Neutrophil-Derived Cathelicidin Promotes Adhesion of Classical Monocytes

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**Rationale:** The leukocyte response in acute inflammation is characterized by an initial recruitment of neutrophils preceding a second wave of monocytes. Neutrophil-derived granule proteins were suggested to hold an important role in this cellular switch. The exact mechanisms by which neutrophils mediate these processes are only partially understood.

**Objective:** To investigate the role of neutrophils and their granule contents in the adhesion of monocyte subpopulations in acute inflammation.

**Methods and Results:** Here, we show that neutrophil-derived cathelicidins (human: LL37, mouse: CRAMP) induce adhesion of classical monocytes but not of nonclassical monocytes in the mouse cremaster muscle and in vitro flow chamber assays. CRAMP is released from emigrated neutrophils and then transported across the endothelium, where it is presented to rolling leukocytes. Endothelial-bound cathelicidin activates formyl-peptide receptor 2 on classical monocytes, resulting in monocytic β1- and β2-integrin conformational change toward an extended, active conformation that allows for adhesion to their respective ligands, vascular cell adhesion molecule 1 and intercellular adhesion molecule 1.

**Conclusions:** These data elucidate a novel mechanism of neutrophil-mediated monocyte recruitment, which could be targeted in conditions where recruitment of classical monocytes plays an unfavorable role. (Circ Res. 2013;112:792-801.)

Key Words: cathelicidin ■ chemokine ■ inflammation ■ monocyte ■ neutrophil ■ recruitment

Neutrophils dominate the initial wave of leukocytes emigrating into inflamed tissues.1 This first wave of neutrophil extravasation precedes a second wave of monocyte extravasation. Recruited neutrophils are thought to contribute to this cellular switch by several mechanisms,2,3 including the release of soluble factors, such as neutrophil granule proteins, that are deposited at the site of inflammation.4 Indeed, supernatants of activated neutrophils from patients with specific granule deficiency show a reduced capacity to attract monocytes, despite normal monocyte chemotaxis in vitro to other stimuli.5 After this observation, several neutrophil granule proteins with monocyte-attracting activity were identified. Among them are cathelicidin (human: LL37, mouse: CRAMP),6 cathespin G,7 α-defensins,8 and azurocidin.9

Peripheral blood monocytes constitute a heterogeneous population of circulating leukocytes in both humans9 and mice.10 Based on the expression of Gr1, classical (inflammatory, Ly6C+) and nonclassical (resident, patrolling, Ly6C−) mouse monocytes can be discriminated. Functionally, nonclassical monocytes are thought to contribute to phagocytosis, wound healing, and tissue remodeling, whereas classical monocytes not only dominate inflammatory processes, such as the early response in infections, but also sterile inflammatory conditions, such as myocardial infarction or atherosclerosis.11 In humans, monocyte subsets are discriminated based on their CD14 and CD16 expression profile with classical monocytes being CD14+CD16−, intermediate monocytes being CD14+CD16+, and nonclassical monocytes being CD14+CD16++.12

Although several studies have shown the important chemotactic activities of neutrophil granule proteins toward monocytes, the role of neutrophil secretory products in early steps of monocyte recruitment is still under investigation.

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of monocyte recruitment has not been studied. Hence, we here investigated the role of neutrophils and their granule constituents in their capacity to differentially induce adhesion of monocyte subsets.

Methods
For detailed Materials and Methods, please see the Online Data Supplement.

Mice
Wild-type C57BL/6 and gene-targeted Lysm gfp/gfp, Cx3cr1egfp/wt, cathelicidin-deficient (Cramp−/−), Fpr1−/−, Fpr2−/−, G-deficient (CG−/−), DARC−/−, and caverolin 1-deficient mice were used for in vivo experiments. All genetically modified animals were on C57BL/6 background. Specific neutrophil depletion was achieved by intraperitoneal injection of monoclonal antibody 1A8 (50 µg/mouse; BioXCell) 8 hours before injection of the stimuli (Online Table I). All animal experiments were approved by the local ethics committee for animal experimentation.

Results
Cathelicidin Induces Firm Adhesion of Classical Monocytes
To study the importance of neutrophils in the adhesion of monocytes, we injected tumor necrosis factor (TNF) into the cremaster of Cx3cr1egfp/wt mice carrying green fluorescent monocytes and recorded the adhesion of monocytes in vivo. To discriminate between classical monocytes and nonclassical monocytes, an antibody to Ly6C was introduced, which labels classical monocytes (Online Figure I). In these experiments, neutropenic mice displayed reduced adhesion of classical monocytes (Ly6C+/gfp+) to postcapillary venules, whereas neutropenic mice exhibited no impaired adhesion of nonclassical monocytes (Ly6C−/gfp+) (Figure 1A and 1B). In contrast, adhesion of nonclassical monocytes (Ly6C+/gfp+) to postcapillary venules, whereas neutropenic mice displayed reduced adhesion of classical monocytes (Online Figure I). In these experiments, to discriminate between classical monocytes and nonclassical monocytes, we performed in vitro flow chamber assays. All genetically modified animals were on C57BL/6 background. Specific neutrophil depletion was achieved by intraperitoneal injection of monoclonal antibody 1A8 (50 µg/mouse; BioXCell) 8 hours before injection of the stimuli (Online Table I). All animal experiments were approved by the local ethics committee for animal experimentation.

