The C5a/C5a receptor 1 axis controls tissue neovascularization through CXCL4 release from platelets

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Platelets contribute to the regulation of tissue neovascularization, although the specific factors underlying this function are unknown. Here, we identified the complement anaphylatoxin C5a-mediated activation of C5a receptor 1 (C5aR1) on platelets as a negative regulatory mechanism of vessel formation. We showed that platelets expressing C5aR1 exert an inhibitory effect on endothelial cell functions such as migration and 2D and 3D tube formation. Growth factor- and hypoxia-driven vascularization was markedly increased in C5ar1−/− mice. Platelet-specific deletion of C5aR1 resulted in a proangiogenic phenotype with increased collateralization, capillarization and improved pericyte coverage. Mechanistically, we found that C5a induced preferential release of CXC chemokine ligand 4 (CXCL4, PF4) from platelets as an important antiangiogenic paracrine effector molecule. Interfering with the C5aR1-CXCL4 axis reversed the antiangiogenic effect of platelets both in vitro and in vivo. In conclusion, we identified a mechanism for the control of tissue neovascularization through C5a/C5aR1 axis activation in platelets and subsequent induction of the antiangiogenic factor CXCL4.
issue homeostasis and healing processes are of fundamental importance in living organisms. The complement system, a central player of the innate immune response, contributes to both tissue homeostasis and dysfunction. Recent clinical and preclinical trials have shown that the complement system may be a promising therapeutic target for the restoration of tissue functionality in different pathological conditions, such as stroke, asthma, and myocardial infarction.

The complement system comprises a group of >30 plasma and cell-bound proteins that are activated canonically by one of the three pathways or non-canonically through plasma or cell-bound proteases. The complement system exerts functions in innate immune defense as an important sentinel system. It is tightly integrated and involved in crosstalk with other humoral and cellular arms of innate immunity, such as the contact system, pattern-recognition receptors, and immunoglobulin G (IgG) Fc receptors. Functions of the complement system include the opsonization of microbial intruders, the clearance of immune complexes, and the induction and regulation of the inflammatory response through the small cleavage fragments of C3 and C5, known as anaphylatoxins. Activation of the terminal pathway of the cascade results in the assembly of C5b–C9, which can nucleate the membrane attack complex and mediate the osmotic lysis of target cells. Previously, components of the complement cascade have been demonstrated to regulate angiogenesis in different pathophysiological settings. Furthermore, the therapeutic value of targeting complement receptors is currently being explored in clinical trials. Nevertheless, the exact mechanisms by which the complement system may interfere with proangiogenic or anti-angiogenic cells or receptors remain to be determined.

Platelets play a decisive role in diseases that are featured by thrombus formation, and thus targeting platelet-associated functions is an established therapeutic principle for treating these diseases. Beyond their classical function in blood hemostasis, platelets contribute to inflammation, immunomodulation, and atherosclerosis. Increasing evidence points to a functional intersection between hemostasis and activation of the complement cascade. Previous studies have reported that complement receptors may be expressed on platelets and that C5a receptor 1 (C5aR1) expressed on platelets modulates endothelial functions that are important for angiogenesis.

C5a-mediated activation of C5aR1 on platelets inhibits endothelial functions that are important for angiogenesis. As both platelets and the complement system have been shown to regulate angiogenesis, we further questioned whether C5aR1 expressed on platelets modulates endothelial functions that are important for angiogenesis. We were able to detect C5aR1 in unstimulated washed platelets (Fig. 2a, b), which was markedly enhanced after collagen-related peptide (CRP) stimulation (Fig. 2c) and correlated with platelet C5aR1 expression (Fig. 2d).

Subsequently, we coincubated endothelial cells with platelets isolated from wild-type (WT) or C5aR1−/− mice. There was no difference in endothelial cell proliferation after the addition of platelets in the presence or absence of C5aR1 using the murine endothelial cell line MHEC-5T (Supplementary Fig. 2a, b). However, endothelial migration was increased by coincubation with C5aR1−/− platelets compared to coincubation with WT platelets using primary mouse lung endothelial cells (MLECs; Fig. 2f). The purity of primary cells was at least 90%, as verified by staining for CD102, CD144, and CD31 (Supplementary Fig. 3). Similarly, endothelial tube formation in an in vitro two-dimensional and three-dimensional (3D) tube-formation assay was increased after coincubation with C5aR1−/− platelets or C5aR1−/− platelet supernatant compared to coincubation with WT platelets or supernatant in MHEC-5T cells and in primary MLECs (Fig. 2g–k). Next, we preincubated human platelets with the C5aR1-specific antagonist PMX3 or control peptide before their addition to human umbilical vein endothelial cells (HUVECs) in the presence of C5a. The results show that endothelial tube formation was significantly enhanced when C5aR1 activity was inhibited by PMX3 (Fig. 2f). In order to better characterize platelet–endothelial interactions in vitro, we assessed whether WT or C5aR1−/− platelets differ with respect to static adhesion to endothelial cells. ADP induced a significant increase in adhesion but there was no difference between WT and C5aR1-deficient platelets (Fig. 2m, n).

Increased ischemia-induced revascularization in C5aR1-deficient mice. Next, we explored the functional role of C5aR1 in vessel formation in vivo by assessing murine embryonic angiogenesis and hindlimb ischemia-induced revascularization. Whole-mount staining was performed on E11.5 WT or C5aR1−/−
embryonic hindbrains. No impact of C5aR1 on developmental angiogenesis could be detected (Supplementary Fig. 4). In contrast, we observed an effect of C5aR1 deficiency on ischemia-driven vessel growth. When we monitored revascularization after femoral artery ligation by repeated laser Doppler imaging (LDI) measurements over 14 days, we found significantly enhanced perfusion in C5ar1−/− animals, indicating faster and more pronounced revascularization when compared to WT control animals (Fig. 3a, b). Next, gastrocnemius muscles were explanted from ischemic hindlimbs after 14 days, and vessel density was quantified by immunofluorescence staining for isoelectin B4 (IB4; green) and 4,6-diamidino-2-phenylindole (DAPI; blue; Fig. 3c). Vessel density of hindlimb muscles from C5ar1−/− mice was significantly higher as compared with that of WT controls after the induction of hindlimb ischemia (Fig. 3c, d). Subsequently, we administered platelet-depleting serum to both WT and C5ar1−/− mice after induction of hindlimb ischemia over 14 days. Platelet depletion was effective and showed a platelet-depletion efficiency >90% measured in peripheral blood of mice (Supplementary Fig. 5) Upon platelet depletion, we observed no significant difference in revascularization between both strains any more, suggesting a critical role of C5aR1 activation on platelets for ischemia-induced angiogenesis (Fig. 3e). Accordingly, vessel density was comparable in both the groups (Fig. 3f).
The increased revascularization in C5aR1-deficient mice is mediated by platelet C5aR1 stimulation-driven collateralization as well as capillary and pericyte growth. To further define the role of platelet C5aR1 in vivo, we crossed floxed GFP-C5ar1fl/fl mice with Pf4-cre+ mice31 and generated Pf4-cre+ C5ar1fl/fl mice, lacking C5aR1 in platelets (Supplementary Fig. 6). When we induced hindlimb ischemia and performed repeated LDI measurements over 14 days in Pf4-cre+ C5ar1fl/fl mice, we found stronger revascularization in platelet-specific C5ar1-knockout mice than in Pf4-cre- C5ar1fl/fl littermate controls (Fig. 4a, b). Similar to C5ar1−/− mice, we detected an increased vessel density in the ischemic muscle tissue of Pf4-cre+ C5ar1fl/fl mice (Fig. 4c, d). To rule out that a difference in platelet deposition upon C5ar1 deficiency in ischemic tissues is responsible for the observed effects, we assessed platelet abundance and microthrombi size in whole-muscle sections of ischemic Pf4-cre+ C5ar1fl/fl mice and Pf4-cre- C5ar1fl/fl controls (Fig. 4e). We found no significant differences between both the groups (Fig. 4f). As CXCL4 (PF4) is known to be expressed by monocytic cells, which are important mediators of ischemia-induced revascularization32, we next assessed whether Pf4-cre+ C5ar1fl/fl mice showed altered C5aR1 expression on monocytes or macrophages compared to Pf4-cre- C5ar1fl/fl mice but found no differences (Supplementary Fig. 7).

To obtain insights into the mechanisms underlying the increased revascularization observed in Pf4-cre+ C5ar1fl/fl mice in vivo, we performed micro-computed tomographic (microCT) analysis of platelet-specific C5ar1-knockout mice subjected to the hindlimb ischemia model at day 9. After 3D reconstruction, the occluded vessel could be identified by comparison with the contralateral control hindlimb (Fig. 5a, b, arrows). Furthermore, collateral artery formation could be observed (Fig. 5a, b and Supplementary Movies 1a–d). By virtual reconstruction, the vascular tree was extracted from the images and analyzed (Supplementary Fig. 8a, b). Vessel size distribution in both the groups (Pf4-cre+ C5ar1fl/fl and Pf4-cre- C5ar1fl/fl) was assessed. The analysis yielded an increase in small- and medium-sized vessels, which comprise arterial collateral vessels, in the proximal section of the hindlimb (Fig. 5c). We also considered the possibility that platelet C5aR1 impacts on the formation of pre-existent collateral vessels, which are known to be important for the initial phase of ischemia-induced revascularization33. Therefore, the vascular tree in nonischemic hindlimbs of both genotypes was quantified. No difference in mean vessel size could be observed (Supplementary Fig. 8c).

In the distal hindlimb, we stained for smooth muscle actin (SMA) and NG2 expression, which denotes arterial vessels. We then quantified the size of the largest arteries and arteriolar density within the gastrocnemius muscle (Fig. 5d, e). However, no significant differences could be detected between Pf4-cre+ C5ar1fl/fl mice and Pf4-cre- C5ar1fl/fl mice in this distal muscle. NG2 staining allowed us to determine pericyle abundance and pericycle coverage of vessels. Comparing ischemic hindlimb muscles from platelet-specific C5aR1-deficient mice with Pf4-cre+ C5ar1fl/fl control mice at day 14 after induction of ischemia, we observed increased pericycle density and enhanced pericycle coverage in platelet-specific C5aR1-deficient mice (Fig. 5f, g). This effect may explain the amplified vessel density in Pf4-cre+ C5ar1fl/fl mice (Fig. 4c, d).

Our observations that platelet C5aR1 inhibits endothelial cell functions, which are relevant for angiogenesis in vitro, and our findings that platelet-specific C5ar1−/− animals exhibit increased ischemia-induced revascularization prompted us to investigate the regulatory mechanisms underlying this process.

Platelet C5aR1 mediates the release of antiangiogenic, platelet-derived CXCL4. To elucidate the mechanisms underlying platelet C5aR1-mediated inhibition of vessel growth and endothelial functions, we stimulated isolated platelets with C5a or vehicle control. A membrane-based antibody array was performed using the harvested supernatants to obtain insights into the regulation of secreted antiangiogenic factors (Fig. 6a). Interestingly, the platelet α-granule component CXCL4 was strongly induced after C5a stimulation (Fig. 6b). Bonferroni’s post hoc analysis showed significant differences compared to all factors except Angiopoietin-1, for which C5a induced a downregulation, as well as endostatin (Supplementary Fig. 9).

To further approach the mechanism of C5a-induced CXCL4 secretion, we stainedstimulated platelets for the α-granule components P-selectin and CXCL4 (Fig. 6c). Low-dose ADP and C5a stimulation induced platelet activation, as indicated by P-selectin upregulation, and P-selectin mobilization toward the cell periphery (ring-like staining pattern, arrows; Fig. 6c, d). C5a induced an altered secretion response compared to ADP, as indicated by a decrease in CXCL4 intensity relative to P-selectin intensity after C5a stimulation compared with ADP (Fig. 6e).

