Hyperglycemia and PPARγ Antagonistically Influence Macrophage Polarization and Infarct Healing After Ischemic Stroke

Michael Gliem, MD; Luisa Klotz, MD; Nico van Rooijen, PhD; Hans-Peter Hartung, MD; Sebastian Jander, MD

**Background and Purpose**—Secondary intracerebral hemorrhage (sICH) is a potentially serious complication of ischemic stroke, in particular under concomitant oral anticoagulation. Previous studies in murine stroke models defined a novel vascular repair function of hematogenous monocytes/macrophages (MO/MP), which proved essential for the prevention of oral anticoagulation–associated sICH. Here, we addressed the question whether hyperglycemia as a clinically relevant prohemorrhagic risk factor and peroxisome proliferator-activated receptor gamma (PPARγ) activation affect MO/MP differentiation and the risk of sICH after ischemic stroke.

**Methods**—Oral anticoagulation–associated sICH was induced by phenprocoumon feeding to mice undergoing transient middle cerebral artery occlusion. Hyperglycemia was induced by streptozotocin treatment. The role of PPARγ-dependent MO/MP differentiation was addressed in mice with myeloid cell–specific PPARγ-knockout (LysM-PPARγ(KO)). Pharmacological PPARγ activation via pioglitazone was tested as a treatment option.

**Results**—Hyperglycemic mice and normoglycemic LysM-PPARγ(KO) mice exhibited abnormal proinflammatory skewing of their hematogenous MO/MP response and abnormal vascular remodeling in the infarct border zone, leading to an increased rate of oral anticoagulation–associated sICH. Pharmacological PPARγ activation in hyperglycemic mice corrected the inflammatory response toward an anti-inflammatory profile, stabilized neovessels in the infarct border zone, and reduced the rate of sICH. This preventive effect was dependent on the presence of macrophages, but independent from effects on blood glucose levels.

**Conclusions**—Hyperglycemia and macrophage-specific PPARγ activation exert opposing effects on MO/MP polarization in ischemic stroke lesions and, thereby, critically determine the risk of hemorrhagic infarct transformation. (Stroke. 2015;46:2935-2942. DOI: 10.1161/STROKEAHA.115.010557.)

**Key Words:** anticoagulation ▪ cerebral ischemia ▪ inflammation ▪ intracerebral hemorrhage ▪ macrophage

Cardioembolic stroke, mostly caused by atrial fibrillation, accounts for at least a quarter of ischemic strokes, and 3% to 13% of patients with cardioembolic infarctions will develop a second embolism within the first 2 weeks of symptom onset,1–5 which will double their mortality rate.6 Oral anticoagulation (OAC) is an effective medical treatment for stroke prevention in patients with atrial fibrillation. The optimal time to start anticoagulation therapy after acute cardioembolic ischemic stroke is currently unknown.7 The uncertain risk of secondary intracerebral hemorrhage (sICH) keeps many physicians from initiation of OAC, especially at early stages after cardioembolic stroke where the infarction may be particularly prone to hemorrhagic transformation. As a prerequisite to clinical advance, pathomechanistic evidence regarding the process of infarct demarcation and sICH is urgently needed.

Inflammatory processes critically influence the evolution of brain damage on ischemic stroke and may exert harmful and beneficial effects.8,9 Cells of the innate immune system, in particular hematogenous monocytes/macrophages (MO/MP), dominate the posts ischemic inflammatory response.10 In a variety of disease models, the balance of inflammatory and noninflammatory MO/MP subpopulations11,12 determines the development of tissue damage and functional outcome.13,14 It was controversial to what extent the sequence of inflammatory and reparative MO/MP responses depends on sequential recruitment of respective cellular subgroups of MO/MP or, alternatively, on the initial recruitment of inflammatory...
monocytes with subsequent differentiation in the target tissue.13–15 We recently showed that inflammatory monocytes invade ischemic brain parenchyma within 24 h of stroke onset and subsequently differentiate within the ischemic brain tissue toward noninflammatory macrophages, which promote early lesion repair, in particular vascular remodeling essential for the prevention of sICH.16,17 Thus, the hematogenous recruitment of monocytes and their appropriate differentiation into macrophages seems critical for the net effect of stroke-induced brain inflammation.