Neutrophil-Derived Cathelicidin Is Transported in a Basal–Apical Direction Across the Endothelium
To study the dynamics of cathelicidin release and in vivo presentation, we injected monocyte-depleted Lysm gfp/gfp mice (thus creating mice with fluorescent neutrophils only) with TNF intrascrotally and recorded the adhesion and extravasation of neutrophils. In addition, microbeads conjugated with an antibody to CRAMP were injected to visualize endothelial-bound CRAMP (Figure 2A). Although adhesion of neutrophils occurred rapidly and peaked at 2 hours, endothelial-bound CRAMP was not detected in significant numbers until 4 hours after TNF stimulation, thus correlating more closely with the number of emigrated neutrophils (Figure 2B and 2C). Detection of endothelial CRAMP peaks between 4 and 12 hours after TNF injection. During this time, neutrophil adhesion decreases, whereas the number of emigrated neutrophils reaches its maximum (Figure 2B and 2C). Depletion of neutrophils reduced the number of adherent anti-CRAMP–conjugated microbeads to a level observed in Cramp−/− mice, thus confirming that CRAMP in this location is neutrophil-derived (Figure 2D). To further investigate alternative CRAMP sources, we analyzed intracellular CRAMP expression in endothelial cells and in neutrophils obtained from enzymatically digested cremaster muscles. To discriminate between adherent and extravasated neutrophils, an antibody to Ly6G was injected intravenously 5 minutes before euthanization.21 In these experiments, minor amounts of CRAMP were detected in endothelial cells (Online Figure II). In contrast, we detected large amounts of CRAMP in adherent neutrophils that were decreased in emigrated neutrophils, suggesting discharge of CRAMP after emigration (Online Figure III).

Because our in vivo experiments suggested that cathelicidins are transported across the endothelium in a basal–apical direction, we aimed at studying such mechanism in vitro. HUVEC were grown on tissue culture filter inserts and stimulated with TNF. Fluorescein isothiocyanate–LL37 was added to the bottom well, and LL37 transportation was assessed by 2-photon microscopy. In these experiments, LL37 was found to accumulate on the endothelial cell surface (Figure 2E). To quantify the amount of transcytosed LL37, we assessed the fluorescence resulting from antibody-stained, surface-bound LL37. In time course experiments, we found that LL37 accumulated on the HUVEC surface not until 2 hours (Figure II).
The transendothelial transportation was abrogated at 4°C (Figure 2G) and by pretreatment with colchicine or cytochalasin B (Figure 2H). Cathelicidins were reported to act through formyl-peptide receptor (FPR) 2, CXCR2, and P2X7.24,25 However, pretreatment of HUVEC with boc-PLPLP (antagonist to FPRs), SB225002 (antagonist to CXCR2), or suramin (antagonist to P2X7) did not impact on transendothelial LL37 transportation (Figure 2H). As highly cationic polypeptide, we suspected that LL37 might interact with endothelial proteoglycans. To this end, we cleaved endothelial heparin sulfate or chondroitin sulfate side chains by use of heparinase or chondroitinase and recorded its effect on LL37 transcytosis. Interestingly, pretreatment with either enzyme markedly reduced the transendothelial transportation across the endothelium (Figure 2H). Finally, targeting caveolae-mediated transportation by pretreatment with filipin resulted in clear-cut decreases of LL37 transcytosis. Because duffy antigen/receptor for chemokines facilitates transport of chemokines across the endothelium26 we also wanted to address its involvement in the transendothelial transportation of CRAMP. However, in our hands, HUVEC activated with TNF did not express duffy antigen/receptor for chemokines and we could not detect an interaction between duffy antigen/receptor for chemokines and LL37 (Online Figure IV).

We next injected CRAMP intrascrotally into neutropenic WT mice and detected its luminal appearance by intravital microscopy. In WT mice, luminal CRAMP was detected 4 hours after intrascrotal TNF injection, a response abrogated in caveolin 1–deficient mice (Figure 2I). Immobilization of anti-CRAMP microbeads was not seen when PBS was used instead of CRAMP (not shown). However, the transportation of CRAMP across the endothelium was maintained in Darc−/− mice.
abrogated LL37-mediated increases in classical CD14++CD16− monocyte adhesion. Because boc-PLPLP blocks activation of all FPR family members, we further used specific antagonists to FPR1 and FPR2. In these experiments, the FPR2-specific antagonist spinorphin (not shown) abrogated LL37-dependent antagonist quin-C7 (Figure 3A) but not the FPR1-specific antagonist CRAMP. Furthermore, the FPR2 expression on CD14++CD16+ monocytes in mice also carries higher levels of FPR2 compared with nonclassical monocytes. Analysis of FPR2 expression revealed that FPR2 is expressed to a higher degree on classical CD14++CD16− monocytes compared with nonclassical monocytes. In these experiments, inhibition of FPRs by quin-C7 clearly reduced classical monocyte adhesion in vivo depending on the use of FPR2, whereas the interaction with FPR1 or CXCR2 was not different compared with empty liposomes. For P2X7, we found no interaction with LL37 (not shown).

