CCN1/CYR61-mediated meticulous patrolling by Ly6C^{low} monocytes fuels vascular inflammation

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Inflammation is characterized by the recruitment of leukocytes from the bloodstream. The rapid arrival of neutrophils is followed by a wave of inflammatory lymphocyte antigen 6 complex (Ly6C)-positive monocytes. In contrast Ly6C^{low} monocytes survey the endothelium in the steady state, but their role in inflammation is still unclear. Here, using confocal intravital microscopy, we show that upon Toll-like receptor 7/8 (TLR7/8)-mediated inflammation of mesenteric veins, platelet activation drives the rapid mobilization of Ly6C^{low} monocytes to the luminal side of the endothelium. After repeatedly interacting with platelets, Ly6C^{low} monocytes commit to a meticulous patrolling of the endothelial wall and orchestrate the subsequent arrival and extravasation of neutrophils through the production of proinflammatory cytokines and chemokines. At a molecular level, we show that cysteine-rich protein 61 (CYR61)/CYR61 connective tissue growth factor nephroblastoma overexpressed 1 (CCN1) protein is released by activated platelets and enables the recruitment of Ly6C^{low} monocytes upon vascular inflammation. In addition endothelium-bound CCN1 sustains the adequate patrolling of Ly6C^{low} monocytes both in the steady state and under inflammatory conditions. Blocking CCN1 or platelets with specific antibodies impaired the early arrival of Ly6C^{low} monocytes and abolished the recruitment of neutrophils. These results refine the leukocyte recruitment cascade model by introducing endothelium-bound CCN1 as an inflammation mediator and by demonstrating a role for platelets and patrolling Ly6C^{low} monocytes in acute vascular inflammation.

inflammation | CCN1 | monocyte | neutrophil | platelet

Upon infection or tissue injury, circulating leukocytes leave the bloodstream and migrate into the inflammatory site. One determining parameter is the luminal side of the vascular endothelium, which locally presents stimulating molecules to establish adhesive contacts with leukocytes and promote their intravascular crawling and extravasation into the tissue. Neutrophils are typically the first leukocytes to be recruited to an inflammatory site, within a few hours, followed by the arrival of inflammatory monocytes (1, 2).

Two phenotypically and functionally distinct subsets of blood monocytes have been defined in mice according to the cell-surface markers they present. The first group is known as "lymphocyte antigen 6 complex (Ly6C)-positive monocytes" (Ly6C$^+$ monocytes) because they express high levels of Ly6C$^+$, but they also express CX_3CR1, CCR2, and CD62L. These Ly6C$^+$ monocytes are called "inflammatory" monocytes because they are recruited to sites of inflammation. The other group comprises the Ly6C^{low} monocytes which express higher levels of CX_3CR1 and lack CCR2 and CD62L. These Ly6C^{low} cells are termed "patrolling" monocytes (3–5). Ly6C$^+$ monocytes functionally resemble human CD14$^+$ monocytes, whereas the Ly6C^{low} monocytes are homologs of the human CD14^{dim}CD16$^+$ subset (6). Ly6C$^+$ monocytes are selectively recruited to inflamed tissues (3). They produce high levels of proinflammatory cytokines and differentiate into macrophages and dendritic cells in several infectious disease models as well as in atherosclerosis (3, 7, 8). In contrast, the main characteristics of Ly6C^{low} monocytes are their ability to patrol along the endothelium of blood vessels in the steady state, independently of the direction of the blood flow, and to scavenge microparticles attached to the endothelium (6, 9, 10), acting as luminal blood macrophages (11). Ly6C^{low} monocytes exhibit anti-inflammatory properties, as evidenced in the *ApoE*$^{-/-}$ and *ldlr*$^{-/-}$ mouse models of atherosclerosis (12, 13). They are also proinflammatory, as their number is increased in lupus patients and animal models (6, 14).

Cysteine-rich protein 61 (CYR61)/CYR61 connective tissue growth factor nephroblastoma overexpressed 1 (CCN1) is a matricellular protein produced and secreted by endothelial cells and fibroblasts, among others (15). First described in cardiovascular development and carcinogenesis, CCN1 now is recognized as a mediator in leukocyte migration and inflammatory processes (15, 16) and in the development of the thymus (17). CCN1 exhibits chemoattractant properties for human and murine inflammatory monocytes by promoting their adhesion and migration through integrin CD11b and the cell-surface heparin sulfate proteoglycan syndecan-4 (15, 16, 18–21). CCN1 treatment of murine macrophages induces transcriptional changes characteristic of M1 polarization including the up-regulation of proinflammatory cytokines, such as TNF-α, IL-1β, or IL-6 (21). In addition, CCN1 is upregulated in response to bacterial or viral infections in vitro and in atherosclerotic lesions in both humans and mice (15, 16) and is released from activated platelets in experimentally induced sepsis (22, 23).

To date, the functions of CYR61/CCN1 in inflammation have been elusive, notably because of the embryonic lethality of *Ccn1*-null mice, which have impaired vascular integrity and dysfunctional

Significance

Upon infection, circulating leukocytes leave the bloodstream and migrate into the inflammatory site. Neutrophils are the first leukocytes to be recruited within a few hours, followed by inflammatory lymphocyte antigen 6 complex (Ly6C)-positive monocytes. This study refines the model of the leukocyte recruitment cascade. We demonstrate that upon Toll-like receptor 7/8-mediated vascular inflammation, platelet activation drives the rapid mobilization of Ly6C^{low} monocytes to the luminal side of the endothelium. Accumulated Ly6C^{low} monocytes do not extravasate into the tissue. Instead, they meticulously patrol the endothelium and control the subsequent recruitment of neutrophils. Moreover, we show that endothelium-bound cyteine-rich protein 61 (CYR61)/CYR61 connective tissue growth factor nephroblastoma overexpressed 1 (CCN1) protein provides a molecular support for adequate patrolling of Ly6C^{low} monocytes in the steady state and under inflammatory conditions.

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cardiovascular development (24). The chemoattractant properties of CCN1 and its ability to bind to monocytes through CD11b led us to examine the role of endothelium-bound CCN1 on the functions of Ly6C^{low} monocytes in a steady state and under inflammatory conditions. In this paper we demonstrate that platelets and the luminal meticulous patrolling of Ly6C^{low} monocytes are important in the initiation of Toll-like receptor 7/8 (TLR7/8)-mediated inflammation and in the recruitment of neutrophils. We also show that CCN1/CYR61 protein is released by platelets and acts as a chemoattractant for Ly6C^{low} monocytes. Moreover endothelium-bound CCN1 is required for adequate patrolling of Ly6C^{low} monocytes in the steady state and under inflammatory conditions. Collectively, our data refine the model of the leukocyte recruitment cascade.

Results

Endothelium-Bound CCN1 Sustains Patrolling of Ly6C^{low} Monocytes in the Steady State. CYR61/CCN1 binds to several leukocytes, and in particular to inflammatory monocytes and macrophages, via integrin CD11b (18, 20, 25). However, the effect of CCN1 on the functions of Ly6C^{low} monocytes has not been investigated so far. We first examined the binding of CCN1 to Ly6C^{low} monocytes. We observed that recombinant human CCN1-Fc binds to both Ly6C^{low} and Ly6C^{+} murine monocytes but not to neutrophils (Fig. 1*A* and Fig. S1). Identical results were obtained with recombinant murine CCN1-His. Importantly, binding to monocytes could be inhibited with a CCN1-blocking antibody (Fig. 1*A*).

Endothelial cells produce, secrete, and bind to CCN1 through integrin $\alpha_V\beta_3$ (15, 16). By live microscopy we examined whether CCN1 secreted by endothelial cells coats their surface. A strong CCN1 signal was detected on the surface of live nonfixed bEnd-5 cells labeled with PECAM1 (platelet endothelial cell adhesion molecule 1) after overnight culture (Fig. 1*B*). For technical reasons (signal vs. noise), detection of CCN1 coating on the surface of mesenteric veins by intravital confocal microscopy was not achieved. Therefore we adapted a technique previously used to detect endothelium-bound CRAMP (cathelin-related antimicrobial peptide) (26) to investigate whether secreted CCN1 may form dense CCN1-rich areas on the luminal side of mesenteric endothelium. To this purpose, intravital confocal microscopy imaging of mesenteric veins was performed on C57BL/6J mice that had been i.v. injected with fluorescent microbeads conjugated with a CCN1-nonblocking antibody (Fig. S2). In this way we were able to detect CCN1 hot spots but not low levels of CCN1 coating. We observed that CD115^{+} cells (CD115 is an exclusive marker for blood monocytes) were positioned close to CCN1-rich spots (Fig. 1*C* and Movie S1). Injection of CCN1-blocking antibodies did not modify the detection of endothelium-bound CCN1 spots (Fig. S3); therefore anti-CCN1 antibody inhibits CCN1 binding only to monocytes and not to endothelial cells.

