Lack of Neutrophil-Derived CRAMP Reduces Atherosclerosis in Mice

Yvonne Döring,* Maik Drechsler,* Sarawuth Wantha, Klaus Kemmerich, Dirk Lievens, Santosh Vijayan, Richard L. Gallo, Christian Weber, Oliver Soehnlein

Rationale: Neutrophils have been reported to contribute to early atherosclerotic lesion formation. Mechanisms of neutrophil-driven atherosclerosis remain unclear so far.

Objective: Investigation of the role of the neutrophil granule protein cathelicidin (CRAMP in mouse, LL37 in human) in atherosclerosis.

Methods and Results: Compared to Apoe−/− mice, Cramp−/− Apoe−/− mice exhibit reduced lesion sizes with lower macrophage numbers. In atherosclerotic aortas, we could detect CRAMP specifically in neutrophils, but not in monocytes or macrophages. By use of intravital microscopy, CRAMP was found to be deposited by activated neutrophils on inflamed endothelium of large arteries. In this location cathelicidins promote adhesion of classical monocytes and neutrophils, but not nonclassical monocytes in a formyl-peptide receptor-dependent manner.

Conclusions: Cathelicidins promote atherosclerosis by enhancement of the recruitment of inflammatory monocytes. (Circ Res. 2012;110:1052-1056.)

Key Words: atherosclerosis • monocyte recruitment • neutrophil

Atherosclerosis is a chronic inflammation of the arterial vessel wall with relatively well-defined roles for leukocytes such as macrophages and lymphocytes.1,2 Recent studies, however, have revealed that neutrophils infiltrate atherosclerotic lesions at various time points,3–5 and depletion studies provide evidence for a proatherogenic role of neutrophils.5,6 Nevertheless, mechanistic insights into how neutrophils promote early atherosclerotic lesion formation remain elusive. Neutrophils contain granules with more than 300 different proteins that undergo limited exocytosis on neutrophil extravasation.7 Some of these proteins are able to activate and recruit immune cells and thus have been coined alarmins.8 Cathelicidins (CRAMP in mouse, LL37 in humans) residing in neutrophil secondary granules were shown to potentiate activate and recruit monocytes and macrophages,9,10 thus fulfilling alarmin criteria. Because cathelicidins were identified in atherosclerotic lesions,11 we investigated their role in a mouse model of atherosclerosis.
mice a high-fat diet for 4 weeks and assessed atherosclerotic lesion sizes in aortic root sections. Lack of CRAMP led to significantly decreased lesion sizes (Figure 1A) characterized by reduced cellularity and a matching decrease in macrophage content (Figure 1B, C). In addition, no difference was found in the number of TUNEL-positive cells in the aortic root (Figure 1D). Cholesterol and triglyceride levels did not differ between ApoC−/− and Cramp−/−ApoC−/− mice (Online Table I). Moreover, counts of circulating leukocytes subsets were not different between the strains either before or after 4 weeks of high-fat diet (Online Table II).

Although cathelicidins are largely restricted to neutrophils, previous studies have reported the presence of cathelicidins in macrophages and endothelial cells in human plaque specimens.11 Hence, we aimed at investigating the cellular origin of CRAMP in atherosclerotic aortas. Intracellular staining of CRAMP in single-cell suspensions of aortas of ApoC−/− and Cramp−/−ApoC−/− mice revealed that cellular CRAMP expression there is specifically confined to neutrophils (Figure 2A and Online Figure IA). In addition, immunohistochemistry showed that CRAMP also is located in the interstitium of aortic root sections of ApoC−/− mice receiving high-fat diet (Figure 2B), whereas feeding normal chow diet results in markedly reduced detection of CRAMP (not shown). The interstitial location of CRAMP is in line with previous reports showing that cathelicidins interact with extracellular matrix proteins.13 To evaluate the possibility of endothelial CRAMP expression, we injected ApoC−/− mice with microbeads conjugated to an antibody against CRAMP. The immobilization of these beads along the carotid artery was evident in ApoC−/− mice after 4 weeks of high-fat diet (Figure 2C). In addition, neutrophil depletion clearly reduced the number of immobilized beads to a level close to what was found in Cramp−/−ApoC−/− mice (Figure 2C), suggesting that CRAMP is released by activated neutrophils and immobilized on the endothelium, a circumstance likely favored by its cationicity.13 Finally, no CRAMP was detected in B and T lymphocytes of spleens and inguinal lymph nodes (Online Figure IB, IC).

