Critical Role of Platelet Glycoprotein Ibα in Arterial Remodeling


Objective—Arteriogenesis is strongly dependent on the recruitment of leukocytes, especially monocytes, into the perivascular space of growing collateral vessels. On the basis of previous findings that platelets are central players in inflammatory processes and mediate the recruitment of leukocytes, the aim of this study was to assess the role of platelets in a model of arterial remodeling.

Approach and Results—C57Bl6 wild-type mice, IL4-R/Ilba mice lacking the extracellular domain of the glycoprotein Ibα (GPIbα) receptor, and mice treated with antibodies to block GPIbα or deplete circulating platelets were studied in peripheral arteriogenesis. Using a novel model of intravital 2-photon and epifluorescence imaging, we visualized and quantified the interaction of platelets with leukocytes and the vascular endothelium in vivo. We found that transient platelet adhesion to the endothelium of collateral vessels was a major event during arteriogenesis and depended on GPIbα. Furthermore, leukocyte recruitment was obviously affected in animals with defective platelet GPIbα function. In IL4-R/Ilba mice, transient and firm leukocyte adhesion to the endothelium of collateral vessels, as well as leukocyte accumulation in the perivascular space, were significantly reduced. Furthermore, we detected platelet–leukocyte aggregates within the circulation, which were significantly reduced in IL4-R/Ilba animals. Finally, platelet depletion and loss of GPIbα function resulted in poor reperfusion recovery as determined by laser Doppler imaging.

Conclusions—Thus, GPIbα-mediated interactions between platelets and endothelial cells, as well as leukocytes, support innate immune cell recruitment and promote arteriogenesis—establishing platelets as critical players in this process. (Arterioscler Thromb Vasc Biol. 2015;35:589-597. DOI: 10.1161/ATVBAHA.114.304447.)

Key Words: adhesion receptor ■ blood platelets ■ leukocytes

Arteriogenesis is defined as the growth of pre-existing arteriolar connections into angiographically visible collateral arteries in response to the occlusion of a major axial artery.1 During arteriogenesis, pre-existing interconnecting arterioles become structurally remodeled to form natural bypasses, resulting in an improvement of blood flow capacity.2,4 Increased fluid shear stress in the latent arteriolar anastomoses induces endothelial cell activation, with subsequent upregulation of adhesion molecules, such as intercellular adhesion molecule 1, and release of chemokines. Together, this triggers the recruitment of inflammatory cells into the perivascular space.5,6 Growth factors, cytokines, matrix metalloproteinases, and the serine protease urokinase-type plasminogen activator released locally promote leukocyte...
recruitment and initiate a remodeling cascade that transforms small vessels into arterioles capable of delivering a markedly increased blood flow.7–9 Mouse models have confirmed the important role of leukocytes, especially monocytes, in the process of arteriogenesis,10,11 and it is presently accepted that shear stress–induced endothelial activation leads to upregulation of endothelial adhesion receptors, resulting in adhesion, and perivascular accumulation of leukocytes.7

Platelets are known to support the inflammatory processes, including atherosclerosis and deep vein thrombosis.12–14 Under physiological conditions, platelet activation and platelet–endothelial interactions are inhibited by endothelium-derived factors, including nitric oxide and prostacyclin.15 However, in the presence of endothelial activation/dysfunction, platelets can directly interact with the structurally intact endothelium. Platelet–endothelial cell interactions have been reported to occur during early inflammatory stages of atherosclerosis and venous thrombosis.12–14 In addition, platelets participate in leukocyte extravasation in various models of inflammation.16–18 During early stages of atherosclerosis, the inflamed endothelium develops properties that render activated endothelial cells adhesive for platelets. Interactions of platelets with endothelial cells trigger the secretion of chemokines (eg, monocyte chemotactic protein 1) and the expression of adhesion molecules, including vascular cell adhesion molecule 1, thus promoting the adhesion of leukocytes.12,19 Studies using intravital microscopy confirmed that platelet–endothelial interactions occur on an inflamed arterial endothelium under high shear stress in vivo.12,14 From studies on atherosclerosis, a pathology that like arteriogenesis represents an inflammatory process, we know that platelet accumulation at the dysfunctional endothelium is an early event, which paves the way for the subsequent recruitment of inflammatory cells.12,13,20 Thus, recent data obtained from models of atherosclerosis suggest a role for platelets in the inflammatory process of arteriogenesis. In fact, platelets have the capacity to de novo synthesize and differentially release proinflammatory proteins.21 The interaction of platelets with the endothelial surface might therefore generate signals for recruitment and extravasation of monocytes not only in the setting of atherogenesis but also during collateral vessel growth. Nevertheless, the relevance of platelets in the process of arteriogenesis remains unclear.

