Gas6 Promotes Inflammatory (CCR2<sup>hi</sup>CX3CR1<sup>lo</sup>) Monocyte Recruitment in Venous Thrombosis

Sandrine Laurance, François-René Bertin, Talin Ebrahimian, Yusra Kassim, Ryan N. Rys, Stéphanie Lehoux, Catherine A. Lemarié, Mark D. Blostein

**Objective**—Coagulation and inflammation are inter-related. Gas6 (growth arrest–specific 6) promotes venous thrombosis and participates in inflammation through endothelial-innate immune cell interactions. In innate immune cells can provide the initiating stimulus for venous thrombus development. We hypothesize that Gas6 promotes monocyte recruitment during venous thrombosis.

**Approach and Results**—Deep venous thrombosis was induced in wild-type and Gas6-deficient (−/−) mice using 5% FeCl<sub>3</sub> and flow reduction in the inferior vena cava. Total monocyte depletion was achieved by injection of clodronate before deep venous thrombosis. Inflammatory monocytes were depleted using an anti–C-C chemokine receptor type 2 (CCR2) antibody. Similarly, injection of an anti–chemokine ligand 2 (CCL2) antibody induced CCL2 depletion. Flow cytometry and immunofluorescence were used to characterize the monocytes recruited to the thrombus. In vivo, absence of Gas6 was associated with a reduction of monocyte recruitment in both deep venous thrombosis models. Global monocyte depletion by clodronate leads to smaller thrombi in wild-type mice. Compared with wild type, the thrombi from Gas6−/− mice contain less inflammatory (CCR2<sup>hi</sup>CX3CR1<sup>lo</sup>) monocytes, consistent with a Gas6-dependent recruitment of this monocyte subset. Correspondingly, selective depletion of CCR2<sup>hi</sup>CX3CR1<sup>lo</sup> monocytes reduced the formation of venous thrombi in wild-type mice demonstrating a predominant role of the inflammatory monocytes in thrombosis. In vitro, the expression of both CCR2 and CCL2 were Gas6 dependent in monocytes and endothelial cells, respectively, impacting monocyte migration. Moreover, Gas6-dependent CCL2 expression and monocyte migration were mediated via JNK (c-Jun N-terminal kinase).

**Conclusions**—This study demonstrates that Gas6 specifically promotes the recruitment of inflammatory CCR2<sup>hi</sup>CX3CR1<sup>lo</sup> monocytes through the regulation of both CCR2 and CCL2 during deep venous thrombosis.

**Visual Overview**—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1315-1322. DOI: 10.1161/ATVBAHA.116.308925.)

**Key Words:** growth arrest-specific 6 ● inflammation ● monocyte ● venous thrombosis

Venous thromboembolism, composed of deep vein thrombosis (DVT) and pulmonary embolism, represents a worldwide major health issue and is a leading cause of cardiovascular death. Since the past decade, the prevalence continues to increase, reaching >400 per 100,000.2

See accompanying editorial on page 1263

The activation of proinflammatory mechanisms during DVT has been emphasized by several in vitro and in vivo studies. Markers of inflammation such as interleukin-6, interleukin-8, P-selectin, chemokine ligand 2 (CCL2), and C-reactive protein are associated with thrombus development.3−7 In addition, the important role of leukocytes in the formation of venous thrombosis has recently been demonstrated. Hence, von Brühl et al.8 demonstrated that neutrophils and monocytes are recruited early and actively participate to venous thrombosis formation after partial ligation of the inferior vena cava. They found that neutrophils and monocytes contribute to thrombosis by triggering FXII-dependent coagulation and delivering tissue factor (TF), respectively. They also demonstrated that expression of P-selectin by the endothelium supported the recruitment of leukocytes. Circulating monocytes are the major source of TF-mediated coagulation.8,8 After vessel injury, activated platelet and endothelial cells release cytokines, which trigger TF expression from monocytes. In patients, blood monocyte counts were associated with an increased risk of venous thrombosis.10

Monocyte-associated TF expression was found to be elevated after surgery in patients and preceded the occurrence of venous thromboembolism.11

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The recruitment of monocytes involves the coordinated secretion of chemokines by the activated endothelium and the expression of chemokine counter-receptors by monocytes. CCL2/CCR2 (C-C chemokine receptor type 2), CX3CL1/CX3CR1 (CX3C chemokine receptor 1), and CCL5/CCR5 are the 3 main chemokines and chemokine receptors that have been involved in the recruitment of monocytes in vascular disease. Not all monocytes express CCR2 or the same level of CX3CR1. Inflammatory monocytes express high levels of Ly6C and CCR2 and low levels of CX3CR1 (CX3C chemokine receptor 1). CCR2hiCX3CR1lo monocytes were specifically depleted using a CCR2 antibody 24 hours before thrombus induction. Flow cytometry analysis confirmed that clodronate reduced the number of inflammatory monocytes recruited to the thrombi of WT mice (Figure 2A). This was associated with a reduction of thrombus size, thrombus area, and thrombus weight (Figure 2Bi through 2Biii). Thus, monocyte depletion is associated with a reduction of thrombus formation.