Interestingly, using confocal microscopy (Supplementary Fig. 10) we found α-granules, which contain predominantly CXCL4 over P-selectin as well as granules containing both CXCL4 and P-selectin (Fig. 6f, g). When we stimulated platelets...
with C5a, we observed a release of these CXCL4-predominant granules from platelets measured as the reduction in area fraction (Fig. 6h) but not of granules containing both CXCL4 and P-selectin or granules containing P-selectin but not CXCL4. These findings strongly suggest that C5a induces the secretion of an α-granule subset predominantly holding CXCL4.

Using human cells, conventional enzyme-linked immunosorbent assay (ELISA) measurements of the CXCL4 concentration in the supernatant of C5a-stimulated platelets confirmed a dose-dependent and significant increase compared with vehicle control-treated platelets (Fig. 6i). Next, time and dose dependency in murine platelets were assessed to find the optimal stimulation conditions in murine platelets (Supplementary Fig. 11). Maximum CXCL4 release was observed at a C5a concentration of 200 ng/ml for mouse samples and 2000 ng/ml for human cells (Fig. 6i and Supplementary Fig. 12a). A dose-dependent CXCL4 secretion effect was achieved at stimulation conditions of 10 min at 25°C and 10 min at 37°C (Supplementary Fig. 12). Analyzing megakaryocytes from C5ar1−/− mice, we observed no difference in CXCL4 granule area fraction (Fig. 6j, k) compared to WT cells. Furthermore, there was no difference in Cxcl4 mRNA between WT and C5ar1−/− megakaryocytes as analyzed by real-time PCR (Fig. 6l), indicating regulation of CXCL4 on the platelet rather than on the megakaryocyte level.

In order to compare C5a-induced CXCL4 release with CXCL4 secretion induced by classical platelet agonists, we performed platelet stimulation with different platelet activators and compared the secretion response of WT and C5ar1−/− mice (Supplementary Fig. 13). We found that, while WT and C5ar1−/− mice displayed no significant differences in response to classical agonist-induced CXCL4 release, no significant CXCL4 secretion occurred after C5a stimulation of C5ar1−/− platelets (Supplementary Fig. 13b). In WT mice, CXCL4 level after 200 ng/ml C5a stimulation was comparable to 25 μM ADP stimulation (Supplementary Fig. 13a). WT and C5ar1−/− platelets did not differ
Fig. 2 Platelets express C5αR1 and platelet C5αR1 inhibits various endothelial functions. a Isolated washed murine platelets express C5αR1 as assessed by immune fluorescence microscopy. There was only partial colocalization of C5αR1 (red) with the α-granule marker P-selectin (green). ×630 magnification, scale bars represent 5 μm. Images are representative of four independent experiments. b Histogram showing C5αR1 expression on platelets (gray curve); the black curves show the histogram obtained with an IgG isotype. The histogram is representative of the analysis of four independent platelet samples. c Furthermore, flow cytometry revealed that C5αR1 expression on platelets is dynamic. Upon stimulation with CRP, platelet C5αR1 expression increased in WT platelets. In c-e, data are displayed as the mean ± SEM (n = 4 independent experiments) and are shown as the percentage of control. The expression in the nonstimulated WT group represents 100%. p < 0.05. d Upon stimulation with ADP, WT platelets displayed C5αR1 upregulation at higher concentrations. e Upon treatment with C5α at the indicated concentrations, no significant changes in C5αR1 expression on platelets could be detected. f Primary lung endothelial cells (MLECs) were grown to a confluent monolayer, which was wounded by scratching with a plastic pipette and coincubated with C5αR1−/− or WT platelets. The absence of C5αR1 resulted in increased endothelial migration after 17 h. Data are displayed as the mean ± SEM (n = 6 independent experiments). The area not populated with cells at the start of the experiment minus the area not populated with cells after 17 h of coincubation with WT platelets represents 100%. p < 0.05. g Endothelial tube formation by MHEC-5T cells in vitro was significantly increased after incubation with platelets isolated from C5αR1−/− mice compared with WT platelets. Data are shown as the mean ± SEM (n = 4 independent experiments) and displayed as the total tube length after 6.5 h. The cumulative length of endothelial tubes after incubation with WT control platelets represents 100%. *p < 0.05. h Similarly, endothelial tube formation by MLECcs was increased after exposure to C5αR1−/− platelets compared with WT platelets. Data are shown as the mean ± SEM (n = 4 independent experiments). Tube formation after coincubation with WT control platelets represents 100%. p < 0.05. i Representative images of endothelial tube formation of MHEC-5T after coincubation with WT or C5αR1−/− platelets. j The supernatant of murine WT platelets significantly inhibited tube formation from endothelial spheroids compared with the supernatant of C5αR1−/− platelets. Data are presented as the mean ± SEM (n = 3 independent experiments) and are shown as the percentage of the total tube length after 24 h in controls. Tube formation after incubation with WT control platelet supernatant represents 100%. p < 0.05. k Representative images of sprout formation from endothelial spheroids in both groups. Scale bar represents 100 μm. l Human platelets were preincubated with a C5αR1 antagonist (PMX53) or control peptide. Subsequently, platelets were coincubated with human umbilical vein endothelial cells (HUVECs) on Matrigel. Endothelial tube formation was significantly enhanced by preincubation with PMX53 compared with the control. Data are shown as the mean ± SEM (n = 4 independent experiments with separate donors) of the total tube length after 6.5 h. The cumulative length of endothelial tubes after incubation with platelets preincubated with control peptide represents 100%. p < 0.05. m There was no significant difference in adhesion to endothelial cells under static conditions between WT and C5αR1-deficient platelets under normoxic conditions. Data are shown as the mean ± SEM (n = 4–5 independent experiments) and as the percentage of control. Adherent WT platelets expressed as the area fraction of platelet-specific staining divided by the number of endothelial cells expressed as the DAPI count per area represents 100%. *p < 0.05 compared to control-stimulated platelets. n Representative images of static platelet adhesion of WT and C5αR1−/− platelets to endothelial cells. Scale bars represent 200 μm. Two-sided Student’s t test in b, f-h, j, l ANOVA in c-e, m.

significantly in their aggregation behavior (Supplementary Fig. 14a, b). To further rule out that defects in hemostasis account for the neovascularization phenotype of C5αR1−/− mice, we carried out additional in vivo experiments using ferric chloride-induced vascular injury. We observed no significant difference in time to thrombus formation in the absence of C5αR1 in comparison to WT mice (Supplementary Fig. 14c). Furthermore, C5αR1 deficiency had no significant effect on in vivo bleeding time (Supplementary Fig. 14d). Nevertheless, as the results in Fig. 6c, d already showed, C5α induced platelet activation. We quantified C5αR1-dependent platelet activation using flow cytometry and found that there was significantly increased but modest platelet activation in WT platelets following C5α stimulation but not in C5αR1−/− platelets (Fig. 6m). Furthermore, C5α induced a dose-dependent calcium signal (Supplementary Fig. 14e). However, C5α had no effect on other platelet activation markers like GPVI, CD40L, CD61, and platelet–leukocyte aggregate formation (Supplementary Fig. 14f). Finally, C5α had no impact on classical platelet functions such as adhesion to von Willebrand factor or aggregation (Supplementary Fig. 15). C5αR1 deficiency did not influence C5αR protein content of platelets (Supplementary Fig. 16). C5αR has recently been shown to modulate platelet aggregation.24 Similarly, C5αR receptor expression profile of platelets was not significantly altered in the absence of C5αR1 (Supplementary Fig. 17). Next, we assessed the impact of C5α on the secretion of other angiogenic factors, which are known to be stored in platelets, by conventional ELISA. Compared to other agonists and vehicle control, C5α did not induce secretion of thrombospondin 1 (Supplementary Fig. 18a), endostatin (Supplementary Fig. 18b), or platelet-derived growth factor (PDGF; Supplementary Fig. 18c) from human platelets. Also, C5α stimulation had no effect on the secretion of δ-granule cargo ATP from platelets (Supplementary Fig. 19). The granule content of platelets from WT versus C5αR1−/− or P4-cre− C5αR1fl/fl versus P4-cre+ C5αR1fl/fl mice showed no significant differences, respectively (Supplementary Fig. 20). Then we analyzed the effect of C5 deficiency on C5αR1-dependent CXCL4 secretion from platelets. Platelets isolated from C5-deficient mice expressed C5αR1 (Supplementary Fig. 21a) but displayed C5α-deficient CXCL4 secretion after stimulation with higher C5α concentrations (500 ng/ml; Supplementary Fig. 21b), while no major differences were detected in the activation response of C5αR1−/− platelets compared to WT platelets (Supplementary Fig. 21c, d). C5αR2 is known to modulate C5αR1 function in a number of different cell types.25 However, we could not detect relevant C5αR2 expression on platelets or an effect of C5αR2-specific stimulation on CXCL4 release from platelets (Supplementary Fig. 22).

Furthermore, we analyzed several potential pathways, which could link binding of C5α via C5αR1 to CXCL4 secretion. We tested phosphorylation of Akt, phosphoinositide-3 kinase (PI3K), glycoinosynase kinase 3β (GSK-3β), p44/p42 mitogen-activated protein kinase (MAPK), PLCβ3, PLCβ2, protein kinase A (PKA), and RAP1. Interestingly, we found that the signal is conducted via the Gβγ subunit of C5αR and not by Gαi, as we found C5α-dependent phosphorylation of PI3K and Akt but not PKA (Fig. 6n–q and statistical analysis in Supplementary Fig. 23). PKC activation seems to be central for C5α-induced CXCL4 secretion as we found consistent C5α-dependent PKC phosphorylation and could also show that PKC activation induces CXCL4 secretion (Fig. 6n, o).

Platelet C5αR1-driven CXCL4 confers the inhibitory effect on vessel formation. To confirm the relevance of C5α-induced CXCL4 secretion, we treated endothelial cells with the supernatant of C5α-stimulated platelets. The addition of supernatant from C5α-treated WT platelets to endothelial cells resulted in an
**Fig. 3** C5aR1 deficiency promotes ischemia-induced revascularization in vivo. WT or C5aR1−/− mice were subjected to hindlimb ischemia and analyzed after 2 weeks. 

**a** Revascularization of the hindlimbs after femoral artery ligation was visualized by laser Doppler fluximetry (LDI). We found increased revascularization in C5aR1−/− animals. Data are shown as the mean ± SEM (n = 7 animals per group) and are displayed as the percentage of the perfusion in the contralateral control limb. *p < 0.05.

**b** Representative LDI images of mouse hindlimbs after femoral artery ligation illustrate increased revascularization in C5aR1−/− animals compared with WT controls.

**c** Vessel density in the gastrocnemius muscle of the ischemic limbs was quantified by immunofluorescence staining. Vessels were visualized by isoelectric B4 (IB4 in green, nuclei in blue), and images of whole-muscle sections were acquired as tile scans and analyzed. At 14 days after the induction of ischemia, C5aR1−/− mice exhibited a significantly higher vessel density than WT controls. ×200 magnification, scale bars represent 200 µm.

**d** Quantification of the IB4-positive area fraction in the muscle sections reveals higher vessel abundance in ischemic C5aR1−/− hindlimbs. Data are shown as the mean ± SEM (n = 10 whole-muscle sections per group) and are displayed as the percentage of control. The IB4-positive area fraction in WT control hindlimbs represents 100%. *p < 0.001.

**e** Furthermore, WT or C5aR1−/− mice were subjected to hindlimb ischemia and platelets were depleted systemically by injection of platelet depleting serum starting on the first day post induction of ischemia. We could not detect significant differences (p < 0.05) in revascularization. Data are shown as the mean ± SEM (n = 7 animals per group) and are displayed as the percentage of the perfusion in the contralateral control limb.