Combining, for the first time, a model of hindlimb ischemia with state-of-the-art intravital imaging, we assessed the early cellular events that mediate the formation of collateral vessels. To achieve this, we developed an in vivo fluorescence microscopy setup to directly visualize and quantify the dynamic process of platelet recruitment inside collateral arteries. We identified that the proinflammatory endothelial phenotype induces platelet interactions and leukocyte recruitment, particularly monocytes and neutrophils, within the collateral vessel wall. Using this approach, we show here that firm platelet adhesion does not occur, but to our surprise, a temporary loose contact between circulating platelets and the collateral endothelium is a prerequisite for leukocyte recruitment and collateral growth and is critically dependent on the platelet receptor glycoprotein Ibα (GPIbα).

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Endothelial Recruitment of Platelets in Collateral Growth Is GPIbα-Dependent

In the mouse model of arteriogenesis applied here, functional collateral arteries developed within 3 weeks after surgical ligation and showed typical corkscrew-like patterns11 (Figure 1A, right). In contrast, only small pre-existing collaterals were present at the sham-operated side (Figure 1A, left). To test the potential role of platelets in the process of arteriogenesis, we first assessed platelet–vessel wall interactions after occlusion of the femoral artery. Using intravital microscopy (Figure 1B and 1C), we observed virtually no firm adhesion of platelets to the arterial vessel wall of collaterals (Figure 1D; Figure 1 in the online-only Data Supplement). However, we found an increase in transient platelet–vessel wall interactions in the collateral arteries 24 hours after femoral occlusion, showing significant values on day 3 after induction of arteriogenesis (Figure 1D). Interestingly, occlusion of the femoral artery increased transient platelet adhesion not only in the collateral arteries but also in collateral veins, when compared with the sham-operated side (Figure 1D). Together, these data indicate that platelet adhesion to the endothelium of collateral arteries (and veins) is a frequent event during arteriogenesis.

Next, we evaluated the role of GPIbα for transient platelet adhesion in this model. GPIbα mediates platelet recruitment under high shear rates22 through binding to von Willebrand factor (vWF) and P-selectin23–25 and is crucial for platelet adhesion to dysfunctional but morphologically intact endothelium in inflammatory processes, including atherosclerosis.12,13 To investigate the role of GPIbα in the setting of arteriogenesis, we used IL4-R/Iba mutant mice that lack the extracellular domain of GPIbα on platelets.26 We found that platelet recruitment in both collateral arteries and veins was significantly reduced in IL4-R/Iba mutants 3 days after femoral occlusion (Figure 2A and 2B), suggesting that GPIbα is indeed critical for transient platelet adhesion in this process. To identify a potential counter-receptor for GPIbα on the vessel wall, we performed immunohistochemical staining for vWF. Interestingly, we detected vWF in collateral vessels, suggesting that vWF represents a potential counter-receptor for GPIbα in the course of arteriogenesis (Figure 2C).

IL4-R/Iba Mice Show Reduced Endothelial Leukocyte Transmigration

Leukocytes are considered to be the major blood cells recruited during the process of arteriogenesis. Accordingly, we found adherent leukocytes in the collateral arteries 24
Figure 1. Platelet–endothelial interactions in the early phase of arteriogenesis. A, Ventral aspect of latex-perfused mouse hindlimbs 21 days after femoral artery occlusion. Collaterals at the occluded (occ) side have substantially increased in size and diameter and appear in typical corkscrew formation (arrows). In contrast, at the sham side (sham), the faint appearance of pre-existing collaterals from the deep branch (arrow heads) is demonstrated. Scale bar, 1.5 mm. B, Scheme showing the relevant region of interest (ROI) where we performed intravital epifluorescence microscopy (IVM) of collateral artery and vein. C, Composed images taken by IVM show an overview of collateral vessels branching from the profunda femoris artery (8) and vein (§). Scale bar, 150 μm. D, Top, Representative IVM images of early platelet–endothelial interactions as evaluated in sham-operated (1 and 3 days, sham) and femoral-ligated (1 and 3 days, occ) wild-type (WT) animals after the injection of Rhodamine B–stained platelets (red). Arrows show platelet–endothelial transient adhesion. Dotted lines show the vessel walls. Scale bar, 100 μm. D, Bottom, Quantitative analysis of transient platelet adhesion (as absolute increase compared with the sham side) in sham-operated (n=7) and femoral-ligated WT mice (n=7) 1 and 3 days after femoral artery ligation. Data are shown as mean±SEM. *P<0.05 compared with sham-operated mice. c.a. indicates collateral artery; and c.v., collateral vein.