To further investigate whether cathelicidin-mediated monocyte adhesion in vivo depends on the use of FPR2, we injected CRAMP, along with TNF, into the scrotum of neutropenic WT mice. Receptor use was initially studied by use of antagonists to FPRs, P2X7, and CXCR2. In these experiments, inhibition of FPRs by quin-C7 clearly reduced CRAMP-mediated adhesion, whereas the other antagonists were without effects (Figure 3E). To further corroborate this, we injected neutropenic Fpr1−/− or Fpr2−/− mice with TNF and CRAMP. Although CRAMP injection resulted in increased adhesion in Fpr1−/− mice, this response was not observed in Fpr2−/− mice (Figure 3E).

Cathelicidins Induce Integrin Activation on Classical Monocytes

Thus far, we have shown that neutrophil-derived cathelicidins resemble a functional similarity with arrest chemokines. 27

Figure 2. Neutrophil-derived cathelicidin is transported in a basal–apical direction across the endothelium. A to C, Luminal CRAMP accumulation parallels with neutrophil extravasation but not neutrophil adhesion. Lysm−/− mice were intrascrotally injected with tumor necrosis factor (TNF; 50 ng), and the adhesion (B, green line) and transmigration (C, green line) of neutrophils were quantified by intravital microscopy at indicated time points. To assess luminal CRAMP presentation, immobilization of G-protein–coupled fluorescent beads conjugated with an antibody to CRAMP was analyzed (red line in B and C; n=4 for each data point). Representative images for indicated time points are shown in A. For better visibility, immobilized beads were marked with red dots. Scale bar, 50 µm. D, Wild-type (WT) or cathelicidin-deficient (Cramp−/−) mice with intact white blood cell (WBC) count or neutropenia were intrascrotally injected with TNF (50 ng, 12 h). Luminal CRAMP presentation was assessed after injection of G-protein–coupled fluorescent beads conjugated with antibodies to CRAMP. *Significant difference compared with respective mouse strain with intact WBC (n=5). E, LL37 is transported across human umbilical vein endothelial cells (HUVEC) grown on transwell filter inserts. LL37 was added to the bottom well, and analyses were made 4 h later by 2-photon microscopy. LL37 (green) was visualized by a fluorescent tag (top) or by antibody staining of nonpermeabilized endothelial cells (bottom). Cell membrane, red; nucleus, blue. F, Antibody detection of transcytosed LL37 on the apical side of endothelial cells (bottom). Cell membrane, red; nucleus, blue. G, Antibody detection of transcytosed LL37 on the apical side of endothelial cells (bottom). Cell membrane, red; nucleus, blue. H, Transcytosis experiments performed at 4°C (G) or by pretreatment of endothelial cells with indicated inhibitors (H; n=6 for each bar). *Significant difference from ctrl. I, CRAMP transport is impaired in caveolin 1–deficient (Cav1−/−) mice. Neutropenic WT, DarC−/−, or Cav1−/− mice were intrascrotally injected with TNF (50 ng, 4 h) and CRAMP (1 µg). Luminal CRAMP presentation was assessed after injection of G-protein–coupled fluorescent beads conjugated with an antibody to CRAMP. Scale bar is 20 µm in representative images. *Significant difference compared with WT mice (n=4).
The latter mediate firm adhesion through inside-out integrin signaling and subsequent changes in integrin conformation toward an extended, high-affinity state. Classical ligands for very late antigen 4 (CD49d/CD29, \(\alpha_4\beta_1\)-integrins) and macrophage 1 antigen (CD11b/CD18, \(\alpha_M\beta_2\)-integrin) are vascular cell adhesion molecule 1 and intercellular adhesion molecule 1, respectively. To test the importance of these 2 ligands for LL37-mediated adhesion, we perfused classical CD14\(^+\)CD16\(-\) monocytes over plates coated with P-selectin/intercellular adhesion molecule 1 or P-selectin/vascular cell adhesion molecule 1 (Figure 4A and 4B). In these experiments, coimmobilization of LL37 or monocyte chemoattractant protein 1 (MCP1) strongly enhanced monocyte adhesion. This response was fully abrogated by a blocking antibody to FPR2, the MCPI-mediated adhesion of monocytes was blocked by an antagonist to CCR2. To further investigate the activation of \(\beta_2\)- and \(\beta_1\)-integrins by LL37, we used an antibody that specifically recognizes the extended conformation of these integrins. Monocytes were incubated with phorbol-12-myristate-13-acetate, MCP1, or LL37 and stained for the expression of active integrins. Although phorbol-12-myristate-13-acetate induces integrin activation on both monocyte subsets, MCP1 and LL37 only induce significant integrin activation on classical CD14\(^+\)CD16\(-\) and intermediate CD14\(^+\)CD16\(^+\) monocytes but not on nonclassical CD14\(^-\)CD16\(^+\) monocytes (Online Figure V). Similarly, phorbol-12-myristate-13-acetate, MCP1, or CRAMP induced integrin activation on murine classical but...
not nonclassical monocytes, leading to increased binding of intercellular adhesion molecule 1 (ICAM-1; A) or P-selectin/vascular cell adhesion molecule 1 (VCAM-1; B) coated dishes. The increase in adhesion by coimmobilization of LL37 or monocyte chemoattractant protein 1 (MCP1) was set to 100%. Classical CD14++CD16− monocytes were pretreated with anti-CD11b (1 µg/mL), anti-CD49d (1 µg/mL), or antagonists to FPR2 (quin-C7, 1 µmol/L) or to CCR2 (RS504393, 1 µmol/L; n=8). *Significant difference compared with ctrl. C, Based on the CD16 and CD14 staining properties, human monocyte subsets were identified within peripheral blood mononuclear cells. Furthermore, antibodies to activation epitopes of VLA4 (HUTS-21) and CD11b (CRBM1/5) were added. Cells were treated with phorbol-12-myristate-13-acetate (PMA; 50 ng/mL), MCP-1 (50 ng/mL), or LL37 (1 µg/mL) for 15 minutes, and the expression of activated CD11b (left) or VLA4 (right) was assessed on classical CD14++CD16− monocytes. *Significant difference from control group (n=6). D, CRAMP activation enhances ICAM-1 and VCAM-1 binding to classical monocytes. Mouse peripheral leukocytes were treated with PMA (50 ng/mL), MCP-1 (50 ng/mL), or CRAMP (1 µg/mL) in the presence of ICAM-1-Fc (left) or VCAM-1-Fc (right) and an anti-Fc antibody. Monocyte subsets were identified by additional antibody staining (CD45, CD11b, CD115, Gr1). *Significant difference from control group (n=5). E and F, Expression of activated CD11b (E) or VLA4 (F) on classical CD14++CD16− monocytes in response to LL37 (1 µg/mL) or MCP1 (50 ng/mL) was set to 100%. Peripheral blood mononuclear cells were pretreated with antagonists to phospholipase C (U73122, 100 nmol/L), FPR2 (quin-C7, 1 µmol/L), or CCR2 (RS504393, 1 µmol/L; n=4). *Significant difference compared with respective control group.

