ORIGINAL ARTICLE

Gut Microbiota Influence the Development of Abdominal Aortic Aneurysm by Suppressing Macrophage Accumulation in Mice

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BACKGROUND: Abdominal aortic aneurysm (AAA) is a life-threatening cardiovascular disease characterized by dilated abdominal aorta. Immune cells have been shown to contribute to the development of AAA, and that the gut microbiota is associated with numerous diseases, including cardiovascular diseases, by regulating immune systems or metabolic pathways of the host. However, the interaction between the gut microbiota and AAA remains unknown.

METHODS: Apolipoprotein E-deficient male mice were fed a high-cholesterol diet and divided into three groups: the control group was maintained under normal water (control group), the oral AVNM group was maintained under drinking water supplemented with ampicillin, vancomycin, neomycin, and metronidazole, and the i.p. AVNM group was injected AVNM intraperitoneally. After 1 week of pretreatment with antibiotics, these mice were administrated Ang II via subcutaneous osmotic pumps for 4 weeks and euthanized to evaluate AAA formation.

RESULTS: Depletion of gut microbiota by oral AVNM ameliorated the incidence of AAAs (control group: 58.9% versus oral AVNM group: 28.6% versus i.p. AVNM group: 75.0%, \( P = 0.0005 \)) and prevented death due to ruptured aneurysms (control group: 11% versus oral AVNM group: 0% versus i.p. AVNM group: 15%). Oral AVNM suppressed monocyte storage in the spleen, but not in other organs. Despite possessing a higher level of cholesterol, recruitment of monocytes into the suprarenal aorta was suppressed in the oral AVNM group. In AVNM drinking mice, NOD1 ligand, a kind of PRR ligands, increased the development of AAAs and accumulation of macrophages in the aortae.

CONCLUSIONS: The gut microbiota plays a critical role in AAA formation. Therefore, regulation of the microbiota or the immune system can be a therapeutic approach for AAA. (Hypertension. 2022;79:2821–2829. DOI: 10.1161/HYPERTENSIONAHA.122.19422.) • Supplemental Material

Key Words: gut microbiota ◼ abdominal aortic aneurysm

Abdominal aortic aneurysm (AAA) is a life-threatening cardiovascular disease among elderly people and is characterized by a weakened and dilated abdominal aorta. AAA is usually asymptomatic until the time of rupture and is, therefore, frequently diagnosed during imaging performed to investigate unrelated symptoms or by ultrasonography screening in developed countries. More than 50% of patients with ruptured AAAs die of sudden cardiovascular collapse before arriving at a hospital. Although technical improvements in surgical or endovascular repair have been achieved, medical preventative therapies to stop the development of AAAs are still lacking. A large cohort study showed that 28% of men with a sub-aneurysmal infrarenal diameter of 25 to...
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29 mm at the age of 65 developed AAAs of ≥55 mm within 15 years.5 However, randomized clinical studies have failed to demonstrate the advantages of early elective surgical or endovascular repair for small AAAs.6–9 Drug therapy or lifestyle interventions should be commenced at an early stage in the development of small AAA. Patients with small AAAs or those deemed unfit for AAA repair currently have no active treatment options.

Innate and adaptive immune cells play a key role in causing chronic inflammation in the aortic wall, which contributes to the development of AAA.4,10 We previously reported that injection of a recombinant mouse IL-2/anti–IL-2 monoclonal antibody complex or UVB irradiation selectively expanded CD4+Foxp3+ Tregs and effectively reduced the development and related mortality inhibition of angiotensin II–induced AAA in apolipoprotein E–deficient (Apoε−) mice.11,12 In addition, alterations in the composition of gut microbiota and its metabolites have a considerable impact on a variety of human diseases via the immune system. Regarding atherosclerosis, we previously demonstrated that microbiota depletion significantly prevented atherosclerotic lesions compared with that in conventional ApoE−/− mice, whereas commensal bacteria were showed to maintain a low level of cholesterol in the plasma via the induction of hepatic bile acid biosynthesis.13 However, studies on the effect of gut microbiota depletion on atherosclerosis is controversial because the gut microbiota composition in conventional mice differ in every facility.14,15 We successively reported that the abundance of Bacteroides vulgatus and Bacteroides dorei was lower in the gut microbiome of patients with coronary artery disease and that oral gavage with live B vulgatus and B dorei could decrease the fecal and plasma lipopolysaccharide concentrations and protect mice against atherosclerosis.16,17 Hazen and colleagues have reported that trimethylamine N-oxide (TMAO), produced from dietary choline, carnitine, or betaine by gut microbiota could predict the risk of developing cardiovascular diseases in an independent large clinical cohort. Supplementing the diet with choline or trimethylamine N-oxide promoted atherosclerosis in a mouse model, and depletion of gut microbiota cancelled dietary choline–enhanced atherosclerosis.15,18,19 Gut microbiota-derived metabolites or gut microbiota-dependent immune systems have been clarified to have critical impacts on the formation of atherosclerosis.