Inflammatory monocytes were specifically depleted using a CCR2 antibody 24 hours before thrombus induction. Flow cytometry analysis showed that circulating inflammatory monocytes were reduced by clodronate treatment (Figure IIA in the online-only Data Supplement). Flow cytometry analysis confirmed that clodronate treatment significantly reduced CD11b+ monocytes in the blood (Figure IIB in the online-only Data Supplement). Flow cytometry analysis confirmed that clodronate did not affect the number or activity of platelets (Figure IIB in the online-only Data Supplement) nor circulating neutrophil counts (Figure IIC in the online-only Data Supplement). Interestingly, clodronate reduced the number of monocytes recruited to the thrombi of WT mice (Figure 2A). This was associated with a reduction of thrombus size, thrombus area, and thrombus weight (Figure 2Bi through 2Biii). Thus, monocyte depletion is associated with a reduction of thrombus formation. Inflammatory monocytes were specifically depleted using a CCR2 antibody 24 hours before thrombus induction. Flow cytometry analysis showed that circulating inflammatory monocytes were reduced by clodronate treatment (Figure IIA in the online-only Data Supplement). Flow cytometry analysis confirmed that clodronate did not affect the number or activity of platelets (Figure IIB in the online-only Data Supplement) nor circulating neutrophil counts (Figure IIC in the online-only Data Supplement). Interestingly, clodronate reduced the number of monocytes recruited to the thrombi of WT mice (Figure 2A). This was associated with a reduction of thrombus size, thrombus area, and thrombus weight (Figure 2Bi through 2Biii). Thus, monocyte depletion is associated with a reduction of thrombus formation. Inflammatory monocytes were specifically depleted using a CCR2 antibody 24 hours before thrombus induction. Flow cytometry analysis showed that circulating inflammatory monocytes were reduced by clodronate treatment (Figure IIA in the online-only Data Supplement). Flow cytometry analysis confirmed that clodronate did not affect the number or activity of platelets (Figure IIB in the online-only Data Supplement) nor circulating neutrophil counts (Figure IIC in the online-only Data Supplement). Interestingly, clodronate reduced the number of monocytes recruited to the thrombi of WT mice (Figure 2A). This was associated with a reduction of thrombus size, thrombus area, and thrombus weight (Figure 2Bi through 2Biii). Thus, monocyte depletion is associated with a reduction of thrombus formation.

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

Results
Gas6 Promotes the Recruitment of Inflammatory Monocytes During Venous Thrombosis
To investigate the role of Gas6 in the recruitment of monocytes during venous thrombosis, we induced DVT in mice using both the FeCl3, and the flow restriction models in the inferior vena cava. We had previously demonstrated that thrombus size is reduced in Gas6−/− mice compared with wild type (WT) using the FeCl3 model. Using the flow restriction model, we confirmed that absence of Gas6 results in the reduction of venous thrombosis (Figure 1A). Importantly, we showed that monocyte recruitment is reduced in Gas6−/− mice compared with WT mice in both DVT models (Figure 1B and 1C). The absence of Gas6 did not affect neutrophil recruitment in FeCl3-induced venous thrombosis (Figure 1D). We then quantified the inflammatory (CCR2hiCX3CR1lo) and patrolling (CX3CR1hi) monocytes within the thrombus of WT and Gas6−/− mice by flow cytometry. Interestingly, the recruitment of CCR2hiCX3CR1lo monocytes was decreased in thrombi from Gas6−/− compared with WT mice. The absence of Gas6 did not affect the recruitment of CX3CR1hi monocytes (Figure 1E and 1F). Immunofluorescence staining within thrombi confirmed that Gas6 deficiency reduced the recruitment of CCR2-expressing monocytes (Figure 1 in the online-only Data Supplement). These data suggest that Gas6 is involved in the recruitment of inflammatory (CCR2hiCX3CR1lo) monocytes during thrombus formation.

