PLATELETS ARE RELEVANT MEDIATORS OF RENAL INJURY INDUCED BY PRIMARY ENDOTHELIAL LESIONS

Running head: Platelet effects in murine TMA
Subject of manuscript: Renal pathology

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ABSTRACT

Several studies suggest a prominent (pro)-inflammatory and harmful role of platelets in renal disease, while newer work also demonstrated platelet release of pro-angiogenic factors. In this study we investigated the role of platelets in a mouse model of selective endothelial cell injury using either platelet depletion or the pharmacological P2Y12 receptor blocker clopidogrel as interventional strategy.

The Concanavalin A (ConA)/ anti-ConA model was induced in left kidneys of C57bl/6J wild-type mice following initial platelet-depletion or platelet-inhibiting therapy using clopidogrel. FACS analysis of GP IIb/IIIa/ p-selectin double-positive platelets and platelet-derived microparticles demonstrated relevant platelet activation following the induction of selective endothelial injury in mice. Enhanced platelet activation persisted for 5 days after disease induction and was accompanied by increased amounts of circulating platelet-derived microparticles as potential mediators of a prolonged procoagulant state. By immunohistochemistry, we detected significantly reduced glomerular injury in platelet depleted mice compared to controls. In parallel, we also saw reduced endothelial loss and a consequently reduced repair response as indicated by a diminished proliferative activity. The P2Y12 receptor blocker clopidogrel demonstrated efficacy in limiting platelet activation and subsequent endothelial injury in this mouse model of renal microvascular injury.

In conclusion, platelets are relevant mediators of renal injury induced by primary endothelial lesions early on as demonstrated by platelet depletion as well as platelet inhibition via the P2Y12 receptor. While strategies to prevent platelet-endothelial interaction showed protective effects, platelets’ contribution during renal regeneration remains unknown.
Key words: renal microvascular endothelial injury, platelets, thrombotic microangiopathy, clopidogrel, platelet microparticles
INTRODUCTION

Renal microvasculature represents a primary or secondary target in various types of glomerulonephritis. During progression of kidney disease the loss of microvascular capillaries is accompanied by progressive renal scarring (7-9, 22, 41). To complement a variety of humoral factors, inflammatory cells frequently contribute to renal pathophysiology, finally leading to functional deterioration and loss of intrinsic renal cells. Nonetheless, the actual contribution of platelets to renal disease remains a contentious issue, with some studies of experimental glomerulonephritis (GN) finding platelets being a source of inflammatory molecules and an important proinflammatory effector cell, while some other clinical studies were not able to establish platelet inhibition as an effective therapy (3, 4, 26, 27).

Following severe acute endothelial injury, platelets continuously interact with the endothelial surface and thereby play a central role in the development of subsequent thrombotic microangiopathy (TMA). In recent years, our pathophysiological knowledge regarding the development of thrombotic microangiopathies has dramatically improved, and specific alterations of von-Willebrand-Factor (vWF) degradation or the complement system have been identified. In contrast, the influence of platelets has not been systematically investigated. To date, no therapeutic strategies are in existence to successfully modulate platelet or endothelial cell properties following initial endothelial injury as a potential protective approach.

We have previously established an inducible murine model of selective endothelial injury in the kidney (17) demonstrating subsequent features of TMA including platelet activation and formation of microthrombi within microvasculature. In the present study, we sought to define the role of platelets in this acute endothelial disease. We
therefore applied two approaches to interfere with the platelet-endothelial cell interaction: platelet depletion using anti-GPIbα antibodies and pharmacological inhibition of the platelet P2Y12 receptor using the clinically established compound clopidogrel. We thereby prove the relevance of platelets for acute and ongoing endothelial injury and provide evidence that interference with platelet activation represents a protective interventional strategy.

MATERIAL AND METHODS

Animal model and experimental design

All animal experiments were done according to American Physiological Society guidelines and duly approved by local government authorities. Male C57Bl/6J wild-type (wt) mice at the age of 9-12 weeks (Charles River, Sulzfeld, Germany) were used for experiments. All animals were fed standard mice chow (Altromin 1324, Spezialfutterwerke GmbH) and received tap water ad libitum.