Depletion of gut microbiota by antibiotics reduces the incidence of cranial aneurysm by suppressing macrophage infiltration and inflammatory cytokines.20 Another group has just released an article showing supplementation of Akkermansia muciniphila inhibit the formation of AAA in mice.21 However, the interaction between gut microbiota and AAAs in humans and mice is not well understood. As a first step to study the potential contribution of the gut microbiota to the pathophysiology of AAAs, we aimed to examine the impacts of gut microbiota on the formation of AAAs in mice using a well-established method of antibiotic cocktail–induced depletion of gut microbiota.

**METHODS**

**Data Availability**
The data that support the findings of this study are available from the corresponding author upon reasonable request.
Mice
Apolipoprotein E-deficient (ApoE−/) mice with a C57BL/6 background were previously described. Six-week-old male mice were fed high-cholesterol diet containing 0.2% cholesterol, 17.1% fat, and water ad libitum. Eleven-week-old mice were divided into 3 groups: drinking water (sham group), drinking water with antibiotics, and drinking water and intraperitoneally administered antibiotics 5 times a week. To deplete the gut microbiota, an antibiotic cocktail consisting of ampicillin (1 g/L), vancomycin (0.5 g/L), neomycin (1 g/L), and metronidazole (1 g/L) (Life Technologies Corporation, Carlsbad, CA) was administered through drinking water. Twelve-week-old mice received 1.44 mg/kg/day of angiotensin II (Ang II) (Sigma-Aldrich, St. Louis, MO) via subcutaneous osmotic mini-pumps (Model 2001, ALZET) for 4 weeks, as previously described. Nod1 ligand (ie, DAP; tirl-dap; InvivoGen, San Diego, CA) was administered through intraperitoneal injection at 100 ng/mouse 3 times per week each since the beginning of the antibiotic treatment. In brief, the mice were anesthetized with isoflurane (WAKO, Richmond, VA), and osmotic mini pumps containing Ang II dissolved in sterile water were inserted into the subcutaneous space. At 16 weeks, the mice were euthanized by cervical dislocation under anesthesia to evaluate AAA formation; we performed a necropsy on all dead mice and found cutting an abdominal aortic rupture event. Some dead mice also showed evidence of suprarenal aortic rupture. All dead mice were included in the analysis of mortality, incidence, and severity of AAA. Mice were housed in specific pathogen-free animal facilities. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Kobe University Graduate School and conformed to NIH guidelines.

Cells
Total white blood cell count was determined by preparing a 1:10 dilution of (undiluted) peripheral blood obtained from the orbital sinus using heparin-coated capillary tubes in RBC Lysis Buffer (BioLegend, San Diego, CA). After organ harvest, cell suspensions of the spleen, bone marrow were obtained. The aortic tissue from thoracic to iliac bifurcation was harvested. Aortic tissue and lung were cut into small pieces and subjected to enzymatic digestion with 450 U/mL collagenase I, 125 U/mL collagenase XI, 60 U/mL DNase I, and 60 U/mL hyaluronidase (Sigma-Aldrich) for 30 minutes at 37 °C while shaking.