Mutation of LL37 Abrogates Its Arrest Function
To identify the motif within LL37 that interacts with FPR2, we compared the amino acid sequences of known FPR2 ligands, including MKYMVM, MMK1, and humanin, with clustal Omega software. Despite the absence of any strict amino acid homology, we found a pattern of chemical properties present in all FPR2 ligands. More precisely, an aromatic amino acid and 2 hydrophobic residues separated by 2 amino acids was the only similarity between the molecules (Figure 5A). LL37 and the other FPR2 agonists are linear peptides, which simplifies the comparison of their 3-dimensional structure. In their helicoid structure with a turn of 3 amino acids, the aromatic and the hydrophobic residues were nearby and can form a hydrophobic domain. To test the involvement of this pattern in LL37-dependent monocyte adhesion, we synthesized a mutated LL37 (mLL37) with an alanine instead of the phenylalanine in position 17 (F17A) and glycine in replacement of the isoleucine and the valine in positions 20 and 21 (I20G and V21G; Figure 5A).

Figure 4. Cathelicidins induce integrin activation. A and B, LL37 mediates macrophage 1 antigen-dependent (CD11b/CD18) and very late antigen 4 (VLA4)-dependent (CD49d/CD29) adhesion. Classical CD14++CD16− monocytes were perfused over P-selectin/intercellular adhesion molecule 1 (ICAM-1; A) or P-selectin/vascular cell adhesion molecule 1 (VCAM-1; B) coated dishes. The increase in adhesion by coimmobilization of LL37 or monocyte chemoattractant protein 1 (MCP1) was set to 100%. Classical CD14++CD16− monocytes were pretreated with anti-CD11b (1 µg/mL), anti-CD49d (1 µg/mL), or antagonists to FPR2 (quin-C7, 1 µmol/L) or to CCR2 (RS504393, 1 µmol/L; n=8). *Significant difference compared with ctrl. C, Based on the CD16 and CD14 staining properties, human monocyte subsets were identified within peripheral blood mononuclear cells. Furthermore, antibodies to activation epitopes of VLA4 (HUTS-21) and CD11b (CRBM1/5) were added. Cells were treated with phorbol-12-myristate-13-acetate (PMA; 50 ng/mL), MCP-1 (50 ng/mL), or LL37 (1 µg/mL) for 15 minutes, and the expression of activated CD11b (left) or VLA4 (right) was assessed on classical CD14++CD16− monocytes. *Significant difference from control group (n=6). D, CRAMP activation enhances ICAM-1 and VCAM-1 binding to classical monocytes. Mouse peripheral leukocytes were treated with PMA (50 ng/mL), MCP-1 (50 ng/mL), or CRAMP (1 µg/mL) in the presence of ICAM-1-Fc (left) or VCAM-1-Fc (right) and an anti-Fc antibody. Monocyte subsets were identified by additional antibody staining (CD45, CD11b, CD115, Gr1). *Significant difference from control group (n=5). E and F, Expression of activated CD11b (E) or VLA4 (F) on classical CD14++CD16− monocytes in response to LL37 (1 µg/mL) or MCP1 (50 ng/mL) was set to 100%. Peripheral blood mononuclear cells were pretreated with antagonists to phospholipase C (U73122, 100 nmol/L), FPR2 (quin-C7, 1 µmol/L), or CCR2 (RS504393, 1 µmol/L; n=4). *Significant difference compared with respective control group.
The data presented here identify a novel neutrophil-driven mechanism of classical monocyte adhesion. On neutrophil tissue infiltration, cathelicidin is released and transported across the endothelium involving a caveolin 1–mediated transportation process. Cathelicidins are then presented to cells rolling along the endothelium. Classical monocytes recognize endothelial-bound cathelicidins via FPR2. This interaction triggers an intracellular signaling cascade, involving phospholipase C, ultimately leading to activation of monocyte β1- and β2-integrins and subsequent adhesion of classical monocytes.