Because monocytes were positioned close to CCN1-rich areas, we investigated whether CCN1 is a molecular partner for the efficient patrolling of Ly6C^{low} monocytes. To explore this possibility, intravital confocal microscopy imaging was set up in vivo to monitor blood monocytes within mesenteric veins in real time. To do so we used *Cx$_3$cr1$^{gfp/wt}$* mice, in which both Ly6C^{+} and Ly6C^{low} monocytes express eGFP (3, 9, 27). Higher levels of CX$_3$CR1 in patrolling Ly6C^{low} monocytes result in higher GFP levels, enabling Ly6C^{low} monocytes to be differentiated from Ly6C^{+} monocytes by FACS and microscopy (3, 9). After the vasculature was monitored under steady-state conditions, *Cx$_3$cr1$^{gfp/wt}$* mice were injected i.v. with either the CCN1-blocking antibody or control Ig. The impact of CCN1 blocking on monocyte patrolling was assessed 30 min later. Injection of control Ig had no impact on the tracks of crawling monocytes (Fig. 1*D*). In contrast, anti-CCN1 antibody strongly impaired the constitutive crawling-type motility of Ly6C^{low} monocytes in the steady state (Fig. 1 *D* and *E*). The average instantaneous velocity (speed mean) and the track length of crawling monocytes were decreased compared with controls.

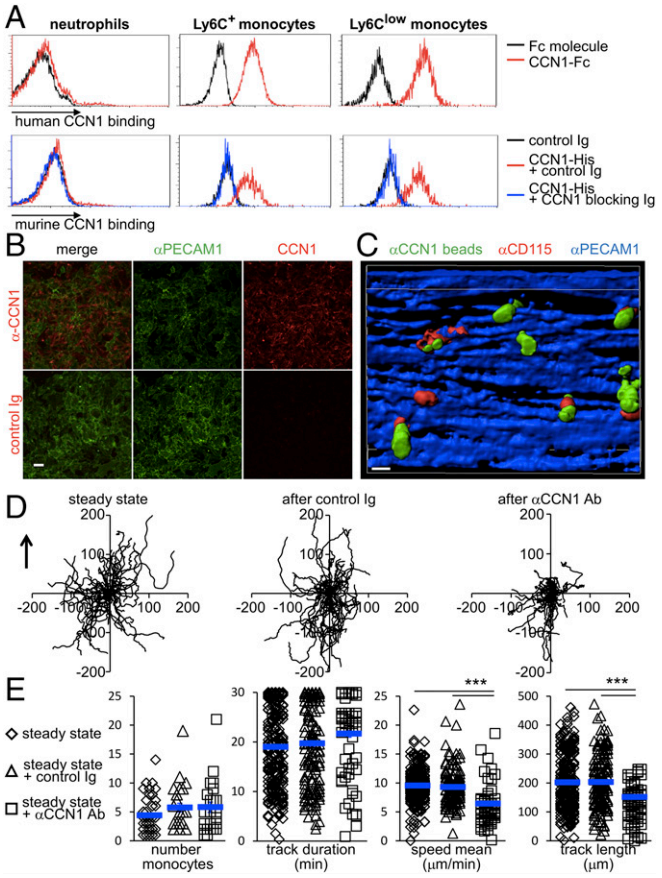

Fig. 1. Endothelium-bound CCN1 sustains the patrolling of Ly6C^{low} monocytes in the steady state. (*A*) Binding of CCN1 (1 μg/mL) was assessed by flow cytometry. For human CCN1, peripheral blood leukocytes were treated with recombinant Fc or human CCN1-Fc for 5 min at room temperature. Human CCN1 binding was detected by flow cytometry using a Dylight 488-conjugated anti-human Fc antibody. For murine CCN1, recombinant CCN1-His (1 μg/mL) was preincubated for 2 h at room temperature with control sheep Ig or blocking polyclonal sheep anti-CCN1 antibodies (50 μg/mL). Then cells were treated with recombinant murine CCN1-His for 5 min at room temperature. Murine CCN1 binding was detected by flow cytometry using a DyLight 650-conjugated anti–His-tag antibody. Data are representative of three experiments. (*B*) Cell surface-bound CCN1 on live bEnd-5 cells. Cells were incubated with sheep anti-CCN1 antibody or control Ig and then with Cy3-conjugated anti-sheep Ig in the presence of AF488-conjugated anti-PECAM1 to detect the vascular wall surface and the presence of CCN1. (Scale bar: 50 μm.) Representative maximal projections of four preparations are shown. (*C*) 3D reconstruction of mouse mesenteric veins obtained by confocal intravital microscopy depicting CD115^{+} patrolling monocytes located next to CCN1 hot spots on the luminal face of mesenteric veins. The view shows the luminal side of the vein. The i.v. injection of AF647-conjugated anti-PECAM1 (CD31, 10 μg) and AF594-conjugated anti-CD115 (1 μg) stained the endothelium blue and monocytes red, respectively. The luminal presence of CCN1-rich areas (green) was assessed after injection of protein A-coupled YFP beads conjugated with a nonblocking antibody to CCN1. (Scale bar: 10 μm.) (Movie S1.) (*D*) Representative tracks of Ly6C^{low} monocytes in the steady state (before) and 30 min after injection of control Ig or CCN1-blocking antibodies (50 μg/mL). Fifty monocytes per condition are represented. The arrow indicates the direction of the blood flow. (*E*) The number of crawling Ly6C^{low} monocytes and their track duration, speed, and length in *D* are quantified. $n = 4$ mice per condition; 25–26 vessels were analyzed in each condition. Data are mean ± SEM; ***$P < 0.005$; Kruskal–Wallis with Dunn's multiple comparisons test.

However, blocking CCN1 did not alter the number of patrolling monocytes nor the duration of their patrolling activity; therefore CCN1 is not involved in the adhesion of Ly6C^{low} monocytes to the endothelial wall (Fig. 1*E*).

Next, because CD11b is one of the known ligands of CCN1 on monocyte surfaces (20, 25), we evaluated the impact of blocking CD11b on the quality of monocyte patrolling in the steady state by intravital microscopy. The i.v. injection of CD11b-blocking antibodies did not affect the number of patrolling Ly6C^{low} monocytes (Fig. S4), as previously reported (9). However, the quality of locomotory behavior was altered (Fig. S4), in a manner close to the blocking of CCN1 (Fig. 1 *D* and *E*). Overall these data show that endothelium-bound CCN1 is required for the efficient patrolling of Ly6C^{low} monocytes in the steady state but is not required for their adhesion to the endothelium.

CCN1 Mediates Luminal Recruitment of Ly6C^{low} Monocytes, Which Precede Neutrophils During TLR7/8-Mediated Inflammation. Ly6C^{low} monocytes survey blood vessels in the steady state (9). Therefore we reasoned that interactions between CCN1 and Ly6C^{low} monocytes might be important in the early steps of blood vessel inflammation. To test this idea, we used the TLR7/8 agonist Resiquimod (R848) (28), because Ly6C^{low} monocytes express high levels of TLR7 and TLR8 (Fig. S5); the homologous human CD14^{dim}CD16^{+} monocytes also have high levels of TLR7/8, which mediate their response to nucleic acids and viruses (6). We first examined changes in CCN1 levels in inflamed mesenteric vessels by intravital confocal microscopy. Luminal endothelium-bound CCN1 was measured in mesenteric vessels before and 20 min after the induction of inflammation generated by applying R848 directly to the imaged vessels. We observed a threefold luminal increase in CCN1 compared with the steady state (Fig. 2*A*), strongly suggesting that CCN1 has a role in inflammation.