In all experiments, site-specific microvascular endothelial injury was induced as described previously (17). The anti-Concanavalin A (anti-con A) serum was generated in New Zealand white rabbits by Seqlab (Sequence Laboratories GmbH, Goettingen, Germany) and tested for antibody concentration to specifically induce the injury model in the wt mice as previously described (17).

In a first experiment, we evaluated the time course of platelet activation in our disease model. Endothelial injury was induced in 10 C57Bl/6 mice and 4 mice served as sham operated (NaCl 0,9% perfusion) controls. Mice were sacrificed 1, 2 and 5 days after disease induction. Repetitive heparinized blood samples were collected by
retroorbital bleedings and immediately processed for measurement of platelet
activation and platelet-derived microparticles (PMPs) from blood samples by FACS.

In a second experiment, 10 wt mice received 100 µg anti-GP Ibα IgG (p0p3/p0p4)
intraperitoneally the day before disease induction as previously described (41), while
5 additional wt mice received equal amounts of rat-IgG (Sigma-Aldrich, Munich,
Germany) and served as controls. These antibodies are known to induce rapid
platelet depletion 24 hours prior to disease induction (5, 36). Mice were sacrificed 24
hours after disease induction. On the day of sacrifice, mice were anaesthetized using
inhaled isoflurane, blood was collected via puncture of the inferior caval vein, and
mice were then perfused via the heart with 0.9% (w/v) NaCl-solution to remove blood
components from both kidneys. Renal tissues were harvested and fixed either in
methyl Carnoy’s or zinc solution.

In a third experiment, 40 wt mice were subjected to clopidogrel treatment or solvent
(placebo) via oral gavage once daily starting the day before disease induction up to
day 1 or day 3 (n=8-12 per group). On the day of sacrifice, we verified treatment
efficacy by measuring tail bleeding time in each animal. Therefore the time until the
first break of the blood stream was measured in pre-warmed PBS solution at 37°C.
Then, tissues were harvested as described above and processed for further analysis.

**FACS analysis**

To analyse platelets and platelet microparticles, platelet-rich plasma (PRP) of
heparinized blood samples was attained following centrifugation at 1500xg for 1.5min
at 21°C and incubated with PE anti-mouse CD41 (Clone MWReg30, ebioscience,
Frankfurt/Main, Germany) and APC anti-CD62 (Clone Psel.KO2.3, ebioscience) at
21°C for 20min. Samples were fixed by addition of equal volume of 1%
paraformaldehyde in PBS and diluted 1:10 with PBS containing 0.1% bovine serum albumin. FACS analysis was acquired using a FACSCanto II from BD and data were analyzed using FlowJo data analysis software (FlowJo, Ashland, OR, USA).

**Tissue processing and immunohistochemical staining**

Methyl Carnoy’s or zinc-fixed tissues were embedded in paraffin and cut into thirty consecutive 3 µm sections spanning the distance of approx. 150 µm enumerated from 1-30. To exclude artifacts due to the heterogeneity of the disease model, for all stainings 3 tissue sections with a distance of at least 10 sectioning layers were used for light or immunofluorescence microscopy. Periodic acid Schiff’s (PAS) and acid fuchsin orange G (AFOG) stainings of three 3 µm interval sections of each biopsy was performed to assess renal injury and fibrin deposition. For immunofluorescence microscopy, further sections were incubated with the following primary and secondary antibodies as indicated and also previously published (17, 19, 20, 22): GPIbα, a rat monoclonal antibody (mAb) against mouse glycoprotein Ib for specific staining of platelets (5); MECA-32, a rat mAb against mouse endothelial cell antigen 32 specific for detecting peritubular endothelial cells (16); CD31, a rat mAb against mouse platelet-endothelial cell adhesion molecule 1 (PECAM-1) specific for detecting endothelial cells (46); PCNA, a murine mAb against proliferating cell nuclear antigen to detect actively proliferating cells (23) (19A2, Merck Millipore); and F4/80, a rat mAb (Invitrogen, Life Technologies GmbH) to detect mouse macrophages/monocytes as inflammatory cell types. All antibodies were diluted in sterile PBS containing 1% (w/v) bovine serum albumin. All tissue sections were incubated with primary antibodies overnight at 4°C in a wet chamber and negative controls included omission of the referred antibodies. Afterwards, fluorescent secondary antibodies (Invitrogen, Life Technologies GmbH) including Alexa Fluor F488 and/or F555 goat
anti-rat IgG were applied at room temperature in the dark. Cell nuclei were counterstained with 4′,6′-diamino-2-phenylindole (DAPI, 1 μg/ml; Applichem).