Monocyte subsets harbor crucial albeit differential functions during inflammation. Classical monocytes originate from the bone marrow and spleen to accumulate at sites of inflammation, where they differentiate into macrophages. In the absence of inflammation, classical monocytes are thought to convert into nonclassical monocytes, although this is still under debate. Nonclassical monocytes patrol the luminal side of postcapillary venules, where they sense damage or infection and trigger inflammatory reactions. However, here we show that classical but not nonclassical monocytes sense cathelicidins, which are known to be potent danger signals, and subsequently adhere to the endothelial lining. Classical monocytes exert potent proinflammatory functions, including release of reactive oxygen species, TNF, interleukin-6, and type I interferons, which have been identified as powerful proinflammatory mediators. In addition, classical monocytes disturb the resolution of inflammation, thus perpetuating inflammatory processes.

With the discovery of monocyte subsets, a concept has emerged wherein the relative expression of adhesion molecules or chemokine receptors governs their recruitment behavior. In this context, it was shown that classical monocytes use CCR2, CX3CR1, CXCR2, as well as CCR5, to adhere and migrate to inflammatory sites. Similarly, P-selectin glycoprotein ligand 1 is expressed to a higher degree on classical monocytes and was hence found to be a major determinant in their recruitment. In this study, we demonstrate that FPR2 is dominantly expressed on classical monocytes and almost absent on nonclassical monocytes, hence explaining the exclusive adhesion of classical monocytes in response to immobilized cathelicidins. In addition, recent studies point toward the importance of FPR1 in the recruitment and locomotion of neutrophils toward danger signals. Thus, it remains to be identified how FPR1 ligands differentially affect adhesion and recruitment of monocyte subsets.

Beyond the induction of classical monocyte adhesion as shown here, cathelicidins may promote monocyte recruitment through direct and indirect chemotactic effects. The direct chemotactic effect is primarily mediated via FPR2 with a maximum activity at concentrations of 0.5 to 50 µg/mL (0.1–10 µmol/L). Notably, the direct chemotactic activity of LL37 is not affected by serum, thus contrasting the effects of serum on the microbicidal effects of LL37. This discrepancy may be a result of the fact that the chemotactic activity is
receptor-mediated and not dependent on a peptide–membrane interaction. Presumably, the part of the LL37 peptide that activates FPR2 is not hidden or altered by association of serum components, indicating that recruitment of leukocytes by LL37 is an important biological mechanism that is maintained along the vascular lumen. Besides its direct chemotactic activities, cathelicidins contribute to the inflammatory accumulation by stimulation of chemokine and cytokine production through effector cells. In this context, LL37 induces the production and release of interleukin-8 from monocytes.48 In addition, LL37 was also shown to interact with the purinergic receptor P2X7, a receptor that is predominantly expressed on monocytes, macrophages, and dendritic cells. LL37 stimulation via P2X7 of lipopolysaccharide–primed monocytes induced processing and release of the potent cytokine interleukin-1β.49 This proinflammatory cytokine upregulates adhesion molecules on endothelial cells and hence promotes leukocyte adhesion.

Fundamental to the accumulation of monocytes and macrophages at sites of inflammation is, however, not just their recruitment but also the control of their mobilization from sites of production, as well as their survival at the inflammatory site.42 Because counts of circulating leukocyte subsets are not altered in Cramp−/− mice,43 it is to be assumed that cathelicidins do not play a role in the mobilization of monocytes from the bone marrow. In contrast, cathelicidins were shown to exert divergent effects on apoptotic cell death. Although LL37 promotes apoptosis in various T cells, smooth muscle cells, and epithelial cells,44,45 it was shown to inhibit apoptotic cell death in neutrophils.24 A consequence of the inhibition of apoptosis of neutrophils may be a survival strategy, leading to an increase of viable neutrophils at the site of infection, which is beneficial for the host during bacterial invasion. Although such data are not readily available for monocytes, one may assume that similar mechanisms could contribute to the cathelicidin-mediated accumulation of monocytes at sites of inflammation.

As detailed above, cathelicidin-dependent mechanisms of monocyte adhesion, chemotaxis, and homeostasis may promote accumulation of classical monocytes at inflammatory sites. That such mechanisms importantly contribute to monocyte accumulation is indicated by observations from air pouch models where the instillation of cathelicidins results in pronounced accumulation of monocytes.5,46 In disease models of vascular inflammation, lack of CRAMP reduces the adhesion of classical monocytes, as well as the number of macrophages in atherosclerotic lesions.43 In a model of arterial injury, neutrophil-derived CRAMP was found to promote adhesion of angiogenic monocytes, thereby limiting neointima formation.47 In addition, CRAMP-deficient mice exhibit reduced accumulation of classical monocytes into the lungs of mice treated with hypochloric acid or lipopolysaccharide (unpublished data) and into the peritoneum on stimulation with TNF, the latter being rescued by local application of CRAMP (unpublished data). Taken together, CRAMP may be an important facilitator of the accumulation of classical monocytes at sites of inflammation, independently of the underlying stimulus. Because the chemotactic activity of cathelicidins is independent of receptors that are typically used by classical chemokines (eg, CCR2 for CCL2/MCP-1), we suspect that cathelicidins exert a nonredundant role in the extravasation of monocytes. In line with this concept, intracellular Ca2+ mobilization in response to neutrophil secretory products is conserved in Cer2−/− classical monocytes.4 This notion, along with the fact that many chemokines require de novo synthesis whereas granule proteins are in general preformed, led us to propose a concept where the initial monocyte efflux is primarily mediated by alarmins (ie, preformed proteins with chemotactic activity), whereas later phases of monocyte emigration rely on chemokines.2