Flow Cytometry
Antibodies used for flow cytometric analyses are provided in Table S1 in the Supplemental Material. Data were acquired on an LSRFortessa X-20 flow cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed with FlowJo v8.8.6 (Tree Star, Inc., San Carlos, CA). Cells were treated with FcBlock (BD Biosciences) for 15 min before incubation with the antibody cocktail for 30 minutes. Samples were fixed before flow analysis. Cell populations were identified as follows: (description of cell markers used).

Histology
Mice were euthanized, and the aorta was perfused with PBS. AAA lesions were cut and embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA), and cross-sections (10 μm) were prepared. Immunohistochemistry was performed on acetone-fixed or formalin-fixed cryosections (10 μm) of the maximum AAA lesions, using antibodies to identify nuclear (DAPI, 1:400; BioLegend) and macrophages (CD68, 1:100; BioLegend). The antibodies used for immunohistochemistry were the same as those listed in Table S1.

Blood Pressure Measurement
Systolic blood pressure was measured using the noninvasive tail-cuff method (BP-98 Softron), as described previously. Systolic blood pressure was measured at least 7 times at baseline and 3 weeks after Ang II pump implantation. The mean systolic blood pressure for each group was determined by averaging the systolic blood pressure of each mouse included in that group. Data from 1 day of measurement at each time point were used.

Quantitative-PCR Analysis for Gut Microbiota
Gut microbial 16S rDNA was extracted from each fecal sample. Quantitative PCR was performed using SYBR Premix Ex Taq (Takara Shigata, Japan) and a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. Genomic DNA of Phocaeicola vulgatus JCM5826T was used to create standard curves to determine target gene copy numbers. Primers used in this experiment are listed in Table S2 in the Supplemental Material.

Real-Time Reverse Transcription PCR Analysis
Total RNA was extracted from the tissues using NucleoSpin RNA (Takara). For reverse transcription (RT), a PrimeScript RT reagent kit (Takara) was used. Quantitative RT-PCR was performed using SYBR Premix Ex Taq (Takara) and a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific), according to the manufacturer's protocol. Amplification reactions were performed in duplicate, and fluorescence curves were analyzed using the included software. For quantitative PCR analysis of gene expression of chemokines Csf-1, Gm-csf, and Mcp-1, we used 4 primer sets including GAPDH for normalization. Primers used in this experiment are listed in Table S2.

Statistical Analysis
Results are expressed as mean ± SEM (standard error of the mean). Statistical tests included Mann–Whitney U test, Student's t test, Kruskal-Wallis test with Dunn's post-hoc analysis, 1-way ANOVA followed by Tukey's post-hoc correction for multiple comparisons, and chi-squared analysis were used to compare proportional data. P≤0.05 was considered to denote significance.
**RESULTS**

**Depletion of Gut Microbiota Decreased the Incidence of AAA and Suppressed the Enlargement of Abdominal Aortae**

We assessed the effects of gut microbiota depletion on AAA formation. The oral administration of a cocktail of antibiotics consisting of ampicillin, vancomycin, neomycin, and metronidazole (oral AVNM group), intraperitoneal injection of a cocktail of antibiotics (i.p. AVNM group), or vehicle (control group) was started 1 week before Ang II implantation and continued until 4 weeks after aneurysm induction (Figure 1A). Depletion of gut microbiota by oral AVNM ameliorated the incidence of AAAs and prevented death due to ruptured aneurysms, although i.p. AVNM did not affect the incidence of AAAs (Figure 1B through 1D). Similarly, the progression of the diameter of suprarenal aorta was significantly suppressed by oral AVNM administration compared with that in vehicle and i.p. AVNM group (Figure 1E). Blood pressure was similar between the control and oral AVNM group; however, blood pressure was higher in the i.p. AVNM group than in the control group, although the cause for it is not clear (Figure S1A). Total cholesterol level was higher in the oral AVNM group, as previously reported (Figure S1B).13 We confirmed a significant depletion of gut microbiota by oral administration of a cocktail of antibiotics, but not by intraperitoneal injection, as previously described (Figure 1F).13,22

