A Prolinflammatory Gut Microbiota Increases Systemic Inflammation and Accelerates Atherosclerosis

Eelke Brandsma, Niels J. Kloosterhuis, Mirjam Koster, Daphne C. Dekker, Marion J.J. Gijbels, Saskia van der Velden, Melany Ríos-Morales, Martijn J.R. van Faassen, Marco G. Loreti, Alain de Bruin, Jingyuan Fu, Folkert Kuipers, Barbara M. Bakker, Marit Westerterp, Menno P.J. de Winther, Marten H. Hofker, Bart van de Sluis,* Debby P.Y. Koonen*

Rationale: Several studies have suggested a role for the gut microbiota in inflammation and atherogenesis. A causal relation relationship between gut microbiota, inflammation, and atherosclerosis has not been explored previously.

Objective: Here, we investigated whether a proinflammatory microbiota from Caspase1−/− (Casp1−/−) mice accelerates atherogenesis in Ldlr−/− mice.

Method and Results: We treated female Ldlr−/− mice with antibiotics and subsequently transplanted them with fecal microbiota from Casp1−/− mice based on a cohousing approach. Autologous transplantation of fecal microbiota of Ldlr−/− mice served as control. Mice were cohoused for 8 or 13 weeks and fed chow or high-fat cholesterol–rich diet. Fecal samples were collected, and factors related to inflammation, metabolism, intestinal health, and atherosclerotic phenotypes were measured. Unweighted Unifrac distances of 16S rDNA (ribosomal DNA) sequences confirmed the introduction of the Casp1−/− and Ldlr−/− microbiota into Ldlr−/− mice (referred to as Ldlr−/−(Casp1−/−) or Ldlr−/−(Ldlr−/−) mice). Analysis of atherosclerotic lesion size in the aortic root demonstrated a significant 29% increase in plaque size in 13-week high-fat cholesterol–fed Ldlr−/−(Casp1−/−) mice compared with Ldlr−/−(Ldlr−/−) mice. We found increased numbers of circulating monocytes and neutrophils and elevated proinflammatory cytokine levels in plasma in high-fat cholesterol–fed Ldlr−/−(Casp1−/−) compared with Ldlr−/−(Ldlr−/−) mice. Neutrophil accumulation in the aortic root of Ldlr−/−(Casp1−/−) mice was enhanced compared with Ldlr−/−(Ldlr−/−) mice. 16S-rDNA-encoding sequence analysis in feces identified a significant reduction in the short-chain fatty acid–producing taxonomies Akkermansia, Christensenellaceae, Clostridium, and Odoribacter in Ldlr−/−(Casp1−/−) mice. Consistent with these findings, cumulative concentrations of the anti-inflammatory short-chain fatty acids propionate, acetate and butyrate in the cecum were significantly reduced in 13-week high-fat cholesterol–fed Ldlr−/−(Casp1−/−) compared with Ldlr−/−(Ldlr−/−) mice.

Conclusions: Introduction of the proinflammatory Casp1−/− microbiota into Ldlr−/− mice enhances systemic inflammation and accelerates atherogenesis. (Circ Res. 2019;124:94-100. DOI: 10.1161/CIRCRESAHA.118.313234.)

Key Words: atherosclerosis • cholesterol • diet • fatty acids, volatile • feces • inflammation

In September 2018, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.06 days. From the Department of Pediatrics (E.B., N.J.K., M.K., D.C.D., M.R-M., M.G.L., A.d.B., J.F., F.K., B.M.B., M.W., M.H.H., B.v.d.S., D.P.Y.K.), Department of Laboratory Medicine, University Medical Center Groningen (M.J.R.v.F., F.K.), and Department of Genetics (J.F.), University of Groningen, the Netherlands; Department of Pathobiology, Dutch Molecular Pathology Center, Utrecht University, the Netherlands (M.K., A.d.B.); Experimental Vascular Biology, Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, the Netherlands (M.J.J.G., S.v.d.V., M.P.J.d.W.); Department of Pathology and Department of Molecular Genetics, CARIM, Maastricht University, the Netherlands (M.J.J.G.); and Institute for Cardiovascular Prevention (IPEK), Ludwig Maximilian’s University, Munich, Germany (M.P.J.d.W.).