Figure 2. Transient endothelial interaction of platelets via GPIbα during the early phase of arteriogenesis. A, Representative images taken by intravital epifluorescence microscopy showing early platelet–endothelial interactions within 3 days of collateral growth as evaluated in wild-type (WT) and IL4-R/Ilbα animals using injected Rhodamine B–stained platelets (red). Arrows show platelet–endothelial transient adhesion. Dotted lines show the vessel walls. Scale bar, 100 μm. B, Quantitative analysis of transient platelet adhesion (as absolute increase compared with the sham side) in IL4-R/Iba (n=5) and WT mice (n=7) 3 days after femoral artery ligation. Data are shown as mean±SEM. *P<0.05 compared with sham-operated mice. C, Sections from adductor tissue of sham-operated mice were stained for von Willebrand factor (vWF; green), isolectin-B4 (red), and nuclei (purple). VWF specific staining was observed in the collateral arterioles compared with the control (inset) by confocal microscopy. Scale bar, 20 μm. c.a. indicates collateral artery; and c.v., collateral vein.

hours after femoral occlusion but not after sham operation (Figure 3A). In collateral veins, a moderate increase in leukocyte–vessel wall interactions occurred, which did not reach statistical significance (data not shown). We next dissected the recruitment pattern of individual leukocyte subsets. To achieve this, we used CX3CR1-eGFP-knock in mice, in which monocytes express enhanced green fluorescent protein under control of the endogenous CX3CR1 chemokine receptor locus.27 Neutrophils were identified by in vivo staining using a nonblocking phycoerythrin-labeled anti-Ly6G antibody (Materials and Methods in the online-only Data Supplement). Three days after femoral occlusion, short-term adhesive events of both monocytes and neutrophils mainly occurred in collateral veins and less frequently in collateral arteries (Figure 3B and 3C). Monocytes showed a significantly enhanced transient adhesion, whereas neutrophils were only moderately increased in arteriogenic collateral veins compared with the dormant collateral veins after sham operation (Figure 3B and 3C). Firm adhesion was not increased compared with the sham side (data not shown). We further investigated the localization of transmigrated leukocytes. We detected transmigrated monocytes and a few neutrophils predominantly along collateral arteries and along collateral veins at their juxta-arterial aspect (Figure 3D), suggesting that in addition to collateral arteries,26,29 collateral veins might serve as exit points for leukocyte recruitment during arteriogenesis.

Because platelets can foster leukocyte aggregation and recruitment under inflammatory conditions in a GPIbα-dependent manner,12,14 we next addressed the role of platelet GPIbα for leukocyte recruitment during arteriogenesis. In collateral veins, we observed a significant transient leukocyte adhesion 3 days after femoral occlusion. They were mainly recruited as individual tethering cells (Figure 4A). Transient leukocyte adhesion was markedly decreased in collateral veins of IL4-R/Iba mice (Figure 4A). Histological analyses further corroborated these findings: in serial sections, we found a significant reduction in the number of cluster...
of differentiation (CD)45-positive leukocytes recruited into close proximity of collaterals in IL4-R/Iba mutants when compared with wild-type mice (Figure 4B). This further supports the concept that during arteriogenesis, platelets foster inflammatory leukocyte recruitment. Indeed, when we addressed the local expression of inflammatory molecules during arteriogenesis, we found that upregulation of urokinase-type plasminogen activator, but not intercellular adhesion molecule, was abolished in IL4-R/Iba mice (Figure 4C).

Hence, transient GPIb–dependent platelet interactions boost the local inflammatory response during the early phase of arteriogenesis.

GPIbα-Dependent Platelet–Leukocyte Interactions in Arteriogenesis

On the basis of the above observation that platelet GPIbα is important not only for platelets but also for leukocyte recruitment, we next addressed direct interactions between platelets and monocytes, as well as neutrophils, in detail. Briefly, we analyzed the proportion of circulating neutrophils or monocytes in whole blood that were complexed with platelets at 1 and 3 days after femoral artery ligation. Twenty-four hours after femoral occlusion, we detected a significant increase in the number of both types of leukocyte–platelet complexes compared with sham. However, 3 days after induction of arteriogenesis, their number declined, indicating that the formation of leukocyte–platelet complexes represents an early event in collateral growth possibly to support cell adhesion to the endothelium (Figure 5; Figure II in the online-only Data Supplement). In contrast, leukocyte–platelet interactions were rarely seen in IL4-R/Iba mice or in sham-operated animals (Figure 5; Figure II in the online-only Data Supplement). Together, these findings indicate that monocytes and neutrophils directly interact with platelets during arteriogenesis.