With no differences in white blood cell counts in ApoC−/− and Cramp−/−ApoC−/− mice, as well as equal numbers in TUNEL-positive cells in the aortic root, reduced accumulation of macrophages is likely attributable to impaired recruitment rather than a shift in monocyte/macrophage homeostasis. Hence, we investigated the in vivo adhesion of leukocytes by use of intravital fluorescence microscopy of the carotid artery. In Cramp−/−ApoC−/− mice, we found reduced adhesion of rhodamine 6G-labeled leukocytes (Figure 3A). Injection of antibodies to Gr1 and CD11b revealed that most of these adherent cells were inflammatory myeloid cells. Their adhesion was found to be significantly diminished in Cramp−/−ApoC−/− mice compared to ApoC−/− mice (Figure 3A). To further investigate whether cathelicidins induce adhesion of a specific myeloid cell subset, we perfused classical monocytes, nonclassical monocytes, and neutrophils over tumor necrosis factor-activated monolayers of human aortic endothelial cells. Whereas LL37 induced adhesion of classical monocytes and neutrophils, nonclassical monocytes

Non-standard Abbreviations and Acronyms

| FPR | formyl-peptide receptors |
| HFD | high-fat diet |

Figure 1. Lack of CRAMP reduces atherosclerotic lesion size. The ApoC−/− or Cramp−/−ApoC−/− mice were fed a high-fat diet for 4 weeks. A. Quantification of Oil-Red O-stained sections. Representative images are shown. B. Enumeration of DAPI-positive cells. C. Quantification of Mac2-positive cells indicating macrophage accumulation. Representative images are displayed underneath. D. Quantification of TUNEL staining indicating apoptotic cells. **P<0.01 between the groups and ***P<0.001 between the groups as assessed by unpaired t test.
were not affected (Figure 3B). The LL37-mediated adhesion was abrogated by an antagonist to formyl-peptide receptors (Figure 3C), a class of chemotactic receptors that previously was found to be involved in LL37-mediated leukocyte recruitment.9,14

### Discussion

Because recent studies have provided evidence for the activation and accumulation of neutrophils in atherosclerosis as well as the importance of neutrophils during early stages of atherosclerotic lesion formation in mice, mechanisms underlying neutrophil-driven atherogenesis need to be investigated. Neutrophil activation results in rapid release of preformed granule proteins, some of which are known to shape vascular inflammatory responses.14 Here, we identified neutrophil-derived cathelicidins as important mediator of neutrophil-dependent monocyte recruitment and macrophage accumulation in early atherosclerotic lesions. The activity of these effectors is likely based on the interaction of cathelicidins with monocyte formyl-peptide receptors, thus facilitating arterial monocyte influx (Online Figure II).

Besides its antimicrobial activity, neutrophil-derived cathelicidin previously has been appreciated for its immune cell-activating properties. In this context, cathelicidins also have been shown to be chemotactic. The direct chemotactic activity of human and mouse cathelicidins was reported to be mediated through formyl-peptide receptors.9,14 These receptors are primarily found on phagocyte subsets, and it was evidenced that LL37 attracts neutrophils and monocytes.9,14 In addition, to direct chemotactic activity, cathelicidins can exert indirect chemotactic activity by stimulating chemokine and cytokine secretion from a variety of cell types through receptor-dependent mechanisms.15 LL37 synergistically enhances the IL-1β-induced production of cytokines (IL-6, IL-10) and chemokines such as MCP1, MCP3, and IL-8 in monocytes.15 In line with the recently demonstrated decrease in aortic macrophage content in enzymatically digested aortas of neutropenic mice compared to respective controls,5 neutrophil-derived cathelicidins may enhance both monocyte adhesion and monocyte extravasation into atherosclerotic arteries by direct or indirect chemotactic activities and, thus, stand out as important facilitators of monocyte/macrophage accumulation during early atherosclerosis.