*These authors contributed equally to this article.

The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.118.313234.

Correspondence to Debby P.Y. Koonen, PhD, Department of Pediatrics, University Medical Center Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. Email d.p.y.koonen@umcg.nl

© 2018 The Authors. Circulation Research is published on behalf of the American Heart Association, Inc., by Wolters Kluwer Health, Inc. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial-NoDerivs License, which permits use, distribution, and reproduction in any medium, provided that the original work is properly cited, the use is noncommercial, and no modifications or adaptations are made.

Circulation Research is available at https://www.ahajournals.org/journal/res DOI: 10.1161/CIRCRESAHA.118.313234

Editorial, see p 12
In This Issue, see p 2
Novelty and Significance

What Is Known?

- Atherosclerosis, the main underlying cause of cardiovascular disease, is influenced by both—the innate and adaptive immune systems.
- Gut microbiota shape the immune system during early life and play a role in regulating inflammation by influencing the differentiation of inflammatory cell types, the production of cytokines, and hematopoiesis.
- Inflammation and atherosclerosis are linked to changes in gut microbiota composition; however, there is little evidence to support a proinflammatory role of gut microbiota in atherosclerosis.

What New Information Does This Article Contribute?

- The presence of a proinflammatory microbiota derived from Caspase1−/− (Casp1−/−) mice is sufficient to promote inflammation and atherosclerosis in antibiotic-treated Ldlr−/− mice, a mouse model with a human-like lipoprotein profile.
- The gut microbiota of Casp1−/− mice increases inflammation in antibiotic-treated Ldlr−/− mice, reflected by increased blood leukocyte numbers, particularly monocytes and neutrophils, proinflammatory plasma cytokines, and neutrophil accumulation in atherosclerotic plaques.
- The gut microbiota of Casp1−/− mice reduces the microbiota-derived anti-inflammatory short-chain fatty acids in antibiotic-treated Ldlr−/− mice, whereas plasma lipid, trimethylamine-N-oxide levels, and gut integrity are unaffected.

Several human studies have provided evidence that links the gut microbiota to cardiovascular disease. Nevertheless, the evidence supporting a causal role of the gut microbiota in cardiovascular disease is limited to the understanding of the importance of trimethylamine-N-oxide in atherosclerosis. Recent findings suggest a pivotal role of the gut microbiota in regulating inflammation. Here, we provide a novel, alternative mechanism by which the gut microbiota may contribute to atherosclerosis, independent of plasma lipids and trimethylamine-N-oxide levels. We show that introduction of a proinflammatory gut microbiota into a mouse model with a human-like lipoprotein profile increases systemic inflammation and accelerates atherosclerosis. This was associated with a reduction in microbiota-derived anti-inflammatory short-chain fatty acids, implying a causal relationship between microbiota composition, inflammation, and atherosclerosis. Collectively, these findings indicate that manipulation of the gut microbiota composition may be potentially effective treatment strategy to protect against inflammation and atherosclerosis and thereby reduce the risk of cardiovascular disease.

Gut microbiota is known to be involved in the shaping of the immune system during early life. Recent studies have suggested a role for the gut microbiota in the regulation of inflammation by influencing differentiation of inflammatory cell types, cytokine production and hematopoiesis. A leaky gut and alterations in gut microbiota composition can both lead to leakage of endotoxins into the circulation that promotes systemic inflammation and to the development of obesity and related metabolic diseases. Symptomatic atherosclerosis is associated with an altered gut metagenome in the human population, and bacterial DNA has been detected in atherosclerotic plaques. Furthermore, a high blood concentration of the microbiota-dependent metabolite trimethyl-amine-N-oxide (TMAO) has been linked to an increased risk of atherosclerosis, indicating a pivotal role for the gut microbiota in atherogenesis. In addition, germ-free ApoE-deficient (ApoE−/−) mice showed lower circulating lipopolysaccharide levels, reduced systemic inflammation, and decreased atherogenesis compared with conventionally raised ApoE−/− mice. Taken together, these findings suggest a triangular relationship between the gut microbiota, host immunity, and atherogenesis; however, proof to support a proinflammatory role for the gut microbiota in atherogenesis is lacking.