We next evaluated the importance of CCN1 in the kinetics of monocyte and neutrophil recruitment to inflamed mesenteric veins. We i.v. injected CellTracker Orange-labeled murine neutrophils into *Cx$_3$cr1$^{gfp/wt}$* mice to visualize monocytes in green and neutrophils in red under steady-state and inflammatory conditions by intravital confocal microscopy (27). After monitoring the locomotory behavior of Ly6C^{low} monocytes in the steady state (precondition), mice were i.v. injected with either the CCN1-blocking antibody or control Ig. At the same time, inflammation was generated by directly applying R848 onto the imaged vessels. Time-lapse series of 30 min were recorded, and the number of patrolling monocytes and neutrophils was determined (Fig. 2 *B* and *C*). R848 stimulation rapidly recruited crawling monocytes to the endothelium; the wave of neutrophils occurred later (Fig. 2 *B* and *C* and Movie S2). Monocytes were actively captured from the flowing blood (Movie S3) or crawled into the field. Interestingly, blocking the binding of CCN1 to monocytes dampened the recruitment of Ly6C^{low} monocytes and almost abolished the arrival of neutrophils (Fig. 2*D*, Fig. S6, and Movie S2). The phenomenon occurred both in large mesenteric veins and in the microvasculature (Fig. S6).

Anti-GR1 antibody recognizes both Ly6C and Ly6G antigens. Therefore, Ly6C^{+} and Ly6C^{low} monocytes can be distinguished by their GFP and Ly6C/GR1 expression (4). Although recruited monocytes were GFP^{high}, intravital imaging was combined with i.v. injection of labeled anti-GR1 antibody, which stains both neutrophils and Ly6C^{+} monocytes but not Ly6C^{low} monocytes. All recruited monocytes that crawled on the endothelium were GR1^{-}, confirming that they were Ly6C^{low} monocytes (Fig. 2*E* and Movie S4).

Overall our results demonstrate clearly that CCN1 is required for the recruitment of Ly6C^{low} monocytes and that this recruitment precedes the arrival of neutrophils upon TLR7/8-mediated inflammation.

Recruited Neutrophils Extravasate While Accumulated Ly6C^{low} Monocytes Meticulously Patrol the Luminal Side of the Endothelium upon TLR7/8-Mediated Inflammation. CD11b is present on the surface of all monocytes and neutrophils. Therefore an i.v. injection

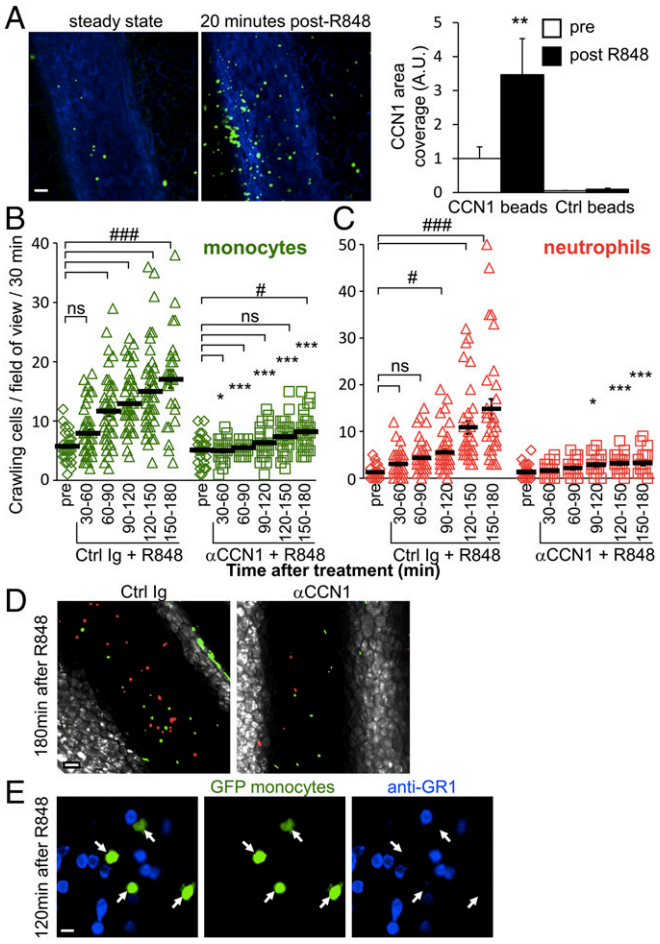

Fig. 2. CCN1 mediates luminal recruitment of Ly6C^{low} monocytes, which precedes the arrival of neutrophils during TLR7/8-mediated inflammation. (*A*) Luminal CCN1 accumulation during inflammation. Representative confocal intravital microscopy images of mesenteric vein in the steady state (before) and 20 min after induction of inflammation by treatment with R848 (100 μg). AF647-conjugated anti-PECAM1 (CD31, 10 μg, i.v.) stained the endothelium blue. Luminal CCN1 accumulation was assessed after i.v. injection of protein A-coupled YFP beads conjugated with a nonblocking antibody to CCN1. (Scale bar: 50 μm.) Quantification is provided on the right. "Pre" denotes steady-state conditions before R848 treatment and antibody injection. Protein A-coupled YFP beads conjugated with rabbit Ig were used as controls. Eight vessels from two mice were analyzed. $^{**}P < 0.01$; Wilcoxon matched-pairs signed rank test. (*B* and *C*) Kinetics of recruitment of Ly6C^{low} monocytes (green) (*B*) and neutrophils (red) (*C*) in response to R848 stimulation (100 μg) in *Cx$_3$cr1*$^{gfp/wt}$ mice transferred with CellTracker Orange-labeled neutrophils. Mice were administered control sheep Ig or CCN1-blocking antibodies (50 μg/mL, i.v.) after steady-state conditions were achieved. Simultaneously, R848 was applied to the monitored vessels to induce inflammation. $n = 5$–8 mice per condition; 28–50 vessels were analyzed per time point. Data are mean ± SEM; $^{*}P < 0.05$, $^{***}P < 0.005$, control (Ctrl) Ig vs. αCCN1 treatment; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.005$, antibody and R848 treatment vs. the precondition; two-way ANOVA with Tukey's multiple comparisons test. (*D*) Representative confocal intravital microscopy images of mesenteric vein 180 min after R848 treatment in the presence of control sheep Ig or CCN1-blocking antibodies (50 μg/mL) (from Movie S2 and experiments shown in Fig. 3 *B* and *C*). (Scale bar: 50 μm.) (*E*) Representative confocal intravital microscopy images of mesenteric vein from Movie S4. Mice were administered BV421-conjugated GR1 antibody (1 μg, i.v.) 120 min after R848 treatment. Endogenous neutrophils are labeled in blue, and all monocytes are green. Inflammatory Ly6C^{+} monocytes would appear in blue and green, and Ly6C^{low} monocytes only in green. All observed monocytes were GR1^{-}, indicating that they were Ly6C^{low} monocytes. White arrows indicate the position of monocytes. Fifteen vessels from three mice were analyzed. (Scale bar: 10 μm.)

of labeled anti-CD11b antibodies will stain all cells located in the lumen but not extravasated cells. Two hours after R848 stimulation, i.v. injection of labeled anti-CD11b antibodies (blue) stained around 50% of transferred red neutrophils, indicating the rapid extravasation of neutrophils into the surrounding tissue after their recruitment to the endothelial wall (Fig. 3*A*). In contrast to the neutrophils, the crawling monocytes were located inside the mesenteric veins, as indicated by their nearly 100% positive labeling with anti-CD11b (Fig. 3*B*).

Analysis of patrolling tracks 60 min after R848 treatment, a time point when the number of Ly6C^{low} monocytes was already increased (Fig. 2 *B* and *C*), revealed a more "meticulous" patrolling behavior of Ly6C^{low} monocytes than seen in the steady state (Fig. 3 *C* and *D*). We observed that R848 increased the duration of attachment of monocytes to the endothelium and that the average instantaneous velocity (speed mean) and the linearity and the length of the track of crawling monocytes were decreased. The more muddled locomotory behavior was also witnessed by the increase of the confinement ratio (track length/track displacement) (Fig. 3 *C* and *D*). In contrast, anti-CCN1 antibody prevented the establishment of meticulous patrolling of Ly6C^{low} monocytes, resulting in an intermediate quality of patrolling (Fig. 3 *C* and *D*). These data show that Ly6C^{low} monocytes remain patrolling on the luminal side of the endothelium upon R848 treatment whereas recruited neutrophils exit the vessel.

Luminal Recruitment of Patrolling Ly6C^{low} Monocytes Relies on Chemokine Receptors. We next wondered whether CCN1 and chemokine receptors use the same pathways for the recruitment of Ly6C^{low} monocytes and neutrophils. To this end, we performed the intravital imaging experiments in *Cx$_3$cr1$^{gfp/wt}$* mice depicted in Fig. 2 in the presence of pertussis toxin (PT), an inhibitor of chemokine receptor signaling. The i.v. injection of PT or CCN1-blocking antibody alone reduced the recruitment of monocytes and neutrophils (Fig. 4 *A* and *B*). Administration of both treatments together completely suppressed the arrival of monocytes and neutrophils in response to R848 (Fig. 4 *A* and *B*), showing that CCN1 acts independently of chemokine receptors.