**Quantitative analysis of immunostaining and capillary rarefaction**

Computer assisted image analysis (ImageJ software, NIH USA) was used to quantify immunostained kidney sections after image acquisition with a digital microscope (BIOREVO BZ9000, Keyence GmbH, Neu-Isenburg, Germany) under 200 or 400-fold magnification.

**Kidney injury and inflammation**

Glomerular platelet infiltration was assessed after staining for GPIbα using a semiquantitative scoring system from 0 to 4, where 0 means absence of platelets, 1 refers to the presence of glomerular platelets in less than 10% of all glomeruli, 2 characterizes the presence of platelets in up to 50% of glomeruli, 3 that more than 50% of glomeruli contained platelet thrombi often with peritubular capillary involvement, and 4 refers to severe glomerular and peritubular thrombosis up to 100% (22). Three distant sections of each kidney were evaluated reflecting 15 cortical fields of vision at x200 magnification. Glomerular injury was evaluated individually in at least 50 randomly selected glomeruli under x400 magnification on PAS stained tissue sections using a similar scoring system from 0 to 4. Specifically, 0 characterizes normal glomeruli without structural damage, 1 means glomerular matrix expansion and edema formation of less than 25% of glomerulus, 2 means increased intraglomerular cell count and swelling up to 50%, 3 refers to obliteration or collapse of capillaries in up to 75% of the glomerular cross-section, and 4 characterizes complete capillary loss and thrombosis. In AFOG stained tissue
sections (37) we quantified glomerular fibrin deposition (intense orange-red colour) in at least 50 randomly selected glomeruli under x400 magnification. Therefore, we used a scoring system from 0-4 analogous to the evaluation of platelet infiltration. All values are given as score±SD per glomerular cross-section. Furthermore, we counted all F4/80 and DAPI double positive infiltrating monocytes and macrophages in 15 randomly selected cortical fields under x400 magnification to assess the cell count per renal cortex excluding glomeruli.

**Endothelial injury and cell proliferation**

After assessment of injury and inflammation, we evaluated the peritubular capillary rarefaction on digital images using a grid overlay (ImageJ software, NIH) consisting of 625µm²-sized squares in at least 15 cortical images sparing glomeruli. This evaluation method has been published previously by our group using a special ocular with exactly the same grid size (17). Squares containing no MECA-32 positive capillary structures were counted. Capillary rarefaction is given as negative positive area ±SD per mm². This data directly reflects peritubular endothelial injury, whereby higher values represent increased loss of capillaries (maximum is 100) and lower values indicate better capillary preservation. Glomerular capillary rarefaction was determined using a semiquantitative scoring system from 0 to 4 in at least 50 random glomeruli per murine kidney. Thereby, 0 means no capillary loss, 1 means less than 25%, 2 means up to 50%, 3 means up to 75% and 4 up to 100% absence of glomerular capillary structures. Values are given as mean score±SD per glomerular cross-section. In the next step, we assessed the proliferation rate during the time course of disease by counting all glomerular PCNA positive cells in 50 randomly selected glomeruli and tubulointerstitial PCNA + cells in 15 cortical areas. In parallel
all MECA-32/PCNA double positive proliferating endothelial cells. Data are given as mean score±SD per glomerular and (tubulo)interstitial cross section.

**Terminal Deoxynucleotidyl Transferase—Mediated dUTP-Biotin Nick-End Labeling Assay**

Necrototic cells were detected by the terminal deoxynucleotidyl transferase—mediated dUTP-biotin nick end labeling (TUNEL) assay, as described previously, according to manufacturer’s instructions (30). The number of TUNEL-positive apoptotic cells was counted in 50 sequentially selected glomeruli and given as the mean number either per gcs or per mm2 after counting 20 peritubular areas of each section.