To examine whether introduction of a proinflammatory gut microbiota accelerates atherogenesis, we exposed female Ldlr−/− mice to the proinflammatory gut microbiota of Casp1−/− mice, as previous reports have demonstrated that alterations in their microbiota sensitize mice to the development of several inflammatory diseases. The gut microbiota of Casp1−/− mice promoted atherosclerosis and increased blood leukocyte numbers, proinflammatory plasma cytokines, and neutrophil accumulation in atherosclerotic plaques, whereas plasma lipid and TMAO levels, and gut integrity were unaffected. The Casp1−/− microbiota reduced microbiota-derived anti-inflammatory short-chain fatty acids (SCFAs).

Methods

The authors declare that all data supporting the findings of this study are available in its Online Data Supplement.

Results

Casp1−/− Microbiota Successfully Introduced into Ldlr−/− Mice

To study whether a proinflammatory microbiota accelerates atherogenesis, we exposed antibiotic-treated Ldlr−/− mice to the gut microbiota of Casp1−/− mice through fecal microbiota transplantation via a cohousing approach (Figure 1A). Autologous transplantation of fecal microbiota from Ldlr−/− mice into antibiotic-treated Ldlr−/− mice via a cohousing approach served as control. Analysis of fecal microbiota composition at time of sacrifice revealed both cohousing and diet-associated changes in gut microbial ecology (Figure 1B). Unweighted UniFrac distances of 16S-rDNA (ribosomal DNA) sequences, a measure for β-diversity, demonstrated clustering between the Ldlr−/− mice receiving Casp1−/− microbiota.
Circulation Research
January 4, 2019

Ldlr−/− [Casp1−/−] mice) and the Casp1−/− donor mice (Figure 1B; Online Table II). Analogously, we observed clustering between Ldlr−/− mice receiving the autologous microbiota transplantation (referred to as Ldlr−/−(Ldlr−/−) mice) and their respective donor mice. We also observed a clear separation between mice fed chow or high-fat cholesterol (HFC) diet (Figure 1B; Online Table II), and this was consistent for all donor and recipient mice. As expected, α-diversity was not different between Ldlr−/−(Ldlr−/−) and Ldlr−/−(Casp1−/−) mice (Online Figure IB). Altogether, these data demonstrate that Casp1−/− and Ldlr−/− microbiota were successfully transferred into Ldlr−/− mice.

**Figure 1.** Transplantation of Casp1−/− microbiota into Ldlr−/− mice via a cohousing approach. Female Ldlr−/− mice aged 12 wk were exposed to fecal microbiota derived from Casp1−/− or Ldlr−/− mice for 8 or 13 wk while fed a chow diet or high-fat cholesterol (HFC) diet. A, Experimental setup of the cohousing approach. Female Ldlr−/− mice were orally gavaged with a cocktail of broad-spectrum antibiotics for a period of 10 d to suppress intestinal microbes. This was followed by daily transfer of used bedding material from cages housing nonantibiotic-treated Ldlr−/− (donor) or Casp1−/− (donor) mice to cages housing the antibiotic-treated Ldlr−/− mice for 1 wk. During this period the mice were kept on chow diet or switched to an HFC diet for the remainder of the study. The antibiotic-treated Ldlr−/− mice were then cohoused with nonantibiotic-treated Casp1−/− mice (referred to as Ldlr−/−(Casp1−/−) mice) or Ldlr−/− mice (autologous transplantation, referred to as Ldlr−/−(Ldlr−/−) mice) in a 3:2 ratio for a period of 8 or 13 wk. B, Principal coordinate analysis plot of Unweighted UniFrac distance on the basis of 16S-rDNA (ribosomal DNA)-encoding sequences in feces collected from chow- and HFC-fed Ldlr−/− mice exposed to Casp1−/− or Ldlr−/− microbiota for 13 wk. Chow: Ldlr−/− mice (donor), n=8; Ldlr−/−(Ldlr−/−) mice, n=15; Casp1−/− mice (donor), n=9; Ldlr−/−(Casp1−/−) mice, n=14. HFC: Ldlr−/− mice (donor), n=7; Ldlr−/−(Ldlr−/−) mice, n=13; Casp1−/− mice (donor), n=8; Ldlr−/−(Casp1−/−) mice, n=14. PC indicates principal coordinate.