**Depletion of Gut Microbiota Suppressed Monocytes/Macrophages Accumulation in Aortae During Aneurysm Formation**

To clarify the relationship between aneurysmal progression and monocytes or macrophages, we assessed immune cells in the aortae through fluorescent immunostaining and flow cytometry. First, we compared CD68+ monocyte or macrophage areas of cross-sectioned suprarenal aortae between the control and oral AVNM
groups in each AAA- and AAA+ group, which were categorized by the presence of AAA. Oral AVNM treatment significantly suppressed the accumulation of CD68+ monocytes or macrophages in the AAA- group. While the incidence of AAA induced a large wave of monocyte infiltration or macrophage proliferation in the aortae, CD68+ monocytes or macrophage areas in only true lumen as well as in the total false and true lumens in the AAA+ group were not significantly different between the control and oral AVNM (oral) group (Figure 2A and 2B). Flow cytometry analysis revealed that oral AVNM treatment suppressed the accumulation of immune cells in the aortae (Figure 2C through 2E). In CD45+ cells, we observed a significant reduction in CD11b+ F4/80+ macrophages, but not Ly6G+ neutrophils (Figure 2C and 2D).

**Administration of Antibiotics Affected Monocyte Depletion Only in the Spleen, but not in Other Organs**

To determine why fewer monocytes or macrophages accumulated in the aortae in the AVNM group, we assessed the effect of AVNM treatment on the number of pooled or generated monocytes. While bone marrow produces and contains numerous monocyte progenitors or monocytes, the spleen or lung contains extramedullary pooled monocytes. In the mice treated with AVNM for a week, the number of total and each subset of splenic reservoir monocytes, including Ly6C<sup>high</sup>, intermediate, and negative subsets, dramatically decreased in the oral AVNM group (Figure 3A). A reduction in monocytes or macrophages was not observed in the bone marrow or lung in the oral AVNM group (Figure 3C and 3D). Additionally, the same result was observed even at the 4-week time point where fewer monocytes were observed in the spleen in the oral AVNM group compared with the control or i.p. AVNM group (Figure 3E). Other populations, such as neutrophils, B cells, and T cells, in the blood or spleen were not affected by oral AVNM treatment (Figure S2). Oral AVNM did not affect the expression of chemokines, such as Csf-1, Gm-csf, and Mcp-1 in the spleen (Figure S3A through S3C).
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Depletion of Gut Microbiota or Bacterial Signal Suppressed the Angiotensin II Induced Mobilization of Monocytes into Aortae

We investigated the time course of Ly6C<sup>high</sup> blood monocytes after Ang II implantation to bridge the gap between splenic monocytes and macrophage accumulation in the aortae. Blood monocytes increased in the control group, but not in the oral AVNM group, 2 days after Ang II implantation (Figure 4A). CD68 positive monocyte macrophage area in the suprarenal aorta significantly increased in the control group from Day 4 to Day 10 after Ang II administration, whereas there was no increase in the AVNM group (Figure 4B and 4C). At Day 10, the accumulation of monocytes or macrophages tended to be suppressed in the AVNM group compared with that in the control (Figure 4B and 4C). A previous report has revealed that the reduction of Pattern Recognition Receptor ligands relates to the perturbation of splenic Ly6Chigh monocytes.23 In particular, it has been reported that the presence of Nod1 ligand, a type of Pattern Recognition Receptor ligands, affected the absolute number of Ly6Chigh monocytes in the spleen. Interestingly, intraperitoneal injection of Nod1 ligand tended to increase splenic reservoir monocytes, significantly increased the accumulation of CD68 positive monocyte macrophage...
areas, and enlarged the diameters of suprarenal aortae in the oral AVNM group, indicating that Nod1 ligand cancelled the effect of depletion of gut microbiota (Figure 4D and 4E).

**DISCUSSION**

Recently, gut microbiota has been shown to regulate several cardiovascular diseases, including atherosclerosis, through the immune system.\(^{17,24}\) Herein, to the best of our knowledge, we have provided the first evidence of gut microbiota as a therapeutic target for AAAs by demonstrating that oral administration of AVNM suppressed the development or progression of AAAs in mice. Intraperitoneal injection of AVNM did not suppress the development or progression of AAAs, suggesting that the presence of gut microbiota in the intestinal tract, not migration into the blood stream, affected the pathogenesis of AAAs.