LL37 is a molecule that belongs to the alarmin family, a group of structurally heterogeneous endogenous molecules that are rapidly released on damage and exert immune cell

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**Figure 2.** CRAMP associated with atherosclerotic lesions is neutrophil-derived. A, Intracellular CRAMP staining in monocytes (SSC<sup>+</sup>CD4<sup>+</sup>CD11<sup>b</sup>Ly6G<sup>+</sup>), macrophages (SSC<sup>+</sup>CD4<sup>+</sup>CD11<sup>b</sup>F4/80<sup>+</sup>), and neutrophils (CD4<sup>+</sup>CD11<sup>b</sup>Ly6G<sup>+</sup>) of enzymatically digested aortas from Apoe<sup>−/−</sup> or Cramp<sup>−/−</sup> Apoe<sup>−/−</sup> mice having received high-fat diet (HFD) for 4 weeks (n=5). B, Immunohistochemical detection of CRAMP in aortic root sections of Apoe<sup>−/−</sup> (top) or Cramp<sup>−/−</sup> Apoe<sup>−/−</sup> (bottom) mice after 4 weeks of HFD. Stainings also were made by use of an isotype control antibody (middle). Pictures were taken with a 25× objective, insert was taken with a 100× objective. C, Luminal arrest of G-protein-coupled fluorescent beads conjugated with antibodies to CRAMP in the carotid artery of Cramp<sup>−/−</sup> Apoe<sup>−/−</sup> mice or Apoe<sup>−/−</sup> mice with intact white blood cell count or neutropenia. All mice received HFD for 4 weeks (n=8). ***P<0.001 compared to mice with intact white blood cell count as calculated by Kruskal-Wallis test with post hoc Dunn test.
recruiting and activating properties. Other molecules that fulfill these criteria recently have received attention in atherosclerosis research. Antibody neutralization of HMGB1, for example, was shown to result in reduced atherosclerotic lesion sizes with lower macrophage numbers as well as the lesional inflammatory profile. Similarly, the alarmins S100A8 and S100A9 are expressed by phagocytes and secreted at sites of inflammation. Previous studies have shown that lack of S100A8 and S100A9 reduces atherosclerotic lesion sizes in response to vascular injury. Thus, alarmins may have an important contribution to early atherosclerotic lesion formation, and interference with these molecules themselves or their respective receptors may harbor valuable opportunities for prevention and treatment.

Sources of Funding
This study was supported by grants from the Deutsche Forschungsgemeinschaft (SO876/3-1, FOR809, SFB914 TP B08), Leducq Transatlantic Network of Excellence CVGeneF(x), and the German Heart Foundation.

Disclosures
None.

References


Novelty and Significance

What Is Known?

- Neutrophils infiltrate aortas rapidly after initiation of feeding a saturated fat-enriched diet in mouse models.
- Activated neutrophils secrete granule proteins that exert antimicrobial activities and stimulate recruitment and activation of immune cells.
- Cathelicidins (CRAMP in mouse, LL37 in humans) have potent effects on recruitment and activation of immune cells, such as monocytes and dendritic cells.

What New Information Does This Article Contribute?

- Endothelial-bound CRAMP is recognized by formyl-peptide receptors expressed on classical monocytes and neutrophils, hence mediating their firm adhesion.