**Casp1−/− Dysbiosis Promotes Atherosclerosis in Ldlr−/− Mice Fed an HFC Diet**

We analyzed atherosclerotic lesion size in the aortic root, and we found that Casp1−/− microbiota did not affect atherosclerotic lesion size in Ldlr−/− mice fed chow or an HFC diet for 8 weeks (Figure 2A and 2B). However, atherosclerotic lesion size was increased by 29% in Ldlr−/−(Casp1−/−) mice compared with Ldlr−/−(Ldlr−/−) mice after 13 weeks of HFC feeding (Figure 2A and 2B; P<0.05). The collagen and macrophage content in aortic root sections was not different between the mice (Online Figure IIA), indicating that lesion size but not severity was increased. In the aortic arches, gene
expression of several macrophage-related and inflammatory markers was similar between HFC-fed mice with the exception of a significant increase in \( \text{IL-10} \) expression in \( \text{Ldlr}^{-/-}(\text{Casp1}^{-/-}) \) mice (Online Figure IIB). Body weight, plasma triglyceride, and cholesterol levels (Online Figure IIIA–IID) also did not differ, and no alteration was observed in plasma levels of TMAO, its TMA precursors (choline, \( \text{l-carnitine} \), betaine, and \( \gamma \)-butyrobetaine; Online Figure IIIE), and TMAO-producing taxonomies (Online Figure IIIF). Altogether, these results exclude plasma lipid levels and TMAO as factors that contribute to the increased atherosclerotic lesions in \( \text{Ldlr}^{-/-}(\text{Casp1}^{-/-}) \) mice.

Casp1\(^{-/-}\) Dysbiosis Promotes Inflammation

Next, we assessed whether \( \text{Casp1}^{-/-} \) dysbiosis accelerates atherosclerosis by increasing plasma inflammatory cytokines. We found a significant elevation in the plasma levels of IL-1\( \beta \), IL-2, IL-10, and IFN (interferon)-\( \gamma \) in \( \text{Ldlr}^{-/-}(\text{Casp1}^{-/-}) \) mice after 13 weeks of HFC diet (Figure 3A), whereas CXCL1 (chemokine [C-X-C motif] ligand 1), TNF (tumor necrosis factor)-\( \alpha \), IL-5 and IL-6 were not affected (Online Figure IVB). Flow cytometry analysis showed an increase in the number of blood Ly6C\(^{hi}\) and Ly6C\(^{lo}\) monocytes and neutrophils in \( \text{Ldlr}^{-/-}(\text{Casp1}^{-/-}) \) mice compared with \( \text{Ldlr}^{-/-}(\text{Ldlr}^{-/-}) \) mice (Online Figure IVA), with leukocyte percentages being unchanged (Online Figure IVC). We also observed increased neutrophil accumulation in atherosclerotic plaques of \( \text{Ldlr}^{-/-}(\text{Casp1}^{-/-}) \) mice compared with \( \text{Ldlr}^{-/-}(\text{Ldlr}^{-/-}) \) fed an HFC diet for 13 weeks (Figure 3C and 3D). These data suggest that \( \text{Casp1}^{-/-} \) dysbiosis enhances IL-1\( \beta \) plasma levels resulting in monocytosis and neutrophilia and increased neutrophil accumulation in atherosclerotic plaques.

