Novel platelet glycoprotein VI and CLEC-2 targeting strategies

Summary belonging to the dissertation:

Novel platelet glycoprotein VI and CLEC-2 targeting strategies: studies in humanized mouse models

Antiplatelet therapy plays an important role in reducing or preventing (the risk of) thrombotic events, thus increasing life quality and expectancy of patients with a heart or brain infarction. Nowadays, the gold standard for such patients is the use of aspirin and $P2Y_{12}$ receptor inhibitors, targeting platelet activation processes dependent on thromboxane A2 and ADP, respectively, both of which are important second mediators of platelet activation. However, treatment with dual antiplatelet drugs coincides with an increased risk of bleeding events. In this thesis, my hypothesis is that the targeted modulation in platelets of ligand-induced clustering of the collagen receptor glycoprotein VI (GPVI) or the podoplanin receptor (C-type lectin-like receptor 2, CLEC-2) provides anti-thrombotic protection with minimal effect on normal hemostasis. To address this, I have characterized the thrombosis and hemostasis profiles of CRISPR-Cas9-modified mice with platelets expressing the human form of GPVI or CLEC-2 receptors, using both *in vitro* and *in vivo* methods. In addition, I have used these mouse models to validate novel inhibitors directed against human GPVI or CLEC-2.

Chapter 1 provides a general introduction to the work presented in this thesis, with emphasis on the functions of various receptors in platelet adhesion, aggregation and thrombus formation in health and disease. In addition, this chapter gives an introduction into the current standards of antiplatelet therapy, and it summarizes recent advances in the targeting of GPVI and CLEC-2.

In **chapter 2**, I show for the first time that the anti-mouse GPVI antibody JAQ1 does cross-react with human GPVI (huGPVI), but not with the GPVI orthologues of other tested mammalian species, such as rats, rabbits, guinea pigs, swine, and dogs. I further show that the JAQ1 antibody affects in part differently the functions of mouse and human platelet GPVI. Similarly, to mouse platelets, JAQ1 inhibits the activation process of human platelets, when induced by collagen-related peptide (CRP). However, unlike mouse platelets, it does not inhibit the collagen-induced adhesion, activation and aggregate formation of human platelets, but it instead causes an increased response. Furthermore, my results indicate that this enhanced effect is also present in the platelets from human GPVI knock-in (*hGP6*$^{tg/tg}$) mice.

In **chapter 3**, I have studied the temporal roles of different pathways of human platelet and coagulation activation, triggered by collagen and tissue factor (TF), respectively, in whole-blood thrombus formation. For this purpose, I adapted the microfluidics whole-blood assay using the Maastricht flow chamber to acutely intervene pharmacologically in the thrombus formation process at desired time points. With the help of this method, my experiments revealed time-restricted, yet crucial, roles of GPVI and Syk protein kinase as well as TF/factor VIIa-induced coagulation during the first two minutes of thrombus buildup. In order to block

GPVI, I employed a novel anti-GPVI Fab, EMF-1. On the other hand, it appeared that the platelet activation processes through thrombin receptors (protease-activating receptors 1 and 4) and integrin αIIbβ3 were prolongedly active, and continued during later stages of thrombus formation. Finally, this work demonstrated a prominent role of the GPVI- and Syk-dependent signaling pathway in the generation of coagulation-active platelets, exposing the negatively charged phospholipid phosphatidylserine.

Chapter 4 is a comprehensive study on the depletion of human GPVI (huGPVI) in mice using transgenic *hGP6*$^{tg/tg}$ mice. First, I show that treatment of the mice with the monoclonal antibody JAQ1 leads to the partial depletion of GPVI *in vivo*, thereby generating low-density GPVI (GPVILO) platelets, which were no longer responsive to the ligands CRP or convulxin. Blood from mice treated with JAQ1 showed an impaired capacity to generate platelet aggregates on collagen at arterial shear stress conditions in whole-blood flow studies. Additional studies with another anti-mouse GPVI monoclonal antibody JAQ4, in combination with wild-type mice, proved that the generation of the GPVILO platelet phenotype is dependent on low-affinity antibodies. Furthermore, in this chapter, I have demonstrated that the specific and high-affinity antihuGPVI antibodies EMF-1 and EMF-2 are able to fully downregulate the receptor in *hGP6*$^{tg/tg}$ mice *in vivo*. In different experimental models, the EMF-1 IgG-treated mice appeared to be profoundly protected against occlusive arterial thrombus formation, whereas tail bleeding times were only minimally affected. With these results, I show that *in vivo* down-regulation of huGPVI has the potential to become a safe and effective strategy to target platelet activation and to treat thrombotic conditions. The work presented in

chapter 5 describes the first characterization of a mouse line humanized for CLEC-2 (hCLEC-2KI), and illustrates that these mice can help in the evaluation of novel therapeutics targeting CLEC-2. First, I show that the transgenic mice only expressing hCLEC-2 are indistinguishable from wildtype mice (with mouse CLEC-2 receptors), in terms of blood-lymph vessel separation and organ development and morphology. I also prove that the platelets from hCLEC-2KI mice become activated, aggregated and spread in a comparable way as wildtype platelets. An important finding is that the novel anti-hCLEC-2 antibody HEL-1 can completely downregulate hCLEC-2 *in vivo;* and furthermore, that this downregulation has no effect on hemostasis. On the other hand, I show that the hCLEC-2KI mice present with an impaired thrombus formation

after FeCl3-induced injury of mesenterial arterioles, in contrast to wildtype mice where complete vessel occlusion was observed in all tested arterioles. Paradoxically, the hCLEC-2 depletion in hCLEC-2KI animals reverts the phenotype to a wildtype-like phenotype.

Finally, in **chapter 6** I discuss the results of this thesis, and critically compare these to the current literature. In particular, I emphasize the relevance and importance of platelet research on different GPVI-targeting strategies, the possibilities of the use of humanized

mouse models in translational research, as well as the implications and challenges of future therapeutic strategies targeting CLEC-2.