Functional Role of Platelets During Arteriogenesis

Finally, we evaluated the biological significance of platelets in the process of arteriogenesis. To address this, we depleted platelets by antibody treatment, resulting in a >95% reduction of circulating platelets in wild-type mice, and then, we quantified collateral artery growth noninvasively by laser
Doppler imaging. Baseline hindlimb perfusion and blood flow reduction immediately after femoral artery ligation were similar in both groups with a strong relative drop in blood flow ratio (occluded/sham; Figure 6). After 1 week of ligation, platelet-depleted mice showed a significant defect in restoring perfusion when compared with mice treated with isotype control, suggesting that the presence of platelets is critical for arteriogenesis (Figure 6A). On the basis of our earlier findings that platelet GPIbα was important for interactions of platelets and leukocytes with the vessel wall, we next examined the functional contribution of GPIbα to arteriogenesis. Importantly, the GPIbα antibody blockade and genetic ablation of the external GPIbα domain in IL4-R/Iba mice each resulted in reduced perfusion recovery 1 week after femoral artery ligation (Figure 6B and 6C). This suggests that platelets through their GPIbα receptor contribute to collateral growth. Correspondingly, when we examined the collateral vessel growth macroscopically by a latex perfusion technique, it was found that platelet-depleted and IL4-R/Iba mice developed collateral arteries of smaller diameter, revealing a less pronounced corkscrew pattern compared with wild-type mice, consistent with reduced vessel growth (Figure 6D). Collectively, these findings suggest that platelets are important players in arteriogenesis by interacting with the endothelium and leukocytes, triggering endothelial activation and leukocyte recruitment.

**Discussion**

Several studies using animal models of femoral artery occlusion have shown that arteriogenesis can be considered as an inflammatory process that supports collateral growth. Here, we show that platelets are critical players in this process supporting monocyte and neutrophil recruitment to collateral vessels. We report that GPIbα is the key adhesion molecule leading to local platelet interactions that boost inflammatory cell recruitment and thereby support early arteriogenesis. Correspondingly, platelet depletion and inhibition of GPIbα are associated with defective arteriogenesis.

Local leukocyte recruitment, especially that of monocytes, is critical for collateral artery growth. Correspondingly, monocyte invasion through the arteriolar wall is currently considered a key feature of arteriogenesis. However, this concept is largely based on histological analysis, whereas direct in vivo visualization of this process has been lacking. As a consequence, the exact localization of leukocyte recruitment and transmigration in arteriogenesis and the potential contribution of other cell types that may escape histological detection, for example, platelets, still remain obscure. Here, we have established a novel model allowing visualization of leukocyte and platelet trafficking in a mouse model of arteriogenesis using intravital 2-photon and epifluorescence microscopy. Using this model, we have made...
Important observations: (1) We observed that a large proportion of leukocytes is recruited in early arteriogenesis to collateral veins. Hence, our findings identify a novel role of veins escorting the collateral arterioles in establishing an inflammatory process that supports arteriogenesis. (2) We found that in addition to leukocytes, platelets also interact with collateral vessel walls, providing an initial stimulus for leukocyte adhesion, transmigration, and the subsequent development of functional collateral arteries. (3) Apart from platelet–endothelial cell interactions, we also observed platelet–leukocyte interactions.

The number of short-lived interactions between platelets and the vessel wall was increased in the collateral vessels after the onset of femoral occlusion. In contrast, we observed almost no firm platelet adhesion to the vessel wall of collateral arterioles in vivo. However, it has been demonstrated earlier that even short-lived interactions between platelets and endothelial cells are sufficient to modulate endothelial properties. Activated platelets release proinflammatory chemoattractants, platelet-derived microparticles, and cytokines from their granules into the local microenvironment, thereby altering chemotactic and adhesive properties of the endothelium. Recruited platelets in turn are capable of producing inflammatory molecules, such as interleukin (IL)-1β or regulated on activation, normal T cell expressed and secreted (RANTES), thereby altering the microenvironment.