Gas6 Is Required for Thrombin-Induced CCR2/CCL2 Expression
Given the role of Gas6 in venous thrombosis and the importance of inflammatory monocytes described above, we analyzed the role of Gas6 in thrombin-induced expression of CCR2 in bone marrow–derived monocytes and CCL2 in endothelial cells in vitro.
Thrombin upregulated CCR2 mRNA and protein expression in WT but not Gas6−/− monocytes (Figure 3Ai and 3Aii). mRNA expression of CCL2 was induced in WT but not in Gas6−/− endothelial cells after thrombin treatment (Figure 3Bi). CCL2 production was increased by thrombin in culture media from WT but not from Gas6−/− endothelial cells (Figure 3Bii). These data suggest that Gas6 is required for thrombin-induced CCR2 and CCL2 expression. Thus, we tested whether the thrombin receptor, PAR-1 (protease activated receptor 1), was involved in Gas6-dependent CCR2 and CCL2 expression. Treatment of WT monocytes and endothelial cells with a PAR-1 antagonist, SCH79797, inhibited the thrombin-induced CCR2 and CCL2 expression. Treatment of WT monocytes and endothelial cells with a PAR-1 antagonist, SCH79797, inhibited the thrombin-induced CCR2 and CCL2 expression. PAR-1 mRNA expression was similar between Gas6−/− and WT monocytes or endothelial cells (Figure 3Bii). These data suggest that thrombin induces CCL2 in endothelial cells and CCR2 in monocytes through PAR-1 in a Gas6-dependent manner but not through the regulation of PAR-1 expression.

Gas6 Is Involved in CCR2/CCL2-Dependent Monocyte Migration

As demonstrated above, thrombin failed to induce CCR2 expression in monocytes lacking Gas6. Thus, we hypothesize that the migratory capacity of Gas6−/− monocytes would be affected. To address the role of Gas6 in the migration of monocytes in response to thrombin, we performed in vitro experiments using Boyden chambers. WT and Gas6−/− monocytes were incubated with thrombin. Results showed that thrombin increased the migration of WT but not Gas6−/− monocytes in a CCR2-dependent manner as demonstrated by incubation with a CCR2-blocking antibody (Figure 3C).

We subsequently analyzed the role of the CCL2 secreted by endothelial cells to trigger monocyte migration. Conditioned media from WT and Gas6−/− endothelial cells were collected and then incubated with WT monocytes. Using Boyden chambers, we found that conditioned media from thrombin-treated WT but not Gas6−/− endothelial cells promoted migration of WT monocytes. Incubation with a CCL2-blocking antibody reduced the migration of WT monocytes treated with conditioned media from thrombin-treated
WT endothelial cells (Figure 3D). Interestingly, we also found that PSGL-1 expression was increased in monocytes incubated with conditioned media from thrombin-treated WT endothelial cells. Incubation with a CCL2 antibody reduced the expression of PSGL-1 in WT monocytes treated with conditioned media (Figure V in the online-only Data Supplement). These data demonstrate that Gas6 plays an important role in endothelium-mediated monocyte migration through CCR2/CCL2 interactions and PSGL-1 expression.

**Gas6-Dependent CCL2 Expression Is Mediated via JNK**

Both Gas6 and thrombin have been shown to activate JNK (c-Jun N-terminal kinase).23–25 Thus, we assessed the role of JNK in Gas6-dependent CCL2 expression and monocyte migration. Thrombin treatment induced the phosphorylation of JNK in WT but not in Gas6−/− endothelial cells (Figure 4A). Incubation with a CCL2 antibody reduced the expression of PSGL-1 in WT monocytes treated with conditioned media (Figure V in the online-only Data Supplement). These data demonstrate that Gas6 plays an important role in endothelium-mediated monocyte migration through CCR2/CCL2 interactions and PSGL-1 expression.

We collected conditioned media from thrombin-treated WT endothelial cells in the presence or absence of SP600125. In Boyden chambers, conditioned media from thrombin-treated WT endothelial cells promoted the migration of WT monocytes. Monocyte migration was inhibited by conditioned media from WT endothelial cells treated with thrombin and SP600125 (Figure 4D). These data suggest that JNK is required for Gas6-mediated CCL2 secretion from endothelial cells thereby promoting monocyte migration.

**Discussion**

In the current study, we show that Gas6 is required for the recruitment of inflammatory (Ly6CεCCR2CX3CR1) monocytes, which contribute to venous thrombosis. Gas6 promotes the recruitment of such monocytes through a CCR2/CCL2-dependent mechanism.