Previous work has shown the importance of neutrophils in early stages of experimental atherosclerosis. Mechanisms underlying neutrophil promotion of atherosclerosis remain unclear. Activated neutrophils secrete preformed granule proteins, some of which induce recruitment and activation of immune cells. This study shows that neutrophil-derived cathelicidin is deposited within atherosclerotic lesions and on the arterial endothelium. Endothelial-bound cathelicidin is recognized by monocytes and neutrophils via involvement of formyl-peptide receptors, thus promoting their adhesion and transmigration. The cathelicidin–formyl-peptide receptor axis may be a novel target for treatment of early phases of atherosclerosis.
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*Circ Res.* 2012;110:1052-1056; originally published online March 6, 2012; doi: 10.1161/CIRCRESAHA.112.265868

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/110/8/1052

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Supplemental Material

Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice

Döring: CRAMP promotes atherosclerosis

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Supplementary methods

Mice
Cramp<sup>−/−</sup> mice (1) were crossed with Apoe<sup>−/−</sup> mice to generate Cramp<sup>−/−</sup>Apoe<sup>−/−</sup>. Apoe<sup>−/−</sup> and Cramp<sup>−/−</sup> Apoe<sup>−/−</sup> were fed a high-fat diet (HFD) containing 21% fat (ssniff) for 4 weeks. Neutrophils were depleted by i.p. injection of mAb 1A8 (100µg/mouse, BioXCell). Mouse strains were backcrossed in the C57Bl/6 background for ten generations. All animal experiments were approved by the local ethical committee.

Immunohistochemistry
The extent of atherosclerosis was assessed in aortic roots by staining for lipid depositions with oil-red-O, quantified by computerized image analysis (Diskus Software) and Leica Qwin Imaging software (Leica Ltd.). Aortic roots were stained with an antibody to Mac2 (BD Biosciences). After incubation with secondary FITC conjugated antibody (Jackson Immuno Research) for 30 min at room temperature, sections were analyzed using a Leica DMLB fluorescence microscope and charge couple device (CCD) camera. In addition, roots were immunelabeled with anti-CRAMP polyclonal antibody (1:100; Innovagen). A Leica DM6000 light microscope with a 25/×0.95 water emersion objective (Leica Microsystems) was used to detect the presence of CRAMP. Images were captured using a Leica DFC 365FX camera. Furthermore, TUNEL staining was performed using In Situ Cell Death Detection Kit, TMR red (Roche) to assess the number of apoptotic/necrotic cells within aortic root sections.

Intracellular Cramp
For FACS analysis of aortic single-cell suspensions whole aortas were 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, Ly6G, F4/80, CD19, and CD3 (all ebioscience). Intracellular labeling of CRAMP (rabbit anti-mouse CRAMP, provided by R. Gallo) 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. Samples were analyzed in a FACSCanto II and FlowJo software (Tree Star).

Lipid detection
Serum levels of cholesterol or triglycerides were assessed by EnzyChrom™ Assay Kits (BioAssay Systems).

Intravital microscopy
Leukocyte adhesion to the carotid artery was analyzed in Cramp<sup>−/−</sup>Apoe<sup>−/−</sup> or Apoe<sup>−/−</sup> with intact white blood cell count or with neutropenia. All mice received high fat diet for 4 weeks. The right jugular vein was canulated with a catheter for antibody and dye injection. The left carotid artery was exposed with intact white blood cell count or with neutropenia. Antibody/bead complexes were allowed to circulate for 5 min and immobilized complexes were detected by intravital microscopy. Intravital microscopy was performed using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera and a 10x saline-immersion objective. For image acquisition and analysis Olympus cell' software was used.

Cell culture and flow adhesion assay
Human aortic endothelial cells (HAoEC) were maintained as described (3). For isolation of monocytes, buffy coats were diluted at 1:1 with PBS. Mononuclear cells were isolated by density gradient isolation using Biocoll Separating Solution (Biochrom AG). 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. Neutrophils were isolated by one-step density gradient centrifugation using Polymorphprep. Myeloid cell subsets were then labeled with calcein, resuspended in medium and perfused over TNF-activated (20 ng/ml, 12 h) HAoEC at a shear rate of 1.5 dyne/cm². LL-37 was added to endothelial medium 15 minutes prior to perfusion. Adherent cells were quantified in six randomly chosen fields.