Exposure to \( \text{Casp1}^{-/-} \) Microbiota Does Not Impair Intestinal Barrier Function in \( \text{Ldlr}^{-/-} \) Mice Fed an HFC Diet

A disturbance in microbiota composition may affect intestinal integrity and subsequently promote systemic inflammation. To investigate the effect of \( \text{Casp1}^{-/-} \) microbiota on intestinal barrier function, we analyzed the gut microbiota composition using the linear discriminant analysis (LDA) effect size (LEfSe) method. We identified 34 microbial taxonomies that differed in abundance between \( \text{Ldlr}^{-/-}(\text{Ldlr}^{-/-}) \) and \( \text{Ldlr}^{-/-}(\text{Casp1}^{-/-}) \) mice (Online Figure VA). \( \text{Casp1}^{-/-} \) dysbiosis resulted in a significant expansion of the genera \( \text{Bilophila} \), \( \text{Streptococcus} \), and \( \text{Mucispirillum} \) (Online Figure VB–VD) under both chow- and HFC-diet conditions. Although these genera are associated with intestinal inflammation, and are known to expand under inflammatory conditions, we did not observe any differences in intestinal barrier function, for example, inflammation and epithelial injury (Online Figure VIA–VIC). In addition, mucus
layer thickness of the colon (Online Figure VID and VIE) and Muc-2 expression (Online Figure VIF) in the colon were not altered between groups, suggesting that the integrity of the mucus layer of the colon was not different between \( \text{Ldlr}^{-/-} \) (\( \text{Ldlr}^{-/-} \)) and \( \text{Ldlr}^{-/-} \) (\( \text{Casp1}^{-/-} \)) mice. Although intestinal permeability was significantly impaired by HFC feeding, only \( \text{Ldlr}^{-/-} \) (\( \text{Casp1}^{-/-} \)) mice fed chow diet displayed increased permeability compared with \( \text{Ldlr}^{-/-} \) (\( \text{Ldlr}^{-/-} \)) mice (Online Figure VIG). These results indicate that \( \text{Casp1}^{-/-} \) microbiota does not change the intestinal barrier function under HFC-diet conditions and, therefore, cannot explain the increase in plasma inflammatory cytokines.

Exposure to \( \text{Casp1}^{-/-} \) Microbiota Lowers SCFA-Producing Microbial Taxonomies and Cecum Concentration of SCFAs

We observed a significant reduction in the abundance of the SCFA-producing taxonomies \( \text{Akkermansia} \) (Figure 4A), \( \text{Christensenellaceae} \) (Figure 4B), \( \text{Clostridium} \) (Figure 4C), and \( \text{Odoribacter} \) (Figure 4D) in \( \text{Ldlr}^{-/-} \) (\( \text{Casp1}^{-/-} \)) mice. As previous studies have shown that SCFAs reduce inflammation,18,19 we measured the concentrations of acetate, propionate, and butyrate in the cecum of the mice. Consistent with the lower abundance of SCFA-producing taxonomies, a significant reduction was observed in the cumulative levels of these SCFAs in \( \text{Ldlr}^{-/-} \) (\( \text{Casp1}^{-/-} \)) mice compared with \( \text{Ldlr}^{-/-} \) (\( \text{Ldlr}^{-/-} \)) mice (Figure 4E) and this was mainly because of lower acetate levels in the \( \text{Ldlr}^{-/-} \) (\( \text{Casp1}^{-/-} \)) mice. Thus, it is conceivable that a reduction in the anti-inflammatory SCFAs may have contributed to the increased levels of inflammatory plasma cytokines of mice exposed to \( \text{Casp1}^{-/-} \) microbiota.

