Spatiotemporal Expression Dynamics of Selectins Govern the Sequential Extravasation of Neutrophils and Monocytes in the Acute Inflammatory Response

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Objective—Leukocyte recruitment to the site of inflammation is a key event in a variety of cardiovascular pathologies. Infiltrating neutrophils constitute the first line of defense that precedes a second wave of emigrating monocytes reinforcing the inflammatory reaction. The mechanisms initiating this sequential process remained largely obscure.

Approach and Results—Using advanced in vivo microscopy and in vitro/ex vivo techniques, we identified individual spatiotemporal expression patterns of selectins and their principal interaction partners on neutrophils, resident/inflammatory monocytes, and endothelial cells. Coordinating the intraluminal trafficking of neutrophils and inflammatory monocytes to common sites of extravasation, selectins assign different sites to these immune cells for their initial interactions with the microvascular endothelium. Whereas constitutively expressed leukocyte L-selectin/CD62L and endothelial P-selectin/CD62P together with CD44 and P-selectin glycoprotein ligand-1/CD162 initiate the emigration of neutrophils, de novo synthesis of endothelial E-selectin/CD62E launches the delayed secondary recruitment of inflammatory monocytes. In this context, P-selectin/CD62P and L-selectin/CD62L together with P-selectin glycoprotein ligand-1/CD162 and CD44 were found to regulate the flux of rolling neutrophils and inflammatory monocytes, whereas E-selectin/CD62E selectively adjusts the rolling velocity of inflammatory monocytes. Moreover, selectins and their interaction partners P-selectin glycoprotein ligand-1/CD162 and CD44 differentially control the intraluminal crawling behavior of neutrophils and inflammatory monocytes collectively enabling the sequential extravasation of these immune cells to inflamed tissue.

Conclusions—Our findings provide novel insights into the mechanisms initiating the sequential infiltration of the perivascular tissue by neutrophils and monocytes in the acute inflammatory response and might thereby contribute to the development of targeted therapeutic strategies for prevention and treatment of cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.305143.)

Key Words: endothelium | immune system | inflammation | leukocytes | microcirculation
Nonstandard Abbreviations and Acronyms

PSGL-1  P-selectin glycoprotein ligand-1
TNF-α  tumor necrosis factor-α

Selectins represent a family of 3 closely related carbohydrate-binding proteins. L-selectin/CD62L is constitutively expressed on leukocytes, whereas E-selectin/CD62E is synthesized de novo in activated endothelial cells. In contrast, preformed P-selectin/CD62P is rapidly translocated to the surface of platelets and endothelial cells on cell activation. Through transient, reversible, adhesive interactions with different binding partners (eg, P-selectin glycoprotein ligand-1 [PSGL-1/CD162] and CD44), selectins have been reported to regulate the initial attachment of leukocytes to the inflamed endothelium and to promote the subsequent activation of these immune cells.14 We therefore hypothesized that these adhesion and signaling molecules play a critical role for the initiation of subtype-specific leukocyte responses.

Here, we report that the complex spatiotemporal expression dynamics of selectins govern the sequential extravasation of neutrophils and inflammatory monocytes in the acute inflammatory response: whereas constitutively expressed leukocyte L-selectin/CD62L and endothelial P-selectin/CD62P together with their interaction partners PSGL-1/CD162 and CD44 initiate the migration of neutrophils to the site of inflammation, de novo synthesis of endothelial E-selectin/CD62E results in the delayed secondary recruitment of inflammatory monocytes. Consistent with the individual expression patterns of L-selectin/CD62L and E-selectin/CD62E in the inflamed microvasculature, neutrophils and monocytes use different sites for their initial interactions with endothelial cells, thereby coordinating the intraluminal trafficking of these leukocyte subsets to common sites of transendothelial migration. In this context, P-selectin/CD62P and L-selectin/CD62L together with PSGL-1/CD162 and CD44 were found to regulate the flux of rolling neutrophils and inflammatory monocytes, whereas E-selectin/CD62E selectively adjusts the rolling velocity of inflammatory monocytes. Moreover, we demonstrate that selectins and their interaction partners PSGL-1/CD162 and CD44 differentially control the intraluminal crawling behavior of neutrophils and inflammatory monocytes collectively enabling the sequential extravasation of these immune cells to the inflamed tissue. Our experimental data provide novel insights into the subtype-specific mechanisms underlying the leukocyte recruitment process uncovering previously unappreciated functions of selectins in the extravasation cascade of these immune cells.

Materials and Methods

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

Results

Effect of Different Chemokines and Cytokines on the Recruitment of Myeloid Leukocytes

In a first set of experiments, we sought to establish an experimental model that enables us to analyze the sequential recruitment of neutrophils and monocytes in the acute inflammatory response. Using flow cytometry, the effect of a C-C motif chemokine (CCL2/monocyte chemoattractant protein-1), C-X-C motif chemokine (CXCL1/KC), and different cytokines (interleukin-1β and tumor necrosis factor [TNF-α]) on the recruitment of different myeloid leukocyte subsets to the peritoneal cavity was characterized. On 6 hours of intraperitoneal stimulation with CCL2, CXCL1, interleukin-1β, or TNF-α, there was a significant, comparable increase in numbers of total leukocytes recruited to the peritoneal cavity when compared with PBS-treated control mice (data not shown). The number of resident monocytes (CD45+CD11b−GR-1lowCD115highF4/80− cells) in the peritoneal lavage fluid was not significantly altered by stimulation with CCL2, CXCL1, interleukin-1β, or TNF-α (Figure IA in the online-only Data Supplement). Whereas all of these inflammatory stimuli potently induced extravasation of neutrophils (CD45+CD11b+GR-1highCD115lowF4/80+ cells; Figure IB in the online-only Data Supplement) to the peritoneal cavity, only stimulation with CCL2 leads to a concomitant extravasation of inflammatory monocytes (CD45+CD11b+GR-1highCD115highF4/80− cells; Figure IC in the online-only Data Supplement).

In line with our results, the C-C motif chemokine CCL2 has previously been reported to be critically involved in the recruitment of neutrophils and monocytes (>80% reduction on blockade of CCL2 or its receptor CCR2) in experimental ischemia reperfusion injury or sepsis.15,16 In this context, CCL2 is known to attract both immune cell populations by mast cell–dependent activation of endothelial cells, whereas cytokines (eg, TNF-α) or lipid mediators (eg, platelet-activating factor, leukotriene B4) are thought to directly activate neutrophils downstream of C-C motif chemokine engagement.11,17 With respect to these previous reports and our present findings, the C-C motif chemokine CCL2 was used as an inflammatory stimulus in further experiments.