Beyond its role in platelet–endothelial interactions, GPIbα is an established molecular determinant of platelet–leukocyte binding. Leukocytes initially adhere to platelets via P-selectin glycoprotein ligand-1–P-selectin interactions, and this adhesion is stabilized by binding of Mac-1 antigen to GPIbα. Engagement of P-selectin glycoprotein ligand-1 and Mac-1 induces an inflammatory cascade in monocytes and facilitates the interaction of the resulting platelet–leukocyte complexes with endothelial cells. On leukocytes, integrin Mac-1 (CD11b/CD18) represents an important counter-receptor for platelet GPIbα. Strikingly, using IL-4Rα-GPIbα animals, we observed a strong reduction in leukocyte–endothelial interactions after femoral occlusion, and immunohistochemical examination of collateral vessels showed reduced numbers of leukocytes in the perivascular space, supporting the notion that leukocyte transmigration is dependent on platelets and GPIbα. Furthermore, in GPIbα-deficient animals, collateral vessel walls displayed a reduction in urokinase-type plasminogen activator expression, which is linked to decreased leukocyte extravasation.

Our results are in line with recent observations in other inflammatory processes, such as venous thrombosis or myocardial infarction, demonstrating that the absence of platelet GPIbα results in reduced leukocyte accumulation and transmigration. Hence, GPIbα-dependent interactions of platelets with leukocytes and the endothelium exert a dual function during collateral development, (1) supporting leukocyte recruitment and (2) facilitating leukocyte transmigration into the perivascular space. In conclusion, our study shows that arteriogenesis is enhanced by platelets, and thus, it uncovers a previously unrecognized protective action of platelets.
platelets during collateral growth. In response to femoral artery ligation, both thrombocytopenic mice and GPIbα-deficient animals showed a poor recovery in blood perfusion, suggesting that platelets are contributing to leukocyte transmigration, which is indispensable for the development of functional collateral vessels. Platelets also play a potential role in the formation of new capillaries, a process termed angiogenesis, that occurs in response to hypoxia and tumor growth. We used arteriogenesis to describe the pathophysiological process induced in the mouse model presented here. Notably, various forms of arterial or vascular remodeling exist, which has led to some terminological confusion in this field as discussed elsewhere.

In the past years, many efforts have been made to understand the molecular mechanisms of leukocyte transmigration during collateral growth. In our present study, we show that platelets, in addition to their well-known function in hemostasis and thrombosis, regulate the inflammatory response during collateral growth by facilitating leukocyte transmigration and hence play an important role in arteriogenesis. Ideally, this knowledge of the novel role of platelets could be integrated to identify therapeutics that specially target the proarteriogenic activities of platelets. Our findings also raise the important question to what extent the antiplatelet medication might inhibit collateral growth in patients. In fact, it has been shown previously that the use of aspirin leads to reduced monocyte transmigration and decreased reperfusion recovery after femoral occlusion. Thus, further studies are required to examine the potential effect of antiplatelet strategies on collateral growth.

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Disclosures

None.

References


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Significance

The mechanisms underlying arteriogenesis remain incompletely understood. We established intravital imaging in a mouse model of arteriogenesis and found that platelets are central players in this process. Via glycoprotein glycoprotein Ibα, they mediate both transient and firm leukocyte adherence to the endothelium of collateral vessels, as well as leukocyte accumulation in the perivascular space. Importantly, platelet deactivation and loss of glycoprotein Ibα function resulted in poor reperfusion recovery of the affected limb. This work introduces a new model for the study of arteriogenesis in mice and establishes platelet glycoprotein Ibα as a major determinant in this process. The findings are likely to be of importance for the development of therapies that support the formation and growth of collateral vessels.

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Supplementary Figure I

Figure S I. Quantitative analysis of firm platelet adhesion (as absolute increase compared to the sham side) in sham operated (n = 7) and femoral ligated WT mice (n = 7) one and three days after femoral artery ligature. Data are shown as mean ± SEM. *P < 0.05 compared with sham-operated mice.
Figure S II. Platelet-leukocyte complexes in whole blood. FACS analysis of whole blood from wildtype (C57Bl6) and IL4-R/Iba mice 24h after femoral artery ligature. Neutrophils were identified using SSC/FSC and a Ly6G (clone 1A8) antibody. Monocytes were identified using CD115 and CD11b antibodies. Following gating on monocytes or granulocytes, complexes formed with platelets were identified using a CD41 antibody and indicated in percent.
Materials and Methods

**Animals.** Specific pathogen-free wild type C57BL/6J were obtained from Charles River. IL4Rα/GP Ibα-tg (mGPIb-/-, hIL4-R/hGPIb+) on C57BL/6J background were generated as described. For intravital visualization heterozygous CX3CR1-eGFP/+ and LysM-eGFP/+ knockin mice were used. All mice used in the experiments were 8-14 weeks old, weight- and sex-matched. All procedures performed on mice were approved by the local legislation on protection of animals (Regierung von Oberbayern, Munich).