To establish the importance of Ly6C^{low} monocyte patrolling in the recruitment of neutrophils, we next performed intravital experiments in *Cx$_3$cr1*-deficient mice (*Cx$_3$cr1$^{gfp/gfp}$*), which have decreased numbers of Ly6C^{low} monocytes patrolling the endothelium in the steady state (9) as compared with *Cx$_3$cr1$^{gfp/wt}$* mice (Fig. 4*A*), although the binding of Ly6C^{low} monocytes to CCN1 is normal (Fig. 4*C*) (9, 29). The recruitment of *Cx$_3$cr1*-deficient monocytes to the vascular wall was reduced in response to R848 (Fig. 4*A*). Interestingly, the recruitment of neutrophils in *Cx$_3$cr1*-deficient mice also was compromised (Fig. 4*B*). Flow cytometry analyses revealed that the expression of fractalkine (CX_3CL1), the ligand of chemokine receptor CX_3CR1, was not significantly increased on the surface of mesenteric endothelial cells 60 min after topical stimulation of mesenteric vessels with R848 (Fig. 4*D*). These results confirm that the presence of Ly6C^{low} monocytes on the vasculature and their ability to patrol efficiently are required to achieve an adequate recruitment of neutrophils in response to R848.

Finally, intravital imaging showed that i.v. injection of antibodies blocking CD11b or CCN1 after R848 stimulation did not affect the attachment of Ly6C^{low} monocytes (Fig. 4 *E* and *F*), as already observed in the steady state (Fig. 1*E* and Fig. S4*B*). Therefore, CCN1 and its ligand CD11b are not involved in the attachment of Ly6C^{low} monocytes even under inflammatory conditions. On the contrary, CD11b blockade after R848 stimulation detached red transferred neutrophils from the endothelial wall (Fig. 4*E*), indicating the importance of integrin CD11b for the adhesion of neutrophils. We infer that the remaining neutrophils were those that had extravasated and thus were not affected by anti-CD11b injection (Fig. 4*A*). Because CCN1 does not bind to neutrophils, anti-CCN1 injection had no effect on their attachment (Fig. 4*F*).

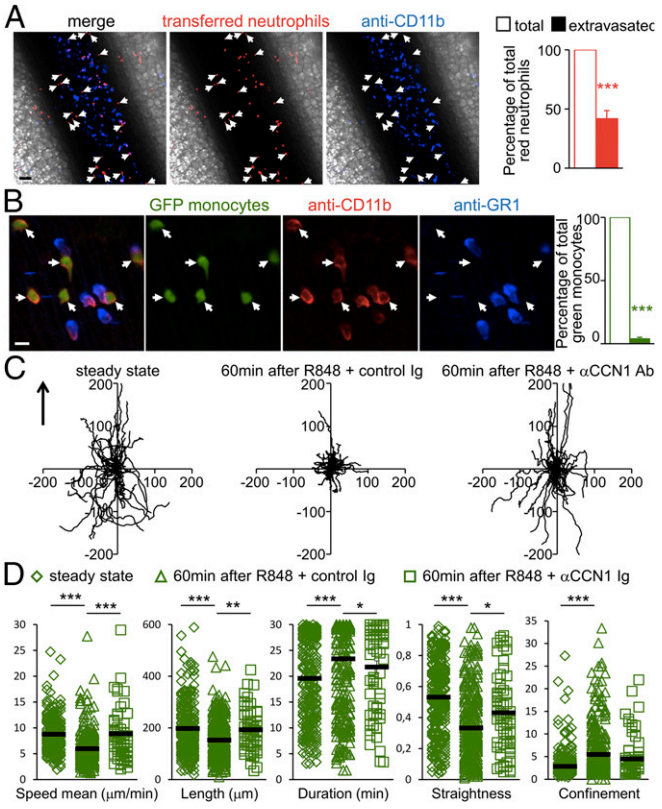

Fig. 3. Recruited neutrophils extravasate while accumulated Ly6C^{low} monocytes meticulously patrol the luminal side of the endothelium upon TLR7/8-mediated inflammation. (*A*) Recruited neutrophils exit the vessel and invade the surrounding tissue. Shown are representative confocal intravital microscopy images of the mesenteric vein. Mesenteric veins of mice transferred with CellTracker Orange-labeled neutrophils were treated with R848 (100 μg). After 120 min, mice were injected with AF647-conjugated anti-CD11b antibody (1 μg, i.v.). All transferred neutrophils are CD11b$^+$ cells. Endogenous neutrophils are blue. Transferred red neutrophils crawling on the luminal side of the endothelium are in contact with circulating labeled anti-CD11b antibody (red and blue). Extravasated red neutrophils do not have contact with the labeled anti-CD11b antibody and appear only in red (white arrows). (Scale bar: 50 μm.) Quantification is provided on the right. $n = 3$ mice; 20 vessels were analyzed. ***$P < 0.005$; Wilcoxon matched-pairs signed rank test. (*B*) Ly6C^{low} monocytes patrol the luminal side of the endothelium. Shown are representative confocal intravital microscopy images of mesenteric vein. AF594-conjugated anti-CD11b and BV421-conjugated anti-GR1 antibodies were administered (1 μg, i.v.) to mice 120 min after R848 treatment. Ly6C^{low} monocytes are GFP$^+$ (green) and GR1$^-$ (not blue). Arrows indicate monocytes. All observed cells were CD11b$^+$ (red), indicating that they are located on the luminal side of the endothelium. (Scale bar: 10 μm.) Quantification is provided on the right. ***$P < 0.005$; Wilcoxon matched-pairs signed rank test. $n = 3$ mice; 16 vessels were analyzed. (*C* and *D*) Meticulous patrolling of Ly6C^{low} monocytes after R848 treatment. (*C*) Representative tracks of Ly6C^{low} monocytes in the steady state (before) and 60 min after R848 treatment that were injected with control sheep Ig or antibodies blocking CCN1 (from experiments in *B*). Fifty monocytes per condition are represented. The arrow indicates the direction of the blood flow. (*D*) Track speed, length, duration, linearity, and confinement ratio of recruited patrolling Ly6C^{low} monocytes from experiments in *B*. Data are mean ± SEM; *$P <$ 0.05; **$P < 0.01$; ***$P < 0.005$; Kruskal–Wallis with Dunn's multiple comparisons test.

We then examined whether locally necrotic endothelial cells could account for the accumulation of Ly6C^{low} monocytes. To examine this possibility, we assessed the integrity of mesenteric veins after R848 stimulation. Mice were i.v. injected simultaneously with Sytox, a nucleic acid stain that can be used as an