**Statistical analysis**

All data are expressed as mean±SD. We first tested for normal data distribution using D’Agostino and Pearson omnibus normality-test. FACS analysis data was analysed using a 2way ANOVA with Bonferroni post-testing. For all other data, For all other data with normal distribution, a F-test was used to compare variances followed by the unpaired Student’s t-test (PRISM software version 5.0, GraphPad, USA). A p value of less than 0.05 was considered statistically significant (*). Data failing the normality test were analysed by a two-sided non-parametric Mann-Whitney-U test and values less than 0.05 were considered significant (#).
RESULTS

Rapid and long-lasting platelet activation after selective endothelial injury

To prove the persistent activation of platelets in our disease model, we first investigated the extent of platelet activation and the subsequent generation of platelet microparticles (PMP) in our murine renal microvascular injury model using FACS analysis of repetitively drawn blood samples. After incubation with antibodies directed against glycoprotein IIb/IIIa (CD41) and p-selectin (CD62), we were able to identify three distinct populations including larger immune-complexes containing platelet fragments (IC), activated platelets (PL) and microparticles (MP) (figures 1A and 1B). Active disease led to a drastic decrease of platelet counts as depicted in figure 1B, showing mice 2 days after initial EC injury versus figure 1A, where data from a sham perfused control is shown. Quantification demonstrated the significant increase of p-selectin positive, activated platelets (figure 1C) and the generation of significant amounts of microparticles (figure 1D) compared to sham controls. While these effects were clearly related to the onset of acute renal endothelial injury and subsequent thrombotic microangiopathy (by 2way ANOVA), no significant differences occurred due to the clopidogrel treatment. This significant degree of platelet activation and generation of platelet-derived microparticles was detected up to day 5 after disease induction.

We then decided to deplete platelets in order to prove the relevance of these cells for the initial microvascular injury in our disease model. Platelet depletion was induced one day before the initiation of endothelial injury, which led to a relevant hemorrhagic diathesis making renal arterial perfusion in platelet depleted mice very challenging. While all mice primarily survived the surgery, 50% died 3-4 hours after end of anesthesia. Since we could not detect intra-abdominal bleedings, we believe that this
Platelet depletion reduced renal injury and preserved microvascular capillaries

On the day of sacrifice, blood was drawn into heparin and immediately analyzed using an auto-analyzer (Beckman Instruments, Brea, CA, USA) to verify efficacy of platelet depletion. A significant reduction of the platelet count (normal values approximately 800 to 1200 platelets per nanoliter (nl)) was seen in platelet-depleted mice (64.5±6.3 per nl; p<0.01 versus controls), but also in non-depleted ConA/anti-ConA controls (101±10.2 per nl).

We then investigated the effect of our platelet depletion strategy on renal histology. First, we assessed platelet activation and thrombus formation after staining for GPIbα (fig. 2A, B) and AFOG staining (fig. 2C, D), respectively. Data clearly showed the reduced deposition of platelets (fig. 2B) and fibrin thrombi (fig. 2D) in glomeruli of platelet-depleted mice compared to ConA/anti-ConA controls (p<0.01). Both findings also demonstrated that thrombocytopenia of control mice was due to the development of thrombotic microangiopathy and platelet consumption, while in platelet-depleted mice it was predominantly a consequence of platelet depletion. To evaluate any possible protective effects on the microvascular endothelium, we performed fluorescence staining for MECA-32 (fig. 3A), showing a better preservation of peritubular capillaries after 24 hours in platelet depleted mice (fig. 3B p=0.056, f<0.05).

Since the primary endothelial cell injury needs to be compensated by a subsequent repair response, we then evaluated cell proliferation as a potential major intrinsic repair mechanism. In a first step all proliferating tubulointerstitial (PCNA+) cells were counted and subsequently all MECA-32/PCNA double-positive proliferating
endothelial cells were assessed. In platelet depleted mice, the proliferative response of endothelial cells was significantly reduced compared to ConA/anti-ConA controls (fig. 3C, p<0.01), which was a consequence of reduced endothelial injury as depicted in figure 3B. In contrast to the endothelium, the overall proliferative response of tubulointerstitial cells was unchanged (fig. 3D).