Discussion

We examined whether a proinflammatory microbiota accelerates atherogenesis in female \( \text{Ldlr}^{-/-} \) mice, a mouse model exhibiting dyslipidemia, inflammation, and atherosclerosis, when fed a western style diet.20 We found that \( \text{Casp1}^{-/-} \) microbiota increased atherosclerosis in the aortic root in HFC-fed \( \text{Ldlr}^{-/-} \) mice (Figure 1A and 1B). This was accompanied by increased proinflammatory plasma cytokines (Figure 3A), increased blood leukocyte numbers, particularly monocytes and neutrophils (Figure 3B), increased neutrophil accumulation in atherosclerotic plaques (Figure 3C and 3D), and reduced levels of SCFAs in the cecum (Figure 4E). These results imply a causal relationship between microbiota composition, inflammation, and atherosclerosis.

We found that in particular the plasma levels of IFN-\( \gamma \), IL-2, and IL-1\( \beta \) were increased in \( \text{Ldlr}^{-/-} \) mice with \( \text{Casp1}^{-/-} \) dysbiosis, suggesting that accelerated atherosclerosis in these mice is partially driven by these cytokines, which is supported by previous studies.21–23 Furthermore, we showed an increase in peripheral blood leukocytes, which have previously been linked to cardiovascular disease.24 Within the leukocyte population, neutrophils and monocytes are important contributors to atherogenesis.25 Increased monocytes and neutrophils in the circulation may lead to infiltration of monocytes and neutrophils into atherosclerotic plaques and further promoting plaque growth.25

We observed that exposure to \( \text{Casp1}^{-/-} \) microbiota lowers SCFA-producing taxonomies and cumulative cecum concentrations of SCFAs. SCFAs have anti-inflammatory properties and
can suppress NF-κB (nuclear factor κB) activity in immune cells,18 resulting in reduced production of proinflammatory cytokines including IFN-γ, IL-1β, and IL-2.26 Furthermore, SCFAs may act as modulators of immune homeostasis by acting as HDAC (histone deacetylase) inhibitors.19 Oral butyrate supplementation has recently been shown to attenuate the adhesion and migration of macrophages and to decrease proinflammatory cytokines in atherosclerotic plaques.27 Thus, it is tempting to speculate that the reduction in SCFAs in Ldlr−/− mice after exposure to Casp1−/− microbiota may have contributed to increased levels of proinflammatory cytokines and leukocytes in the circulation and neutrophil accumulation in the atherosclerotic plaque.

It is well recognized that microbial transplantation can be transient.11 Thus, we cannot exclude that certain effects on TMAO, although not present at time of sacrifice, may have been lost throughout the length of the study. Future studies, therefore, should include more frequent and earlier time points to rule out these possibilities.

Whereas previous studies have shown a decreased abundance of Akkermansia muciniphila on high-fat diet feeding, our data show on opposing effect on A. muciniphila after HFC feeding. A similar effect on high-fat and high-carbohydrate diet feeding in mice was recently shown28 and warrants further investigation. Nevertheless, promising results have been obtained with the administration of A. muciniphila resulting in protection against atherogenesis in ApoE−/− mice by strengthening the gut barrier and preventing metabolic endotoxemia-induced inflammation.29 Likewise, metformin’s reported beneficial effects on atherosclerosis in humans with type I and II diabetes mellitus and nondiabetic dysglycaemia may be related to its ability to enhance the growth of A. muciniphila and promote SCFA production.30 Together with our findings, this indicates that manipulation of the gut microbiota composition is an interesting treatment strategy to protect against inflammation and atherosclerosis and reduce cardiovascular disease risk.

Acknowledgments
We dedicate this article to M.H. Hofker. His ideas laid the groundwork for this article. We thank Mihai Netea (Radboud University Nijmegen Medical Center, Department of General Internal Medicine, Nijmegen, the Netherlands) for the generous gift of the Casp1−/− mice and Theo Boer (University of Groningen, University Medical Center Groningen, Department of Laboratory Medicine, Groningen, the Netherlands) for technical assistance. Editorial services were provided by Kate McIntyre (University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, the Netherlands). Additional information: Coauthor M.H. Hofker died September 12, 2016.

Sources of Funding
E. Brandsma, J. Fu, F. Kuipers, M. Hofker, and D.P.Y. Koonen are supported by a grant from CardioVascular Onderzoek Nederland.
Disclosures

None.

References