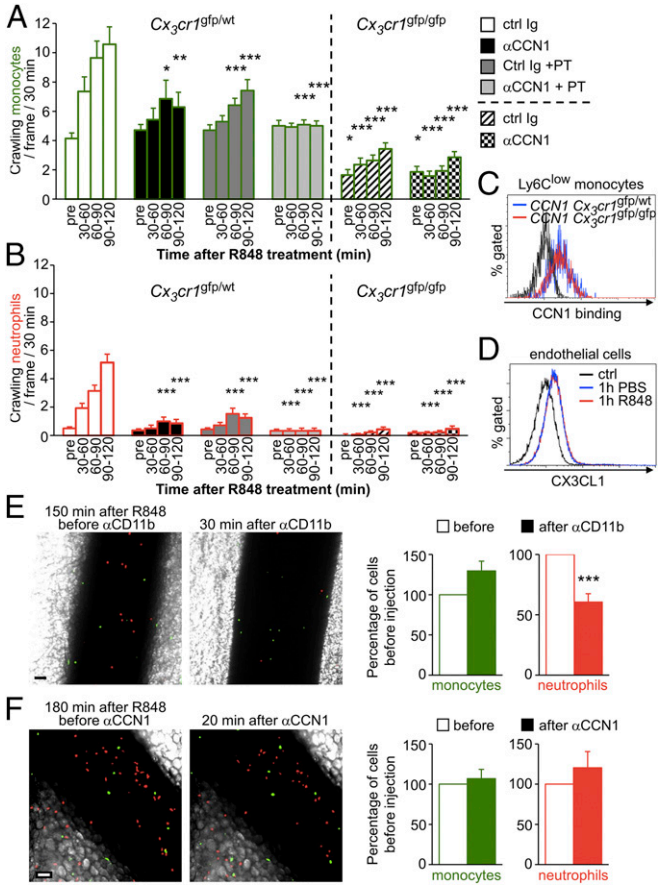

Fig. 4. Recruitment of patrolling Ly6C^{low} monocytes relies on chemokine receptors. (*A* and *B*) Kinetics of recruitment of Ly6C^{low} monocytes (bars outlined in green) (*A*) and neutrophils (bars outlined in red) (*B*) in response to R848 stimulation (100 μg) in *Cx$_3$cr1*$^{gfp/wt}$ (*Left*) and *Cx$_3$cr1*$^{gfp/gfp}$ (*Right*) mice transferred with CellTracker Orange-labeled neutrophils. After steady-state conditions were recorded, R848 was applied to the monitored vessels to induce inflammation. Simultaneously, mice were i.v. injected with control sheep Ig or antibodies blocking CCN1 in PBS containing PT (50 μg). "Pre" denotes steady state conditions before R848 treatment and antibody injection. $n = 3$ mice per condition; 14–21 vessels were analyzed per condition. $^*P < 0.05$, $^{**}P < 0.01$. $^{***}P < 0.005$; two-way ANOVA with Tukey's multiple comparisons test. (*C*) FACS analysis of CCN1 binding to Ly6C^{low} monocytes of *Cx$_3$cr1*$^{gfp/wt}$ and *Cx$_3$cr1*$^{gfp/gfp}$ mice. (*D*) Mesenteric vasculature of C57BL/6J mice was treated with PBS or R848 (100 μg). Then mesenteric endothelial cells were analyzed by FACS for CX$_3$CL1 surface expression. $n = 3$ mice per condition. (*E*) Representative confocal intravital microscopy images of mesenteric vein. Mice were injected with anti-CD11b antibody (50 μg, i.v.) 150 min after R848 treatment (100 μg). Thirty minutes later, attached monocytes (green) and neutrophils (red) were quantified. (Scale bar: 50 μm.) Quantification is provided on the right. $n = 3$ mice; 20 vessels were analyzed. $^{***}P < 0.005$; Wilcoxon matched-pairs signed rank test. (*F*) Representative confocal intravital microscopy images of mesenteric vein. Mice were injected with anti-CCN1 antibody (50 μg, i.v.) 180 min after R848 treatment (100 μg). Twenty minutes later, attached monocytes (green) and neutrophils (red) were quantified. (Scale bar: 50 μm.) Quantification is provided on the right. $n = 2$ mice; 14 vessels were analyzed. Data are mean ± SEM.

indicator of dead cells, and with anti-PECAM1 to label the vascular wall. 3D reconstruction of vessels revealed no dead endothelial cells even 120 min after stimulation. However, a few dead cells could be observed deep in the tissue surrounding the stimulated vessels (Movie S5). As a positive control for Sytox staining, mesenteric veins were treated with H_2O_2 to induce cell death. In that case, dead endothelial cells were observed all around the vasculature (Movie S6).

Our data point out that the recruitment of Ly6C^{low} monocytes involves a synergic role of chemokine receptors (including CX$_3$CR1) and CCN1 in response to R848 but not in response to local necrosis of endothelium.

Platelet–Ly6C^{low} Monocyte Interactions Are Required for the Initiation of Inflammation. Platelets are known to be important for hemostasis, but the number of reports regarding their role in immunity is increasing (30–32). Their ability to recognize pathogens and microbial products contributes to the activation and recruitment of immune cells (33, 34). In fact, efficient interaction between platelets and inflammatory monocytes increases the production of cytokines (35, 36).

In vivo labeling of mouse platelets with anti-CD49b was described and validated previously (33, 37). We thus performed intravital microscopy of mesenteric veins with anti-CD49b and found numerous interactions between platelets and crawling monocytes as early as 20 min after R848 stimulation, although such interactions were not detected in the steady state (Fig. 5 *A* and *B* and Movies S7 and S8). Ly6C^{low} monocytes seemed to be less polarized and to exhibit smaller lamellipods than during meticulous patrolling in response to R848 stimulation. An average interaction lasted 19.5 ± 1.3 s, and on average each monocyte interacted with 6.9 ± 0.4 platelets every 3 min. These interactions were further confirmed in vitro. Activation of platelets with thrombin strongly increased the percentage of Ly6C^{low} monocytes interacting with platelets (Fig. 5*C*), but the presence of CCN1 did not affect these interactions (Fig. 5*C*).

We next studied the role of platelets in early monocyte recruitment in response to R848 by intravital microscopy. Mice

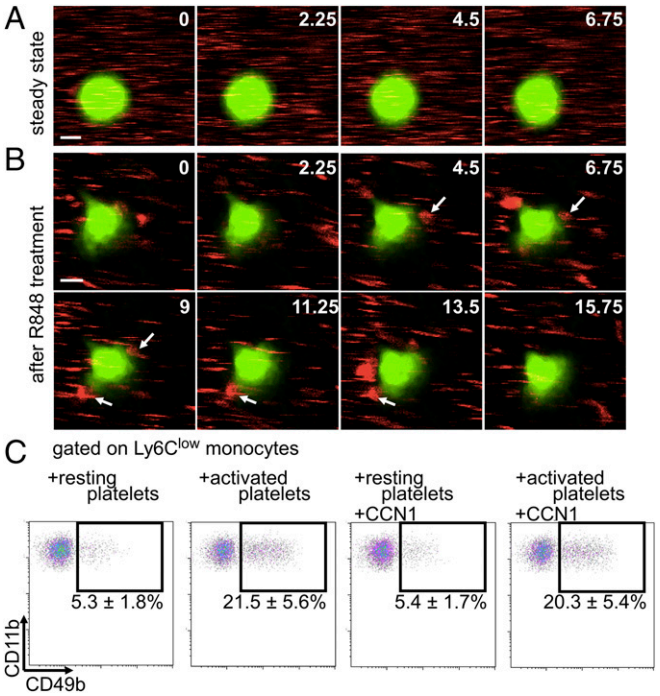

Fig. 5. Early platelet–Ly6C^{low} monocyte interactions. (*A* and *B*) Intravital microscopy of interactions (arrows in *B*) of platelets (labeled with anti-CD49b; red) and Ly6C^{low} monocytes (labeled with GFP, green) in mesenteric veins in the steady state (*A*) and after R848 stimulation (50 μg) of the vasculature (*B*). Images in *A* are from Movie S7. (Scale bar: 5 μm.) Images in *B* are from Movie S8. Arrows indicate platelets interacting with a monocyte. Numbers in corners indicate time (in seconds). (Scale bar: 5 μm.) Images are representative of 71 monocytes analyzed. (*C*) Representative flow cytometry analysis of platelets interacting with Ly6C^{low} monocytes. Peripheral blood leukocytes were incubated with thrombin (0.25 U/mL)-activated platelets for 20 min in the presence of recombinant CCN1 (5 μg/mL). Plots are gated on Ly6C^{low} monocytes. Data are mean ± SEM from four preparations.