Expression Profiles of L-Selectin/CD62L, PSGL-1/CD162, and CD44 in Myeloid Leukocyte Subsets

In a further set of in vitro experiments, we evaluated the expression patterns of L-selectin/CD62L, PSGL-1/CD162, and CD44 in different myeloid leukocyte subsets (Figure 1A). Using confocal microscopy and flow cytometry, these proteins were found to be constitutively expressed by neutrophils and resident/inflammatory monocytes. PSGL-1/CD162 showed the highest surface expression on these immune cells, whereas expression of L-selectin/CD62L and CD44 on these leukocytes was relatively low. In detail, the surface expression levels of L-selectin/CD62L were higher on neutrophils than on inflammatory or resident monocytes, whereas surface expression levels of CD44 and PSGL-1/CD162 were more pronounced on inflammatory monocytes than on neutrophils or resident monocytes. On cell activation, the surface expression of L-selectin/CD62L was significantly diminished in neutrophils and resident/inflammatory monocytes, whereas surface expression of PSGL-1/CD162 was only significantly reduced in neutrophils.

To further characterize the expression profiles of P-selectin/CD62P, E-selectin/CD62E, PSGL-1/CD162, and CD44 in the microvasculature, tissue RNA expression of these adhesion and signaling molecules was analyzed by quantitative real-time reverse transcription polymerase chain reaction. Whereas RNA expression of E-selectin/CD62E (which is only expressed by endothelial cells) immediately increased on stimulation with CCL2, RNA levels of P-selectin/CD62P, PSGL-1/CD162, and CD44 were only slightly enhanced (Figure 1B). To characterize the expression of these adhesion and signaling molecules in more detail, immunostaining and confocal microscopy were performed on cremasteric tissue whole mounts (Figure 1C). In the unstimulated microvasculature, expression levels of P-selectin/CD62P and E-selectin/CD62E, as well as of PSGL-1/CD162 and CD44, on microvascular endothelial cells were low. On stimulation with CCL2, however, expression levels of these proteins significantly increased. Most interestingly, the expression of E-selectin/CD62E was strictly localized to platelet endothelial cell adhesion molecule-1/CD31+ endothelial junctions, whereas P-selectin/CD62P, PSGL-1/CD162, and CD44 were broadly distributed over the entire endothelial surface.

Role of Selectins and Their Interaction Partners PSGL-1/CD162 and CD44 for CCL2-Elicited Recruitment of Neutrophils and Monocytes

As outlined above, intraperitoneal stimulation with CCL2 induced a significant increase in numbers of neutrophils (CD45+ CD11b+ GR-1high CD115low F4/80− cells) and inflammatory monocytes (CD45+ CD11b+ GR-1high CD115high F4/80− cells) extravasated to the peritoneal cavity when compared with PBS-treated control mice (Figure 2A) but did not...
significantly alter the number of resident monocytes (CD45+ CD11b+ GR-1low CD115high F4/80− cells) in the peritoneal lavage fluid.

Next, we analyzed the functional relevance of selectins and their interaction partners PSGL-1/CD162 and CD44 for the recruitment of different myeloid leukocyte subsets. Whereas antibody blockade of L-selectin/CD62L, PSGL-1/CD162, or CD44 significantly reduced the migration of neutrophils and inflammatory monocytes to the peritoneal cavity, blockade of P-selectin/CD62P almost completely abolished these CCL2-dependent leukocyte responses (Figure 2B and 2C).

In contrast, antibody blockade of E-selectin/CD62E (using monoclonal antibodies (mAbs) directed against the consensus repeat region [clone 10E9.6; Figure 2B and 2C] or the lectin and epidermal growth factor–like domains [clone 9A9; Figure IIA and IIB in the online-only Data Supplement] of murine E-selectin) selectively diminished extravasation of inflammatory monocytes without significantly affecting the recruitment of neutrophils.

In addition, we characterized the role of neutrophils and platelets for the CCL2-elicited recruitment of different myeloid leukocyte subsets. Depletion of neutrophils did not

Figure 2. Role of selectins and their interaction partners P-selectin glycoprotein ligand (PSGL-1)/CD162 and CD44 for the recruitment of neutrophils and monocytes. A, Representative flow cytometry plots demonstrating the analysis of the recruitment of different myeloid leukocyte subsets to the peritoneal cavity. Quantitative data for the peritoneal recruitment of resident/inflammatory monocytes and neutrophils in animals receiving blocking monoclonal antibodies (mAbs) directed against L-selectin/CD62L, E-selectin/CD62E (clone 10E9.6), P-selectin/CD62P (B), PSGL-1/CD162 or CD44 (C), and neutrophil- or platelet-depleting mAbs (D) as assessed 6 hours after intraperitoneal stimulation with CCL2 by flow cytometry (means±SEM; n=7 per group; #P<0.05 vs PBS). APC indicates allophycocyanin; and PE, phycoerythrin.
significantly change the number of inflammatory monocytes migrated to the peritoneal cavity (Figure 2D). Furthermore, extravasation of neutrophils and inflammatory monocytes was not significantly altered on depletion of platelets. Depletion of neutrophils and platelets was verified in the peripheral blood by flow cytometry (data not shown).

Individual Characteristics of Intravascular Interactions of Neutrophils and Monocytes With Inflamed Endothelial Cells
To study the mechanisms underlying the recruitment of neutrophils and inflammatory monocytes in more detail, we used in vivo microscopy on the cremaster muscle of CX3CR-1GFP/+ mice exhibiting fluorescence-labeled monocytes/macrophages. Combining transillumination (for the detection of total leukocytes) and fluorescence (for the detection of monocytes) in vivo microscopy, we were able to distinguish GFPlow cells (representing neutrophils), GFPhigh cells (representing resident monocytes), and GFPneg cells (representing inflammatory monocytes; Figure 3A and 3B). Over 98% of rolling or firmly adherent total leukocytes were positive for CD11b (representing leukocytes of myeloid origin; Figure 3A and 3B). In PBS-treated control animals, resident monocytes patrolled inside the microvasculature, whereas only few intravascularly rolling, firmly adherent, intraluminally crawling, and transmigrated neutrophils and inflammatory monocytes were observed. On stimulation with CCL2, however, the number of firmly adherent, intraluminally crawling, and transmigrated neutrophils and inflammatory monocytes were increased, whereas the responses of inflammatory monocytes were delayed by 120 minutes. In contrast, intravascular endothelial cell interactions of resident monocytes remained largely unchanged on onset of stimulation with CCL2 (Figure 3C).