Gas6 has been shown to have either pro- or anti-inflammatory effects depending on the disease and the cell type studied.26 Interestingly, Gas6 has emerged as an important regulator of immune homeostasis by promoting interactions between endothelial cells and leukocytes and leukocyte extravasation in murine models of inflammation (heart transplantation, endotoxinemia, and vasculitis).21,27 It was found that IFNα induced the Gas6 receptor, Axl, in monocyte-derived dendritic cells.
and that Gas6 stimulates the migration of these cells. Gas6 has also been implicated in the migration of neutrophils. Neutrophils have been largely implicated in venous thrombosis especially because of their capacity to activate coagulation through the formation of NETs (neutrophil extracellular traps). Depletion of neutrophils dramatically reduces venous thrombosis. However, in the present study, we did not find any role for Gas6 regarding neutrophil recruitment. Studies have also demonstrated that monocytes play a crucial role in the pathogenesis of venous thrombosis induced by partial ligation of the inferior vena cava in mice. In agreement with this study, we found that monocytes are recruited early during venous thrombosis formation. We found that Gas6 is specifically involved in the recruitment of monocytes in venous thrombosis induced by FeCl₃ or by flow restriction. To establish the role of monocytes in FeCl₃-induced venous thrombosis, we first depleted all circulating monocytes in WT mice. We observed that monocyte depletion reduced venous thrombosis, which is consistent with previously published studies. Thus, our results strongly suggest that Gas6 specifically enhances the recruitment of monocytes during venous thrombosis. Several mechanisms have been identified to explain the role of monocytes in DVT. It has been shown that the major source of TF arises from monocytes in the flow restriction model. Although it was not investigated in the present study, one can speculate that monocyte depletion would be associated with a reduction of TF within a thrombus and thus a reduction of activation of the extrinsic pathway of the coagulation cascade. Monocytes can interact with platelets to form monocyte–platelet complexes, which induce platelet activation. However, depletion of monocytes did not modify platelet activation in our study. Two monocyte subsets, CCR2<sup>hi</sup>CX3CR1<sup>lo</sup> and CX3CR1<sup>hi</sup>, have been described based on the expression of chemokine receptors. Inflammatory monocyte recruitment occurs in other cardiovascular diseases such as atherosclerosis, myocardial infarction, and hypertension. Interestingly, the loss of Gas6 reduces the recruitment of inflammatory monocyte to the thrombus without affecting the recruitment of patrolling monocytes.

Figure 3. Gas6 (growth arrest–specific 6) is required for C–C chemokine receptor type 2 (CCR2)/chemokine ligand 2 (CCL2)–dependent monocyte migration. A, Expression of CCR2 is increased by thrombin in wild-type (WT) but not in Gas6<sup>−/−</sup> monocytes (i) at the mRNA level by quantitative real-time polymerase chain reaction (n=4) and (ii) at protein level showed by immunofluorescent staining (red; n=5–8). Nuclei are counterstained with DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride; blue). B, Expression of CCL2 is induced by thrombin in WT but not in Gas6<sup>−/−</sup> endothelial cells (i) at the mRNA level (n=9) and (ii) in the culture supernatant (n=8–10). C, Thrombin increases the migration of WT but not in Gas6<sup>−/−</sup> monocytes. Migration is inhibited when WT monocytes are incubated with a CCR2-blocking antibody (n=6). D, Migration of WT monocytes is increased in the presence of conditioned media from WT but not in Gas6<sup>−/−</sup> endothelial cells treated with thrombin. Migration is blocked by addition of a CCL2-blocking antibody to the conditioned media (n=5). P<0.05, *vs WT vehicle (Veh), #vs WT thrombin (Thr).
monocytes. Specific depletion of inflammatory monocytes, using a CCR2 antibody, decreases thrombus formation to the same extent as global monocyte depletion. In accordance with our results, CCR2 deficiency was found to be associated with fewer monocytes recruited during DVT. A more recent study demonstrated that Ly6C+ monocytes were recruited in the first 2 days of thrombosis formation after the establishment of flow restriction.