Frequent systemic comorbidities of stroke as diabetes18 or hypercholesterolemia19 may promote proinflammatory skewing of MO/MP functioning in wound healing paradigms.20,21 Interestingly, hyperglycemia is also a risk factor for sICH after stroke.22–24 We therefore addressed the question whether hyperglycemia affects MO/MP-dependent repair mechanisms preventing OAC-associated sICH. We furthermore aimed to identify regulatory pathways of MO/MP differentiation, which might serve as targets of preventive therapy.

Methods
Experimental procedures are detailed in Methods section in the online-only Data Supplement and illustrated in Figure 1A. All animal experiments were approved by local authorities and performed in accordance with international guidelines on handling laboratory animals. Focal cerebral ischemia was induced by 55 minutes of transient middle cerebral artery occlusion (tMCAO). Mice were anticoagulated by oral administration of phenprocoumon.17 To provoke hyperglycemia, streptozotocin (10 mmol/L in citrate buffer, pH 4.6; Sigma-Aldrich, Steinheim, Germany) was injected intraperitoneally 6 weeks before tMCAO induction for 5 consecutive days. Infarct volumes and hemorrhages were visualized by staining with 2,3,5-triphenyltetrazolium chloride.17 LysM-PPARγ(KO) or LysM-PPARγ(WT) mice were used to study the effect of proinflammatory macrophage skewing. Pharmacological PPARγ activation was achieved by oral administration of pioglitazone (20 mg/kg body weight; Takeda Pharmaceutical, Osaka, Japan) starting directly after tMCAO (d0). Clodronate-filled liposomes were used for depletion of hematogenous macrophages.16 Cellular and molecular processes were studied by immunohistochemistry, flow cytometry, and reverse transcription polymerase chain reaction (see Table for primer sequences). All analyses were performed by an investigator blinded for treatment allocation. Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA), and results are presented as mean±SD.

Results
Risk of OAC-Associated sICH After tMCAO Is Increased by Hyperglycemia
To investigate sICH after ischemic stroke, we used a previously described model16 of OAC-associated sICH (Figure 1A and 1B). Feeding the vitamin K antagonist phenprocoumon to mice results in effective OAC with an international normalized ratio of 2.8±0.9 (n=10) 48 h later. Initiation of OAC at 24 h after tMCAO is associated with a rate of 50% hemorrhagic transformation and lack of parenchymal hemorrhage at day 5 after stroke induction (Figure 1B).

To test the influence of hyperglycemia on the rate of OAC-associated sICH, we used the streptozotocin model of type 1 diabetes mellitus. In streptozotocin-treated mice, fasting blood glucose levels were increased to 320±56 mg/dL (n=12), as opposed to 98±10 mg/dL in control mice (n=9; Figure 1C). International normalized ratio testing revealed similar intensity of OAC in hyperglycemic and control animals (Figure 1D). However, hyperglycemic mice showed a significantly increased rate of parenchymal hemorrhage compared with normoglycemic controls (Figure 1B). Volume of infarcted tissue was not significantly different between the groups (ctrl 75±22 mm³ [n=7] versus hyperglycemic 65±12 mm³ [n=9]).

OAC-Associated sICH in Hyperglycemic Mice Is Reduced by Pioglitazone Treatment
The PPARγ agonist pioglitazone is licensed for the treatment of type 2 diabetes mellitus and exerts glucose-lowering effects

![Graph](http://stroke.ahajournals.org/)

**Figure 1.** A, Outline of the experimental design indicating time points of pharmacological treatment and coagulation testing. B, Representative brain slices illustrating parenchymal hemorrhage (top), hemorrhagic transformation (middle), or absence of secondary intracerebral hemorrhage (sICH; bottom) after transient middle cerebral artery occlusion (tMCAO). Scale bar =5 mm, with 2× magnifications shown on the right. Rate and severity of oral anticoagulation (OAC)–associated sICH is increased in hyperglycemic mice (hg) and reduced toward control (ctrl) level via pioglitazone (pio) treatment (n≥9, χ² test). Black bars indicate parenchymal hemorrhage; grey bars, hemorrhagic transformation; and white bars, absence of sICH. C, Blood glucose (n=9) in control (ctrl), streptozotocin-treated (hg), and hyperglycemic pioglitazone (hg+pio)–treated mice. Whiskers of the box-plot indicate extreme values. D, International normalized ratio (INR) measurements indicate similar intensity of OAC in control, hyperglycemic, and hyperglycemic pioglitazone–treated mice (n=10). Bars denote mean±SD. *P<0.05. VKA indicates vitamin K antagonist.
via reduction of insulin resistance. Additionally, pioglitazone has pleiotropic effects on a variety of cellular processes, notably the promotion of anti-inflammatory macrophage differentiation.23 Because insulin resistance does not contribute to hyperglycemia in the streptozotocin model, we found similar blood glucose levels in pioglitazone-treated versus untreated mice (Figure 1C). Nevertheless, the rate of OAC-associated sICH was significantly reduced in pioglitazone-treated hyperglycemic mice (Figure 1B). In particular, severe parenchymal hemorrhage was not observed on pioglitazone treatment. Infarct volumes were not significantly different between groups (control hyperglycemic 65±12 mm³ [n=9] versus pioglitazone-treated hyperglycemic mice 80±15 mm³ [n=7]).