In a next step, the interaction sites of different myeloid leukocyte subsets with endothelial cells were analyzed. Using in vivo microscopy, monocytes were found to almost exclusively roll along platelet endothelial cell adhesion molecule-1/CD31-immunoreactive endothelial junctions of the inflamed microvascular endothelium (Figure 4A; Video I in the online-only Data Supplement), whereas the rolling paths of neutrophils were less defined (Video 2 in the online-only Data Supplement). Interestingly, the intravascular rolling paths of monocytes on microvascular endothelial cells were significantly shortened on blockade of E-selectin/CD62E and no longer restricted to endothelial junctions, whereas the rolling paths of neutrophils remained unaltered (data not shown). In contrast, both inflammatory monocytes and neutrophils were observed to firmly adhere (Figure 4B) and to intraluminally

Figure 3. Extravasation dynamics of neutrophils and monocytes in the acute inflammatory response. A, Representative in vivo microscopy images of postcapillary venules in the CCL2-stimulated cremaster muscle of CX3CR-1GFP/+ mice receiving phycoerythrin-labeled anti-CD11b monoclonal antibodies (mAbs) visualizing the recruitment of myeloid leukocyte subsets (transillumination: total leukocytes; CD11b+: myeloid leukocytes; CX3CR-1GFP+: monocytes). B, Quantitative data for the proportion of intravascular rolling and firmly adherent myeloid leukocytes of total leukocytes (means±SEM; n=3 per group). A and C, Measuring the fluorescence intensity of CX3CR-1GFP+ cells allowed the identification of resident (GFP+) and inflammatory (GFP−) monocytes (means±SEM; n=3 per group). D, Quantitative data for intravascular rolling, firm adherence, and crawling, as well as transmigration of resident/inflammatory monocytes and neutrophils in cremasteric venules within 360 minutes of stimulation with CCL2 as assessed by in vivo microscopy (means±SEM; n=3 per group). GFP indicates green fluorescent protein.
crawled predominantly along junctions of inflamed endothelial cells (Figure 4C). In this context, the majority of firmly adherent monocytes and—to a lesser degree—neutrophils were found to adhere to tricellular junctions of endothelial cells (Figure 4B).

Role of Selectins and Their Interaction Partners PSGL-1/CD162 and CD44 for CCL2-Elicited Intravascular Rolling and Firm Adhesion of Neutrophils and Inflammatory Monocytes

In a next set of experiments, the role of selectins and their ligands PSGL-1/CD162 and CD44 for intravascular interactions of neutrophils and monocytes with endothelial cells was studied. After 6 hours of intrascrotal stimulation with CCL2, baseline measurements of leukocyte rolling and firm adhesion were performed in postcapillary venules of the cremaster muscle of CX3CR-1<sup>GFP</sup>/− mice. No significant differences in numbers of intravascularly rolling or firmly adherent neutrophils and resident/inflammatory monocytes were detected among all experimental groups (Figures 5A and 6A). To directly assess the functional relevance of selectins and their interaction partners PSGL-1/CD162 or CD44 for CCL2-elicited leukocyte responses, blocking antibodies directed against these proteins were subsequently administered and the in vivo microscopy measurements were repeated 5 and 45 minutes later. Antibody blockade of P-selectin/CD62P completely abolished the flux of rolling neutrophils and monocytes. Whereas antibody blockade of L-selectin/CD62L only slightly diminished the flux of rolling inflammatory monocytes and neutrophils, the rolling velocity of these immune cells was not significantly altered. Conversely, blockade of E-selectin/CD62E did not change the flux of rolling leukocytes but selectively enhanced the rolling

Figure 4. Intravascular endothelial cell interactions of neutrophils and monocytes. Representative in vivo microscopy images of intravascularly rolling (A, left), firmly adherent (B, left), and crawling (C, left) resident/inflammatory monocytes (green) and neutrophils (red) in postcapillary venules in the CCL2-stimulated cremaster muscle. Arrows indicate the rolling/crawling routes or adhesion sites of leukocytes relative to platelet endothelial cell adhesion molecule-1/CD31-immunoreactive endothelial junctions (blue). The quantitative analysis of the localization of intravascularly rolling (A, right), firmly adherent (B, right), and crawling (C, right) leukocytes relative to endothelial junctions is shown (mean±SEM; n=3 per group). GFP indicates green fluorescent protein.
velocity of inflammatory monocytes without affecting the rolling velocity of neutrophils (Figure 5A and 5B; Figure III in the online-only Data Supplement). In contrast, antibody blockade of PSGL-1/CD162 or CD44 significantly reduced the flux of rolling neutrophils and inflammatory monocytes and increased the rolling velocity of inflammatory monocytes (Figure 6A and 6B). It is noteworthy that blockade of selectins or their interaction partners PSGL-1/CD162 and CD44 did not significantly alter the intravascular adherence of inflammatory monocytes, whereas only blockade of P-selectin/CD62P significantly diminished the intravascular adherence of neutrophils (Figures 5C and 6C).

**Role of E-Selectin and P-Selectin for Intravascular Rolling and Firm Adherence of Neutrophils and Inflammatory Monocytes in TNF-α-Stimulated Postcapillary Venules**

To confirm our results on the role of E-selectin and P-selectin for intravascular rolling and firm adherence of neutrophils under different inflammatory conditions, endothelial cell
interactions of these immune cells were analyzed in the mouse cremaster muscle on intrascrotal stimulation with TNF-α. In accordance with our observations in the peritonitis model, frequent endothelial cell interactions of neutrophils (Figure IVA in the online-only Data Supplement), but few endothelial cell interactions of inflammatory monocytes (Figure IVB in the online-only Data Supplement), were detected in cremasteric postcapillary venules on stimulation with TNF-α. On blockade of P-selectin/CD62P, but not of E-selectin/CD62E, the number of intravascularly rolling and (subsequently) firmly adherent neutrophils was significantly diminished when compared with isotype control antibody-treated animals (Figure VA and VC in the online-only Data Supplement). The rolling velocity of neutrophils was slightly but not significantly enhanced on blockade of E-selectin/CD62E (Figure VB in the online-only Data Supplement).

Using confocal microscopy, the expression patterns of E- and P-selectin were determined in postcapillary venules of

Figure 6. Role of P-selectin glycoprotein ligand (PSGL-1)/CD162 and CD44 for initial intravascular endothelial cell interactions of neutrophils and monocytes. Quantitative data for intravascular rolling, rolling velocity, and firm adherence of inflammatory monocytes and neutrophils in postcapillary venules of the CCL2-stimulated cremaster muscle in isotype control antibody-treated animals or in animals treated with blocking monoclonal antibodies (mAbs) directed against PSGL-1/CD162 or CD44 as assessed by in vivo microscopy (mean±SEM; n=5 per group; *P<0.05 vs PBS). GFP indicates green fluorescent protein.
the cremaster muscle stimulated with TNF-α (Figure VIA in the online-only Data Supplement). Similarly to our previous findings, expression of E-selectin/CD62E was localized to endothelial junctions, whereas P-selectin/CD62P was broadly distributed over the entire endothelial cell surface. In contrast, 6 hours of stimulation with TNF-α resulted only in a rather weak elevation in the surface expression of E- and P-selectin when compared with CCL2 (Figure VIA in the online-only Data Supplement).