**f** Vessel density in the gastrocnemius muscle of the ischemic limbs was quantified by immunofluorescence staining as described in **c**. At 14 days after the induction of ischemia, WT and C5aR1−/− mice with platelet depletion did not exhibit significant differences in vessel density. ×200 magnification, scale bars represent 200 µm. Image is representative of 10 whole-muscle sections per group analyzed. Two-way ANOVA with Bonferroni’s post hoc test in **a, e**. Two-sided Student’s t test in **d**.
Fig. 4 Platelet-specific C5a receptor deletion inhibits revascularization but does not alter platelet deposition in vivo. Pf4-cre\(^{-}\) C5ar\(^{fl/fl}\) mice were generated as described in the “Methods” section. Hindlimb ischemia was induced, and Pf4-cre\(^{-}\) C5ar\(^{fl/fl}\) mice served as controls. Platelet-specific C5a receptor 1 knockout mice showed increased revascularization. Data are presented as the mean ± SEM (n = 5 animals per group) and are displayed as the percentage of the perfusion in the contralateral control limb. *p < 0.05. b Representative LDI images of mouse hindlimbs after femoral artery ligation illustrate increased revascularization in Pf4-cre\(^{+}\) C5ar\(^{fl/fl}\) mice compared with littermate control animals. c Vessel density in the representative gastrocnemius muscle sections (vessel marker IB4 in green, nuclei in blue) of the ischemic limbs of Pf4-cre\(^{+}\) C5ar\(^{fl/fl}\) showed significantly higher vessel density than Pf4-cre\(^{-}\) C5ar\(^{fl/fl}\) controls. Scale bars represent 200 µm. d Quantification of the IB4-positive area fraction in the muscle sections reveals higher vessel abundance in ischemic Pf4-cre\(^{+}\) C5ar\(^{fl/fl}\) hindlimbs at ×200 magnification. Data are shown as the mean ± SEM (n = 10 whole-muscle sections per group) and are displayed as the percentage of control. The IB4-positive area fraction in Pf4-cre\(^{-}\) C5ar\(^{fl/fl}\) hindlimbs represents 100%. *p < 0.05. e By staining for CD42b (red), platelet deposition was quantified in whole-muscle sections of Pf4-cre\(^{+}\) C5ar\(^{fl/fl}\) or Pf4-cre\(^{-}\) C5ar\(^{fl/fl}\) mice, as described in the “Methods” section. No difference in the number of platelets per area or microthrombi size was detected. Displayed are representative images for both genotypes, IB4 staining (green) depicts vascular structures; DAPI (blue) depicts nuclei. Scale bars represent 100 µm. f CD42b-positive single platelets were quantified by size using automated digital image analysis as described in the “Methods” part in whole-muscle sections. There was no significant difference between Pf4-cre\(^{-}\) C5ar\(^{fl/fl}\) and Pf4-cre\(^{-}\) C5ar\(^{fl/fl}\) mice both with respect of the number of platelets per muscle area as well as the size of the platelets/microthrombi. Data are shown as the mean ± SEM (n = 6 whole-muscle sections per group) and are displayed as the percentage of control. The readings in Pf4-cre\(^{+}\) C5ar\(^{fl/fl}\) hindlimbs represent 100% in both graphs. *p < 0.05. Two-way ANOVA with Bonferroni’s post hoc test in a. Two-sided Student’s t test in d, f.
inhibitory effect on endothelial tube formation (Fig. 7a). This effect was C5aR1 specific, as the platelet supernatant from C5a-stimulated C5ar1−/− platelets had no effect (negative control; Fig. 7a). Addition of CXCL4 to C5a-conditioned C5ar1−/− supernatant restored the effect of C5a-conditioned WT supernatant and the supernatant of C5ar1-Cxcl4-double-knockout mice yielded comparable results with C5ar1−/− supernatant (Fig. 7a).

Moreover, endothelial migration was inhibited by C5a-stimulated WT platelet supernatant (Supplementary Fig. 24a), whereas proliferation was not affected (Supplementary Fig. 24b). Next, we depleted CXCL4 from C5a-stimulated WT platelet supernatant using anti-CXCL4 antibody-coated Sepharose beads. Removal of CXCL4 from C5a-conditioned platelet supernatant resulted in significantly increased endothelial tube formation, suggesting that C5a-driven CXCL4 release is one of the central factors mediating the antiangiogenic role of platelets (Fig. 7b). In C5ar1−/− platelet supernatant, CXCL4 depletion had no effect (Fig. 7b). As C5a was shown to induce phosphorylation of PI3K, Akt, and PKC (Fig. 6n–q), we tested whether preincubation of platelets with the according kinase inhibitors followed by C5a stimulation could...
impede the inhibitory effect of C5a-conditioned platelet supernatant on endothelial tube formation. Indeed, we found no significant differences between tube formation with vehicle control-stimulated platelet supernatant and C5a-conditioned platelet supernatant from platelets preincubated with Akt, P13K, and PKC kinase inhibitors (Fig. 7c).

To further assess the role of CXCL4 in platelet C5aR1-mediated inhibition of angiogenesis, we isolated primary murine lung endothelial cells from Cxcr3−/− mice. CXCR3 has been described as the endothelial receptor that mediates antiangiogenic effects of CXCL4.35 The supernatant from C5a-stimulated WT platelets did not inhibit endothelial migration (Fig. 7d) and tube formation (Fig. 7e) in Cxcr3−/− cells compared with vehicle control-stimulated WT platelet supernatant, whereas this effect was present using WT endothelial cells (Fig. 7d). Moreover, we observed no increased endothelial tube formation of Cxcr3−/− endothelial cells using supernatant from C5a-conditioned versus vehicle control-stimulated C5ar1−/−/ platelets (Fig. 7e).

We continued to study the mechanisms, by which platelets control angiogenesis after C5a stimulation in vivo using a mouse model, which allowed us to selectively add C5aR1+/+ or C5aR1−/− platelets. For this purpose, we used the in vivo Matrigel plug assay, in which vessel ingrowth into an extracellular matrix-like gel, which is subcutaneously injected into mice, is stimulated by endothelial growth factors.32 Similar to ischemic hindlimb tissue (Fig. 1a), we observed complement activation upon induction of vessel growth, as verified by the deposition of C3b (Fig. 7f). Having confirmed the presence of complement activation, we considered the Matrigel plug assay a suitable model to further determine the functional role of platelets in vessel formation. Consistent with our findings of improved revascularization after induction of hindlimb ischemia as a consequence of C5aR1 deficiency (Fig. 3), Matrigel plugs in C5ar1−/− mice displayed a larger area of neovascularization after 7 days, indicating a higher level of angiogenic factor-induced vessel growth (Fig. 7g). To further delineate the cell-specific role of C5aR1 on platelets, we resuspended freshly isolated murine platelets in Matrigel supplemented with basic fibroblast growth factor (bFGF). WT platelets inhibited growth factor-induced angiogenesis in C5ar1−/− mice, which could not be observed for C5ar1+/− platelets (Fig. 7h). Next, we injected Matrigel into C5ar1−/− mice and supplemented it with WT platelets or platelets isolated from Cxcl4−/− animals. Compared to Cxcl4−/− platelets, WT platelets inhibited growth factor-induced angiogenesis in C5ar1−/− mice (Fig. 7i). In another set of experiments, we systemically inhibited CXCL4 by intravenous injection of an anti-CXCL4 blocking antibody or control IgG as previously described.36 Matrigel plugs from C5ar1−/− mice supplemented with WT platelets displayed an increased level of growth factor-induced angiogenesis when CXCL4 was inhibited compared to controls (C5ar1−/− mice supplemented with WT platelets + IgG injection; Fig. 7j). Finally, we examined whether the C5a-C5aR1 axis can be influenced pharmacologically in the context of vessel growth. Matrigel was supplemented with the C5aR1 antagonist PMX53 or the respective control peptide. C5aR1 inhibition resulted in an increase of growth factor-induced angiogenesis as compared to control (Fig. 7k, l).

In the hindlimb ischemia model, we detected CXCL4 deposition in muscle tissue 1 week after induction of ischemia and CXCL4 was deposited outside of vessels (Fig. 7m). Interestingly, we detected significantly lower levels of CXCL4 in homogenized hindlimb muscles of C5ar1−/− mice subjected to hindlimb ischemia than in those of WT littermate controls (Fig. 7o). These findings strongly support our hypothesis that C5a-induced CXCL4 is an important negative regulator of ischemia-induced revascularization in vivo. Analyzing histological sections, we found significantly less CXCL4 deposition in C5ar1−/− mice (Fig. 7m, n). When we specifically targeted C5aR1 with the C5aR1-specific antagonist PMX205 in vitro, C5a-induced CXCL4 secretion from platelets was completely abrogated (Fig. 7p). Moreover, using the C5aR1 antagonist PMX205 in vivo, we observed that revascularization was significantly increased during days 4–10, when C5aR1 was inhibited (Fig. 7q), whereas PMX205 had no effect on tail bleeding time (Supplementary Fig. 25).

In conclusion, we provide evidence for a functional crosstalk between cells of hemostasis with the innate immune system during tissue revascularization. More specifically, we demonstrate

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**Fig. 5 C5ar1 deficiency on platelets induces increased collateral artery formation, capillarization, and pericyte coverage.** Platelet-specific C5ar1-deficient mice were subjected to hindlimb ischemia. To visualize collateral arteries, mice were perfused with a contrast agent at 49 after induction of ischemia and subjected to microCT analysis as described in the “Methods” section. a 3D reconstructions of arteries from Pf4−/− C5ar1−/− mice showed collateral artery formation, while the main femoral artery (arrows) was no longer perfused. a, b A comparison of collateral artery formation in mice with C5ar1-deficient or -competent platelets shows increased collateralization in platelet-specific C5ar1-knockout mice. Images are representative of 3–4 mice, i.e., 3–4 vessel trees analyzed. c The size distribution of vessels within the ischemic hindlimbs of Pf4−/− C5ar1−/− mice and Pf4−/− C5ar1+/− mice was quantified within the microCT data in mouse hindlimbs proximal to the knee in the region of the adductor muscle (for details on methodology, please refer to the “Methods” section and Supplementary Fig. 8). Pf4−/− C5ar1−/− mice displayed larger vessels in both the small vessel range as well as the medium-sized vessel range. Data are shown as overlaid single measurements of each of the 3–4 vessel trees analyzed and as mean ± 95% confidence interval of all vessels quantified within each size region (n = 3–4 animals, i.e., vessel trees per group). Data are stated in pixels/voxels and 1 voxel represents 21.6 µm. Furthermore, the mean vessel diameter shows a clear trend that the proximal part of the ischemic hindlimb are larger in Pf4−/− C5ar1−/− mice compared to controls (right) going along with improved revascularization. d Characterization of arteries in the distal ischemic hindlimb gastrocnemius muscle of Pf4−/− C5ar1−/− or Pf4−/− C5ar1+/− mice reveal no significant differences in large artery size or abundance of arterioles (SMA in red, vessel marker IB4 in green). ×200 magnification, scale bars represent 200 µm, arrows mark large muscle arteries double positive for SMA and IB4. e Large distal muscle arteries were assessed by measuring the perimeter of the 5 largest arteries present in a whole-muscle section acquired by tile scanning at ×200 magnification (left). Furthermore, the area fraction in the muscle sections of SMA-positive vessels was quantified at ×200 magnification. Data are shown as the mean ± SEM (n = 10 whole-muscle sections per group) and are displayed as the percentage of control. The sum of artery diameters of the five largest arteries per section or the area fraction of SMA staining in Pf4−/− C5ar1−/− hindlimb muscle sections represent the 100% control. n.s. = no significant difference was observed. f Platelet-specific C5ar1-knockout mice displayed increased pericyte coverage of vessels in the ischemic tissue at d14 after induction of ischemia (pericyte marker NG2 in red, vessel marker IB4 in green). ×200 magnification, scale bars represent 200 µm. g Pericyte density was assessed by measuring the area fraction of NG2-positive staining in whole-muscle sections acquired by tile scanning at ×200 magnification (left). Furthermore, pericycle coverage was assessed by calculating the spatial colocalization of NG2 and IB4 staining. Data are shown as the mean ± SEM (n = 10 whole-muscle sections per group) and are displayed as the percentage of control. The area fraction of NG2 staining or the pericycle coverage in Pf4−/− C5ar1−/− hindlimb muscle sections represent 100%. “p < 0.05 or <0.01. Two-sided Student’s t test in e, g.
that platelet C5aR1 inhibits endothelial functions in vitro and decreases growth factor-induced angiogenesis and ischemia-induced revascularization in vivo. Mechanistically, we identify platelet-derived CXCL4 production and secretion downstream of C5a/C5aR1 axis activation as a secretory pathway, by which platelets control vessel formation.