Because CRAMP was found abundantly in adherent neutrophils and to far lesser extent in emigrated neutrophils, we conclude that neutrophils are the primary source of cathelicidins in the inflammation model used in this study. However, tissue-resident cells, such as endothelial cells that carry lower amounts of CRAMP, may contribute to luminal CRAMP. The primary importance of neutrophil-derived CRAMP in leukocyte adhesion is further supported by a study showing that mice reconstituted with Cramp−/− bone marrow but not with WT bone marrow exhibit reduced adhesion of monocytic angiogenic cells.47

The unraveled process of cathelicidin transendothelial transportation and monocyte activation described here offers various possibilities for therapeutic interference. Caveolin 1 mediates transportation of chemokines, such as MCP1, across the endothelium, hence being an important facilitator of leukocyte adhesion.48 In atherosclerosis, mice lacking caveolin 1 exhibit strongly reduced atherosclerotic lesion sizes, along with lower amounts of MCP1 presented on the endothelium as well as fewer adherent and transmigrating monocytes,49 thus implying that caveolin 1–dependent shuttling of chemokines is relevant to inflammatory pathologies such as atherosclerosis. As similar observations regarding monocyte adhesion and recruitment are made in atherosclerotic CRAMP-deficient mice,43 caveolin 1–mediated transportation of cathelicidins may not just occur in the microcirculation as shown here but also in large arteries. Hence, tissue-specific targeting of such process may be an interesting approach to control the accumulation of classical monocytes.

Previous studies have shown that the antimicrobial activity of cathelicidin resides within specific domains that are different from the immune cell activating domains.50 Lack of cathelicidins in humans or mice favors the onset of infections and impairs monocyte recruitment.5,15 This study provides evidence for the importance of the central FKRIV motif in FPR2-dependent adhesion of classical monocytes. Several groups have studied the involvement of specific amino acid domains in the LL37 sequence responsible for its antimicrobial51 or other immunomodulatory effects52 usually resulting in an LL37-derived peptide sequence with the FKRIV motif present in it. Although the role of FPR2 was not investigated in these studies, our findings show that strategies specifically preventing the LL37–FPR2 interaction could be applicable in treatment of inflammatory processes where recruitment of classical monocytes plays a nonfavorable role.

Taken together, our data provide a novel mechanism of monocyte recruitment involving the interaction of neutrophil-derived cathelicidin and monocytic FPR2. Therapeutic interference with transendothelial cathelicidin transportation, its
endothelial presentation, or the cathelicidin–FPR2 interaction may be beneficial in circumstances where recruitment of classical monocytes plays an unfavorable role.

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Disclosures
None.

References
Neutrophils contribute to the adhesion of classical monocytes, an effect that is, in part, mediated by neutrophil-derived CRAMP. CRAMP is released from emigrated neutrophils and then transported across the endothelium to be presented to monocytes rolling along the endothelium. CRAMP induces adhesion of classical monocytes by ligating formyl-peptide receptor 2.

Novelty and Significance

What Is Known?

- An acute inflammatory response is characterized by neutrophil infiltration that precedes the recruitment of classical monocytes.
- Activated neutrophils secrete granule proteins that not only exert antimicrobial activities but also instruct immune cells.
- Cathelicidins (CRAMP in mouse, LL37 in humans) have potent effects on the recruitment and activation of immune cells, such as monocytes and dendritic cells.

What New Information Does This Article Contribute?

- Neutrophils contribute to the adhesion of classical monocytes, an effect that is, in part, mediated by neutrophil-derived CRAMP.
- CRAMP is released from emigrated neutrophils and then transported across the endothelium to be presented to monocytes rolling along the endothelium.
- CRAMP induces adhesion of classical monocytes by ligating formyl-peptide receptor 2.

- CRAMP-induced formyl-peptide receptor 2 activation induces conformation changes that lead to the activation of integrins.

During acute inflammation, emigrating neutrophils contribute to monocyte recruitment, but the underlying mechanisms remain unclear. Activated neutrophils secrete preformed granule proteins, some of which recruit and activate immune cells. This study shows that neutrophil-derived cathelicidin is secreted by emigrated neutrophils, transported across the endothelium in a caveolin 1–dependent manner, and presented on the luminal side of the endothelium toward monocytes rolling along the endothelium. Cathelicidin bound to the endothelium is recognized by formyl-peptide receptor 2, which is expressed to a higher degree on classical than nonclassical monocytes. This interaction leads to rapid integrin activation with subsequent adhesion of classical monocytes. Given the importance of classical monocytes in inflammation, the cathelicidin–formyl-peptide receptor 2 axis may be a novel target in the treatment of several inflammatory diseases.
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Supplemental Methods

Intravital microscopy of the cremaster muscle
An inflammatory response was induced by intrascrotal injection of TNF (50ng). The cremaster muscle was exposed and recordings were made using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera (Hamamatsu Photonics) and a 20x water dipping objective. For image acquisition and analysis Olympus cell software (Olympus) was used. An antibody to Ly6C (ebioscience) was injected i.v. to detect adhesion of Ly6C-positive CM. For luminal detection of CRAMP presented on the endothelium, 50µl of Protein G Fluoresbrite® YG Microspheres (Polysciences) were coupled to 50µg of polyclonal Ab to CRAMP (Innovagen). Beads and antibody were reacted for 30min at room temperature, washed twice and subsequently injected i.v.