**Effects of Hyperglycemia and PPARγ Activation on MO/MP Phenotype and Inflammatory Gene Expression**

Because the reduction of sICH by pioglitazone treatment was independent from blood glucose levels, we hypothesized that hematogenous macrophage-targeted effects of pioglitazone might underlie its protective effect in our model. Flow cytometric analysis of intracerebral leukocytes at day 3 after stroke was used to distinguish CD45⁺CD11b⁺ hematogenous MO/MP from CD45⁺CD11b⁺ central nervous system-resident microglia (Figure 2A), as validated previously in bone marrow chimeric animals.16 Hematogenous inflammatory MO/MP were identified by high Ly6c and low F4/80 expression, whereas more differentiated MO/MP reciprocally upregulated F4/80 and downregulated Ly6c (Figure 2A). Overall, population sizes of microglia, granulocytes, hematogenous MO/MP, and their subpopulations, that is, undifferentiated Ly6c⁺F4/80⁻ and differentiated Ly6c⁻F4/80⁺ MO/MP, were similar in control, hyperglycemic, and hyperglycemic pioglitazone–treated mice (exemplified for CD45⁺CD11b⁺ hematogenous MO/MP in Figure 2A). This was confirmed by a similar level of Mac3⁺ phagocyte staining in immunohistochemical analysis (Figure 2B). However, expression of CD80 and tumor necrosis factor (TNF)-α were increased in F4/80⁺, but not in F4/80⁻, MO/MP of hyperglycemic mice, indicating that hyperglycemia induced an abnormal proinflammatory phenotype specifically of differentiated hematogenous MO/MP (Figure 2C). Pioglitazone treatment of hyperglycemic mice reduced CD80 and TNF-α expression by F4/80⁺ MO/MP toward the level of normoglycemic controls (Figure 2C). In all other cell populations, CD80 and TNF-α were not changed by pioglitazone. Quantitative polymerase chain reaction analysis of lesional mRNA confirmed hyperinduction of inflammatory cytokines, such as TNF-α and interleukin-1β, and a reciprocal decrease of anti-inflammatory cytokines, such as transforming growth factor-β and interleukin-10, in hyperglycemic mice which was corrected toward control levels by pioglitazone treatment (Figure 2D). Taken together, hyperglycemia caused abnormal proinflammatory skewing of differentiated hematogenous MO/MP in the infarctions, which was corrected by pharmacological PPARγ activation.

Previous studies showed that myeloid cell–specific PPARγ knockout in LysM-PPARγ(KO) mice induces proinflammatory skewing of MO/MP.26 To substantiate that proinflammatory skewing of MO/MP is sufficient to increase the rate of OAC-associated sICH, we studied the rate of OAC-associated sICH and the differentiation of hematogenous MO/MP in normoglycemic LysM-PPARγ(KO) mice. Compared with wild-type controls, LysM-PPARγ(KO) mice exhibited higher TNF-α expression in differentiated MO/MP and an increased rate of hemorrhagic transformation after tMCAO (Figure 3A).

**Prevention of OAC-Associated sICH Is Macrophage-Dependent**

To corroborate involvement of MO/MP in pioglitazone-driven repair, we tested the effect of pioglitazone treatment on the rate of sICH in MO/MP-depleted mice. Clodronate-filled liposomes deplete bone marrow–derived MO/MP but leave resident central nervous system macrophages/microglia unaffected.27 Confirming previous findings,16 early clodronate liposome administration at day 1 and 2 after tMCAO caused sICH (Figure 3B). Pioglitazone treatment did not reduce the rate of sICH in MO/MP-depleted mice. Thus, the protective effect of pioglitazone depended on the presence of hematogenous MO/MP and was not due to a direct effect on brain-resident cells.