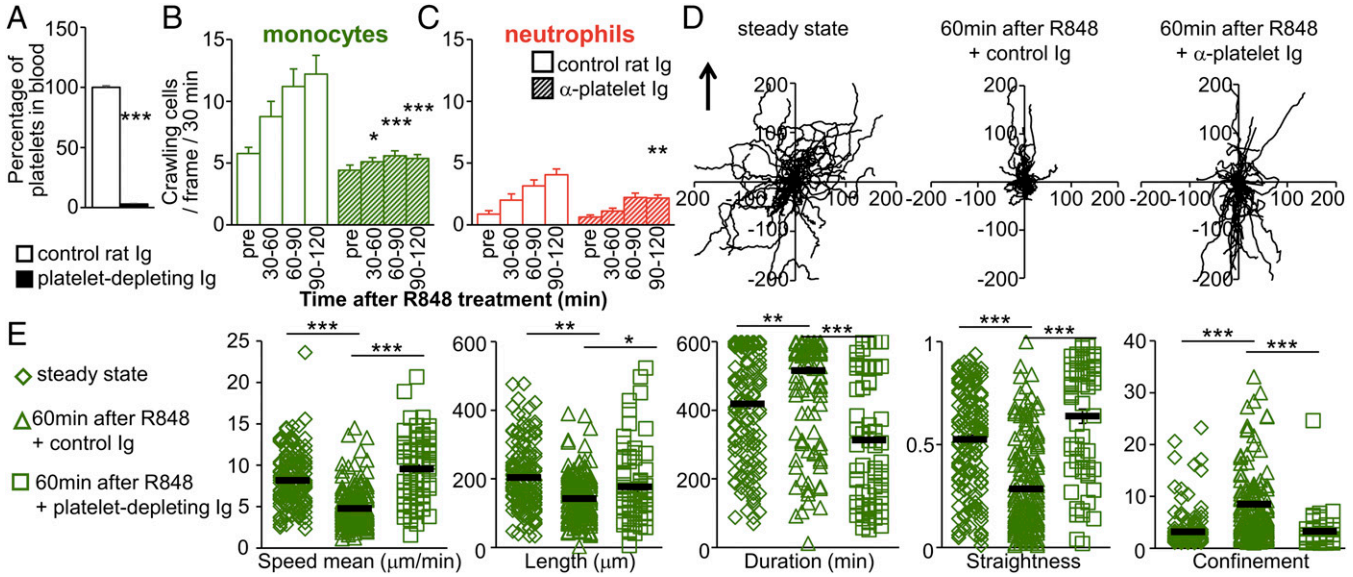

Fig. 6. Platelet–Ly6C^{low} monocyte interactions are required for the initiation of inflammation and meticulous patrolling. (*A*) C57BL/6J mice were administered control rat Ig or platelet-depleting antibodies (50 μg, i.v.). Depletion of platelets from blood circulation was evaluated 1 h later by flow cytometry. Data are expressed relative to control rat Ig-treated mice. Three mice were studied per condition. ***$P < 0.005$; the Mann–Whitney unpaired test was used for statistical analysis. (*B* and *C*) Kinetics of recruitment of Ly6C^{low} monocytes (green) (*B*) and neutrophils (red) (*C*) in response to R848 stimulation (100 μg) in *Cx$_3$cr1$^{gfp/wt}$* mice transferred with CellTracker Orange-labeled neutrophils. Mice were administered control rat Ig or platelet-depleting antibodies (50 μg, i.v.) 1 h before R848 was applied to the monitored vessels. "Pre" denotes steady-state conditions before R848 treatment. Three mice were used, and 19–21 vessels were analyzed per condition. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$; two-way ANOVA with Tukey's multiple comparisons test. (*D* and *E*) Impaired meticulous patrolling of Ly6C^{low} monocytes after R848 treatment (100 μg) in platelet-depleted mice. (*D*) Representative tracks of Ly6C^{low} monocytes in the steady state (before) and 60 min after R848 treatment from experiments in *B* in mice injected with control rat Ig or platelet-depleting antibodies. Fifty monocytes per condition are represented. The arrow indicates the direction of the blood flow. (*E*) Track speed, length, duration, straightness, and confinement ratio of recruited patrolling Ly6C^{low} monocytes from experiments in *B*. Data are mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$; Kruskal–Wallis with Dunn's multiple comparisons test.

were i.v. injected with platelet-depleting antibodies or control Ig. After 60 min (the time needed to eliminate >95% of circulating platelets) (Fig. 6*A*), we monitored the behavior of Ly6C^{low} monocytes in response to R848. Depletion of platelets drastically impaired the recruitment of Ly6C^{low} monocytes and neutrophils (Fig. 6 *B* and *C* and Movie S9). In addition, the locomotory behavior of Ly6C^{low} monocytes from platelet-depleted mice failed to exhibit the characteristics of meticulous patrolling seen after R848 treatment (Fig. 6 *D* and *E*). We did not see protrusions where platelets become trapped in the steady state. In the steady state, patrolling is quick.

We conclude that platelets are important for the recruitment of Ly6C^{low} monocytes and in the setting of meticulous patrolling upon R848 stimulation.

Platelets Contribute to Luminal CCN1 upon TLR7/8-Mediated Inflammation. Platelet depletion and blocking CCN1 have similar effects on leukocyte recruitment and on the locomotory behavior of Ly6C^{low} monocytes (Figs. 2, 3, and 6). Moreover platelets are known to produce and release CCN1 in experimentally induced sepsis (22). Therefore we next investigated whether increased luminal CCN1 upon R848 treatment was dependent on platelets. Intravital confocal imaging revealed that depletion of platelets did not affect endothelium-bound CCN1 in mesenteric vessels in the steady state, but it suppressed the increase of endothelium-bound CCN1 in response to TLR7/8 stimulation (Fig. 7*A*). Treatment of platelets with thrombin alone induced the release of CCN1 within 30 min, whereas R848 had no effect (Fig. 7*B*). No increase in CCN1 release was observed in similarly stimulated peripheral blood mononuclear cells (PBMCs) or in two endothelial cell lines (Fig. 7 *C* and *D*).

Taken together, our data show that platelets are required for luminal CCN1 upon TLR7/8 stimulation in vivo and, based on in vitro experiments, suggest that they directly release CCN1.

Platelets Potentiate the Production of Inflammatory Cytokines and Chemokines by Ly6C^{low} Monocytes. Because Ly6C^{low} monocytes are recruited before neutrophils (Fig. 2), and this process relies on platelets (Figs. 5 and 6), we tested the capacity of Ly6C^{low} monocytes to produce cytokines and chemokines relevant to neutrophil recruitment and activation. To this end, FACS-sorted Ly6C^{low} monocytes were stimulated in vitro with R848 (Fig. 8). The response of Ly6C^{low} monocytes was characterized by the production of chemokines KC (CXCL1), MIP1α (CCL3), and MIP1β (CCL4) and the proinflammatory cytokines TNF-α and IL-6. The production of KC and MIP1α is of particular interest, because they are well known to contribute to neutrophil recruitment (38). Moreover, the presence of platelets or thrombin-activated platelets further stimulated the production of cytokines by Ly6C^{low} monocytes, at least regarding the production of IL-6 and KC (Fig. 8).

Taken together, these data show that interactions between platelets and Ly6C^{low} monocytes favor the recruitment of Ly6C^{low} monocytes, their meticulous patrolling, and their activation upon R848-mediated inflammation.

Discussion

The leukocyte recruitment cascade is often considered to be merely a case of cell transmigration into the injured or infected tissue. Neutrophils are critical in the early steps of the immune response, because they typically are the first leukocytes to be recruited to the inflammatory region, followed by the emigration of Ly6C^{+}/GR1^{+} monocytes (1, 2). These neutrophils actively participate in the subsequent arrival of Ly6C^{+}/GR1^{+} monocytes through the release of granule proteins such as cathelicidins, azurocidin, or cathepsin G (26, 38–40), but not that of Ly6C^{low} monocytes.

TLR7/8-mediated inflammation of the endothelium by R848 up-regulates ICAM-2, P-selectin, E-selectin, and VCAM-1,

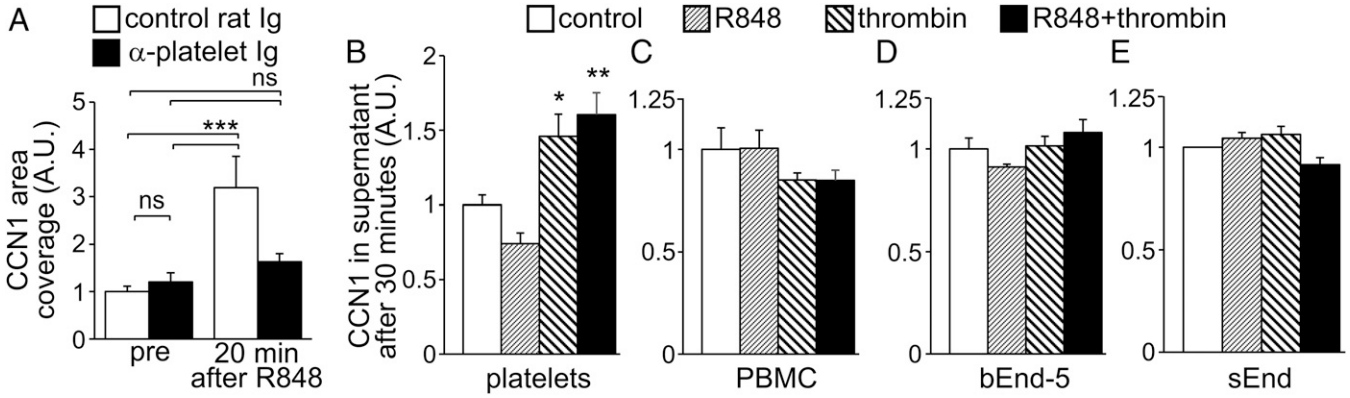

Fig. 7. Platelets contribute to luminal CCN1 upon TLR7/8-mediated inflammation. (*A*) C57BL/6J mice were administered control rat Ig or platelet-depleting antibodies (50 μg, i.v.) 1 h before starting the experiment. Luminal CCN1 accumulation in mesenteric veins in the steady state (before) and 20 min after R848 treatment (100 μg) was assessed after i.v. injection of protein A-coupled YFP beads conjugated with a nonblocking antibody to CCN1. Protein A-coupled YFP beads conjugated with rabbit Ig were used as controls. Data are normalized to the level of CCN1 before R848 treatment of control Ig-treated mice; 19–23 vessels from three mice per group were analyzed. ***$P < 0.005$; two-way ANOVA with Sidak's multiple comparisons test. (*B–E*) CCN1 release by platelets (*B*), PBMCs (*C*), and bEnd-5 (*D*) and sEnd cells (*E*) were assessed by ELISA. Cells were incubated with thrombin (0.25 U/mL) and/or R848 (3 μg/mL) for 30 min. Data are normalized to the level of CCN1 release in the control incubation. Seven independent preparations per condition were used for platelets, and four independent preparations per condition were used for PBMCs and bEnd-5 and sEnd cells. Data are mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$; one-way ANOVA with Holm–Sidak multiple comparisons test.

