, which plays important roles in arterial thrombosis,[9] venous thrombosis,[10] cancer and sepsis.[6,11] On the other hand, its role in hemostasis seems to be dispensable, as suggested by the fact that patients deficient in GPVI do not display a severe bleeding disorder.[12] In this thesis, I investigated the role of GPVI in hemostasis and thrombosis *in vivo*, capitalizing on a mouse model with a human form of *GP6* (*hGP6*$^{tg/tg}$). In addition, I worked on the targeting of human GPVI *in vitro* and *in vivo,* thereby taking advantage of in-house generated antibodies. We tested the effect of the known anti-mouse GPVI antibody JAQ1 for the first time on human GPVI. This led to the discovery that JAQ1 binds to a structurally conserved epitope of mouse and human GPVI, which has been shifted in function during the evolution of the two species. In addition, the study gave proof of concept that the *hGP6*$^{tg/tg}$ mouse model is suitable for testing earlier developed and new anti-human GPVI compounds, as the model fully replicated the

effect of JAQ1 on human platelets (Chapter 2). The demonstration that JAQ1 binds to huGPVI in vitro and that the *hGP6*$^{tg/tg}$ model replicated what observed in human, served as foundation to the development of the pharmacological approach to generate huGPVI^{LO} platelets in vivo using JAQ1, as described in chapter 4.

In order to establish the time-dependent role of GPVI on whole-blood thrombus and clot formation, I modified a previously standardized microfluidic device, with a collagen and tissue factor surface. This allowed me to distinguish between early and late contributions of the thrombus-forming pathways. This effort revealed a crucial early role for GPVI-induced platelet signaling as well as for extrinsic coagulation-induced thrombin generation, which was confined to the first minutes of thrombus buildup. Markedly, my work indicate that this novel microfluidic application, represents a suitable in vitro approach to assess the contribution of different platelet and coagulation inhibitors/activators, and therefore improve pharmacokinetic studies of earlier developed as well as novel drugs for thrombosis and hemostasis.

To block GPVI, I employed a novel anti-GPVI Fab fragment, EMF-1, one clone out of 16 antibodies (EMF 1-16), with a strong GPVI-blocking effect (kindly provided by Emfret Analytics, Germany, on a collaborative basis). In addition, I found that the roles of platelet thrombin receptors and integrin αIIbβ3 were more prolongedly and extended throughout the whole process of thrombus buildup. Overall, this work revealed a more persistent contribution of thrombin-dependent platelet activation, whereas the initial collagen-dependent platelet activation was primarily important for platelet procoagulant activity and the promotion of fibrin deposition (Chapter 3).

In the following Chapter 4, I focused my attention on establishing the *in vivo* downregulation of huGPVI on mouse platelets. It appeared to be possible to partially down-regulate huGPVI from the surface of circulating mouse platelets using antibodies with a high on-off rate, thereby pharmacologically producing GPVI-low platelets, which

showed impaired responses to GPVI-specific agonists. In addition, I demonstrated that the complete depletion of GPVI with the EMF-1 antibody may be a safe, effective, long-term and reversible approach to target this platelet receptor. Finally, due to its long half-life and the low impact on hemostasis, I speculate that the scaled or complete depletion of GPVI may be a good therapeutic approach for the prevention of thrombotic events.

In the last Chapter 5, my work shifted towards the study of the related platelet receptor CLEC-2, also a member of the ITAM-signaling receptor family together with GPVI. We generated a mouse line humanized for CLEC-2 (hCLEC-2^{KI}), which proved to be suitable for the testing of anti-human CLEC-2 therapeutics *in vivo*. The study revealed that human CLEC-2 can fully compensate for mouse CLEC-2 during mouse development, and that hCLEC-2 has a minor role in hemostasis. On the other hand, it became clear that mouse CLEC-2 plays a more important role in thrombus stability than the human CLEC-2. Finally, this work generated useful tools to study the involvement of hCLEC-2 in different (patho)physiological conditions, which will help to better define a possible use of CLEC-2 targeting in therapeutic settings.

Overall, the thesis shows the current progress in the development of GPVI and CLEC-2 targeting strategies using humanized mouse lines. My work presented a modified microfluidic Maastricht flow-chamber as a useful tool for the detailed analysis of thrombus formation *in vitro*. Using the (novel) mouse lines with humanized GPVI and CLEC-2, we characterized several novel antibodies with interesting *in vivo* effects. Finally, this thesis reports for the first time a pre-clinical way of huGPVI depletion as a potential new therapeutic strategy. In this work, the results provide strong evidence that EMF-1 might be a novel promising lead for the generation of anti-GPVI drugs, with unprecedented high affinity and long half-life.

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