Cell culture
Human umbilical vein endothelial cells (HUVEC) (PromoCell) were cultured and propagated on collagen (50 µg/ml, Biochrom) coated tissue culture flask in endothelial growth medium (PromoCell). Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation. Monocyte subsets were then isolated using the CD16 Monocyte Isolation Kit or the Monocyte Isolation Kit II (both Miltenyi Biotech Inc.) for non-classical and classical monocytes, respectively.

Flow adhesion assay
HUVEC cultured in petri dishes were activated with TNF (50ng/ml, 12 h, Peprotech). LL37 (1µg/ml, Anaspec) or mutated LL37 (1µg/ml) were added 15min prior to performance of flow chamber assays. Isolated monocyte subsets were labeled with Calcein AM (Invitrogen) and perfused at 2.5dyne/cm² over HUVEC monolayers. In separate experiments, monocytes were pretreated for 30min with inhibitors detailed in the result section, washed twice and then used for flow chamber assays. Adherent cells were quantified in six randomly chosen fields.

Transcytosis assay
HUVECs were cultured on 24-well polycarbonate transwell filter inserts (5µm, Corning Costar) coated with collagen. HUVECs were activated with human TNF (50ng/ml, 12h, Peprotech). FITC-conjugated LL37 (10µg/ml) was added to the bottom chamber. Alternatively, native (unconjugated) LL37 (10µg/ml) was added to the lower chamber of transwells. In order to detect the transcytosed LL37 on the HUVEC cell surface, a FITC conjugated antibody against human LL37 (Innovagen) was used and the fluorescence intensity assessed by a plate reader. For visual analysis, HUVEC were then counter-stained with DAPI (nuclear dye) and CellMask (plasma membrane stain, Invitrogen) and fixed with 4% PFA at 4°C for 30min. After washing, the samples were analyzed using a Leica SP5 MP system with a water dipping 20x; NA 1.00 objective and a Ti:Sa MaiTai DeepSee laser (Spectra Physics) tuned to 790nm. Three Hybrid detectors (HyD) were spectrally tuned for optimal detection efficiency and low bleed through of signal of the used markers (HyD1: 435-485nm, HyD2: 515-550nm, HyD3: 565-630nm). Furthermore, a dye separation protocol (Leica LAS AF software) was performed for removal of any leftover bleed through of signal. Additional image preparation was performed using ImagePro 3D analyzer 7.0 (Media Cybernetics).

Surface plasmon resonance
Interaction between LL37 and chemotactic receptors were studied by Surface Plasmon Resonance on a Biacore X100 system (GE Healthcare). LL37 or mutated LL37 were immobilized on a CM4 sensor chip at a level of 400 response unit (RU) by amine coupling. Receptors FPR1, FPR2, CXCR2 and P2X7 (Abnova) were used as analyte and diluted in a HBS-N running buffer (0.1M HEPES, 1.5M NaCl pH 7.4). Each experiment was performed with a flow of 10µl/min with running buffer. Sensor chip surfaces were regenerated with 50mM NaOH and equilibrated with running buffer prior to the next injection. Results were analyzed with Bioevaluation software.

Integrin activation assay
Human PBMCs were incubated 15min with PBS, PMA (50ng/ml, Sigma-Aldrich), MCP1 (50ng/ml, PeproTech), LL37 (1µg/ml, AnaSpec), or mutated LL37 (1µg/ml) and then stained with antibody against CD45, CD14, and CD16 for 30min at 4°C (BD Pharmingen). Antibodies to CD29 (HUTS-21,
BD Pharmingen) and CD11b (CBRM1/5, ebioscience) detecting epitopes of activated integrins only were used to assess integrin activation. Integrin activation was measured by flow using a FACSCanto II (BD Biosciences). The results are analyzed with FlowJo Software (Treestar).

Murine peripheral blood cells were isolated from C57Bl6 mice and suspended in Hanks Balanced Salt Solution containing 1mM CaCl₂ and MgCl₂ (Invitrogen). Cells were exposed to PMA (50ng/ml, Sigma-Aldrich), MCP1 (50ng/ml, PeproTech), CRAMP (1µg/ml, Innovagen) or an equal volume of PBS, in the presence of ICAM-1/Fc (10µg/ml, R&D Systems) or of VCAM-1/Fc (10µg/ml, R&D Systems) and PE-conjugated anti–human IgG1 (Fc-specific, Southern Biotechnology) for 5 minutes at 37°C. After washing, cells were labeled with antibodies to CD45, CD115, and Gr1 to identify monocyte subsets. Binding of ICAM-1 or VCAM-1 was measured by flow cytometry.

**Intracellular Cramp detection**

For FACS analysis of cremaster muscle single-cell suspensions, mice were injected intrascrotally with TNF (50ng, 4h). An antibody to Ly6G (1A8, 1µg) was injected 5 minutes prior to sacrifice to label adherent neutrophils. The circulation of mice was flushed with cold PBS and the muscle tissue was excised and digested with 0.25 mg/ml Liberase (Roche) in RPMI1640 + 10% FCS medium at 37 °C for 1h. Surface staining for flow cytometric analysis was conducted using combinations of antibodies against CD11b, Gr1, CD31, and CD45 (all ebioscience). Intracellular labeling of CRAMP (rabbit anti-mouse CRAMP) and an appropriate isotype control (rabbit IgG isotype control, GeneTex) was performed with IC fixation buffer and permeabilization buffer (both from ebioscience) according to the manufacturer’s protocol for staining of cytosolic proteins. Anti-rabbit IgG (ebioscience) served as fluorescently labeled secondary antibody.