Recently, it has been shown that several mediators of innate immune cell trafficking are increased in a murine model of venous thrombosis, among them CCL2, CXCL1, CXCL5, interleukin-6, and P-selectin. Interestingly, it was found that CCL2 expression is increased over time during venous thrombosis induced by flow restriction. CCR2 is the main receptor for CCL2 and has been described to participate in the recruitment of inflammatory monocytes in atherosclerosis. Thus, we tested in vitro whether Gas6 was required for CCR2 expression from monocytes and CCL2 expression from endothelial cells when treated with thrombin. We found that thrombin induced CCR2 expression in WT but not in Gas6−/− monocytes. More importantly, we demonstrated that CCR2 was required for thrombin-induced monocyte migration and that this process was Gas6 dependent. In addition, we found that CCL2 expression was increased by thrombin in WT but not in Gas6−/− endothelial cells. CCL2 secreted by WT endothelial cells was directly associated with an increase of monocyte migration and PSGL-1 expression. Hence, blocking CCL2 in vivo decreases the recruitment of inflammatory monocytes in the thrombus, resulting in the formation of a smaller clot. This is in accordance with a previous study showing that CCL2 blockade reduced monocyte recruitment and neointima formation after wire injury in vivo.

In accordance with other studies, we demonstrate that thrombin induces CCL2 and CCR2 expression through PAR-1 activation. Importantly, this study demonstrates that Gas6 is required for thrombin-mediated CCL2 and CCR2 expression in the context of venous thrombosis. Finally, several studies have demonstrated that the expression of CCL2 was regulated by JNK MAP kinase in endothelial cells and adipocytes. Accordingly, we found that Gas6 mediates thrombin-induced expression of CCL2 through the activation of JNK. Overall, the above data extend previous findings that demonstrate that Gas6 and its receptors have a role in inflammation.

We previously showed that Gas6 from the hematopoietic and the nonhematopoietic compartments was important for venous thrombosis. Here, we demonstrate that Gas6...
contributes to monocyte recruitment through the expression of CCL2 by endothelial cells and the expression of CCR2 by monocytes, which also suggest a contribution of both compartments. Our study emphasizes the role of Gas6 from monocytes and endothelial cells. However, Gas6 is also found in platelets in mice. Platelets are known to support inflammation and interact with endothelial cells, monocytes, and neutrophils. 49 They secrete cytokines stored in α-granules that facilitate leukocyte recruitment. 80 It is possible that the absence of Gas6 in platelets may impair their role in inflammation and monocyte recruitment. Thus, we cannot rule out a role for Gas6 from platelets in our study.

Taken together, our data reinforce the link between inflammation and venous thrombosis by showing that the recruitment of inflammatory monocytes is indispensable in the early stage of venous thrombus formation. Gas6 regulates both inflammatory and thrombotic events. We provide significant insight in the role of Gas6 in the formation of venous thrombosis through inflammatory monocyte recruitment. Deciphering the role of Gas6 and the precise function of inflammatory monocytes in venous thrombosis occurrence can be a powerful tool for the identification of new targets for future anti-thrombotic therapy.

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Disclosures
None.

References


Highlights

- Monocytes are recruited during venous thrombosis.
- Gas6 (growth arrest-specific 6) promotes venous thrombosis and modulates monocyte biology.
- Gas6 promotes the recruitment of inflammatory monocytes during venous thrombosis.
- The Gas6-dependent recruitment of inflammatory monocytes requires C-C chemokine receptor type 2 and chemokine ligand 2.
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Running Title: Gas6 promotes monocyte recruitment.

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Supplemental Figure Legends

Supplemental Figure I. Gas6 promotes the recruitment of inflammatory monocytes. MOMA-2 (red) and CCR2 (green) positive monocytes are reduced in Gas6−/− compared to WT thrombi (N=5). P<0.05, * vs control.

Supplemental Figure II. Clodronate reduces circulating monocytes but not neutrophils and platelets. (A) Clodronate reduces the number of circulating CD11b+ monocytes in WT mice (N=5-7). (B) Clodronate does not modify platelet content (CD41+) or platelet activation (P-selectin+) (N=3). (C) Neutrophil (Ly6G+) content is not alter by clodronate treatment (N=5-6). Numbers indicate percentage of gated live cells. FSC indicates forward scatter; SSC, side scatter. P<0.05, * vs control.

Supplemental Figure III. CCR2 antibody treatment successfully reduced circulating inflammatory monocytes. (A) Representative dot plots of Ly6C^{hi/lo} monocytes from electronically gated CD11b+ mononuclear cells isolated from peripheral blood of WT mice injected with a CCR2 mAb (α-CCR2) or a control isotype. Injection of a CCR2 mAb specifically reduces circulating CD11b+Ly6C^{hi} monocytes (N=3-4). (B) MOMA-2 (red) and CCR2 (green)-positive cells are decreased by injection of a CCL2 blocking antibody (N=4-5). P<0.05, * vs control.