**Increased Rate of sICH in LysM-PPARγ(KO) and Hyperglycemic Mice Is Associated With Defective Vascular Remodeling**

Our previous studies implicated hematogenous MO/MP in the prevention of sICH by promoting collagen-4 deposition around infarct neovessels in a transforming growth factor-β–dependent manner.16 To elucidate the mechanism underlying increased sICH in LysM-PPARγ(KO) and hyperglycemic mice, we performed an immunohistochemical analysis of neovessel morphology and collagen expression in infarcted brain parenchyma. Collagen-4 deposition in the infarct border zone was reduced after tMCAO in LysM-PPARγ(KO) mice (Figure 4A). Moreover, neovessels in LysM-PPARγ(KO) mice were thin-walled and dilated (Figure 4B). An identical
defect of vascular remodeling was found in hyperglycemic mice (Figure 4C) and could be corrected by pioglitazone treatment (Figure 4C and 4D).

Discussion
Our results extend the knowledge concerning hemorrhagic infarct transformation after ischemic stroke in 3 ways: we could identify (1) hyperglycemia as a condition that provokes proinflammatory, antireparative MO/MP polarization, (2) proinflammatory skewed MO/MP as a prohemorrhagic factor in subacute stages after ischemic stroke, and (3) PPARγ in monocyte-derived macrophages as an endogenous regulator of MO/MP polarization and a potential therapeutic target to prevent sICH.
Similar to hyperglycemic mice, we also found abnormal proinflammatory MO/MP skewing, disturbed neovessel formation, and an increased rate of sICH (encompassing parenchymal hemorrhage and hemorrhagic transformation) in mice with myeloid cell–specific PPARγ knockout. Why PPARγ knockout only increased hemorrhagic transformation but not parenchymal hemorrhage is currently an open question. However, we think that both conditions are only different manifestations of the same pathophysiological process. Our findings therefore corroborate the impact of appropriate MO/MP differentiation on controlling the risk of sICH and mechanistically link the observed preventive effect of pharmacological PPARγ agonism with functional differentiation of MO/MP in brain infarction. As a potential limitation, LysM-PPARγ(KO) mice also exhibit altered neutrophil functions, which may contribute to the phenotype observed in our study. Hyperglycemia as such favors a proinflammatory skewing of MO/MP because they use glycolysis for rapid inflammatory responses, whereas repair-associated MO/MP are dependent on fatty acid oxidation for their long-term reparative tasks. Previous studies showed that PPARγ regulates the MO/MP phenotype toward a more reparative phenotype and promotes fatty acid oxidation in MO/MP. Taken together, by counteracting proinflammatory effects of hyperglycemia on MO/MP activity at multiple levels, PPARγ constitutes a master switch between systemic metabolic factors and local actions of MO/MP in tissue injury and disease.

Previous studies described various beneficial effects of PPARγ activation, including direct neuroprotection, as well as broad anti-inflammatory effects, which may synergistically improve stroke outcome. We were not able to detect differences in infarct size between PPARγ agonist–treated animals and controls. Primary end point of our study was the bleeding rate, and selection criteria might underestimate the previously detected differences in infarct sizes. Pleiotropic effects of PPARγ agonism to date hampered the identification of relevant cells that convey its beneficial effects. Previous targeted knockout studies only addressed the role of neurologically expressed PPARγ. Our macrophage-specific knockout and depletion paradigms for the first time identify the impact of macrophage differentiation on vascular repair and bleeding rate. Zhao et al described anti-inflammatory and antioxidative actions of PPARγ in subacute stages of primary ICH, promoting neuroprotection and hematoma resolution and, thereby, limiting brain damage. This concept is extended by our present data, implicating PPARγ not only in the limitation of secondary damage but also in the initial prevention of delayed sICH. In a recent study of primary ICH, hyperglycemia promoted expansion of the intracerebral hematoma by plasma kallikrein–mediated, osmosenstive inhibition of hemostasis. However, in our study, the effect of PPARγ agonism was independent from blood glucose lowering but associated with the protracted process of macrophage differentiation. Accumulating evidence indicates that reshaping deranged macrophage polarization rather than blocking MO/MP recruitment per se is the most promising approach to MO/MP as therapeutic targets.