**Role of Selectins and Their Interaction Partners PSGL-1/CD162 and CD44 for CCL2-Elicited Intravascular Crawling of Neutrophils and Inflammatory Monocytes**

Finally, the role of selectins and their interaction partners PSGL-1/CD162 and CD44 for intraluminal crawling of neutrophils and inflammatory monocytes was analyzed (Figure VIA in the online-only Data Supplement). On stimulation with CCL2, many neutrophils, as well as resident and inflammatory monocytes, were observed to crawl in the inflamed cremasteric microvasculature. Whereas blockade of E-selectin/CD62E, P-selectin/CD62P, L-selectin/CD62L, PSGL-1/CD162, or CD44 did not significantly alter the frequency of intraluminally crawling neutrophils, blockade of P-selectin/CD62P largely changed the crawling directionality of these immune cells (Figure VIIA in the online-only Data Supplement). In contrast, blockade of L-selectin/CD62L or PSGL-1/CD162 significantly diminished the frequency of intraluminally crawling inflammatory monocytes, whereas blockade of P-selectin/CD62P significantly altered the crawling directionality of these inflammatory cells. The crawling velocity of inflammatory monocytes or neutrophils was significantly diminished on blockade of PSGL-1/CD162, whereas blockade of CD44 only reduced the crawling velocity of inflammatory monocytes.

**Systemic Leukocyte Counts and Microhemodynamic Parameters**

To assure intergroup comparability, systemic leukocyte counts and microhemodynamic parameters of analyzed venules, including inner vessel diameter, blood flow velocity, and shear rate, were determined in each experiment. No significant differences were detected among experimental groups (Table I in the online-only Data Supplement).

**Discussion**

Leukocyte recruitment from the microvasculature to the site of injury or infection is a key event in the inflammatory response. Whereas the basic principles of this process have been characterized in the past decades,2–6 the mechanisms regulating the extravasation of distinct leukocyte subpopulations remain poorly understood. Initial in vitro studies suggested that the recruitment of distinct leukocyte subsets is mediated by specific classes of chemokines.7,8 Results of recent in vivo studies, however, point to a more complex regulation of the recruitment process of specific leukocyte subpopulations.9–14 Selectins represent a family of 3 closely related carbohydrate-binding proteins that have been implicated in the initial attachment of leukocytes to the inflamed endothelium and in the subsequent activation of these immune cells.14 We therefore hypothesized that these adhesion and signaling molecules are critical for the initiation of subtype-specific leukocyte responses.

In a first attempt, we sought to characterize the expression profiles of selectins and their principal interaction partners on the surface of different myeloid leukocyte subpopulations and on microvascular endothelial cells. We found that levels of constitutively expressed L-selectin/CD62L (which is the only leukocyte selectin) were higher on the surface of neutrophils than on inflammatory or resident monocytes, whereas surface expression of CD44 (which serves as a interaction partner of endotheliually expressed E-selectin/CD62E18) and PSGL-1/CD162 (which predominantly binds to P-selectin/CD62P19) was more pronounced on inflammatory monocytes than on neutrophils or resident monocytes. In accordance with previous reports,20 endothelial E-selectin/CD62E was mainly synthesized de novo on onset of inflammation, whereas P-selectin/CD62P (which is immediately translocated from intracellular Weibel–Palade bodies to the endothelial cell surface on cell activation21,22) was performed in microvascular endothelial cells. Interestingly, luminal expression of E-selectin/CD62E was strictly localized to endothelial junctions, whereas weakly expressed endothelial PSGL-1/CD162 and CD44 (which serve as interaction partners of leukocyte L-selectin/CD62L23), as well as strongly expressed P-selectin/CD62P, were broadly distributed over the entire luminal surface of inflamed endothelial cells.

Translating the individual expression profiles of these adhesion and signaling molecules into function, we demonstrate that surface translocated P-selectin/CD62P and—not to a lesser degree—constitutively expressed L-selectin/CD62L together with their principal interaction partners PSGL-1/CD162 and CD44 initiate the migration of neutrophils to the site of inflammation, whereas de novo synthesis of E-selectin/CD62E launches the delayed secondary extravasation of inflammatory monocytes. Accordingly, low expression of E-selectin/CD62E on microvascular endothelial cells (as observed on stimulation with TNF-α) was not sufficient for the induction of extravasation of inflammatory monocytes. This selective effect of E-selectin/CD62E on the recruitment of inflammatory monocytes might be related to the different expression levels of the E-selectin/CD62E ligand CD44 on the surface of inflammatory monocytes and neutrophils. Furthermore, we found that the early extravasation of neutrophils and inflammatory monocytes is not dependent on the presence of platelets, indicating that endothelial, but not platelet, P-selectin/CD62P mediates these events.

Recently, secretion products of emigrated neutrophils have been implicated in the extravasation of inflammatory monocytes to the perivascular tissue in prolonged or chronic inflammatory scenarios.1,4,15 In acute inflammation, however, neutrophils did not contribute to the recruitment of inflammatory monocytes pointing to an individual regulation of the extravasation process of neutrophils and monocytes by selectins and their interaction partners PSGL-1/CD162 or CD44. In summary, our experimental findings clearly demonstrate that
the complex spatiotemporal expression dynamics of selectins govern the sequential extravasation of neutrophils and inflammatory monocytes in the acute inflammatory response.

Taking a closer look at the extravasation modes of the different myeloid leukocyte subsets, we sought to explore which sites neutrophils and inflammatory monocytes use for their initial interactions with inflamed endothelial cells. Here, we report for the first time that different immune cell populations use individual paths while rolling in the inflamed microvasculature: in accordance with our previous findings, we show that inflammatory monocytes establish their initial interactions with the inflamed endothelium of postcapillary venules almost exclusively along endothelial junctions, where E-selectin/CD62E is highly expressed. Consistent with a less restricted expression pattern of P-selectin/CD62P, PSGL-1/CD162, and CD44 on the surface of inflamed microvascular endothelial cells, however, the rolling routes of neutrophils were less defined. It is noteworthy that the subsequent adhesion sites and crawling routes of neutrophils and inflammatory monocytes were predominantly localized along endothelial junctions of the inflamed vessel wall strongly, suggesting that these myeloid leukocyte subsets finally pass the endothelial cell layer via common paths by using the paracellular transmigration route. Consequently, this selectin-dependent spatial differentiation of the initial endothelial cell interactions of neutrophils and monocytes coordinates the intraluminal trafficking of these immune cells to endothelial junctions and might thereby enhance the efficacy of the sequential extrava-
sation of these myeloid leukocyte subsets.