Pharmacological platelet inhibition prevents endothelial injury and subsequent thrombotic microangiopathy in the kidney

Having provided evidence that platelets are direct mediators of endothelial injury in this disease model, we decided to use the clinically established compound, clopidogrel, to interfere with platelet activation via the P2Y12 receptor.

Treatment of mice by daily gavage of 75 mg/kg body weight clopidogrel was started 24 hours prior to disease induction, which was continued on a daily basis thereafter. To verify treatment efficacy, tail bleeding times were measured in each animal on the day of sacrifice. All clopidogrel treated mice had a significantly prolonged tail bleeding time compared to control mice (Table 1; day 1: p<0.01; day 3: p<0.05). Evaluation of glomerular changes and thrombus formation were done on PAS (fig.4A) and AFOG stained tissues sections. On days 1 and 3 glomerular injury was significantly reduced in the clopidogrel-treated group (fig. 4B, day 1: p<0.05; day 3: p<0.001) whereas glomerular fibrin deposition was only reduced on day 3 (fig. 4C, p<0.05). To assess potential effects of inflammatory cells, we evaluated the macrophage/monocyte infiltration of the renal cortex after staining for F4/80 as an indicator of the inflammatory response. In clopidogrel-treated mice, we detected a significantly lower number of F4/80+ cells 24 hours after disease induction (fig. 4D, Table 1; p<0.001), while no differences were detectable between both groups Table 1 on day 3.
Since platelet depletion led to preservation of capillaries, we subsequently assessed protective effects of the clopidogrel treatment on glomerular and peritubular capillary injury on stainings of CD31 and MECA-32 (fig. 5A). Analysis of differences showed a significant decrease of endothelial injury in glomeruli of clopidogrel-treated mice versus ConA/anti-ConA controls (fig. 5B; p=0.059, f<0.01) on day 3. In parallel, evaluation of peritubular capillaries on MECA-32 stained tissue sections also demonstrated a significantly decreased endothelial injury in the clopidogrel treated group on day 3 (fig. 5C, p<0.01). Subsequent evaluation of cell proliferation as an indicator of the repair response showed a decreased glomerular proliferation (fig. 5D; p<0.01) on day 3 and a decreased tubulointerstitial cell proliferation on days 1 (fig. 5E; p<0.01) and fig. 3 (f<0.05) as well as a reduced peritubular endothelial cell proliferation on day 3 (fig. 5F; p<0.01). In parallel, we also detected a reduced number of necroptotic cells in glomeruli of clopidogrel treated mice as judged by the TUNEL staining on day 1 (f<0.05; Table 1).

DISCUSSION

Numerous experimental studies have shown a close correlation of microvascular endothelial injury and healing with the restitution of glomerular integrity, renal function and progression of kidney disease (20, 22, 24, 29-32). While protective strategies for the microvascular endothelium are lacking, they might comprise an important approach to change the outcome of inflammatory kidney disease, especially in the presence of acute endothelial lesions. The latter may lead to the development of subsequent thrombotic microangiopathy, indicating that the interaction between endothelial cells and platelets can be very harmful and should be considered as a therapeutic target (17, 28, 34).
The present study was performed to i) define the role of platelets in our ConA/anti-ConA murine model of selective primary endothelial injury (17) and ii) test whether the therapeutic interference with the platelet-endothelial cell interaction through blockade or inhibition of platelet function represents a protective strategy in the kidney. By histology, an early and pronounced platelet influx with subsequent TMA was evident, already (17).

To prove the pronounced systemic platelet activation, we evaluated the extent of platelet activation as reflected by the amount of p-selectin positive platelets and platelet-derived microparticles (PMP). We found a clear-cut increase of platelet activation and generation of PMP up to 5 days after the initial renal endothelial lesions, underscoring the fact that platelet activation is overlapping with the phase, when endothelial repair is already ongoing. Yet their role in kidney disease is widely unclear, PMP have been characterized as catalytic procoagulant surfaces involved in thrombogenesis (13, 39). They have also been shown to contain a variety of inflammatory components, cytokines and growth factors that are related to their involvement in inflammatory processes, immune responses and angiogenesis (39, 40).