Supplemental Figure IV. Thrombin-induced CCL2 and CCR2 expression is mediated by PAR-1. (A) CCL2 (N=6) and (B) CCR2 (N=4) mRNA expression is increased by thrombin in endothelial cells and monocytes, respectively. Inhibition of PAR-1 with SCH79797 prevents this increase. (C) PAR-1 expression in endothelial cells (N=3-6) and (D) monocytes (N=5-6) is independent of Gas6 and is not modulated by thrombin. P<0.05, * vs untreated.

Supplemental Figure V. CCL2 produced by the endothelial cells induces PSGL-1 expression in monocytes. Conditioned media from thrombin-treated endothelial cells induces PSGL-1 expression in monocytes. PSGL-1 expression is inhibited in presence of a CCL2 blocking antibody (N=4-5). P<0.05, * vs WT Veh conditioned media.

Supplemental Figure VI. Thrombin induced CCL2 mRNA expression in a dose-dependent manner in endothelial cells. Thrombin significantly induced CCL2 mRNA expression at doses 0.5 and 1 U/mL (N=7). P<0.05, * vs untreated endothelial cells.
Supplemental Figure I.
Supplemental Figure II.
Supplemental Figure III.
Supplemental Figure IV.
Supplemental Figure V.
Supplemental Figure VI.
Gas6 Promotes Inflammatory (CCR2^{hi}CX3CR1^{lo}) Monocyte Recruitment in Venous Thrombosis

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Methods

Animals

All experiments performed on mice were approved by the Animal Care Committee of McGill University. Gas6−/− male mice, littermate controls and C57BL/6N wild type (WT) between 8 to 12 weeks of age, obtained by in house breeding, were used in all experiments1.

Venous thrombosis models

The ferric chloride (FeCl3) model of thrombosis, on the inferior vena cava (IVC), was used as previously described1. The concentration of FeCl3 used was 0.18 M (equivalent to 5%). When indicated, thrombosis was induced using the flow restriction model as previously described2. Briefly, a blunted 30G needle was placed on the exposed inferior vena cava and a ligation of the vessel around the needle was performed with a permanent ligature (6-0 Perma-Hand Silk, Ethicon). Subsequently, the needle was carefully removed to avoid complete obstruction. Thrombi were harvested 48h after the establishment of flow restriction. Thrombus formation was monitored for 30 min for the FeCl3 model using a high frequency ultrasound system (HFUS), the Vevo770® micro imaging system (Visualsonics, Toronto, Canada) as previously described3, 4. For histology and immunofluorescence staining, the venous wall and thrombus were removed and fixed for 4h with 4% paraformaldehyde. After overnight incubation in 30% sucrose in PBS at 4°C, samples were embedded vertically in OTC compound (Tissue-tek, Sakura) and stored at -80°C until processing. For thrombus dry weight, the venous wall was removed by dissection and the thrombus was weighed.

Monocyte depletion and CCL2 blocking

Clodrosome® (liposomal clodronate suspension, Encapsula Nano Sciences) was used to deplete blood monocytes from mice and Encapsome® control liposome suspension was used as a control. Twenty-four hours prior to the surgery, mice were injected intravenously with 100 µL of liposomal clodronate or control liposomes. Depletion of monocytes expressing CCR2 was achieved by intravenous (IV) injection of a CCR2 antibody 24h prior to the surgery (clone MC-21; a generous gift form Dr Mathias
Mack). The blocking of CCL2 was done by IV injection of 20 µg of CCL2 blocking antibody (R&D systems) just before surgery.

**Histology and immunofluorescence staining of thrombus**

Serial 7 µm frozen sections were cut using cryostat. For histology, slides were stained with hematoxilin and eosin (H&E). Images were acquired at room temperature with a Leica DM 2000 fluorescent microscope (5x magnification) and the Infinity Capture Software (Concord, ON, Canada). Thrombus area was quantified using ImageJ software (NIH, Bethesda, Maryland, USA). For immunofluorescence staining, samples were incubated with 10% Bovine Serum Albumin (BSA) for 30 min at room temperature. Samples were then incubated for 1h at room temperature with a rat anti-mouse MOMA-2 (Abcam), a goat anti-mouse CCR2 (Santa Cruz Biotechnology), a rat anti-mouse Ly6G (R&D) or isotype control antibodies. Finally, appropriate secondary AlexaFluor antibodies were used for detection (anti-rat 555nm, anti-goat 488nm; Molecular Probes). After washing, sections were incubated with the 555- and 488-nm Alexa Fluor-conjugated secondary antibodies for 1 h at room temperature. All antibodies were diluted in 0.3% BSA in PBS. Nuclei were labeled with DAPI. Images were acquired at room temperature with a Leica DM 2000 fluorescent microscope (20x magnification) and the Infinity Capture Software (Concord, ON, Canada). Quantifications of positive cells were performed using ImageJ software (NIH, Bethesda, Maryland, USA).