The uncertain risk of infarct bleeding, the optimal time to start OAC after acute embolic stroke is currently uncertain. Our previous data suggested that initiation of OAC is relatively safe beyond day 3 after stroke when macrophage-dependent vascular remodeling has already resulted in sufficiently stable vessel walls in the infarct border zone. Our present study extends these findings by modeling the influence of hyperglycemia as a frequent comorbidity in stroke patients. Clinical studies have shown a worse prognosis of ICH and an increased rate of hemorrhagic transformation on systemic thrombolysis in hyperglycemic and diabetic stroke patients. Regarding OAC-associated hemorrhage, clinical trials showed an increased rate of bleeding complications in diabetes mellitus, but did not specifically address its relevance for ICH. Our findings in the murine model strongly suggest diabetes mellitus as a risk factor for OAC-associated sICH, at least under the conditions of marked hyperglycemia and subacute brain infarction modeled in our experiments.

Regarding pathomechanisms of sICH, Jickling et al suggested that early sICH is dependent on reperfusion and blood–brain barrier disruption by reactive oxygen species and metalloproteinases, whereas delayed sICH beyond 24 h after ischemia is determined by postischemic inflammation and vascular remodeling. Our previous studies using MO/MP depletion in normoglycemic mice already strongly implicated monocyte-derived macrophages in the prevention of delayed hemorrhagic transformation by promoting vascular collagen-4 deposition in a transforming growth factor-β–dependent manner. Hemorrhagic transformation in the depletion paradigms was associated with the formation of thin-walled and dilated neovessels prone to rupture. In our present study, we found a similar abnormal phenotype of neovessels in hyperglycemic mice, indicating that abnormal proinflammatory skewing of macrophage differentiation is functionally equivalent to a complete lack of macrophages. Of note, MO/MP recruitment per se was not altered in hyperglycemic mice. Thus, in line with accumulating evidence in other models, appropriate differentiation of recruited MO/MP seems to be critical for the outcome of stroke-associated inflammation.
Figure 4. Immunohistochemical analysis of neovessel morphology in the infarct border zone at day 6 after ischemia. A, Reduced collagen-4 expression in LysM-PPARγ(KO) vs WT mice (n=6, Student’s t test). B, Quantitative analysis of vessel wall thickness and diameters revealed comparatively thin-walled and dilated neovessels in LysM-PPARγ(KO) vs WT mice (n=6, Student’s t test). C, Hyperglycemic mice exhibit reduced collagen-4 expression around neovessels, which was restored to normoglycemic control level by pioglitazone treatment (n=6, ANOVA with Bonferroni post hoc test). D, Neovessels in hyperglycemic mice exhibit dilation and wall thinning similar to LysM-PPARγ(KO) mice, which was corrected by pioglitazone treatment (n=6, ANOVA with Bonferroni post hoc test). Bars denote mean±SD. *P<0.05, **P<0.01, ***P<0.001. Scale bars =30 μm.
Taken together, the balance of pro- versus anti-inflammatory differentiation of hematogenous MO/MP determines the risk of secondary hemorrhage after ischemic stroke. Targeting PPARγ might be an effective means to promote protective and repair-enhancing properties of hematogenous MO/MP in subacute brain infarction. As such, myeloid cell–specific PPARγ activation might help to reduce the rate of sICH in high-risk patients with a need of early OAC.

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Disclosures

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References


12.作为基础的科学研究,中国医学科学院的高晓东教授和国际研究团队在实验中进一步验证了这一理论。


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HYPERGLYCEMIA AND PPARγ ANTAGONISTICALLY INFLUENCE MACROPHAGE POLARIZATION AND INFARCT HEALING AFTER ISCHEMIC STROKE

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Supplementary Methods

Animals
We used male wild-type (WT) C57BL/6 mice (from Centre d'Elevage R. Janvier [CERJ], Le Genest-St-Isle, France) weighing 28-32 g and aged 12-18 weeks and LysM-PPARγ(KO) or LysM-PPARγ(WT) mice. 79 mice were used to exclude influences of hyperglycemia on INR guided phenprocoumon treatment. 90 (+12 Bederson score <1 or no reperfusion, +12 blood glucose < 250 mg/dl, +7 INR > 5) mice were used to study infarct development, bleeding rate and hematogenous MO/MP in control, hyperglycemic and hyperglycemic pioglitazone treated mice. In 41 (+2 Bederson score <1, +6 INR > 5) mice the effect of pioglitazone treatment in normoglycemic mice was assessed. 24 (+2 Bederson score <1) mice were treated with clodronate liposomes. 36 (+5 Bederson score <1 or no reperfusion, +2 INR > 5) LysM-PPARγ(KO) and LysM-PPARγ(WT) were analyzed.