We then tested the relevance of platelets in this disease model using well-established anti-GPIb antibodies to specifically induce a more than 90% platelet depletion by Fc-independent mechanisms without systemic side effects. These antibodies had previously been used in long term experiments with repetitive application, by our own group and others (5, 19, 36).

While a decrease of platelets was also evident in non-depleted mice, this latter decrease has to be put in the context of enhanced platelet activation and subsequent thrombus formation in these mice. Therefore, the low platelet count in depleted mice
is related to the antibody application, while it is a consequence of consumption by active thrombotic microangiopathy in non-depleted mice.

To investigate the effect of the reduced platelet-endothelial cell interaction on histology, we evaluated the degree of microvascular injury in the kidney. We found that platelet depletion significantly reduced renal injury in general and effectively preserved microvascular capillaries. Thereby, this is the first study directly demonstrating that platelets mediate loss of endothelium in the kidney and that platelet depletion can protect from capillary rarefaction (28, 47). In recent years, many experimental and clinical studies have proven the efficacy of P2Y12 inhibition to prevent arterial thrombus formation and platelet activation (42). Hence, we subsequently chose this approach to investigate the effect of pharmacological platelet inhibition in our disease model. Since we wanted to know, whether the inhibition of the P2Y12 receptor would also be able to sufficiently interfere with the early platelet-endothelial cell interaction in our disease model, we started gavage of clopidogrel the day before initiation of disease.

In analogy to platelet depletion, P2Y12 inhibition prevented glomerular injury and fibrin deposition. During the time course of disease, glomerular as well as peritubular capillaries were protected by P2Y12 inhibition, which is consistent with our results of the platelet depletion approach. We showed that both, the occurrence of necroptotic cells and (endothelial) cell proliferation, as a relevant intrinsic repair mechanism, were significantly reduced in the kidneys of mice that underwent platelet depletion or inhibition. Taken together, these data indicate the protective effect of platelet inhibition for the endothelium. In line with our finding, platelets are considered as being important effector cells of experimental inflammatory renal diseases. A variety of experimental studies in models of immune complex nephritis, anti-Thy1 nephritis and Habu-snake venom glomerulonephritis demonstrated significant platelet
activation, while few studies have proved this by direct interference with the platelet activation process or platelet depletion (9, 26, 27). Therefore, their exact relevance in distinct types of kidney disease is not clear. We have recently shown that platelets do not play a relevant role during passive crescentic glomerulonephritis in mice (18), a chronic inflammatory disease model with platelet activation. An important study by Johnson et al. used the rat anti-Con A model of renal endothelial injury, showing that platelets play a mediating role in this endothelial disease, while they could not detect relevant, directly protective effects for the microvascular endothelium (28). Using another rat (anti-Thy1) glomerulonephritis model, Peters et al. also showed protective effects using clopidogrel (42).

Experimental studies have also shown that platelets release a broad range of inflammatory mediators and cytokines inducing endothelial cell activation, leukocyte adhesion and transmigration (11). In the present study, we found a reduced number of F4/80+ macrophages/monocytes in kidneys of clopidogrel treated mice indicating reduced inflammation early after injury induction. This finding indicates that protective effects of clopidogrel treatment might also be mediated directly (but also indirectly) via its influence on local inflammation. Abele et al. have previously shown anti-inflammatory, protective effects of platelet inhibition due to clopidogrel treatment in an aortic allograft model (1, 2). Recently, it was proposed that the P2Y12 receptor might also play a role in amplifying the release of platelet’s α-granules (12), since it directly interferes with P-selectin driven platelet activation. The relevance of P-selectin mediated platelet activation has been previously shown in the rat Con-A model (47), and is reflected by the drastic increase of P-selectin positive platelets and PMP in our disease model. However, FACS analysis couldn’t detect a decreased systemic platelet activation by clopidogrel, which might relate to the limited group size of this approach. Moreover, we have previously shown that a large amount of
circulating cytokines is released upon endothelial injury in this disease model (21), which is in accord with studies in other rodent animal models showing platelet's contribution to disease progression and inflammation and the anti-inflammatory as well as anti-fibrotic effects of clopidogrel (14, 35, 38, 42, 45).