Toward a more comprehensive understanding of the recruitment of neutrophils and inflammatory monocytes, we analyzed how selectins and their principal interaction partners are engaged in the different steps of the extravasation cascade of these immune cells. To avoid distraction by cumulative effects arising from a priori blockade of a defined protein in the course of the inflammatory response, we administered blocking antibodies directed against the different target proteins after (and not before) the onset of inflammation and immediately performed in vivo microscopy in the inflamed microvasculature. This unique approach allowed us to characterize the function of selectins and their interaction partners in the extravasation process of leukocytes in previously unknown detail. Our experimental data reveal that P-selectin/CD62P and—to a lesser degree—L-selectin/CD62L together with their interaction partners PSGL-1/CD162 and CD44 regulate the flux of rolling neutrophils and inflammatory monocytes, whereas E-selectin/CD62E exclusively adjusts the rolling velocity (which is a prerequisite for the subsequent adhesion and transmigration) of inflammatory monocytes. Moreover, we found that particularly the lectin and epidermal growth factor–like domains and—to a lesser degree—the consensus repeat region of E-selectin/CD62E are critical for these processes. These findings extend in vitro and ex vivo data implicating L-selectin/CD62L, E-selectin/CD62E, or P-selectin/CD62P as well as their binding partner PSGL-1/CD162 in temporary interactions of monocytic cells/monocytes or neutrophils with activated endothelial cells. Furthermore, our results elucidate conventional in vivo microscopy observations involving adhesive interactions among L-selectin/CD62L, P-selectin/CD62P, or E-selectin/CD62E and their binding partners PSGL-1/CD162 or CD44 in the control of intravascular leukocyte–endothelial cell interactions.

In addition to their adhesive properties, selectins and their interaction partners PSGL-1/CD162 and CD44 are well known to initiate intracellular signaling events in rolling leukocytes, which ultimately result in conformational changes of surface-expressed integrins. Here, we demonstrate that P-selectin/CD62P (but not L-selectin/CD62L, E-selectin/CD62E, PSGL-1/CD162, or CD44) exhibits slight effects on intravascular firm adherence of neutrophils, which is known to strictly rely on tight interactions between leukocyte β2 integrins and endothelial members of the immunoglobulin superfamily (eg, CD54/intercellular adhesion molecule 1).2–6 Because the attenuation of neutrophil intravascular adherence appeared rather delayed on blockade of P-selectin/CD62P, however, it cannot be excluded that this effect simply reflects the consequences of inhibiting preceding P-selectin/CD62P-dependent steps in the extravasation cascade of these inflammatory cells. In contrast, we show that P-selectin/CD62P profoundly regulates the crawling direction of neutrophils and inflammatory monocytes on the inflamed endothelium—a process that requires less-static interactions among β2 integrins and endothelial members of the immunoglobulin superfamily.28 These findings might be explained by previous observations demonstrating that interactions of endothelial P-selectin/CD62P with its binding partners on the surface of rolling leukocytes induce the extended, but not the high affinity, conformation of the leukocyte β2 integrin lymphocyte function–associated antigen 1/CD11a.43 In this context, we further report that leukocyte L-selectin/CD62L together with its interaction partner CD44 controls the frequency of intraluminally crawling inflammatory monocytes, whereas CD44 or PSGL-1/CD162 adjusts the crawling velocity of neutrophils and inflammatory monocytes. In line with these results also L-selectin/CD62L,44 PSGL-1/CD162,44 and CD4445 have recently been identified as signaling molecules that initiate conformational changes of integrins and—according to our findings—promote intravascular crawling of leukocytes to the site of emigration.

In conclusion, our experimental data demonstrate that the complex spatiotemporal expression dynamics of selectins determine the sequential extravasation of neutrophils and inflammatory monocytes in the acute inflammatory response. Whereas endothelial surface translocated P-selectin/CD62P and constitutively expressed leukocyte L-selectin/CD62L together with their interaction partners PSGL-1/CD162 and CD44 induce the migration of neutrophils to the site of inflammation, de novo synthesis of endothelial E-selectin/CD62E initiates the secondary extravasation of inflammatory monocytes. Coordinating the intraluminal trafficking of neutrophils and inflammatory monocytes to common sites of emigration, selectins assign different sites to these immune cells for their initial interactions with the inflamed endothelium. In this context, P- and L-selectin/CD62L together with PSGL-1/CD162 and CD44 control the flux of rolling neutrophils and
monocytes, whereas E-selectin/CD62E exclusively adjusts the rolling velocity of inflammatory monocytes. Moreover, selectins and their interaction partners PSGL-1/CD162 and CD44 participate in the control of the intraluminal crawling behavior of neutrophils and inflammatory monocytes collectively enabling the sequential extravasation of these immune cells to the inflamed tissue. Our experimental data provide novel insights into the mechanisms underlying the recruitment process of different myeloid leukocyte subsets uncovering previously unappreciated functions of selectins in the extravasation cascade of these immune cells (Figure VIII in the online-only Data Supplement). From a translational point of view, these findings might contribute to the development of targeted therapeutic strategies for the prevention and treatment of cardiovascular pathologies.

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Disclosures

None.

References

22. McEver RP, Beckstead JH, Moore KL, Marshall-Carlson L, Bainton DF. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies.
Leukocyte recruitment to the site of inflammation is a key event in the pathogenesis of various cardiovascular pathologies. Infiltrating neutrophils constitute the first line of defense, which precedes a second wave of emigrating monocytes reinforcing the inflammatory reaction. Here, we demonstrate that selectins—a family of 3 closely related carbohydrate-binding proteins—assign different sites to neutrophils and inflammatory monocytes for their initial interactions with the microvascular endothelium, thereby coordinating the intraluminal trafficking of these immune cells to common sites of extravasation. Whereas constitutively expressed leukocyte L-selectin/CD62L and endothelial P-selectin/CD62P together with CD44 and P-selectin glycoprotein ligand-1/CD162 initiate the emigration of neutrophils, de novo synthesis of endothelial E-selectin/CD62E launches the delayed secondary recruitment of inflammatory monocytes. Our findings provide novel insights into the mechanisms initiating the sequential infiltration of the perivascular tissue by neutrophils and monocytes and might thereby contribute to the development of targeted therapeutic strategies for prevention and treatment of cardiovascular pathologies.
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MATERIALS AND METHODS

Animals

Male C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). Male CX3CR1GFP/+ mice were generated as described previously and backcrossed to the C57BL/6 background for 6 to 10 generations. All experiments were performed using mice at the age of 10 to 12 weeks. Animals were housed under conventional conditions with free access to food and water. The experiments were performed according to German legislation for the protection of animals and approved by the local government authorities.