Stroke model
All animal experiments were approved by local authorities and performed in accordance with international guidelines on handling laboratory animals. For tMCAO induction we used a 6–0 standardized suture coated with silicon rubber (6021PK10; Doccol Corporation, Redlands,
CA, USA) which was left in situ for 55 min.\(^1\) Only animals with successful reperfusion (evidenced by blood reflux after suture withdrawal) and with a Bederson score >1\(^2\) at day 1 after tMCAO were used for further experiments. Exclusions based on these criteria were n=19. Infarct volumes and hemorrhages were visualized at the indicated time points by staining with 2,3,5-triphenyltetrazolium chloride according to a previously described protocol.\(^3\)

**Streptozotocin-induced hyperglycemia**

Streptozotocin (10 mmol/L in citrate buffer pH 4.6, Sigma-Aldrich, Steinheim, Germany) was injected intraperitoneally at a dose of 40 mg/kg body weight/day for 5 consecutive days. Each injection was performed after 4 hours fasting. After 6 weeks, blood glucose was tested after 4 hours fasting with a bedside test. Animals with a fasting glucose < 250 mg/dl were excluded from further experiments (n=12).

**Pioglitazone treatment**

Pioglitazone (Takeda Pharmaceutical, Osaka, Japan) was dissolved in PBS and administered once daily via gastric tubing (20mg/kg body weight) starting directly after tMCAO (d0) until sacrifice.

**Oral anticoagulation**

Phenprocoumon tablets (HEXAL AG, Holzkirchen, Germany) were dissolved in phosphate buffered saline (PBS) at a concentration of 0.24 mg/ml. The solution was administered once daily via gastric tubing resulting in a dose of 0.8 mg/kg body weight/day. INR was monitored at day 2 and day 4 after initiation of OAC using a point of care device (CoaguChek®, Roche, Basel, Switzerland). Animals with an INR > 5 at any time point after treatment initiation were excluded from analysis (n=15). 11 were excluded due to death before effective oral
anticoagulation was reached. Mortality rate in orally anticoagulated mice was 10% in control mice, 23% in hyperglycemic and 11% in hyperglycemic pioglitazone treated mice. In mice with effective oral anticoagulation after 48 hours mortality rate was 12% in control and 11% in pioglitazone treated mice. In orally anticoagulated LysM-PPARγ(KO) mice mortality was 18% and 15% in LysM-PPARγ(WT) mice.

Flow cytometry

Intracerebral leukocytes were isolated from ischemic lesions (mean tissue weight 40 mg per animal) or homotopic nonischemic tissue as detailed elsewhere and stained according to an established protocol. In addition, we used FITC-labeled rat anti-mouse TNF-α (clone MP6-XT22, BD Biosciences, San Jose, CA), FITC-labeled hamster anti-mouse CD80 (clone 16-10A1, BD Biosciences) and APC-labeled rat anti-mouse F4/80 (clone C1:A3, Serotec Ltd., Oxford, UK) antibodies.

Immunohistochemistry

Paraffin sections were stained with polyclonal anti-collagen-4 (Meridian Life Science, Saco, ME) followed by the Vectastain Elite kit reagents (Vector Laboratories, Burlingame, CA), and analyzed by ImageJ software (NIH, Bethesda, MD).

Macrophage depletion

For depletion of hematogenous macrophages we used clodronate-filled liposomes as detailed elsewhere.

Quantitative real-time PCR

Analysis of total RNA was performed by quantitative real-time PCR as described previously. Gene-specific primer pairs as indicated in Table 1 were designed by using PrimerExpress 2.0
software (Applied Biosystems, Darmstadt, Germany). Relative gene expression levels were determined according to the $\Delta\Delta C_t$ method.

**Statistical analysis**

Statistical analyses were conducted using GraphPad Prism™ software (GraphPad Software Inc., La Jolla, CA).

**References to Supplementary Methods**