**Mouse model of hindlimb ischemia.** Mice were anesthetized by subcutaneous drug injection as described previously. Arteriogenesis was induced via right femoral artery ligation. Animals were shaved in the femoral region with a hair cutter and depilatory creme (Pilca, GlaxoSmithKline) and then placed in supine position with abduction of the hind limb. Body temperature was maintained between 36°C and 37°C using a heating pad. After incision of the skin, the femoral artery was dissected from the vein and femoral nerve, exposed and a ligature with a silk strand (Ethicon; Johnson & Johnson) was placed distal of the arteria profunda femoris below the circumflex femoral artery and proximal of the descending genus artery. The incision was sutured carefully to prevent self-mutilation of the mouse. The left hind limb of each animal was used as operative control (sham). Here, the surgical procedure was the same, including preparation of the femoral artery and placement of the filament under the vessel, except for ligation of the artery. After the surgical procedure, the animals were medicated with postoperative analgesia and housed individually with free access to water and chow, and were allowed to move freely. There were no signs of any gross impairment or necrosis.

**Preparation and staining of platelets and leukocytes for intravital microscopy.** Mouse platelets were isolated from whole blood and labeled with Rhodamine B as reported earlier. The fluorescent labeled platelet suspension was adjusted to a final concentration of 150x10⁶ platelets / 250µl and injected intravenously via a jugular vein catheter. Firm and transient adhesion of murine platelets was assessed and quantified by in vivo video microscopy. Therefore, Acridine Orange (Sigma-Aldrich) was injected intravenously (i.v.), which resulted in robust in vivo labeling of circulating leukocytes. Slow surface translocation (transient adherence) along the endothelium as well as localization of transmigrated leukocytes in the perivascular space was also assessed by in vivo video microscopy. In CX3CR1-eGFP mice all blood monocytes are GFP positive. A small fraction of natural killer cells also expresses GFP. Cells of the myeloid lineage other then monocytes as well as blood B and T lymphocytes are GFP negative. To differentiate neutrophils and monocytes, we injected CX3CR1-eGFP mice with a PE-labeled anti-Ly6G antibody (10µg/animal; Clone 1A8; eBioscience).

**Intravital epifluorescence microscopy.** The procedure to induce hind limb ischemia was performed as described above, but arteriogenesis was assessed after femoral artery ligation of both limbs. Sham operated animals served as operative control (sham). On day three after femoral artery ligation, animals were again anesthetized and then fixed on a custom-made heating map to maintain a physiological temperature. A polyethylene catheter (Portex) was implanted into the jugular vein and stained platelets and/or fluorescent antibodies were injected i.v.. The skin of the animals was reopened and the vessels of interest were carefully exposed. Beside skin removal, no further preparation was performed to prevent tissue damage. A pre-warmed (37°C) isotonic saline solution (0.9%) was used for continuously wetting the vessel regions during imaging. Measurements were performed with a high-speed widefield Olympus BX51WI fluorescence microscope using a long-distance condenser and a 20x (NA 0.95) water immersion objective with an Olympus MT 20 monochromator and an ORCA-ER CCD Camera (Hamamatsu). For image acquisition and analysis a Dell computer with Cell^R (Olympus) software was used. Cell-endothelial interaction was quantified at the collateral artery and vein close to the junction to the profunda femoris in one field of view (100µm vessel length) per hind limb. Regions of interest (ROI) were analyzed by intravital microscopy, including the collateral artery and vein directly at the branching site from the profunda femoris artery (Fig. 1 B, C). Immotile cells were counted as adherent and moving...
cells were counted as rolling (in veins) within 30 seconds as described\textsuperscript{6} or as transient, touching the vessel wall (in arteries) and calculated for mm\textsuperscript{2} vessel wall. Non-adherent cells were seen as bypassing unsharp cells in the blood flow.

Immunofluorescent imaging of tissue sections
Paraffin embedded mouse adductor tissue sections were de-waxed by incubating for 5 minutes x2 in Histoclear, x1 in 100% ethanol, x1 in 70% ethanol and x2 in H2O. Antigen retrieval was performed by microwaving sections in 10mM sodium citrate buffer (pH 6) for 10 minutes. Slides were allowed to cool and washed x2 with PBS and x1 with 0.5% Tween-20 (PBST) before blocking in 3% BSA PBST for 3 hours. Sections were washed x1 in PBST and stained with rabbit polyclonal antibody to VWF (Dako, Ely, UK) and isoelectin-B4 conjugated with biotin (Vector Labs Peterborough, UK), or rabbit IgG control (Santa Cruz, Dallas, TX, USA) in PBS (1% BSA) overnight at 4°C. Sections were washed x2 in PBS and x1 in PBST and secondary detection was performed by incubating sections in goat anti-rabbit Alexa-Flour-488 and Streptavidin Alexa-Flour-555 (Invitrogen, Paisley, UK) with DRAQ-V-633 (Biostatus, Leicester, UK) for nuclear detection in PBS 1% BSA for 1 hour. Sections were washed x2 in PBS, x1 in PBST and x2 in H2O and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Images were captured using an LSM510 META confocal microscope (Carl Zeiss, Cambridge, UK) using a 40\times objective lens and running version 3.2 of the LSM acquisition software.