leading to peripheral blood leukocyte rolling (41). Here we provide evidence for the role of endothelium-bound CCN1 in leukocyte recruitment. In particular, we demonstrate that activated platelets release CCN1 protein. We show that after R848 stimulation increased endothelium-bound CCN1 mediates the rapid mobilization of Ly6C^{low} monocytes that remain on the luminal side of the vessel, in accordance with its previously described chemoattractant properties for inflammatory monocytes (15, 16). Direct interactions with platelets and endothelium-bound CCN1 commit accumulated Ly6C^{low} monocytes to a meticulous patrolling of the vascular wall and the local production of proinflammatory cytokines and chemokines, thus controlling the initial capture of neutrophils from the circulation. The surrounding inflamed tissue obviously produces strong chemotactic gradients supporting the later rapid extravasation of neutrophils (Fig. 9).

Ly6C^{low} monocytes constitutively patrol the endothelium and rarely extravasate in the steady state (9), whereas they rapidly invade the peritoneal cavity in response to *Listeria monocytogenes* infection (9). In contrast, they are mobilized tardily to the injured myocardium a few days after inflammatory Ly6C^{+} monocytes (42). Here we show that Ly6C^{low} monocytes do not extravasate in response to R848, as also observed in kidney capillaries (10). Instead the Ly6C^{low} monocytes remain in the lumen and meticulously patrol the endothelial wall, a locomotory behavior characterized by enhanced adhesion time, decreased speed, and decreased track length in mesenteric veins. Conversely, in the kidney microvasculature, Carlin et al. (10) found that the track length of Ly6C^{low} monocytes increased upon TLR7 stimulation. They also showed that Ly6C^{low} monocytes are responsible for the luminal recruitment of neutrophils to kill endothelial cells of the kidney microvasculature and that the remaining cell debris is scavenged by monocytes (10). In our study of the mesenteric vasculature, we did not observe endothelial cell death. However, some dead nonendothelial cells could be observed deep in the surrounding tissue 2 h after the initiation of inflammation. We also report that neutrophils extravasate rapidly after Ly6C^{low} monocyte-dependent recruitment. The wave of neutrophils began around 90 min after R848 stimulation, and many had extravasated by 120 min. Neutrophils thus remain crawling on the luminal side of the vessel for a very short period. In contrast, Ly6C^{low} monocytes were significantly increased and remained on the luminal side throughout the course of the experiment. The discrepancy between the studies may result from the functional and physical differences between kidney capillaries and mesenteric veins. Notably patrolling Ly6C^{low} monocytes are 10 times more abundant in kidney microvasculature than in the mesentery (10).

Inflammation increases the number of activated platelets in the circulation (31, 32, 43, 44). We show here that activation of

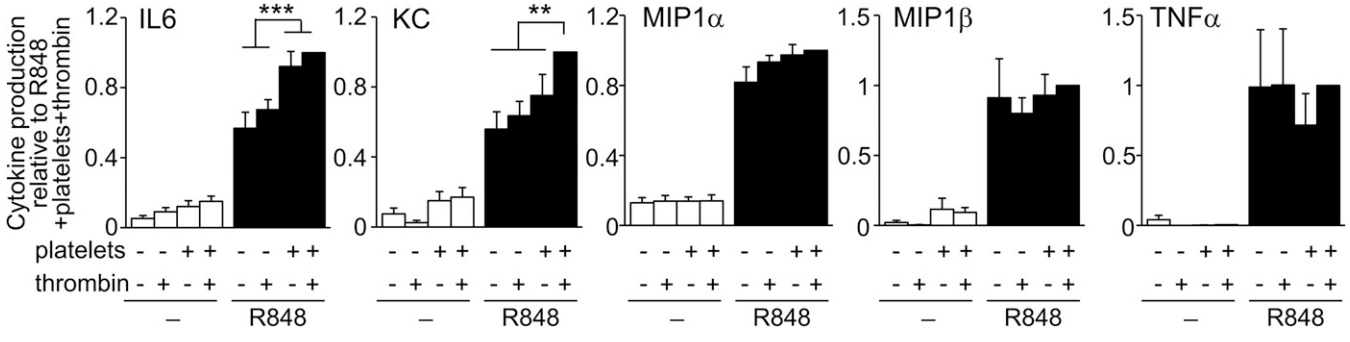

Fig. 8. Platelets potentiate the production of inflammatory cytokines and chemokines by Ly6C^{low} monocytes. Cytokine production by FACS-sorted Ly6C^{low} monocytes treated for 16 h with R848 (3 μg/mL) in the presence of platelets and thrombin (0.25 U/mL). Data are mean ± SEM normalized to cytokine production in the presence of R848, platelets, and thrombin. $n = 4–8$ per condition. **$P < 0.01$, ***$P < 0.005$; one-way ANOVA with Fisher's LSD multiple comparisons test.

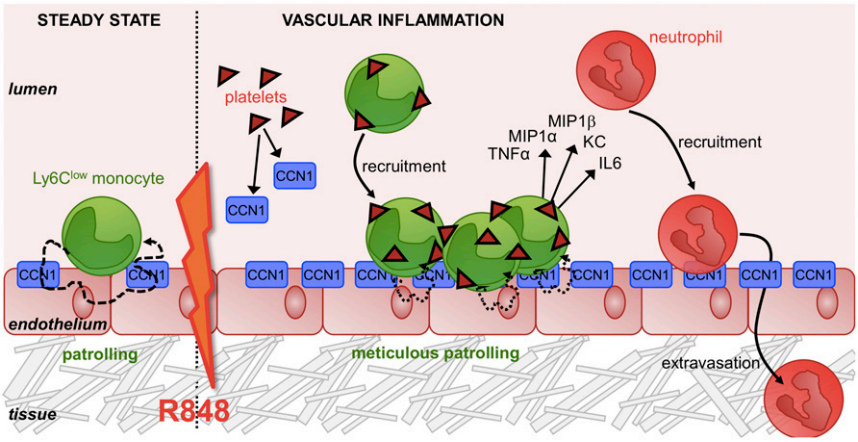

Fig. 9. Model of the CCN1-mediated mechanisms of vascular inflammation. Schematic representation of Ly6C^{low} monocytes' behavior with the endothelium in the steady state and after TLR7/8-mediated vascular inflammation. CCN1 sustains the patrolling of Ly6C^{low} monocytes in the steady state. Under inflammatory conditions, CCN1 is released by platelets. Luminal accumulation of CCN1 stimulates the recruitment of Ly6C^{low} monocytes. While interacting with platelets, mobilized Ly6C^{low} monocytes meticulously patrol the luminal face of the vasculature and orchestrate the recruitment of neutrophils via the production of inflammatory cytokines and chemokines.