**Isolation and treatment of murine endothelial cells and bone marrow-derived monocytes**

Murine endothelial cells were isolated from lungs of 8-weeks old WT or Gas6−/− mice as previously described. Endothelial cells were cultured in 50% endothelial cell basal medium (EBM-2) supplemented with endothelial cell bullet kit (EGM-2) and 50% DMEM/F12 supplemented with 10% fetal bovine serum. For experiments, endothelial cells were used between passage 3 and 6. Cells were treated with 1U/mL thrombin as a pro-thrombotic stimulus. This dose significantly increased CCL2 mRNA expression in WT endothelial cells (Figure VI in the online-only Data Supplement). In addition, we and other groups have tested this dose on platelet activation and on tissue factor expression in endothelial cells and monocytes.
Bone marrow-derived monocytes were isolated from bone marrow of 8-week old WT or Gas6\textsuperscript{-/-} mice. Bone marrow suspensions were isolated by flushing femurs and tibias of WT or Gas6\textsuperscript{-/-} mice with RPMI and penicilline/streptomycine. After two washes, cells were cultured in RPMI supplemented with 10% FBS and penicilline/streptomycine. Two hours after seeding, cells were washed twice with cell media to remove the non-adhering cells. Cells were treated the day after. Endothelial cells and monocytes were treated with thrombin (1U/mL) for 4h. When indicated, cells were pre-treated with PAR-1 antagonist, SCH79797 (250 µM, Tocris), and SP600125 (Sigma Aldrich, 10\textsuperscript{-5} mol/L) for 30 min.

**Immunofluorescence staining of monocytes**

After isolation, WT and Gas6\textsuperscript{-/-} monocytes were plated on glass coverslip on 24 well plates. 24h later cells were treated with or without thrombin for 4h. Cells were then fixed with 4% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, blocked with 10% BSA for 30 min, and incubated with a goat anti-mouse CCR2, a goat anti-mouse PSGL-1 or isotype control antibodies (Santa Cruz Biotechnology) for 1h. Cells were then incubated with an anti-goat Alexa fluor 555nm and an anti-goat Alexa fluor 488nm antibodies for 1h (Thermo Fisher Scientific). Slides were mounted using mounting media containing DAPI (Vector Laboratories). Images were acquired at room temperature with a Leica DM 2000 fluorescent microscope (40x magnification) and the Infinity Capture software (Concord, ON, Canada). Quantification of histological surface area was done using ImageJ Version 1.44p (NIH, Bethesda, Maryland, USA).

**Collection and use of endothelial cells conditioned media**

As described above, WT and Gas6\textsuperscript{-/-} endothelial cells were treated with or without thrombin. After 4h, cell culture media was collected, centrifuged 5 min at 1500 rpm and filtered to remove cells and cell debris. Conditioned media were stored at -20°C until use. For the migration assay, 700 µL of conditioned media was added to the lower compartment of a Boyden chamber and migration of monocytes was assessed as described below.

**Migration assay**
The migration assays were done using Boyden Chambers. In the first set of experiments, 100,000 WT or Gas6−/− bone marrow-derived monocytes were plated in the upper compartment and treated with thrombin for 4h. The lower compartment was filled with either RPMI or conditioned media from WT or Gas6−/− endothelial cells treated with vehicle or thrombin and with or without SP600125 as described above. When indicated, a CCL2 blocking antibody (R&D Systems) was added to the conditioned media at 3 µg/mL. Monocytes were incubated in the presence of a CCR2 antibody (Clone MC-215) at 10 µg/mL when indicated. The Boyden chambers were incubated overnight at 37°C. At the end of the experiment, the semi-permeable membrane of the upper chamber was collected and mounted on a slide with mounting media containing DAPI. The number of cells that have passed through the membrane was counted.