Discussion
Although both complement components and platelet-derived mediators contribute to the regulation of vessel formation, a functional intersection between platelets and the complement system in the context of revascularization has not been investigated so far. Here we characterized...
Fig. 6 Platelet C5aR1 mediates release of the paracrine effector CXCL4 in an Akt- and PKC-dependent fashion. a Washed murine WT platelets carefully isolated with inhibitors of platelet activation were stimulated with C5a. The supernatant was analyzed by a membrane-based antibody array. Specific mediators, such as CXCL4 (red circles), were upregulated after stimulation with C5a (bottom) compared with the vehicle control (top). b Results of four array repeats were quantified. For most factors, C5a stimulation (gray) induced a slight upregulation compared to vehicle control (black). The strongest increase was observed for CXCL4. Data are shown as the mean ± SEM (n = 4 independent experiments) and are displayed as intensity values. *p < 0.05. Bonferroni’s post hoc analysis was performed; results are displayed in Supplementary Fig. 9. c Single-cell staining revealed that murine platelets are activated upon C5a stimulation and release CXCL4. Upon stimulation with ADP and C5a, a ring-like staining pattern was observed, indicating platelet activation (arrows). However, C5a-stimulated platelets exhibit reduced CXCL4 content after stimulation with C5a, which cannot be observed to the same extent in the ADP-stimulated group. ≥630 magnification, scale bars represent 5 µm. Images are representative of four independent experiments. d Quantification of P-selectin intensity in each platelet reveals upregulation, indicating platelet activation upon stimulation with ADP and C5a. However, no significant difference was detected between ADP and C5a. Data are shown as the mean ± SEM (n = 5 images analyzed) and are displayed as the percentage of control. The P-selectin intensity in vehicle control-stimulated WT platelets represents 100%. *p < 0.05 e Quantification of CXCL4 intensity in relation to P-selectin intensity in each platelet reveals relatively stronger secretion of CXCL4 upon C5a stimulation compared to ADP. Data are shown as the mean ± SEM (n = 5 images analyzed) and are displayed as the percentage of control. The CXCL4 intensity in relation to P-selectin intensity in vehicle control-stimulated WT platelets represents 100%. *p < 0.05. f Single platelets from mice were stimulated with C5a or vehicle control. Granules were quantified by calculating the area of predominantly green staining within the platelets (P-selectin-predominant), predominantly red areas (CXCL4-predominant) as well as overlaid yellow areas (P-selectin-CXCL4 double positive). ≥630 magnification, scale bars represent 1 µm. Images are representative of >100 analyzed single platelets. g As described in Supplementary Fig. 10, granule area fractions were quantified. We observed a distinct distribution pattern of granules, with a similar amount of CXCL4-predominant as well as P-selectin-CXCL4 double-positive granules and a significantly lower number of P-selectin-predominant granules, which contain P-selectin over CXCL4. Data are shown as the mean ± SEM (n = 112 single platelets analyzed) and are displayed as the area fraction. p (CXCL4 versus double positive) < 0.001, p (CD62P versus double positive) = 0.0022. h Upon stimulation with C5a, analysis revealed a reduction in CXCL4-predominant granules, while the area fraction of double-positive granules remains unchanged, while the relative area fraction of P-selectin predominant granules increases, thus demonstrating C5a-induced secretion of a subset of granules, which contain CXCL4 over P-selectin. Data are shown as the mean ± SEM (n = 166 single platelets analyzed) and are displayed as the area fraction. p (CD62P versus CXCL4) < 0.001. i Conventional ELISA confirmed a significant dose-dependent increase in CXCL4 secretion from human platelets after C5a stimulation. Maximum CXCL4 release is reached at a C5a concentration of 2000 ng/ml. Data are shown as the mean ± SEM (n = 8 independent experiments containing separate donors) and are displayed as the percentage of control. The CXCL4 protein level of vehicle-stimulated platelet supernatant represents 100%. *p < 0.05. j Murine megakaryocytes from WT versus C5ar1−/− mice were assessed for distribution of CXCL4-CXCL4-predominant granules (red). Displayed are representative images, nuclei are shown in green. ≥630 magnification, scale bars represent 10 µm. k As described in Supplementary Fig. 10, granule area fractions were quantified in megakaryocytes from WT versus C5ar1−/− mice. No significant difference could be detected for CXCL4-predominant granules. Data are shown as the mean ± SEM (n = 10–12 single megakaryocytes analyzed) and are displayed as the area fraction. l RT-PCR analysis detected similar mRNA levels of CXCL4 in megakaryocytes from WT versus C5ar1−/− mice. Data are presented as the mean ± SEM (n = 5 independent experiments) and are shown as relative expression in relation to GAPDH. m Citrated whole blood from WT and C5ar1−/− mice was stimulated using different concentrations of C5a (20, 200, 2000 ng/ml) and vehicle control and assessed for platelet activation markers by flow cytometry. For the gating strategy, please refer to the “Methods” section. Activated platelets are detected as CD62P/JONA+−, whereas JONA detects activated GPIbIIa. C5a induced platelet activation in WT platelets but not in C5ar1−/− mice. Data are shown as the mean ± SEM (n = 4 independent experiments) and are displayed as the percentage of control. The percentage gated CD62P/JONA+− platelets in the vehicle-stimulated group represents 100%. *p < 0.05. n In order to uncover the signaling downstream of C5ar1 leading to CXCL4 secretion, lysates of WT platelets were generated after vehicle control or C5a stimulation and samples were probed by equal protein concentrations for phospho-proteins as well as non-phosphorylated controls. Platelet C5ar1 ligation induced reproducible PKC substrate phosphorylation (PKC phosphor Ser). As a control, we used the PKC activator PMA. PKC activation was quantified by dividing the phosphorylation signal by the total protein signal as well as unphosphorylated PKC α (Supplementary Fig. 23). Displayed are representative images of at least four independent experiments. o We also checked whether PKC activation also entails CXCL4 release by stimulating washed platelets with C5a and PMA. Both C5a and PMA induced significant CXCL4 release. Data are shown as the mean ± SEM (n = 5–16 independent experiments) and are displayed as the percentage of control of CXCL4 concentration in platelet supernatant measured by ELISA. The mean fluorescence intensity (MFI) of platelets in the vehicle-stimulated group represents 100%. *p < 0.01. p Furthermore, C5a induced an upregulation of phosphorylated Akt (Ser473), PI3K (Ser 47), GSK-3β (Ser 9), p44/42 MAPK (Thr202/Tyr204), PLCβ3 (Ser537). Displayed are representative images of at least four independent experiments. For quantification of phosphorylation, please refer to Supplementary Fig. 23. q Interestingly, C5a induced no regulation of phosphorylated PLCβ2 (Tyr1271) and PKA (α/β/γ catalytic subunit phospho T197). Activated Rap1 (Rap1-GTP) was analyzed using a Rap1 pulldown assay. No significantly altered amounts of Rap1-GTP could be detected. Displayed are representative images of at least four independent experiments. One-way ANOVA with Bonferroni’s post hoc test in b, d, e, g, i, m. Two-sided Student’s t test in k, l, o.

a role for the anaphylatoxin receptor C5aR1 expressed on platelets in ischemic and revascularizing tissue. We found that (i) platelets modulate specific endothelial functions relevant for angiogenesis through C5aR1, (ii) platelet C5aR1 mediates an inhibitory effect on collateral artery formation in ischemia-induced revascularization and (iii) capillary formation and pericyte coverage, (iv) platelets release CXCL4 upon C5a stimulation, and (v) the platelet C5aR1-mediated effect on vessel formation is mediated by preferential CXCL4 release in vitro and in vivo.

C5a receptor 1 is expressed on immune cells, epithelial cells, and endothelial cells. Interestingly, complement anaphylatoxin receptors have been reported to be expressed on platelets, which are cells of the hemostatic system, and their expression correlates with platelet activation markers in atherosclerosis, a disease featuring vascular inflammation. Platelets express a variety of complement-regulating proteins (reviewed in ref. 44). In this study, we observed an increased presence of platelets in revascularizing tissue, and we detected the anaphylatoxin receptor C5aR1 on platelets within angiogenic tissue. Future studies should address whether platelets or platelet-derived factors can be useful as biomarkers to detect or characterize angiogenic tissue. Importantly, we demonstrated the importance of platelet C5aR1 for endothelial functions in vitro and neovascularization in vivo using approaches of platelet reconstitution and pharmacological...
inhibition. Furthermore, we generated a platelet-specific C5aR1-deficient mouse model to specifically assess the platelet C5aR1-mediated effects.

The antiangiogenic relevance of platelets or platelet-derived mediators has been suggested for tumor angiogenesis. During the early stages of tumor growth, it was observed that platelet-derived thrombospondin 1 serves as a negative regulator of angiogenesis. Here we identified a complement-driven release mechanism for the platelet-derived chemokine CXCL4 as an inhibitor of vessel formation in vivo as well as endothelial functions in vitro. Italiano et al. and others showed that various proangiogenic and antiangiogenic factors are stored in distinct
subpopulations of platelet α-granula and that these factors can be released differentially in response to specific stimuli.\(^{25,27}\) However, our understanding of the mechanisms underlying this tailored secretion is poor. Angiostatin release from platelets, for example, follows different kinetics than VEGF secretion during thrombus formation.\(^{46}\) Furthermore, proteinase-activated receptors 1 and 4 can counter-regulate endostatin and VEGF release from human platelets.\(^{47}\) Here we identified the C5a/C5AR1 axis not only as a potent inducer of platelet activation but also demonstrate that this pathway drives the release of CXCL4, an α-granule component, which only partially colocalizes with other α-granule components, such as P-selectin.

Platelets contain both proangiogenic and antiangiogenic factors.\(^{27}\) Accordingly, platelets have also been described as...
proangiogenic mediators in conditions, such as ovarian cancer. In fact, platelet-derived products are used by clinicians to foster healing in orthopedic patients, partially due to their proangiogenic properties. Here we observed a specific inhibitory effect of platelet C5aR1 in ischemia-induced revascularization. Furthermore, platelets are important for the prevention of excessive hemorrhage from newly formed vessels. The net effect that platelets have on vessel formation may depend on the surrounding microenvironment or other factors, which should be further characterized in future studies.

Complement activation in ischemia-reperfusion injury (IRI) is well characterized (reviewed in ref. 50). However, this work shows complement deposition in hindlimb ischemia tissue without reperfusion. Reactive oxygen species (ROS) production is regarded as the main driver of complement activation in IRI by the classical pathway. Most likely, this mechanism also applies to the hindlimb ischemia model, where ROS production plays a critical role even in the absence of reperfusion. Furthermore, the activated endothelium in the ischemic tissue may bind gC1qR and thereby activate the classical complement pathway.