platelets is required for the recruitment of Ly6C^{low} monocytes and to stimulate their cytokine production. However, we did not observe platelet accumulation on the endothelial surface or thrombus formation. We presume that platelet-activating signals do not reach a threshold necessary for thrombus formation. Confocal intravital microscopy also revealed transient but repeated interactions with Ly6C^{low} monocytes after R848 treatment. Trapping in monocyte protrusions may participate in the establishment of meticulous patrolling. However, we did not investigate the molecules responsible for these interactions. Although TLR7 is expressed by human and murine platelets and mediates platelet aggregation to leukocytes (34), R848 treatment alone did not stimulate CCN1 release. In contrast, thrombin elicits changes in the shape of platelets, the release of the platelet activators ADP, serotonin, and thromboxane A2 (43–45), and also the release of CCN1, as shown here and previously (22). Activated platelets are able to bind to leukocytes, preferentially to monocytes through the expression of P-selectin (32, 35), which is mobilized to the surface of TLR7 agonist-stimulated and thrombin-activated platelets (34, 43, 44). In this way, they participate in the activation and recruitment of immune cells (30, 31, 33, 46), acting as chaperones. Activated platelets were reported to bind more quickly and more intensely to monocytes than to neutrophils (31). However, we observed no interaction between Ly6C^{low} monocytes and platelets in the steady state, perhaps because the setup of our microscope allowed the detection only of interactions lasting longer than 2 s. Therefore, we cannot entirely rule out brief interactions in the steady state, such as the "touch-and-go" events observed with Kupffer cells in liver sinusoids (33). We also report that Ly6C^{low} monocytes are responsive to R848 in terms of cytokine and chemokine production, as is consistent with findings on their human homologs, CD14^{dim}CD16^{+} monocytes, which respond to nucleic acids and viruses via TLR7/8 (6). In addition, interactions with platelets further improve the production of KC and IL-6, as would be expected from platelets' role in stimulating cytokine production by inflammatory monocytes (35, 36). Therefore, platelets have at least three functions in our model: (*i*) recruitment of Ly6C^{low} monocytes; (*ii*) commitment to meticulous patrolling of Ly6C^{low} monocytes; and (*iii*) promotion of cytokine production by Ly6C^{low} monocytes via direct interaction and/or release of CCN1.

Matricellular protein CYR61/CCN1 has been studied extensively in cancer development and wound healing (15, 16, 47, 48). In addition to confirming the chemoattractant properties of CCN1 on monocytes/macrophages (18, 20, 21), we also show that CCN1 sustains the patrolling of Ly6C^{low} monocytes and is important for the initiation of inflammation. We used i.v. injection of CCN1-blocking antibodies in WT mice to circumvent the lethality of *Ccn1*-null mice, which results from the impairment of vascular integrity and cardiovascular development dysfunction (24). Patrolling of Ly6C^{low} monocytes is dependent on CD18, CD11a, ICAM1, ICAM2, and CX_3CR1 (9, 10). Here we report that endothelium-bound CCN1 constitutes a molecular support for monocyte patrolling in the steady state but is not required for monocyte adhesion to the endothelium. CD11b is an integrin able to engage multiple ligands, counter receptors, or products of coagulation and complement (49), one of which is CCN1 on monocytes and macrophages (15, 16, 20, 21, 25). A role of CD11b in the patrolling of Ly6C^{low} monocytes was ruled out initially (9), based solely on the observation that anti-CD11b injection does affect the adhesion of crawling monocytes to the endothelium but without examining the locomotory behavior (9). Here we show that blocking CD11b recapitulated in part the phenotype of CCN1 blocking, affecting the locomotion of monocytes without affecting their adhesion in the steady state.

In this study, we focus on the early steps of inflammation. We pinpoint platelets as a source for endothelium-bound CCN1 after R848 stimulation, because activated platelets released CCN1 in vitro and depletion of platelets suppressed inflammation-induced endothelium-bound CCN1 increase in vivo. Because platelets were observed only in contact with Ly6C^{low} patrolling monocytes and not attached to the endothelium, we speculate that activated platelets release CCN1 in the circulation which then binds to the inflamed endothelium. Neither murine PBMCs nor endothelial cells released CCN1 on a short-term basis, although endothelial cell-derived CCN1 coats the surface of these cells in the steady state and 18-h treatment with TNF-α and bafilomycin A1 stimulated CCN1 release by human PBMCs (18). These results are in accordance with the chemoattractant properties of CCN1 for monocytes/macrophages in the early phase of inflammation, whereas PBMC-derived CCN1 locally immobilize recruited leukocytes in the invaded tissue after extravasation at later time points (18, 19, 50–53).

Live imaging of endogenous neutrophils may be tricky at long-time points as antibodies used to detect them have depleting and/or blocking properties. In order to avoid functional interference with neutrophil behavior, we preferred to inject negatively selected neutrophils labeled with Cell-Tracker Orange. We observed no difference between the rolling of transferred

and endogenous neutrophils (Movie S10). Another issue with live imaging is the detection of chemokines. In our case, live detection of CCN1 would certainly require an antibody with a higher affinity because the background signal was too high in mesenteric veins. To circumvent the detection problem, we adapted a technique previously used to detect endothelium-bound CRAMP (26) that allowed the monitoring of the luminal accumulation of CCN1 in response to R848, which is a ligand for TLR7/8 (28). Such a result reinforces the idea that CCN1 has a role in antiviral responses and TLR7 recognition of viral single-strand RNA (54). Indeed, hepatitis C virus, coxsackievirus B3, and herpes simplex virus type1 were shown to induce CCN1 expression in vitro and in vivo (15). CCN1 accumulation mediates the rapid mobilization of Ly6C^{low} monocytes to the endothelium in response to TLR7/8-mediated local inflammation of the vasculature. Mobilization of Ly6C^{low} monocytes and adequate patrolling motion are mandatory for the arrival of neutrophils, as evidenced by experiments with anti-CCN1 and antiplatelet antibodies. The recruitment of monocytes and neutrophils also was reduced in *Cx$_3$cr1*-deficient mice that exhibit lower numbers of patrolling Ly6C^{low} monocytes, underlining the early importance of patrolling in leukocyte recruitment in response to R848. However, *Cx$_3$cr1*-deficient mice have no defects in neutrophil recruitment when challenged with the complex TLR7-independent pathogens *Clostridium difficile* and *Candida albicans* and or in recruitment at later time points (55, 56). This finding argues in favor of a local and timely impact of CCN1 and Ly6C^{low} monocytes or a role restricted to TLR7/8-mediated inflammation.

Our results have important implications for pathologies involving inflammation. Circulating activated platelets promote inflammation in rheumatoid arthritis patients (57). Interactions with monocytes and the endothelium of the vessel wall in *ApoE*$^{-/-}$ mice enable the deposition of chemokines on the cell surface and the recruitment of monocytes and thus accelerate the development of atherosclerotic plaques (58) that exhibit intense CCN1 expression (25). Platelet activation markers and soluble adhesion molecules are also observed in patients with systemic lupus erythematosus (SLE) (59). Increased recruitment of human CD16^{+} monocytes into synovial tissues and glomeruli is also seen in rheumatoid arthritis and lupus patients, respectively (6, 60–63), and murine Ly6C^{low}/Gr1^{low} monocytes are expanded in the Yaa model of SLE (14). Interestingly, increased soluble CCN1 levels are observed in synovial fluid from rheumatoid arthritis patients (15) and in the serum of SLE patients (64). Hence, the chemoattractant properties of CCN1 for human and murine monocytes and macrophages (15, 16, 18, 19) strongly implicates CCN1 in the numerous pathologies in which TLR7 activity plays an important role.

Collectively, our data provide evidence for refining the model of the leukocyte recruitment cascade and the TLR7/8-mediated response. The commitment of Ly6C^{low} monocytes to surveying the endothelial wall reinforces the proposition that they should be considered luminal blood macrophages (11). The accumulation and meticulous patrolling of Ly6C^{low} monocytes on the luminal side of the endothelium and their interaction with platelets also demonstrates that leukocytes can play an active role in inflammation without extravagating. The therapeutic targeting of Ly6C^{low} monocytes and CCN1 to control leukocyte recruitment and the course of inflammation in human pathologies remains an interesting possibility for future investigation.

Materials and Methods

Detailed methods are provided in *SI Materials and Methods*.

Animals. *Cx$_3$cr$^{gfp/gfp}$* mice were initially obtained from Charles River Laboratories. C57BL/6J mice were from Charles River Laboratories or from the animal facility of the University of Geneva. All animals were kept on a C57BL/6J background in the specific pathogen-free animal facility of the University of Geneva.

Statistics. Statistical calculations were carried out in Prism 6 (GraphPad). Mann–Whitney unpaired test, Wilcoxon matched-pairs signed rank test, Kruskal–Wallis with Dunn's multiple comparisons tests, one-way ANOVA with Fisher's least significant difference (LSD), Holm–Sidak multiple comparisons tests, and two-way ANOVA with Tukey's or Sidak's multiple comparisons tests were used as appropriate. All statistical tests are two-sided. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

Study Approval. Animal procedures were performed in accordance with the guidelines of the Institutional Ethical Committee of Animal Care in Geneva, Switzerland and the Cantonal Veterinary Office.

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