In contrast, George et al. demonstrated that platelets are also required for the maintenance of vascular integrity in inflammation and proposed that platelets locally deliver vasoactive mediators by release of their storage granules during transient interaction with the inflamed vessel wall (15). Subsequent, experimental studies have described active sequestration of pro-angiogenic factors and molecules in platelet α-granules (25, 33) and in vitro platelets stimulate endothelial cells and promote the assembly of capillary-like structures (10, 43). Central repair mechanisms such as cell proliferation have been linked with platelet activation and secreted factors such as PDGF (6). Therefore, it is possible that the beneficial effect of platelet inhibition in our disease model could be harmful in situations, when such factors are necessary to support proper regeneration.

In summary, here we demonstrated that the depletion of platelets as well as the blockade of the P2Y12 receptor reduce the microvascular injury in a mouse model of selective endothelial injury and subsequent TMA in the kidney. This is a further proof of the general concept to interfere with platelet activation and the platelet-endothelial cell interaction in distinct kidney diseases. Further studies will be necessary to investigate and differentiate early and late effects of platelets on endothelial cell injury and repair.
Acknowledgements

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18. Hohenstein B, Daniel C, Johnson RJ, Amann KU, and Hugo CP. Platelets are not critical effector cells for the time course of murine passive crescentic glomerulonephritis. *Platelets.*


Figures

Figure 1  Platelet activation and generation of platelet-derived microparticles following selective endothelial cell (EC) injury. In sham operated mice (A) as well as in mice with selective EC injury (B), FACS analysis detected three distinct populations consisting of microparticles (MP), platelets (PL) and immune complexes (IC). The number of p-selectin positive, CD41 positive platelets was significantly higher in both groups undergoing EC injury compared to sham controls, while no differences could be detected between the groups with and without clopidogrel treatment (C, data table). In parallel, large amounts of MP were generated in mice after induction of selective EC injury (D, data table), without difference between clopidogrel treated and untreated mice. ***p<0.001; **p<0.01 by 2way ANOVA..

Figure 2  Reduced glomerular thrombus formation due to reduced injury after platelet depletion. Staining for GPIbα, in control mouse 24 h after disease induction (A). Platelet-derived thrombi and fibrin positive glomeruli were assessed after AFOG staining (B). The deposition of platelets (C) and fibrin rich thrombi (D) in glomeruli of PLT depleted animals compared to PBS injected controls was reduced. **p<0.01.

Figure 3  Reduced capillary rarefaction and necessity of repair after platelet depletion. Renal tissue sections were double-stained with anti-MECA-32 and anti-PCNA antibodies to identify proliferating endothelial cells as shown in figure A, depicting a control mouse 24 hours after disease induction. Endothelial rarefaction
was reduced in platelet depleted mice (B). Proliferating endothelial cells were assessed as PCNA+/MECA-32+ (C). Proliferative response of tubulointerstitial cells was evaluated after PCNA staining (D). **p<0.01; # f<0.05.

**Figure 4** Clopidogrel treatment protects from glomerular injury and reduces the inflammatory response. For evaluation of glomerular injury we used PAS stained tissue sections as shown in figure A and a semiquantitative scoring system from 0 to 4 representing degree of injury (B). Fibrin thrombus formation was evaluated after AFOG staining (C). Monocytes /macrophages were evaluated after staining for F4/80 (D). *p<0.05, ***p<0.001 and Mann-Whitney-U #p<0.05.

**Figure 5** Clopidogrel treatment protects renal capillaries. Staining of CD31 (A) was used to evaluate glomerular capillary rarefaction (B). Tubulointerstitial capillary rarefaction was assessed after staining for MECA-32 as described in the methods section (C). Glomerular (D), tubulointerstitial (E) and endothelial cell proliferation (MECA-32+/PCNA+ cells) were evaluated after double staining for MECA-32 and PCNA (F). *p<0.05, **p<0.01; and Mann-Whitney-U: ##p<0.01.
Figure 1
Figure 2
Figure 3

A

B

C

D

**Peritubular capillary loss score 0 to 100**

**MECA-32⁺/PCNA⁺ cells per mm² renal cortex**

**PCNA⁺ TI cells per mm² renal cortex**
Figure 4
Figure 5
Table 1

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*P<0.05, **P<0.01, ***P<0.001 vs. Placebo, *p<0.05 by F-test