*Real-time quantitative polymerase chain reaction (qPCR)*

CCR2 expression was evaluated in monocytes and CCL2 in endothelial cells by quantitative real-time qPCR. Total RNA from cultured cells was extracted using a commercial kit according to the manufacturer’s instructions (Genaid; Froggabio). Five hundred nanogram of total RNA was reverse-transcribed as per the manufacturer’s instructions (Quanta; VWR). The SYBRgreen intercalant was used to detect the amplification with the PerfeCta SYBRGreen Fast (Quanta; VWR). Primers were designed using Primer Express Software (Applied Biosystems). The S16 gene was used for normalization. Fold changes were calculated using the ΔCt method and results were expressed as a fold change compared to untreated WT. Primer sequences were designed as follows:

S16 forward: 5’-ATCTCAAAGGCCCTGGTAGC-3’
S16 reverse: 5’-ACAAAGGTAAACCCCGATCC-3’
CCR2 forward: 5’-TGCTGTGTTTGGCTCTCTA-3’
CCR2 reverse: 5’-CCTACAGCGAAACAGGGGTG-3’
CCL2 forward: 5’-TTAAAAACCTGGATCGGAACCAA-3’
CCL2 reverse: 5’-GCATTAGCTTCAGATTACGGGT-3’
PAR-1 forward: 5’-TGAACCCCCGTCAATCTTTTC-3’
PAR-1 reverse: 5’-CCAGCAGGACGTTCATTTT-3’
**CCL2 ELISA**

CCL2 concentrations in endothelial cell supernatants were determined by ELISA following the manufacturer’s instructions (R&D Systems).

**Western Blot**

For western blot analysis, endothelial cells were homogenized in lysis buffer containing 1% Nonidet P40, 0.5% deoxycholic acid (sodium salt), 0.1% SDS, 1% Triton, and anti-proteases inhibitor cocktail. Twenty-five micrograms of protein were loaded onto a 10% polyacrylamide-SDS gel, subjected to electrophoresis, and transferred to nitrocellulose membranes. Membranes were incubated overnight with a rabbit anti-mouse phospho-JNK and a rabbit anti-mouse JNK antibodies (Cell signaling technology). Signals were revealed by chemiluminescence (PerkinElmer) with the molecular imager chemidoc XRS system (BioRad) and quantified by densitometry with Quantity one software (BioRad).

**Flow Cytometry**

Thrombi of WT and Gas6−/− mice were incubated 1h at 37°C with Liberase (Roche, 0.125 µg/mL). Single cell suspension was prepared by passing the sample 5 times through a 20G needle and a 100 µm cell strainer. Cells were resuspended in PBS containing 2% FBS and stained with a brilliant violet 421-conjugated anti-mouse CD11b (BioLegend), an AlexaFluor 488-conjugated anti-mouse Ly6G (clone 1A8; BioLegend), an APC-conjugated anti–mouse CCR2 (clone 47301; R&D Systems) and a PE-conjugated anti–mouse CX3CR1 (clone SA011F11; BioLegend) antibodies. Quantification of CCR2hiCX3CR1lo and CX3CR1hi monocytes within thrombi was done on gated CD11b+Ly6G− cells to exclude neutrophils.

Mononuclear cells were isolated from whole blood by Ficoll Density gradients (Histopaque®-1083, Sigma-Aldrich). Cells were resuspended in 2% FBS in PBS, and stained for the surface markers CD11b, Gr1 and Ly6C (eBioscience). Gating was first performed on forward versus side scatter to remove cell debris and doublets before selection of live cells based on exclusion of a viability dye. Blood monocytes were
identified as CD11b\(^+\)Ly6C\(^{hi/lo}\). Quantification of Ly6C\(^{hi}\) and Ly6C\(^{lo}\) blood monocytes was done on electronically gated CD11b\(^+\) cells. Each cell population was considered positive or negative compared with the degree of fluorescence when stained with appropriate isotype control antibody.

For analysis of neutrophils, whole blood was drawn from WT mice injected or not with clodronate. Red blood cells (RBC) were lysed using RBC lysis buffer (BioLegend). Blood neutrophils were stained using a PE-conjugated anti-mouse Ly6G (BD Bioscience). Platelets from whole blood were stained using a PE-conjugated anti-CD41 and a FITC-conjugated P-selectin antibody (BD Bioscience).

Flow cytometry was performed on the BD LSR Fortessa (BD Biosciences). Fluorescence minus one controls was used to determine fluorescence background and positively. Data analysis was performed using Flow Jo software (Tree Star Inc.).

**Statistical Analysis**

As indicated in the legend of each figure, data are presented as mean ± SEM or as dot for individual experiments with a line representing the median. D’Agostino and Pearson omnibus test was used to test the normal distribution of the groups. Significant difference between groups was assessed by a t-test. A one-way analysis of variance followed by a Tukey’s multiple comparison were also performed.

**References**