Reagents

Recombinant murine CCL2 (0.45 µg in 600 µl PBS intraperitoneally (i.p.) or 0.3 µg in 400 µl PBS intrascrotally (i.s.); R&D Systems, Nordenstadt, Germany), CXCL1 (0.3 µg in 600 µl PBS i.p.; R&D Systems), interleukin-1β (IL-1β; 0.05 µg in 600 µl PBS i.p.; R&D Systems) or tumor necrosis factor-α (TNF-α; 0.45 µg in 600 µl PBS i.p. or 0.3 µg in 400 µl PBS i.s.; R&D Systems) were used to induce expression of endothelial adhesion/signaling molecules and/or (subsequent) leukocyte recruitment. An anti-Ly-6G monoclonal antibody (mAb; clone 1A8; 150 µg intravenously (i.v.); 24 h and 6 h prior to induction of inflammation; BD Biosciences, San Jose, CA, USA) was used for the depletion of neutrophils. An anti-GPIbα (CD42b) mAb (clone Xia.B2; 50 µg i.v.; 24 h and 6 h prior to induction of inflammation; emfret Analytics, Eibelstadt, Germany) was used for the depletion of platelets. Leukocyte responses were analyzed upon administration of blocking mAbs: anti-CD62E mAbs [clone 10E9.6 (BD Biosciences) or clone 9A9 (gift from M. Sperandio); 50 µg in 100 µl PBS intra-arterially (i.a.) or i.v.; BD Biosciences], anti-CD62P mAb (clone RB40.34; 50 µg in 100 µl PBS i.a. or i.v.), anti-CD62L mAb (clone MEL-14; 50 µg in 100 µl PBS i.a. or i.v.; Biolegend, San Diego, CA, USA), anti-CD44 mAb (clone IM7; 50 µg in 100 µl PBS i.a. or i.v.; Biolegend), or anti-CD162 mAb (clone 4RA10; 50 µg in 100 µl PBS i.a. or i.v.; BD Biosciences).

Peritonitis assay

After 6 h of i.p. stimulation with CCL2, mice were sacrificed and their peritoneal cavity was washed with 10 ml of ice-cold PBS as described previously. The total number of leukocytes recovered from the peritoneal lavage fluid was analyzed by using a Coulter A C T counter (Coulter Corp.). Samples were then labeled with anti-CD45 APC-Cy7 mAb (clone 30-F11; BD Bioscience), anti-CD11b FITC mAb (clone M1/70; eBioscience, San Diego, CA, USA), anti-GR-1 PE mAb (clone RB6-8C5; eBioscience), anti-CD115 APC mAb (clone AFS98; eBioscience), and anti-F4/80 eFluor450 mAb (clone BM8; eBioscience) for 30 minutes on ice. Erythrocytes were lysed with lysing solution (1:10; BD FACS lysing solution; BD Biosciences). After two washing steps, leukocytes were resuspended in 250 µl PBS. Using flow cytometry (Galilus; Beckman Coulter Inc, Brea, CA, USA), myeloid leukocytes were detected by expression of CD45 and CD11b as well as by the absence of F4/80. Thereof, neutrophils were identified by high expression of Gr-1 and low expression of CD115, inflammatory monocytes by high expression of Gr-1 and CD115, and resident monocytes by high expression of CD115 as well as by low expression of Gr-1 (see gating strategy in Fig. 2A).

In vivo microscopy on the cremaster muscle

Surgical procedure

The surgical preparation of the cremaster muscle was performed as originally described by Baez with minor modifications. Mice were anesthetized using a ketamine/xylazine mixture (100 mg/kg ketamine and 10 mg/kg xylazine), administrated by i.p. injection. The left femoral artery was cannulated in a retrograde manner for administration of microspheres and
antibodies. The right cremaster muscle was exposed through a ventral incision of the scrotum. The muscle was opened ventrally in a relatively avascular zone, using careful electrocautery to stop any bleeding, and spread over the pedestal of a custom-made microscopy stage. Epididymis and testicle were detached from the cremaster muscle and placed into the abdominal cavity. Throughout the procedure as well as after surgical preparation during in vivo microscopy, the muscle was superfused with warm buffered saline.

Experimental protocols

In a first set of experiments, three postcapillary vessel segments were randomly chosen in a central area of the spread-out cremaster muscle among those that were at least 150 µm away from neighboring postcapillary venules and did not branch over a distance of at least 150 µm. In vivo microscopy measurements of leukocyte intravascular rolling, adherence, and crawling as well as transmigration were performed after i.s. injection of PBS and after 60, 120, 180, or 360 min of i.s. stimulation with CCL2 (n=3 per group). In further experiments, baseline in vivo microscopy measurements of leukocyte rolling and adherence were performed in three postcapillary vessel segments 360 min after i.s. stimulation with CCL2. Subsequently, blocking mAbs were applied and the in vivo microscopy measurements were repeated 5 and 45 min after the administration of mAbs. Additionally, the intraluminal crawling behavior of leukocytes was analyzed for 30 min in a single postcapillary venule 15 minutes after the administration of mAbs. After in vivo microscopy (~ 60 min after administration of mAbs), blood samples were collected by cardiac puncture for the determination of systemic leukocyte counts using a Coulter ACT Counter (Coulter Corp., Miami, FL, USA). Anesthetized animals were then killed by bleeding to death.

In selected experiments, non-blocking PE-labeled anti-CD11b mAbs (visualization of myeloid leukocytes), PE-labeled anti-Ly-6G mAbs (visualization of neutrophils), or PE-labeled / Alexa488-labeled anti-CD31 mAbs (visualization of endothelial junctions) were used.

In vivo microscopy

The setup for in vivo microscopy was centered around an AxioTech-Vario 100 Microscope (Zeiss MicroImaging GmbH, Goettingen, Germany), equipped with a Colibiri LED light source (Zeiss MicroImaging GmbH) for fluorescence epi-illumination microscopy as described previously ². Light was directed onto the specimen via filter set 62 HE (Zeiss MicroImaging GmbH) fitted with dichroic and emission filters [TFT 495 + 610 (HE); TBP 527 + LP615 (HE)]. Microscopy images were obtained with an AxioCam Hsm digital camera using a 20x water immersion lens (0.5 NA, Zeiss MicroImaging GmbH). The images were processed with AxioVision 4.6 software (Zeiss MicroImaging GmbH).

Quantification of leukocyte kinetics and microhemodynamic parameters

In vivo microscopy records were analyzed offline using the imaging software ImageJ (National Institutes of Health, Bethesda, MD). GFP<sup>neg</sup>, GFP<sup>high</sup>, and GFP<sup>low</sup> cells were distinguished by the analysis of their fluorescence intensities as described previously ⁶ with minor modifications. Rolling leukocytes were defined as those moving slower than the associated blood flow and quantified for 60 s per venule. Firmly adherent cells were determined as those resting in the associated blood flow for 30 seconds and related to the luminal surface per 100 µm vessel length. Transmigrated cells were counted in regions of interest (ROI), covering 75 µm on both sides of a vessel over 100 µm vessel length. For the analysis of leukocyte intravascular crawling, leukocytes were tracked in the vessel lumen within a time period of 30 min. The frequency of intravascular crawling leukocytes was determined by dividing the total number of adherent leukocytes by the number of intravascularly crawling leukocytes. Parameters including migration velocity, directionality, and accumulated distance were calculated automatically by the software. Accumulated
distance is the total distance presented as a line connecting the positions of the selected leucocyte at each time point.