Two-photon \textit{in vivo} microscopy. The collateral arteries and veins were visualized \textit{in vivo} using a LaVision Biotech TrimScope connected to an upright Olympus microscope, equipped with a MaiTai Laser and a 20x water immersion objective (numerical aperture 0.95, Olympus). Pictures were acquired at 800nm excitation wavelength in a 500x500µm frame with 512x512 pixels and a z-step of 3µm and detected by PMTs (G6780-20, Hamamatsu). Imsector (LaVision Biotech) was used as acquisition software. Three days after femoral artery ligation, the mice were again anesthetized as described and fixed on a custom built-stage which maintained a physiological temperature. CX3CR1-eGFP mice and PE labeled anti-Ly6G antibody (10µg/mouse, Clone 1A8 eBioscience) were used to visualize neutrophils and monocytes. In addition, 2 MD TRITC-Dextran (Invitrogen) was infused for visualization of the vessel lumen. The two-photon microscopy sessions (sham and occluded) had the same total duration. 3D reconstruction and volume rendering was performed using Volocity (Perkin Elmer).

Depletion of platelets \textit{in vivo}. Platelet depletion was induced using a rat anti-mouse GPIb\textalpha (CD42b) antibody (2 mg/kg body weight, Emfret analytics), which was given i.v. 24h before and every 48 h after femoral ligation by s.c. injection. Isotype antibody injection served as control\textsuperscript{7, 8}. The absence of platelets was confirmed with an automatic hematology analyzer (Sysmex) from whole blood.

GPIb\textalpha-inhibitor treatment. The monoclonal anti-GPIb\textalpha antibody was described previously\textsuperscript{7}. GPIb\textalpha-blocking, non-deleting Fab fragments (p0p/B) were prepared as described\textsuperscript{7} and kindly provided by B. Nieswandt (Wuerzburg, Germany). C57BL/6J mice were treated with either p0p/B or control Fab fragments (Emfret Analytics) subcutaneously 24h and immediately before femoral ligation, and every 48 hours thereafter. The blocking Fab fragment was administered in a dose of 150mg/kg body weight and stability of platelet numbers was monitored by platelet count measurements (Sysmex) in EDTA-anticoagulated whole blood. For control experiments control rat IgG Fab was used.

Visualization of collateral arteries by latex perfusion. For gross imaging of collateral arteries in limb muscles, the abdominal aorta was cannulated using a polyethylene catheter (Portex), and both hind limbs were perfused first with phosphate-buffered saline (PBS) containing 0.1% adenosine (Fluka, Milwaukee, WI, USA) and 0.05% bovine serum albumin (BSA, from Sigma, St. Louis, MO, USA), then with latex flexible compound (Chicago Latex) to visualize superficial collateral arteries and veins.

Histology. Three days after femoral ligation the animals were sacrificed and both hindlimbs were perfused with phosphate-buffered saline (PBS) containing 0.1% adenosine (Fluka, Milwaukee, WI, USA) and 0.05% bovine serum albumin (BSA, from Sigma, St. Louis, MO, USA), then 4 min with fixing solution (4% buffered paraformaldehyde) via cannulation of the
The limb muscles were excised and paraffin-embedded. Serial cross-sections or longitudinal sections of the limb muscle were cut (6 µm). For detection of leukocytes, slides were incubated with a CD45 antibody. In detail, antigen retrieval was performed in a microwave (750W) using Target Retrieval Solution (Dako). After rinsing with Tris-buffer (pH 7.5), and 7.5% hydrogen peroxide to block endogenous peroxidase activity, slides were incubated with a rat anti-mouse CD45 antibody (BD Pharmingen, dilution 1:600) at room temperature for 60 minutes. Subsequently, slides were rinsed with Tris-buffer and detection was performed using the Vectastain ABC Kit elite Rat IgG (Vector) and DAB+ (Dako). Images were acquired using a Leica DMRB epifluorescence microscope with a Zeiss AxiosCam and processed by AxioVision 4.6 software (Zeiss).