The complement system is known to be involved in the regulation of angiogenesis. An inhibitory role of C5aR1 on revascularization is in accordance with previous reports. In an earlier study, we showed that C5aR1 inhibits angiogenesis through the secretion of soluble VEGF receptor 1 (sVEGF1) from macrophages. Complement-mediated release of sVEGF1 has been described to be clinically relevant in placental dysfunction. How does this reconcile with our current finding that the complement-mediated inhibition of neovascularization is also dependent on platelets? Vessel formation and growth are ubiquitous mechanisms in organisms and require a finely balanced equilibrium of proangiogenic and antiangiogenic stimuli from different cells. Thus, several cell types may utilize the complement system as a means to modulate angiogenesis and revascularization. The complement system is one of the ontogenetically oldest plasma protein systems and mediates crosstalk among various cell systems in immunity and tissue repair processes.

Other studies have reported a decreased level of angiogenesis in C5ar1−/− mice and in mice deficient in complement factor 3. These studies investigated the role of the complement system in laser-induced choroidal neovascularization, a model for age-related macular degeneration, in which the retinal pigment epithelium plays a unique pathophysiological role. Given the versatility of the complement system, it is possible that it regulates angiogenesis in a context-dependent manner, such as stimulating angiogenesis in the eye but eliciting different effects in other tissues and experimental models.

In the present study, we identified a mechanism underlying the inhibitory role of platelet C5aR1 in revascularization. The antiangiogenic factor CXCL4 is released from platelets upon stimulation with C5a and mediates this effect in a receptor-specific manner as verified by in vitro and in vivo approaches. At this point, we cannot rule out that other platelet factors besides CXCL4 released upon C5a stimulation mediate the observed revascularization-modulating effects. These factors will have to be identified and studied in future investigations.

CXCL4 is a pro-coagulant, which binds and neutralizes heparins and thus impacts not only on hemostasis but also acts as an inhibitor of angiogenesis. The functions of CXCL4 in vascular homeostasis are complex. In vivo studies using various transgenic mouse models demonstrated an important role for CXCL4 in thrombosis. Neutralization of CXCL4 identified its central role for the anticoagulant effect of heparins. Others have shown in a primate model that physiologically relevant concentrations of CXCL4 stimulate the generation of activated protein C (APC), which suggests that CXCL4 plays a previously underappreciated role in the soluble coagulation cascade. As one of the underlying mechanisms, it was suggested that CXCL4 binds with relative high specificity and high affinity to thrombomodulin and protein C, interactions which may enhance the affinity of the thrombin–thrombomodulin complex for protein C, thereby promoting the generation of APC. Interestingly, CXCL4 can alter the structure of fibrin, thereby contributing to the sealing of blood clots.

Previous findings identified C3a and C5a as activators of platelet activation, aggregation of gel-filtered human platelets as well as serotonin release. For C5a, we detected increased platelet activation; however, we found no significant dense granule release from murine platelets. At this point, we cannot entirely explain why C5a induces a2b/b3 integrin activation but not aggregation. Further profound studies are needed to characterize this observation. It is well appreciated that C5 deficiency protects mice from lethal thrombosis. Mice lacking C5 experience milder thrombocytopenia, consumptive coagulopathy, and liver injury than C5-competent mice in a model of histone-induced liver injury. We found that C5-deficient platelets secrete CXCL4 upon C5a stimulation. Interestingly, the absence of C5aR1 had no effect on in vivo hemostasis or bleeding time in our study suggesting an effect on revascularization, which is distinct from the hemostatic functions of activated platelets.

Strikingly, we observed that targeting CXCL4 was sufficient to block the effect of platelet C5aR1 on vessel formation. These findings suggest that C5aR1-mediated effects can be blocked pharmacologically and that CXCL4 is the predominant angiogenic factor released upon C5a stimulation of platelets. However, at this point, we cannot entirely rule out that in addition to CXCL4 other platelet factors are released upon C5a stimulation and contribute to the observed revascularization-modulating effects.

The C5a receptor 1 is a seven transmembrane G protein-coupled receptor, which transmits its signals via the Gq subunit that induces PI3K-dependent Akt phosphorylation and the Gαq subunit, leading to PKA activation. Overall, C5aR1-dependent signaling is complex involving PI3K and Akt-dependent pathways, PKC and MAPKs, IkBα, NFκB, and sphingosine 1 phosphate (SIP). In T cells, it has been shown that C5aR1 transmits its signal via PI3K and Akt. In neutrophils, C5a-induced PI3K signaling has been demonstrated in macrophages PI3K, Akt, MEK1/2, and ERK1/2 signaling. Furthermore, PLCβ has been implicated in C5aR1 signaling, particularly if PKC is involved. Here we demonstrate a signaling pathway involving PI3K, Akt, PLCβ, PKC, and Erk1/2. No C5a-dependent signal in platelets was detected for PLCγ2, PKA, and Rap1. This is important, as the C5aR has been shown to signal via Rap1 in platelets, recently. A PI3K-Akt-dependent pathway leading to PKC activation has already been observed for platelets, as in PAR-1-driven CXCL12 release.

We found an increased abundance of CXCL4 in ischemic muscle tissue of C5aR1-deficient mice. Platelets are the major source of CXCL4 in organisms. In vitro, we identified CXCL4 as the major component released from platelets in response to C5a stimulation mediating the inhibitory effect on endothelial functions, such as tube formation and migration. This effect of CXCL4 is in accordance with previous findings. In vivo, arteriogenesis or collateral artery outgrowth is considered the primary mechanism responsible for hindlimb revascularization after femoral artery ligation. Indeed, we observed increased collateral artery formation in platelet-specific C5aR1-deficient mice associated with their phenotype of enhanced revascularization. We observed differences in hindlimb revascularization between the assessed genotypes (WT versus C5ar1−/− or Pf4-cre+/− C5ar1fl/fl) after the onset of ischemia. Although we have quantified the vascular network in nonischemic control limbs, we cannot, at this point, entirely rule out that platelet C5aR1 impacts also on the formation of pre-existent collaterals in the mouse hindlimb.
While in the distal hindlimb no differences in artery size or number could be detected, we noted differences in vessel density indicating an additional angiogenesis-related process. This is in accordance with recent reports attributing an important additional role to angiogenesis in hindlimb revascularization. A key to understanding the phenotype of ameliorated revascularization in platelet-specific C5aR1-deficient mice might be the increased presence and an amplified coverage of vessels with pericytes, which we observed in platelet-specific C5aR1-deficient animals. Pericytes have been shown to express CXCR3, the receptor for CXCL4. Exploring the effect of the C5aR1-CXCL4 system is recognized as a promising strategy in drug discovery. The response in ischemic diseases such as coronary or peripheral arteries will have to be addressed in further studies.

Here we successfully targeted C5aR1 in vivo using a C5aR1 antagonist (avacopan) and a C5aR1-deletion mouse line expressing Cre-recombinase under control of the platelet- and megakaryocyte-specific promoter to generate platelet-specific C5aR1-knockout mice. For characterization of this new strain, platelets, leukocytes, and red blood cells were counted using a Sysmex cytometer (Sysmex KX-21N, Görlitz, Germany).

**Genotyping of floxed G-Fsp-C5ar1−/− mice.** For genotyping, we used ear biopsies. DNA was extracted from the tissue using a Qiagen Blood and Tissue DNA kit. PCR was run under the following conditions: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. Then the samples were loaded onto a 1.5% TAE-agarose gel. The amplification products were detected by ethidium bromide staining.

**Mouse hindlimb ischemia model.** We used a previously described protocol to induce hindlimb ischemia. Briefly, the femoral artery of mice aged 10–12 weeks was ligated immediately distal to the branch point of the caudal femoral artery and the epigastric artery. Tissue perfusion was assessed preoperatively, immediately post-ligation, and at 2, 4, 6, 8, 10, and 14 days after the surgical intervention, as previously described. Blood flow in the hindlimb was analyzed using an infrared LDV (Moor Instruments, Axminster, UK) at the site under measurement.}

**Methods.**

**Mice.** All animal procedures were approved by the regional animal care and use committee of the District of Tübingen, Baden-Württemberg (Konrad-Adenauer-Straße 20, 72072 Tübingen, Germany). All animal experiments were performed in accordance with the German law guidelines of animal care. C57BL/6 (WT) mice were originally acquired from Jackson Laboratories but were bred in our own animal facility. Here the light cycle was 12 h, temperature 20–22 °C, humidity 40–60%. C5aR1-deficient mice (CD88, C5ar1−/−) have been previously described elsewhere. They were kindly provided by Dr. C. Gerard (Harvard Medical School, Boston, MA, USA) and bred in our animal facility on the C57BL/6 background. Furthermore, we used mice deficient for C5 (B6/Cg-TgIns2-GP/pto) bred in the animal facility of the University of Lübeck (Germany), which have been described previously. Mice deficient in C5aR2 as well as tdTomato-C5aR2 KO mice were described previously. Furthermore, C5aR-deficient mice (B6.129x1-C5aR1tm1Raw) were used. Mice deficient for the CXCR chemokine receptor 3 (CD183, Cxcr3−/−) were purchased from Jackson Laboratories and have been described elsewhere. Mice deficient in CXCL4 (Cxcl4−/−) were bred in our own animal facility (on a C57BL/6 background). G-Fsp-C5ar1−/− mice have been described previously. Briefly, GFP-C5aR1-knock in mice were generated by gene targeting. (Ac)GFP and an internal ribosomal entry site (IRES) were inserted adjacent to the coding exon of C5aR1. C5aR1-knockout mice, the AcGP IRES C5ar1 cassette was ranked with two loxP sites. We then crossed these mice with a Pf4-cre strain expressing Cre-recombinase for characterization of this new strain, platelets, leukocytes, and red blood cells were counted using a Sysmex cytometer (Sysmex KX-21N, Görlitz, Germany).

**Sample preparation for microCT.** For microCT analysis, 9 days after induction of ischemia, mice were injected with 50 μl heparin (i.p.). After 10 min, the mice were euthanized, the thorax was opened, and the aorta was exposed. A catheter was carefully inserted into the descending aorta and manually fixed with sutures, and
the liver incised several times to permit drainage of blood. Then mice were per-
fused with PBS (37 °C, 80 ml) containing heparin and nitroglycerin to remove the blood. The liver, containing the Fusedeca AG (Münz AG, Switzerland) was prepared according to a previously published protocol102 and then injected through the catheter in the aorta until leakage from the liver was observed. Thereafter, it was left to polymerize for 1 h at room temperature (RT). Legs were collected and immersion-fused in 2% PFA–PBS for 48 h followed by fixation in 70% ethanol. For tomographic imaging, samples were immersed in paraffin (3 h) before they were covered by a thin layer of paraffin and let air dry.

Image acquisition by microCT. For tomographic imaging, we shaped the samples minimally with a scalpel, wrapped the samples in X-ray transparent melamine resin foam (Basotec, SWILO GmbH, Sta. Maria, Switzerland) and mounted them in a standard sample holder inside a Bruker SkyScan1172 high-resolution micro-
tomography machine (Bruker microCT, Kontich, Belgium).

The X-ray source was set to a voltage of 70 kV and a current of 142 µA, with a 0.5-mm Al filter in the beam path. For the relatively low-resolution scans we show here, we recorded a set of 315 projections of 820 × 1242 pixels at every 0.6° over a 180° sample rotation. Every projection was exposed for 1410 ms, and three projections were averaged to one to reduce image acquisition noise. To cover the entire leg, we performed an overscan with three subscons stacked along the long axis of the leg. This resulted in approximately 35 min of imaging per sample and an isometric voxel size of 21.6 µm in the final data sets.