By measuring the distance between several images of one fluorescent bead under stroboscopic illumination, centerline blood flow velocity was determined. From measured vessel diameters and centerline blood flow velocity, apparent wall shear rates were calculated, assuming a parabolic flow velocity profile over the vessel cross-section.

**Experimental groups**

Animals were assigned randomly to the following groups: C57BL/6 mice undergoing 360 min of i.p. stimulation with recombinant murine CCL2 and receiving an i.v. administration of isotype control antibodies or blocking mAbs directed against P-selectin/CD62P, E-selectin/CD62E, L-selectin/CD62L, PSGL-1/CD162, or CD44 (n=7 per group). In additional experiments, C57BL/6 mice received neutrophil or platelet depleting mAbs prior to 360 min of i.p. stimulation with recombinant murine CCL2/MCP-1 (n=7 per group).

In a further set of experiments, CX3CR1GFP+/− mice received an i.s. injection of PBS or were i.s. stimulated for 60, 120, 180, or 360 min with CCL2 (n=3 per group). In final experiments, CX3CR1GFP+/− mice received an i.a. administration of isotype control antibodies or blocking mAbs directed against P-selectin/CD62P, E-selectin/CD62E, L-selectin/CD62L, PSGL-1/CD162, or CD44 after 360 min of i.s. stimulation with CCL2 (n=5 per group). Furthermore, CX3CR1GFP+/− mice received an i.a. administration of isotype control antibodies or blocking mAbs directed against P-selectin/CD62P or E-selectin/CD62E after 360 min of i.s. stimulation with TNF-α (n=4 per group).

**Confocal microscopy**

For the analysis of cell surface molecules on blood leukocytes, blood was taken from the vena cava of male C57Bl/6 mice, heparinized, incubated with CCL2 (10 ng / 100 µl), and PBS for control. Samples were incubated with rat anti-mouse Ly-6C/G (GR-1) Alexa Fluor 647 mAb (clone RB6-8C5; Molecular Probes, Frederick, USA) and anti-mouse CD115 (c-fms) Alexa Fluor 488 mAb (clone AFS98, eBioscience), as well as with rat anti-mouse CD62L (Biolegend), rat anti-mouse CD162 (BD Bioscience), or rat anti-mouse CD44 mAb (Biolegend) for 30 minutes on ice. After washing, samples were incubated with an Alexa Fluor 555-linked goat anti-rat antibody (Molecular Probes) for 30 minutes on ice. Then, erythrocytes were lysed with lysing solution (1:10; BD FACS lysing solution; BD Bioscience) and cells were plated on fibronectin (AppliChem, Darmstadt, Germany) coated coverslips. Confocal z-stacks typically covering 30 µm (z-spacing 0.5 µm) were acquired using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with an oil-immersion lens (Leica; 63x; NA 1.40).

For the analysis of PECAM-1, P-selectin/CD62P, E-selectin/CD62E, CD44 and PSGL-1/CD162 expression on endothelial cells of postcapillary venules, excised mouse cremaster muscles (6 h after intrascrotal injection of CCL2, TNF-α, or PBS) were fixed in 2 % paraformaldehyde. Tissues were then blocked and permeabilized in PBS, supplemented with 10 % goat serum (Sigma Aldrich) and 0.5 % Triton X-100 (Sigma Aldrich). After incubation at 4° C for 12 hours with antibodies directed against PECAM-1 (CD31 goat IgG; Santa Cruz Biotechnology, Dallas, Texas, USA) and CD62P (rat anti-mouse; BD Biosciences), PECAM-1 (CD31 goat IgG; Santa Cruz Biotechnology) and CD62E (rat anti-mouse; BD Biosciences), PECAM-1 (CD31 goat IgG; Santa Cruz Biotechnology) and CD44 (rat anti-mouse; Biolegend), or PECAM-1 (CD31 goat IgG; Santa Cruz Biotechnology) and CD162 (rat anti-mouse; BD Bioscience), tissues were incubated for 180 min at room temperature with an Alexa Fluor 633-linked donkey anti-goat antibody (molecular probes) and then with an Alexa Fluor 488-linked goat anti-rat antibody (molecular probes). Immunostained tissues were mounted in PermaFluor (Beckman Coulter, Fullerton, CA) on glass slides. Confocal z-stacks typically covering 30 µm (z-spacing 0.5 µm) were acquired using a Leica SP5 confocal laser-
scanning microscope (Leica Microsystems, Wetzlar, Germany) with an oil-immersion lens (Leica; 63x; NA 1.40). The fluorescence signal of CD62P, CD62E, CD44, and CD162 was only quantified on the surface of endothelial cells of postcapillary venules not measuring the fluorescence signal of these adhesion and signaling molecules on leukocytes and platelets (easily identified by expression of PECAM-1 and morphological characteristics).

Flow cytometry

To analyze the effect of CCL2 or PMA on the expression profiles of L-selectin/CD62L, PSGL-1/CD162, or CD44 on murine neutrophils and monocytes, anticoagulated whole blood samples were incubated (30 min; 37 °C) with CCL2 (100 ng/ml), PMA (50 ng/ml), or PBS as negative control. After washing, cells were incubated with primary antibodies directed against CD45, CD11b, GR-1, CD115, CD62L, CD44, and CD162 on ice. Isotype-matched controls were used in all experiments. After lysis of erythrocytes, stained cells were analyzed on a flow cytometer (Gallios, Beckmann Coulter). Approximately 20,000 gated events were collected in each analysis.

Quantitative realtime RT-PCR (qRT-PCR)

mRNA expression levels of selectins and their respective interaction partners were measured by qRT-PCR analyses of total cremaster RNA preparations. RNA isolation and qRT-PCR were performed as described before 7. Briefly, cremaster muscles were explanted at the indicated times after i.s. CCL2 injection and total RNA was extracted by the TRIzol method (Life Technologies, Karlsruhe, Germany). 1 µg of RNA was subjected to reverse transcription with 200 units RevertAid reverse transcriptase in the presence 50 µM random hexamers, 5 µM Oligo(dT)18, 400 µM dNTPs, and 1.6 units/µl Ribolock RNase inhibitor (all from Fermentas, St. Leon-Rot, Germany). The resulting cDNA (20 ng per reaction) was applied to qRT-PCR runs (20 µl final volume) with 300 nM primers (synthesized by Sigma Aldrich, Taufkirchen, Germany) in 1x Maxima SYBR Green qPCR Mastermix (Fermentas) and a standard cycling protocol (10 min 95°C, 45x (15 s 95°C, 30 s 60°C)) on an LC480 qPCR cycler (Roche Applied Science, Penzberg, Germany). The following primer pairs were used: CD62E Forward 5'- CCC CTC CTG CCA AGT GGT A-3', CD62E Reverse 5'-TCA TGT TCA TCT TTC CCG GG-3', CD62P Forward 5'-ATG TGC AGA GCG GTC AAA TG-3', CD62P Reverse 5'-CGT TCA GTG ACT GCC CCT CT-3', CD162 5'-TGC TGT GGT GGG CAC GGT A-3', CD162 Reverse 5'-GAT GGT CAG CAC CAC AAG GAA-3', CD44 Forward 5'-TCC AAT GCC TCA GCC CCT C-3', CD44 Reverse 5'-TGG GTA CTG GTC TGA CTG ATG TAC A-3', 18S rRNA Forward 5'-CGG CTA CCA CAT CCA AGG AA-3', 18S rRNA Reverse 5'-GCT GGA ATT ACC GCC GCT GCT-3', beta-2-microglobulin Forward 5'- CGG CCT GTA TGC TAT CCA GAA A-3', beta-2-microglobulin Reverse 5'-AAT GTG AGG CGG GTG AAG CT-3', delta-aminolaevulinate-synthase Forward 5'-ATC ATC CCT GTG CGG GTT G-3', delta-aminolaevulinate-synthase Reverse 5'-TAA TTG ATG GCC TGG ACG TAG ATA TT-3'. Relative quantification was performed by employing the standard curve method, and the results were normalized on a matrix of 18S rRNA, beta-2-microglobulin, and delta-aminolaevulinate-synthase. Untreated controls (t=0) served as calibrator.