**Statistics**

All data are expressed as mean±SD. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software Inc.). Mann-Whitney test, one way ANOVA with Dunnett post-hoc test, or Kruskal-Wallis test with posthoc Dunn tests were used as appropriate. * indicates a *p*-value < 0.05.
**Online Figure 1: In vivo labeling of classical monocytes.** To discriminate between monocyte subsets an antibody to Ly6C (1 µg) was introduced i.v. into Cx3cr1^egfp/wt mice. 

**A:** Example of intravital microscopy of the cremaster muscle using a beam splitter to allow recording of two emission wavelengths. CM, classical monocyte; NCM, non-classical monocyte. 

**B:** Blood was drawn from Cx3cr1^egfp/wt mice injected with an antibody to Ly6C and stained with antibodies to CD45 and CD11b. Classical monocytes (Ly6C^gfp^+, orange) and non-classical monocytes (Ly6C^gfp^−, green) also appear CD11b^SSC^hi. In contrast, gfp^− leukocytes (grey) are either CD11b^SSC^lo (neutrophils) or CD11b^− SSC^lo (lymphocytes). 

**C:** Cx3cr1^egfp/wt mice were injected i.v. with a PE-conjugated antibody to NK1.1. Labeling of NK cells was confirmed by counterstaining with an antibody to CD122 ex vivo (left). In *vivo*, the antibody to NK1.1 did not label adherent gfp^+ cells (right). 

**D:** Intravenous injection of an antibody to Ly6C efficiently labels classical monocytes. Anti-Ly6C-PE (1 µg) was introduced i.v. into Cx3cr1^egfp/wt mice. To assess *in vivo* labeling efficiency, white blood cells were counterstained ex vivo with antibodies to CD45, CD11b, and CD62L to allow for identification of monocyte subsets. Anti-Ly6C binding was then quantified on non-classical (I) and classical (II) monocytes.
Online Figure II: LL37 does not induce endothelial cell adhesion molecule expression. Human umbilical vein endothelial cells were treated with buffer (ctrl), LL37 (1 µg, 15 min), TNF (20 ng, 12 h), or a combination of both. Expression of E-selectin, ICAM-1, or VCAM-1 was measured by use of a fluorescence plate reader and directly conjugated antibodies towards the respective adhesion molecule. n= 3 for each bar.
Online Figure III: Neutrophils release CRAMP during extravasation. Mice were injected intrascrotally with TNF (50ng, 4h) and an antibody to Ly6G (1A8, 1µg) was injected 5 minutes prior to sacrifice to label adherent neutrophils. Cremaster muscles were excised and enzymatically digested. Intracellular CRAMP was measured in adherent and emigrated neutrophils as well as in endothelial cells. Representative histograms of three independent experiments display specific CRAMP staining as well as the respective isotype control fluorescence.
Online Figure IV: Cathelicidin does not interact with duffy antigen/receptor for chemokines (DARC). 

**A:** TNF does not induce expression of DARC on human umbilical vein endothelial cells (HUVEC). HUVEC were treated with TNF (20 ng, 12 h) and the expression of DARC was analyzed by fluorescence plate reader. n = 3. **B:** LL37 does not bind to DARC. DARC-expressing Madin-Darby canine kidney (DARC-MDCK) or control MDCK cells were incubated with fluorescent LL37 at various concentrations and binding was assessed by flow cytometry. Displayed is one representative experiment.
Online Figure V: LL37 activates integrins on human intermediate but not on non-classical monocytes. Based on the CD16 and CD14 staining properties, CD14^{++}CD16^{+} intermediate monocytes (A) and CD14^{+}CD16^{++} non-classical monocytes (B) were identified within peripheral blood mononuclear cells. Moreover, antibodies to activation epitopes of VLA4 (HUTS-21) and CD11b (CBRM1/5) were added. Cells were treated with PMA (50 ng/ml), MCP-1 (50 ng/ml), or LL37 (1 µg/ml) for 15 minutes and the expression of activated CD11b (left) or VLA4 (middle) was assessed on intermediate and non-classical monocytes. Representative histograms are displayed to the right. * indicates significant difference compared to ctrl.
Online Figure VI: CRAMP does not activate integrins on murine non-classical monocytes. Mouse peripheral leukocytes were treated with PMA (50 ng/ml), MCP-1 (50 ng/ml), or CRAMP (1 µg/ml) in the presence of ICAM-1-Fc (left) or VCAM-1-Fc (right) and an anti-Fc antibody. Monocyte subsets were identified by additional antibody staining (CD45, CD11b, CD115, Gr1). n = 3. * indicates significant difference compared to ctrl.
Online Table I: Differential leukocyte counts in mice with intact WBC and in neutropenic mice. All values are given in count/ml venous blood.

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<th>neutrophils</th>
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<th>resident monocytes</th>
<th>T-lymphocytes</th>
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<tr>
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<td>0.6x10⁵ +/- 0.2x10⁵</td>
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