**Statistics**
All data are expressed as mean±SD. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software Inc.). Unpaired Student’s t-test, Mann-Whitney, one way ANOVA with Bonferroni’s Multiple Comparison Test or Kruskal-Wallis test with posthoc Dunn tests were used as appropriate. $p$-values < 0.05 were considered significant.
Supplementary references


Supplementary data

Online Table I: Serum lipid levels are not different in Apoe<sup>−/−</sup> and Cramp<sup>−/−</sup> Apoe<sup>−/−</sup> mice. Serum cholesterol and triglyceride levels were measured in Apoe<sup>−/−</sup> and Cramp<sup>−/−</sup> Apoe<sup>−/−</sup> mice after four weeks of high fat diet. All values are displayed as mean ± SD.

<table>
<thead>
<tr>
<th>Serum lipids</th>
<th>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Cramp&lt;sup&gt;−/−&lt;/sup&gt; Apoe&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholesterol</td>
<td>596.9 ± 292.0</td>
<td>626.8 ± 98.02</td>
</tr>
<tr>
<td>triglycerides</td>
<td>173.8 ± 79.21</td>
<td>162.3 ± 59.40</td>
</tr>
</tbody>
</table>
Online Table II: White blood cell counts are not different in Apoe<sup>−/−</sup> and Cramp<sup>−/−</sup> Apoe<sup>−/−</sup> mice. Absolute counts of indicated circulating leukocyte subsets were measured before and four weeks after high fat diet (HFD). All values are displayed as mean ± SD.

<table>
<thead>
<tr>
<th>Blood cells</th>
<th>x10&lt;sup&gt;5&lt;/sup&gt; cells/ml</th>
<th>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Cramp&lt;sup&gt;−/−&lt;/sup&gt; Apoe&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>before HFD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>monocytes</td>
<td>2.57 ± 0.85</td>
<td>2.85 ± 0.78</td>
<td></td>
</tr>
<tr>
<td>- classical</td>
<td>1.25 ± 0.43</td>
<td>1.92 ± 0.57</td>
<td></td>
</tr>
<tr>
<td>- non-classical</td>
<td>1.17 ± 0.36</td>
<td>0.94 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>neutrophils</td>
<td>5.98 ± 2.60</td>
<td>4.15 ± 1.57</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>12.10 ± 2.60</td>
<td>11.01 ± 2.18</td>
<td></td>
</tr>
<tr>
<td><strong>4 weeks HFD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>monocytes</td>
<td>4.84 ± 0.82</td>
<td>4.36 ± 1.08</td>
<td></td>
</tr>
<tr>
<td>- classical</td>
<td>3.65 ± 0.62</td>
<td>2.89 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>- non-classical</td>
<td>1.30 ± 0.32</td>
<td>1.20 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>neutrophils</td>
<td>9.67 ± 4.76</td>
<td>7.48 ± 2.27</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>6.57 ± 1.56</td>
<td>7.67 ± 2.47</td>
<td></td>
</tr>
</tbody>
</table>
Online Figure I: CRAMP is not expressed in B and T lymphocytes in the aorta or secondary lymphoid organs. Intracellular CRAMP staining in B (CD45^+CD11b^-CD19^+CD3^-) and T lymphocytes (CD45^+CD11b^-CD19^-CD3^+) of enzymatically digested aortas (A), or single cell suspensions from spleen (B) or inguinal lymph nodes (C). Analyses were made in Apoe^-/- or Cramp^-/- Apoe^-/- mice having received HFD for 4 weeks. n=4. Red dashed line indicates CRAMP fluorescence generated by intracellular CRAMP staining in aortic neutrophils (see Figure 2B).
Online Figure II: Mechanisms of cathelicidin-mediated monocyte recruitment in large arteries. Activated neutrophils secrete cathelicidin which is immobilized on arterial endothelium due to its cationic nature. In this location cathelicidins interact with formyl-peptide receptors on classical monocytes thereby promoting firm adhesion. In addition, cathelicidins are secreted by emigrated neutrophils thus facilitating recruitment of inflammatory monocytes.