MicroCT reconstruction and vessel visualization. The projection images were then subsequently reconstructed into a 3D stack of 8-bit gray value PNG images with NRecon (Bruker, Version: 1.7.4.2). After reconstruction, we visualized the legs with MeVisLab (Version 3.1 (2018-06-26 Release), MeVis Medical Solutions AG, Bremen, Germany). We extracted the bone with a gray value threshold-based region growing algorithm with manually placed seed points inside the bone. The vessels were extracted with a “Vessels” filter, which is calculated as a function of the Hessian matrix. Threshold-based segmentation of this Vesselsness measure provided the vessels for a visualization with the MeVis Path Trace, which is a completely refactored fork of the ”ExposureRender” framework by Thomas Kroes103. For quantitative analysis of the vascular network within the hindlimbs, we performed an isometric 3D segmentation of the vessels in the beam path. For the relatively low-resolution scans we show here, we recorded a set of 315 projections of 820 × 1242 pixels at every 0.6° over a 180° sample rotation. Every projection was exposed for 1410 ms, and three projections were averaged to one to reduce image acquisition noise. To cover the entire leg, we performed an overscan with three subscons stacked along the long axis of the leg. This resulted in approximately 35 min of imaging per sample and an isometric voxel size of 21.6 µm in the final data sets.

Quantitative real-time PCR (qPCR). Total RNA was isolated from the hindlimb muscles of mice 1 week after the induction of hindlimb ischemia; total RNA was also isolated from the nonischemic contralateral control hindlimb muscles. The tissue was perfused with PBS and ground cryogenically, and RNA was isolated using TriFast (p gastro, WVR, Radnor, PA, USA) followed by purification with the RNeasy Mini Kit (Qiagen, Hilden, Germany) including DNase I treatment (Qiagen) according to the manufacturer’s instructions. The RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA quality was evaluated by an Agilent Bioanalyzer 2100 (Agilent Techno-
logy, Santa Clara, CA, USA). To generate cDNA, total RNA was converted into cDNA using the Transcripter First Strand cDNA Synthesis Kit (Roche Diagnostics, Rotkreuz, Switzerland). The cDNA samples were subjected to qPCR amplification using the QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler 480 (Roche). The primers were used as follows: actin, 5′-ctgcgttctcgccgcca-3′ and 5′-gaggctcacgctctggc-3′; Gapdh, 5′-ccctcatt-gacctcaactcaatgg-3′ and 5′-ggaggctcacgcctctggctc-3′; CD242b, 5′-ctgctcattcacaacaag-3′ and 5′-ttctgaaggtcctgcaca-3′; CD41, 5′-ttgcttcgctcggcagca-3′ and 5′-ttctttgacggtctcaga-3′; CSaR1, 5′-tagatctggctgccctct-3′ and 5′-ctaccatt-ggaccggct-3′ (see also Supplementary Data 1). The following PCR cycling parameters were used: 95 °C for 15 min; 50 cycles of 15 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C. Dissoication curve analysis was performed on all PCR products to ensure that specific PCR products were generated. PCR was performed in triplicate for every product, and the data were analyzed using the LightCycler software (v.1.5, Roche) with the Ct advanced relative quantification function.

For the detection of CXCL4 mRNA in megakaryocytes, RNA was isolated as described for hindlimb muscle tissue. Transcription into cDNA was performed using Random hexamer primer mix (Carl Roth GmbH, Karlsruhe, Germany). MMLV RT (Thermo Fisher Scientific), and RiboLock (Thermo Fisher Scientific). Equal amounts of cDNA were applied for qPCR amplification using Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA) and 7900 HT Real-Time PCR System (Applied Biosystems). The following primers were used: cxcl4 5′-tcctgaggtccggctcag-3′ and 5′-cttccaagttcgcggtc-3′; gapdh 5′-ggcgacacatcgctcaca-3′ and 5′-cccttcgctctgggatgac-3′. The following PCR cycling parameters were used: 50 cycles of 10 s at 94 °C and 60 s at 60 °C, and finally 15 s at 95 °C, 15 s at 60 °C, and 15 s at 95 °C. The data were analyzed using the Delta-Delta-CT-Method.

In vivo Matrigel plug assay. The in vivo Matrigel plug assay was performed as previously described104 with some modifications. Two aliquots of Matrigel (0.5 ml, Becton Dickinson, Franklin Lakes, NJ, USA) were injected in the back of each mouse. At 48 h after injection, mice were sacrificed, and the entire hindlimb was isolated and imaged with the CT scan as described above. Tomograms were used to calculate the volume of the plugs using an in-house homemade software. Other Matrigel experiments involved intravenous injection of a blocking anti-CXCL4 antibody (10 µg/mouse, rat-anti-mouse IgG2b, Clone 1409/10, R&D, Minneapolis, MN, USA) or control IgG2b (BioLegend, San Diego, CA, USA) as previously described105. Furthermore, in some experiments, Matrigel was supplemented with the CSaR1 antagonist PMX33 (AcF[OPdChaWR], Tocris Bioscience, R&D) or a control peptide (PMXControl, AcF[OPdChaADR], Tocris) at 5 µg for each Matrigel plug (50 µl), which was shown to be a successful dose for local injection, previously106. After 7 days, mice were sacrificed, and the Matrigel plugs were fixed with 4% PFA, processed for histology (frozen sections), and stained with hematoxylin and eosin using standard staining protocols and reagents. Bright-field images were obtained with a Nikon Optiphot-2 microscope equipped with a ×2 plan-apochromat (N.A. 0.08) objective lens and a digital sight DS-5M camera using the Nikon NIS elements BR software (v.3.2, Nikon Instruments, Tokyo, Japan) for image acquisition and analysis. For readout, the ratio of the vessel area to the total Matrigel plug area was calculated. Furthermore, immunofluorescent staining using IB4 was performed to visualize plug neovascularization as described in immunofluorescence microscopy studies.

Intravital microscopy and bleeding time. Bleeding time experiments were carried out principally as described before107. Briefly, mice were anesthetized, and a 3-mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gentle absorption of the blood with filter paper at 20-s intervals without making contact to the wound site. Intravital microscopy and induction of platelet thrombus formation in vivo were carried out as described before108.

Human samples. For experiments with human material, written informed consent was received from participants prior to inclusion in the study. The study was approved by the institutional ethics committee (270/2011BO31) and complies with the Declaration of Helsinki and the good clinical practice guidelines.

Flow cytometry. Most flow cytometry experiments were performed in citrated whole blood, which was drawn from mice as described below. Blood was diluted 1:5 using Tyrode’s solution (with 1 mM CaCl2 and 0.5 mM MgCl2). In some experiments, blood was diluted at the ratio of blood to filter paper at 20-s intervals without making contact to the wound site. Intravital microscopy and induction of platelet thrombus formation in vivo were carried out as described above108.

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a buffer containing ionomycin (3 μg/ml), nigericin (2 μg/ml), and CCP (10 μM). After addition of calcium, level was acquired for 30 s, then the stimulant was added. In another experiment, platelets isolated from Matrigels were stimulated with ADP (0.1 mM, Chrono-log Corporation) for 30 min at 37 °C and stained with CD62p-FITC (rat anti-mouse P-selectin, clone Wug. E9) or an appropriate isotype control (rat IgG1, both from Emfret Analytics) for the assessment of platelet activation.

Most flow cytometry experiments were performed using a Beckman Coulter Cytoflex S 4-laser instrument (Beckman Coulter, Krefeld, Germany) immediately after sample preparation and staining. Unless otherwise stated, specific mAb binding was expressed as the mean fluorescence intensity (MFI) of 25,000 events in the absence of specific antibody. Data were analyzed using the CytExpert software (v.2.4, Beckman Coulter).

For the characterization of C5aR1 expression on immune cells in Pf4cre-GFP-C5ar1flPfl and Pf4cre-GFP-C5ar1flPfl mice, 200 μl of citrate whole blood were used per sample. Ten microliters of FcB Block (Millenium Biotech) was added to each sample followed by 50 μl of antibody dilution of pre-titrated antibodies. Antibodies were F4/80-BV785, CD11b-BV605, and CD14-APC (all BioLegend) and CD45 PE-Cy5.5 (eBioscience) and incubated for 20 min in the dark. Samples were washed and resuspended in FACS-Buffer for analysis. Analysis was performed on a LSRIIFortessa flow cytometer (BD Bioscience) with a violet (400 nm), blue (488 nm), yellow/green (561 nm), and red (640 nm) laser. Specific mAb binding was expressed as the MFI, and data were analyzed using the Flojo software (v.10, Tree Star, Ashland, OR, USA).

ATP release. Aggregation of platelets was estimated using light transmission aggregometry using a lumiaggregometer Model 700 (ChronoLog). Washed murine platelets were adjusted to a concentration of 250 × 10^3 platelets per μl in Tyrode buffer pH 7.4 and were preincubated with PBS or 20 ng/ml C5a for 10 min. After adjusting the measurement according to the manufacturer’s protocol, platelets were activated with 0.25 μg/ml CRP or with 2.5 μM/ml thrombin for 10 min at 37 °C and a stirring speed of 1000 rpm. Analysis was performed using the aggreglink8 software (ChronoLog).

**Aggregometry.** Aggregation of platelets was estimated using light transmission aggregometry using a lumiaggregometer Model 700 (ChronoLog). Washed murine platelets were adjusted to a concentration of 250 × 10^3 platelets per μl in Tyrode buffer pH 7.4 and were preincubated with PBS or 20 ng/ml C5a for 10 min. After adjusting the measurement according to the manufacturer’s protocol, platelets were activated with 0.25 μg/ml CRP, or with 2.5 μM/ml thrombin, or further agonists for 10 min at 37 °C and a stirring speed of 1000 rpm. Analysis was performed using the aggreglink8 software (ChronoLog). For human samples, platelet-rich plasma (PRP) was generated as described above and used for aggregometry with platelet-poor plasma as a control; stimulation was performed using ADP and CRP as well as C5a at defined concentrations.

**Immunofluorescence microscopic studies.** From ischemic murine hindlimb muscle tissue, 8-μm-thick sections of the gastrocnemius muscle were processed for immunofluorescence staining. Matrigel sections were processed accordingly. For visualization, the following primary antibodies were used: Donkey F(ab')2 anti-mouse IgG (H + L, 1:50), rat anti-mouse CD88 antibody (C5aR1, BioLegend, sc-8419, Santa Cruz, 1:50), rat anti-mouse CD62P antibody (CSaR1, BioLegend, #158185 1:60), and rabbit polyclonal CXCL4 antibody (PA1A72Mu01, Cloud Clone Corporation, Houston, TX, USA, 1:12). For visualization, the following secondary antibodies were used: donkey anti-mouse preadsorbed IgG Alexa Fluor 488 (ab150109, Abcam, 1:200), donkey anti-rabbit highly cross-adsorbed IgG Alexa Fluor 647 (Thermo Fisher, 1:100), and donkey anti-rat preadsorbed IgG Alexa Fluor 568 (ab175475, Abcam, 1:200). Images were acquired using a Zeiss LSM 800 confocal laser scanning microscope with Zeiss ZEN 2.3 (blue edition) software or an Olympus Fluoview 1000. Subsequent image analysis was performed with Image Pro Plus (Ver. 7.0). For analysis of single platelet granule composition, we took snapshots of single platelets from confocal immunofluorescence images, and granules were differentiated by distinct regions of interest in a fluorescence scatter plot using Image Pro Plus (Supplementary Fig. 10).