Non-invasive in vivo measurement of limb perfusion. Blood flow measurements were conducted in WT vs. IL4-R/Iba (n=10/group) using the Laser Doppler Imaging technique (Moor LDI 5061 and Moor Software Version 3.01, from Moor Instruments Instruments, Remagen, Germany) as previously described9. In brief, mice were anesthetised and kept at 37°C body temperature in a heating chamber. Measurements were performed before, immediately after surgery and on postoperative day 3 and 7. The right-to-left ratio was calculated for each mouse and background tissue values were subtracted to eliminate tissue bias. For investigation of the role of platelets and their GPIb-receptor a subset of animals was treated with either a platelet depleting antibody (n=10) or the Fab fragment p0p/B targeting the GPIb receptor (n=10). The depleting antibody or the blocking Fab fragment was administered 24h before and every 48h during the time course of the experiment.

qRT-PCR. 24 h after occlusion of the femoral artery, mRNA levels of uPa, and ICAM-1 were quantified in collateral arteries of WT and IL4-R/Iba mice (n = 3/group). For tissue sampling, the abdominal aorta was cannulated, and both hind limbs were perfused first with physiological saline solution, then with latex flexible compound (Chicago Latex, Chicago, IL, USA). Two collateral vessels per animal per side were isolated. All samples were snap frozen in liquid nitrogen and stored at –80°C until further processing. For quantitative real-time polymerase chain reaction total RNA was extracted from isolated collateral vessels as previously described10. Residual genomic DNA was removed by on-column DNase I digestion using the RNase-Free DNase Set (Qiagen, Hilden, Germany) and 1 µg RNA was reverse transcribed into cDNA using random nonamers (Roche, Mannheim, Germany) and the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche). qRT-PCR was performed with a Light Cycler 1.5 (Roche) in a reaction volume of 10 µl using a SYBR Green I Kit (Light Cycler® FastStart DNA MasterPlus, Roche) according to the manufacturer’s protocol. Each primer (uPA: fw 5’-CTGCTATCATGGAAATGGTGACTC-3’ rev 5’-CTAGGCTAATAGCATCAGGTCTG-3’; ICAM-1: fw 5’-GAAGTCTGTCAAACAGGAG-3’ rev 5’-CAGTACTGGCACCAGAATG-T3’; 18S: fw 5’-GGACAGGATTGACAGATTGATAG–3’ rev 5’-CTCGTTCGTTATCGGAATTAAC–3’) was used in a concentration of 50 pM. At least two independent qRT-PCR reactions were performed on each template as follows: an initial denaturation step at 95°C for 10 minutes (min) was followed by 40 cycles of denaturation (95°C, 10 seconds [sec]), annealing (5 sec, temperatures: 68°C (18S); 62°C (uPA), 58°C (ICAM-1)), and extension (72°C, 15 sec). Specific amplification was controlled by melt curve analyses and expression levels of targets were related to the expression levels of 18S rRNA.

Flow cytometry. Whole blood from each animal was collected from the facial vein11 and assayed for flow cytometry by standard techniques. Fc receptors were blocked using rat anti-mouse CD16/CD32 (clone 2.4G2, BD Pharmigen). Neutrophils were identified using a PE labelled rat anti-mouse Ly6G antibody (clone 1A8, BD Pharmigen) and characteristic side scatter. Monocytes were detected by double positive staining with rat anti-mouse CD115-PE (clone AF589, eBioscience) and rat anti-mouse CD11b-APC (clone M1/70, eBioscience). Platelets were detected by CD41-FITC positive staining (clone eBioMWReg30, eBioscience). Platelet-leukocyte aggregates were double positive for both populations. Antibody specificity was verified using an appropriate isotype labelled antibody (anti-rat IgG2a or anti-rat IgG2b). Erythrocytes were depleted from the preparations with BD FACS Lysing solution (BD Biosciences). Flow cytometric measurement was performed on a Becton Dickinson FACSCalibur or a Beckman Coulter Gallios. Data were processed using FlowJo software.
Platelet-leukocyte aggregate formation was considered a sensitive marker for assessment of platelet activation.\(^{12}\)

**Statistical Analysis.** All data are shown as mean ± SEM, unless indicated otherwise. Statistical analyses were carried out using SigmaPlot® 12.0 (Systat Software). Results were tested for normality, and conferring to the results analyses of two groups were performed with unpaired student’s t-test or Mann-Whitney rank sum test, respectively. More than two groups were compared using the ANOVA-LSD post hoc test. A value of P less than 0.05 was considered significant.

**References**