Statistics

Data analysis was performed with a statistical software package (SigmaStat for Windows; Jandel Scientific). T-test (2 groups) or One-way ANOVA followed by the Dunnett test (> 2 groups) were used for the estimation of stochastic probability in intergroup comparisons. Mean values and SEM are given. P values <.05 were considered significant.
References


Effect of different chemokines and cytokines on the recruitment of neutrophils and monocytes. Panels show quantitative data for the peritoneal recruitment of resident monocytes (A), neutrophils (B), and inflammatory monocytes (C) in C57BL/6 mice as assessed 6 h after i.p. stimulation with CCL2, CXCL1, IL-1β, or TNF-α by flow cytometry (mean±SEM; n=4 per group; #p<0.05, vs. unstimulated).
Effect of the anti-E-selectin/CD62E mAb ‘clone 9A9’ on CCL2-elicited recruitment of neutrophils and inflammatory monocytes. Panels show quantitative data for the peritoneal recruitment of neutrophils (A) and inflammatory monocytes (B) in C57BL/6 mice treated with the anti-E-selectin/CD62E mAb ‘clone 9A9’ or isotype control antibody as assessed 6 h after i.p. stimulation with CCL2 by flow cytometry (mean±SEM; n=4 per group; *p<0.05, vs. isotype control).
Effect of the anti-E-selectin/CD62E mAb ‘clone 9A9’ on CCL2-elicited endothelial cell interactions of neutrophils and inflammatory monocytes. Panels show quantitative data for the relative changes in numbers of intravascularly rolling and firmly adherent inflammatory monocytes (A) and neutrophils (B) as well as in their intravascular rolling velocities as compared to baseline conditions in postcapillary venules of the cremaster muscle of CX3CR1GFP/+ mice treated with the anti-E-selectin/CD62E mAb ‘clone 9A9’ or isotype control antibody as assessed 6 h after i.s. stimulation with CCL2 by in vivo microscopy (mean±SEM; n=4 per group; *p<0.05, vs. isotype control).
TNF-α-elicited endothelial cell interactions of neutrophils and monocytes. Panels show quantitative data for the proportion of intravascularly rolling and firmly adherent as well as transmigrated neutrophils (A) and inflammatory monocytes (B) of total leukocytes in postcapillary venules of the cremaster muscle of CX3CR-1\(^{GFP/+}\) mice as assessed 6 h after i.s. stimulation with CCL2 or TNF-α by in vivo microscopy (mean±SEM; \(n=4\) per group).
Role of E- and P-selectin for TNF-α-elicited endothelial cell interactions of neutrophils. Panels show quantitative data for the relative changes in numbers of intravascularly rolling (A) and firmly adherent neutrophils (C) as well as in their intravascular rolling velocities (B) in cremasteric postcapillary venules as compared to baseline conditions after i.a. application of mAbs directed against E-selectin/CD62E (clones 10E9.6 and 9A9) or P-selectin/CD62P as assessed 6 h after i.s. stimulation with TNF-α by in vivo microscopy (mean±SEM; n=4 per group; *p<0.05, vs. isotpye control).
Supplemental Figure VI

Effect of TNF-α on surface expression of E- and P-selectin in microvascular endothelial cells. Representative confocal microscopy images (A) and quantitative analysis (B) of E-selectin/CD62E and P-selectin/CD62P expression in endothelial cells of cremasteric postcapillary venules after stimulation with TNF-α (mean±SEM; n=4 per group; #p<0.05, vs. unstimulated).
Role of selectins and their interaction partners PSGL-1/CD162 and CD44 for intravascular crawling of neutrophils and monocytes. Representative migration plots of intravascularly crawling inflammatory monocytes and neutrophils in postcapillary venules of the CCL2-stimulated cremaster muscle in isotype control antibody treated animals or upon blockade of P-selectin/CD62P, L-selectin/CD62L, E-selectin/CD62E (clone 10E9.6), PSGL-1/CD162, or CD44 (A). Panels show quantitative data for the crawling frequency, directionality, or velocity of inflammatory monocytes and neutrophils as assessed by in vivo microscopy (B; mean±SEM; n=4 per group; #p<0.05, vs. isotype control).
Role of selectins for extravasation of neutrophils and monocytes. A schematic overview on the role of selectins and their interaction partners PSGL-1/CD162 and CD44 for different steps in the extravasation cascade of neutrophils and monocytes is shown (no (-), intermediate (+), strong (++), very strong (+++) effect).
## Supplemental Tables

### Supplemental Table I

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<th>Stimulus</th>
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<th>Inner vessel diameter [µm]</th>
<th>Vmean [mm s(^{-1})]</th>
<th>Wall shear rate [s(^{-1})]</th>
<th>Systemic leukocyte counts (x 10^3 \mu l^{-1})</th>
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**Microhemodynamic parameters and systemic leukocyte counts.** Systemic leukocyte counts as well as microhemodynamic parameters, including inner vessel diameter, blood flow velocity, and wall shear rate were obtained as detailed in *Material and Methods* (mean±SEM for n=4-5 per group).
Supplemental videos

**Supplemental video 1.** Postcapillary venule in the inflamed cremaster muscle as observed by combining transillumination and fluorescence *in vivo* microscopy. Rolling CX3CR-1<sup>GFP/+</sup>-positive monocytes (green) predominantly interact with CD31-immunoreactive endothelial junctions (blue).

**Supplemental video 2.** Postcapillary venule in the inflamed cremaster muscle as observed by combining transillumination and fluorescence *in vivo* microscopy. Interactions of rolling Ly-6G-positive neutrophils (red) with endothelial cells are not restricted to CD31-immunoreactive endothelial junctions (blue).