**For the characterization of platelet abundance in ischemic hindlimb tissue, CD42b-positive signals were quantified according to size characteristics in an automated fashion as single platelets or micro thrombi using Image Pro Plus (Ver. 7.0).**

**ATP staining of megakaryocytes,** we blocked samples with 5% donkey serum and used the following primary antibodies: purified rat anti-mouse CD62P (BD Pharmingen, San Jose, CA, USA, 1:50) and rabbit polyclonal CXCL4 antibody (PA1A72Mu01, Cloud Clone Corporation, Houston, TX, USA, 1:12). The following secondary antibodies were used: Donkey F(ab')2 anti-rat IgG (H + L)–Alexa Fluor 647 (#712-606-153, Diana, Hamburg, Germany, 1:250) and Donkey anti-rabbit IgG (H + L)–Alexa Fluor 568 (#715-655-152, Diana, Hamburg, Germany, 1:250). Cell nuclei were stained with DAPI (green signal). Images were acquired using a Zeiss LSM 800 confocal laser scanning microscope with Zeiss ZEN 2.3 software, and analysis was performed by the Image Pro Plus software as illustrated in Supplementary Fig. 10.

**Isolation of human and murine platelets and generation of platelet releasate.** Human platelet and venous washed platelet solutions were prepared from human volunteers. Blood was drawn from the antecubital vein into acid–citrate–dextrose (ACD) buffer and centrifuged at 430 × g for 20 min. PRP was removed and added to HEPS-buffered Tyrode’s solution (2.5 mM HEPS, 150 mM NaCl, 1 mM KCl, 0.35 mM CaCl2, 0.36 mM MgCl2, 5.5 mM glucose, and 1 μg/ml BSA) and subsequently centrifuged at 900 × g for 10 min. The resulting platelet pellet was resuspended in HEPS-buffered Tyrode’s solution (pH 7.4, supplemented with 1 mM CaCl2 and 1 mM MgCl2). Platelets were then preincubated with a C5aR1 antagonist (10 μM; PMX3X, AfC[OPaChA][AD], Tocris) for 30 min at 37 °C or control peptide (AfC[OPaChA][AD]) as previously described. Subsequently, platelets were coincubated with endothelial cells to assess the effect of platelet C5aR1 blockade on endothelial tube formation in vitro.

To isolate murine platelets, blood was drawn from the heart or the retro-vascular plexus, collected in ACD buffer, and centrifuged at 120 × g for 20 min. PRP was removed and added to HEPS-buffered Tyrode’s solution (pH 7.4) and subsequently centrifuged at 14,000 × g for 10 min. The resulting platelet pellet was carefully resuspended in HEPS-buffered Tyrode’s solution (pH 7.4). HEPS-buffered Tyrode’s solution was supplemented with 1 mM CaCl2 and 1 mM MgCl2. The platelet content was quantified using a Sysmex cytometer (Sysmex KX-21N, Götitz, Germany) and was adjusted to the required concentration. Subsequently, either the platelets were stimulated, the supernatant was collected or lysed, and the platelets were used for single-cell staining or the platelets were used at specific concentrations for co-incubation with endothelial cells, resuspended in Matrigel for injection into mice, or used in flow cytometric analyses. For some experiments, platelet isolation was performed using activation inhibitors prostacyclin (0.5 μg/ml, Sigma Aldrich) and apyrase (0.02 U/ml, Sigma Aldrich). As agonists for stimulation of murine as well as human platelets, we used CRP (CmbCol) at specified concentrations, ADP (Chrono-log Corporation), C5a (R&D), thrombin (Roche), TRAP-6 (TRAP, Dasyus, Holzheim, Germany), thromboxane A2 (U46619, TxA2, R&D), phorbol 12-myristate 13-acetate (Abcam) at 100 ng/ml (approximately 160 nM), and the CsA2 agonist P32 (used at 1 μM), which has been previously described. For some experiment, platelets were preincubated with the CsA2 agonist PMX205 (used at 15 μM for 30 min at 37 °C, Tocris) or control peptide. Subsequently, platelets were stimulated with CsA. If not otherwise stated, stimulation with CsA (R&D) was performed at a concentration of 20 nM for 30 min at 37 °C as previously described for other cell types. Following CsA stimulation, the supernatant was collected by centrifugation at 14,000 × g for 4 °C and analyzed or used to stimulate endothelial cells. The pellet was lyzed using RIPA buffer supplemented with protease/phosphatase inhibitor (PK, Thermo Fisher) and antagonized by western blotting.

For single platelet staining, 5 μM ADP was used along with C5a for 5 min or in other experiments for 10 min at 37 °C. After stimulation, platelets were fixed with PFA. Resting, CsA-stimulated, or ADP-stimulated fixed platelets were immobilized on poly-L-lysine coated coverslips and stained using the antibodies and staining conditions specified above.
For lysate preparation, 100 x 10^6 platelets were lysed using RIPA buffer and protease inhibitor (Halt, Thermo Fisher) in a volume of 100 μl. In other experiments, the amount of input protein for western blot analysis was determined following CXC4 following a protocol that was previously described. Briefly, supernatants were incubated overnight at 4°C with 100 μg/ml anti-CXC4 antibody (rat IgG2b, MAB 395, R&D) or IgG2b control (BD Biosciences, San Jose, CA, USA). Then washed protein G-coupled sepharose beads (4 Fast Flow, GE Healthcare, Little Chalfont, Buckinghamshire, UK) were added to the supernatant and incubated for 2 h at 4°C, and then immune complexes were removed by centrifugation at 14,000 × g for 5 min. The resulting CXC4-depleted supernatant was subsequently incubated with endothelial cells.

Enzyme-linked immunosorbent assay. Platelet releasates were analyzed by ELISA using a mouse PF4/CXC4 QuantiKine ELISA Kit (R&D), a membrane-bound antibody array (Proteome Profile Mouse Angiogenesis Array Kit, AR0105, R&D), a Serotonin ELISA Kit (BA E-8900, LDN, Nordhorn, Germany), a Hexasamidinase B (HEXB) ELISA Kit (SEA57Mu, Cloud Clone Corporation), a Mouse VEGF ELISA (ab209882, Abcam), a Thrombospinomin 1 ELISA Kit (THBS1, ABIN6574175, Antibodies-Online, Aachen, Germany), an endostatin COL1A1/ES ELISA Kit (Mouse collagen type XVIII a 1 Endostatin ELISA Kit, MBS701673, MyBioSource.com, San Diego, USA), and a TIMP-1 ELISA Kit (Mouse TIMP-1 QuantiKine ELISA, MTA100, R&D). For human samples, we used a human CXC4/PF4 QuantiKine ELISA Kit (R&D), a human thrombospomin-1 Quantikine ELISA Kit (R&D), a human PDGF BB ELISA Kit (ab100624, Abcam), and a human Endostatin ELISA Kit (RayBiotech, Peachtree Corners, GA, USA).

For ex vivo analysis, tissue homogenates were prepared from ischemic and nonischemic hindlimbs of C57Bl/6 mice by cryo-grinding the tissue with liquid nitrogen, and pellets were then washed in PBS, resuspended in PBS, and incubated for 2 h at 4°C. The resulting suspension was pelleted by centrifugation at 12,000 g for 10 min, and supernatants were used for the ELISA.

Western blot analysis. Platelet lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing and non-reducing conditions using a 12% gel and subjected to western blot. Whole-protein detection was performed, and nitrocellulose membranes were incubated with primary rat IgG2b CD88-specific antibody (10/92, Hycut Biotech, Uden, Netherlands). We used goat anti-rat 800 CW (LI-COR, Bad Homburg, Germany) as a secondary antibody and REVERT total protein stain (LI-COR) for normalization. Membranes were scanned with the Odyssey Infrared Imaging System (LI-COR) and analyzed.

For other experiments, platelets were lysed in RIPA buffer (150 mM NaCl, 50 mM TRIS, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, and protease inhibitor cocktail (PI)) and boiled in SDS sample buffer for 10 min at 95°C. Membranes were probed with the following primary antibodies: anti-mouse C3aR (Clone D12; 2 μg/ml); thrombospondin 1 (rabbit polyclonal anti-T197, Abcam, #ab216572, 1:500); catalytic subunit phospho T197, Abcam, #ab975991, 1:1000; phospho PKA (Ser) catalytic subunit T197, Abcam, #ab216572, 1:500; phospho Akt (Ser473, Cell Signaling Technology, #9271S, 1:1000), total Akt (Rabbit monoclonal Akt pan C677, Cell Signaling Technology, #4691S, 1:1000); phospho P38 MAPK (Phospho Thr180/Tyr182, Cell Signaling Technology, #4076, 1:1000); phospho PI3K (Phospho PI3 Kinase p85 Thr458/p55 Thr419, Cell Signaling Technology, #22288, 1:500); phospho P44/42 MAPK (Erk1/2, Cell Signaling Technology, #4690, 1:1000); Schematic diagrams for signaling pathways were constructed using the Servier Medical Art (www.schematicdiagram.com).

Cell proliferation assay, scratched wound assay, tube-formation assay, and spheron tube-formation assay. Endothelial cell proliferation was determined by measuring the number of adherent cells at different time points. Briefly, MHEC-5T (3 x 10^5 cells/well) or MLECs (3 x 10^5 cells/well) were plated onto 96-well plates with fibrinogen (4 μg/ml, Merck Millipore) at 2 mg/ml at 4°C overnight. Murine WT or Cxcr1−/− platelets were isolated as described and stimulated with ADP (10 μM) and washed with PBS, fixed (4% PFA), and stained with DAPI. For analysis of cell migration using a microscope (BX41, Nikon, Japan), images were captured every 5 min, and the cells were then transferred to T75 culture flasks coated with 0.4% gelatin. A second puncturation step was performed when the cells were confluent, and the cells were then transferred to T75 flasks and harvested when near confluence. Cell proliferation was verified by flow cytometric analysis for the expression of CD105, CD31, and CD144 (Supplementary Fig. 2). Antibodies used in MLECs were not included as described and performed according to the manufacturer's instructions. Readouts were taken after 18 h.

A scratched wound assay was performed as previously described. Briefly, MHEC-5T (2 x 10^5 cells/well) or MLECs (3 x 10^5 cells/well) were plated onto 24-well plates and incubated for 24 h. Subsequently, the confluent monolayer was wounded with a plastic pipette tip, generating a 1-mm-wide gap. Cells were then incubated with washed platelets resuspended in medium (2 x 10^5 cells/well) or vehicle control. In other experiments, cells were coincubated with the supplement of platelets generated as described earlier. As a measure of endothelial migration, the area repopulated with MHEC-5T or MLECs after 16 or 28 h was quantified by phase-contrast microscopy.
For the in vitro tube-formation assay, MHEC-ST, HUVECs, or MLECs (3 × 10^4 cells/well) were plated onto Matrigel-coated 48-well tissue culture plates in endothelial culture medium containing 2% FBS or on ibidi slides (ibidi, Planegg, Germany) with medium containing 2% FBS. Cells were coincubated with washed platelets (2 × 10^5 cells/well), vehicle control, or platelet supernatant. After 6.5, 12, or 24 h, depending on the cell type, tube formation was imaged by phase-contrast microscopy. Furthermore, preincubation of platelets with antagonists, such as PMAx5 (30 min at 37°C, Tocris), was performed as described in the figure legends. Using Axiovision software (v.3.2, Zeiss), the total tube length in multiple high-power fields per well was assessed and quantified as the average total tube length per well.

For the spheroid tube-formation assay, endothelial cell spheroids of defined cell numbers were generated as previously described[116]. In brief, HUVECs were suspended in culture medium containing 0.2% (wt/vol) carboxymethylcellulose (Sigma-Aldrich) and seeded in round-bottom 96-well plates to form a single spheroid per well (400 cells/spheroid). The spheroids were then embedded into rat collagen I (BD) and the spheroid-containing collagen was rapidly transferred into warm 24-well plates and allowed to polymerize for 30 min. Then 100 μL of endothelial cell medium supplemented with platelet supernatant or vehicle control was added to the wells. After 24 h, pictures were acquired using an Axiovert 100 microscope and a Plan-NEOFLUAR 10x objective. In vitro capillary sprouting was quantified by measuring the cumulative length of sprouts per spheroid using the Axiovision software. The mean cumulative sprout length per spheroid was calculated after the evaluation of 10–15 spheroids/condition.

**Embryonic hindbrain angiogenesis model.** At day E11.5, the hindbrains of murine WT and C5aR1−/− fetuses were successfully prepared, isolated, and stained as whole mounts using a previously published protocol[117]. Briefly, isolated hindbrains were fixed in 4% PFA for 2 h at RT and washed three times. Afterwards, they were blocked using 0.1% Triton-X (Sigma-Aldrich) and 10% normal donkey serum (Sigma-Aldrich) in PBS at 4°C overnight and then incubated with Alexa-488-conjugated Griffonia simplicifolia IB4 (1200, Thermo Fisher) for 3 h at RT. The ventricular plexus was analyzed using a Zeiss LSM5 EXCITER confocal laser scanning microscope (Zeiss) with a ×10 objective. 3D confocal images were reconstructed with ImageJ (NIH). Analysis of the vascular networks was performed using a previously described method[118] and AngioTool software (National Cancer Center, Rockville, MD, USA) as recently described[119].

**Data presentation and statistics.** The results are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad Prism 9. Comparisons between two groups were performed using Student’s t test. Comparisons between more than two groups were conducted using analysis of variance (ANOVA), and comparisons between two groups with several time points were performed using two-way ANOVA. Bonferroni’s post hoc test was performed for all ANOVA analyses. p < 0.05 was considered statistically significant.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** All relevant data are available upon request from the corresponding author. Source data are provided with this paper.

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37. Etafani, J. et al. Control of angiogenesis by galectins involves the release of platelet-derived proangiogenic factors. PLoS ONE 9, e96402 (2014).

38. Zaslavsky, A. et al. Platelet-derived thrombospondin-1 is a critical negative regulator and potential biomarker of angiogenesis. Blood 115, 4605–4613 (2010).

39. Kisucka, J. et al. Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. Proc. Natl. Acad. Sci. USA 103, 855–860 (2006).

40. Packham, I. M., Watson, S. P., Bicknell, R. & Egginton, S. In vivo evidence for platelet-induced physiological angiogenesis by a COX driven mechanism. PLoS ONE 9, e107503 (2014).

41. Stellos, K. et al. Platelets in regeneration. Semin. Thromb. Hemost. 36, 175–184 (2010).

42. Klement, G. L. et al. Platelets actively sequester angiogenesis regulators. Blood 113, 2835–2842 (2009).

43. Rutkowski, M. J., Sughrue, M. E., Kane, A. J., Mills, S. A. & Parsa, A. T. Cancer endothelial cell migration without affecting endothelial cell proliferation and monocyte recruitment. J. Thromb. Haemost. 9, 209–219 (2011).

44. Rabinet, M. J., Huet, E. & Boulay, F. The N-formyl peptide receptors and the complement cascade. Nat. Rev. Immunol. 10, 1407–1418 (2010).

45. Egan, K. et al. Platelet adhesion and degranulation induce pro-survival and pro-angiogenic signalling in ovarian cancer cells. PLoS ONE 6, e26125 (2011).

46. Bone, C. et al. Angiogenic properties of sustained release platelet-rich plasma: characterization in vitro and in the ischemic hind limb of the mouse. J. Vasc. Surg. 50, 870–879 (2009).

47. Noris, M. & Remuzzi, G. Overview of complement activation and regulation. Semin. Nephrol. 33, 479–492 (2013).

48. Carlucci, E. et al. Oxidative stress-induced miR-200c disrupts the regulatory T cells. Adv. Wound Care 2, 515–519 (2013).

49. Yn, W., Ghebrehiwet, B., Wekslers, B. & Peerschke, E. I. Classical pathway complement activation on human endothelial cells. Mol. Immunol. 44, 2228–2234 (2007).

50. Carmeliet, P. Angiogenesis in life, disease and medicine. Nature 438, 932–936 (2005).

51. Pelosi, P. et al. Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. J. Immunol. 174, 491–497 (2005).

52. Thurnham, D. J. et al. Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. J. Biol. Chem. 284, 16939–16947 (2009).

53. Mihara, Y., Sato, T. & Tsuzuki, H. Effect of anti-inflammatory drugs on angiogenesis in breast cancer. J. Cell. Biochem. 115, 1332–1341 (2015).

54. Rikfalvi, A. Platelet factor 4: an inhibitor of angiogenesis. Semin. Thromb. Hemost. 30, 379–385 (2004).

55. Myler, H. A. & West, J. L. Heparanase and platelet factor-4 induce smooth muscle cell proliferation and migration via hFGF release from the ECM. J. Biochem. 131, 913–922 (2002).

56. Thurnham, D. J. et al. Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. J. Biol. Chem. 284, 16939–16947 (2009).

57. Stuppia, M. et al. Genetic and functional characterization of patients with vascular endothelial growth factor receptor-2 deficiency. J. Clin. Invest. 122, 2837–2848 (2008).

58. Scherer, T. et al. Platelet activation with recombinant CXCL4(L1) enhances vascular endothelial growth factor (VEGF) release from human platelets. PLoS ONE 9, e85121 (2014).

59. Zhang, J. et al. Platelet-derived proangiogenic factors. J. Appl. Physiol. 93, 3–11 (2002).

60. Schröder, F. et al. Platelet factor 4 prevents expression of COX-2 and releases proinflammatory cytokines in human endothelial cells. J. Immunol. 181, 5790–5797 (2008).

61. Ward, Peter A. & Sarma, V. J. New developments in CsA receptor signaling. Cell Health Cytoskelet. 4, 57–62 (2012).

62. Lallès, P. N. et al. Locally produced C5a binds to T-cell expressed CsAR to enhance effector T-cell expansion by limiting antigen-induced apoptosis. Blood 112, 1759–1766 (2008).

63. Strainic, M. G. et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. Immunology 128, 425–435 (2012).

64. Wrann, C. D. et al. The phosphatidylinositol 3-kinase signalling pathway exerts protective effects during sepsis by controlling CsA-mediated activation of innate immune functions. J. Immunol. 178, 5940–5947 (2007).

65. Bosmann, H. B., Latina, G., Zouzou, F. S. & Ward, P. A. Evidence for anti-inflammatory effects of CsA on the innate IL-17A/IL-23 axis. FASEB J. 26, 1650–1651 (2012).

66. Kwan, W., van der Touw, W., Paz-Artal, E., Li, M. O. & Heeger, P. S. Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells. J. Exp. Med. 210, 257–268 (2013).
von Hundelshausen, P. et al. Chemokine interactome mapping enables tailored intervention in acute and chronic inflammation. Sci. Transl. Med. 9, 1–15. (2017).

Limbourg, A. et al. Evaluation of postnatal arteriogenesis and angiogenesis in a mouse model of hind-limb ischemia. Nat. Protoc. 4, 1737–1748 (2009).

Qi, X. et al. 2-Hydroxypropyl)-β-cyclodextrin is a new angiogenic molecule for therapeutic angiogenesis. PLoS ONE 10, 1–16 (2015).

Li, Y. et al. The effect of heparin administration in animal models of sepsis: a systematic review and meta-regression analysis of published studies. Crit. Care Med. 39, 1104 (2011).

Kumar, V. et al. Preclinical pharmacokinetics of complement C5a receptor antagonists PMX53 and PMX205 in mice. ACS Omega 5, 2345–2354 (2020).

Schädl, L. et al. Comparative imaging of the murine hind limb vasculature and muscle tissue by microCT and light microscopy. Sci. Rep. 7, 41842 (2017).

Kroes, T., Post, F. H. & Botha, C. P. Exposure render: an interactive photorealistic volume-rendering framework. PLoS ONE 7, e38586 (2012).

Girardi, G., Redecha, P. & Salmon, J. E. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. Nat. Med. 10, 1222–1226 (2004).

Abe, T. et al. Local complement-targeted intervention in periodontitis: proof-of-concept using a C5a receptor (CD88) antagonist. J. Immunol. 189, 5442–5448 (2012).

Lonsdorf, A. S. et al. Engagement of αββ3 (GPIIb/IIIa) with αβ3 integrin mediates interaction of melanoma cells with platelets. J. Biol. Chem. 287, 2168–2178 (2012).

Assinger, A., Volf, I. & Schmid, D. A novel, rapid method to quantify intraplatelet calcium dynamics by ratiometric flow cytometry. PLoS ONE 10, 1–15. (2015).

Zaglia, T. et al. Optimized protocol for immunostaining of experimental GFP-expressing and human hearts. Histochem. Cell Biol. 146, 407–419 (2016).

Nording, H. et al. Platelet bound oxLDL shows an inverse correlation with plasma anaphylatoxin C5a in patients with coronary artery disease. Platelets 27, 593–597 (2016).

Langer, H. et al. Adherent platelets recruit and induce differentiation of murine embryonic endothelial progenitor cells to mature endothelial cells in vitro. Circ. Res. 99, e2–e10. (2006).

Reis, E. S. et al. C5a receptor-dependent cell activation by physiological concentrations of desarginated C5a: insights from a novel label-free cellular assay. J. Immunol. 189, 4797–4805 (2012).

Croker, D. E. et al. Discovery of functionally selective C5aR2 ligands: novel modulators of C5a signalling. Immunol. Cell Biol. 94, 787–795 (2016).

van Triest, M., de Rooij, J. & Bos, J. L. Measurement of GTP-bound Ras-like GTAPases by activation-specific probes. Methods Enzymol. 333, 343–348 (2001).

Choi, E. Y. et al. Del-1, an endogenous leukocyte-endothelial adhesion inhibitor, limits inflammatory cell recruitment. Science 322, 1101–1104 (2008).

Schönberger, T. et al. Pivotal role of phospholipase D1 in tumor necrosis factor-α-mediated inflammation and scar formation after myocardial ischemia and reperfusion in mice. Am. J. Pathol. 184, 2450–2464 (2014).

Carmona, G. et al. Role of the small GTAPase Rap1 in integrin activity regulation in endothelial cells and angiogenesis. Angiogenesis 113, 488–497 (2009).

Fantin, A., Vieira, J. M., Plein, A., Maden, C. H. & Ruhrberg, C. The embryonic mouse hindbrain as a qualitative and quantitative model for studying the molecular and cellular mechanisms of angiogenesis. Nat. Protoc. 8, 418–429 (2013).

Zudaire, E., Gambardella, L., Kurcz, C. & Vermeren, S. A computational tool for quantitative analysis of vascular networks. PLoS ONE 6, e27385 (2011).

Luna, R. L. et al. Placental growth factor deficiency is associated with impaired cerebral vascular development in mice. Mol. Hum. Reprod. 22, 130–142 (2016).

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Author contributions
H.N. and H.F.L. designed the research study. H.N., M.S., L.B., F.E., J.P., M. Mezger, O.B., E.C., D.H., R.H., and H.F.L. conducted the experiments. H.N., F.E., J.P., M. Mezger, O.B., E.C., H.F.L., A.N., C.M.K., R.M.-S., and K.K. analyzed the data. D.S., O.B., A.V., P.H., and J.K. provided reagents. H.N., R.S., F.E., J.P., D.S., O.B., R.F., B.P., E.C., J.E., I.E., T.C., P.H., J.K., M.G., and H.F.L. wrote the manuscript.

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