Pathological features of AAAs in humans include extracellular matrix degradation and loss of vascular smooth muscle cells associated with immune cell infiltration, contributing to vascular remodeling and weakening of the aortic wall.\(^{1,4}\) Among the innate and adaptive immune cells, macrophages have been shown to play a critical role in the formation of AAAs and are a major source of proteolytic enzymes, such as matrix metalloproteinases, which compromise the integrity of the vessel wall by degrading the extracellular matrix.\(^{4}\) Monocyte-derived macrophages have an important contribution to macrophage accumulation in AAAs.\(^{25}\) Depletion of all types of aortic macrophages reduces the incidence of AAA, while selective depletion of lymphatic vessel endothelial receptor-1\(^+\) (Lyve-1\(^+\)) resident type adventitial aortic macrophages had no protective effects.\(^{26}\)

We clearly showed the accumulation of macrophages in the suprarenal aorta in the control group in the early phase from Day 4 to Day 10 after Ang II administration, as is characteristic of this murine AAA model.\(^{27}\) However, the accumulation of macrophages was not observed after Ang II administration in the AVNM group. To clarify the source of monocytes infiltrating aortic walls, we counted monocytes by flow cytometry in the bone marrow, lung, and spleen. The structure of the spleen enables the
removal of older erythrocytes and results in the efficient removal of microorganisms and cellular debris from the circulation. Although monocytes have been considered circulating in the bloodstream, additional function of the spleen as a reservoir of undifferentiated monocytes has been clarified. Splenic monocytes serve as a reservoir of monocytes in a steady state and accommodate them until a rapid onset of inflammation after myocardial infarction or atherosclerosis.

In addition, splenic reservoir monocytes were demonstrated to mobilize from the spleen to the aorta in response to Ang II and contribute to the vascular inflammatory response and forming AAA in Apoe−/− mice. Splenectomy suppressed the mobilization of monocytes from the spleen into the blood and inhibited the development of aneurysm, suggesting a direct link between splenic monocytes and aneurysmal inflammation. Clinical studies have also reported that monocytes contribute to the development of aneurysms. One report has shown that patients with AAAs have a high proportion of circulating CD14++CD16+ monocytes, which are thought to be categorized as Ly6Cintermediate monocytes in mice. Another report has demonstrated that intermediate CD14++CD16+ and nonclassical CD14+CD16+ monocyte subsets increased in patients with large AAAs.

Oral administration of AVNM suppressed the number of splenic reservoir monocytes but did not affect the number of monocytes in the bone marrow nor lung, which was consistent with a previous report. Additionally, intraperitoneal injection of AVNM did not reduce the number of splenic reservoir monocytes, which excludes the possibility of immune modulating effect of antibiotics themselves. Additionally, a reduction in circulating Ly6Chigh monocytes in the blood in the early phase indicates that monocytes and macrophages that accumulate in the abdominal aorta are mobilized from the spleen by Ang II. In the present study, intraperitoneal injection of AVNM did not suppress the number of splenic reservoir monocytes. Our findings showed that gut microbiota, not bacteria leaked into the blood stream, regulated the total number of splenic reservoir monocytes. Therefore, we suggested that the development of AAAs was accelerated by monocyte mobilization from the spleen into the aorta, independent of blood pressure or cholesterol levels.

We found that the spleen was significantly affected by the presence of the gut microbiota. Depletion of gut microbiota did not influence the expression of chemokines such as Csf-1, Gm-csf, and Mcp-1. Therefore, a decrease in the number of reservoir monocytes in the spleen is not affected by these chemokines. It has been reported that reduction of Nod1 ligands, including gut bacterial peptidoglycan, by abolishing the entire gut microbiota is associated with reduced numbers and perturbed function of splenic Ly6C<sup>high</sup> monocytes, while LPS, TLR4 ligand, do not affect the absolute number of splenic Ly6C<sup>high</sup> monocytes. Indeed, our results demonstrated that administration of Nod1 ligand tended to increase splenic reservoir monocytes, suppressed the reduction of monocyte macrophage accumulation in the suprarenal aortae, and showed a correlation with increased diameter of the suprarenal aortae in gut microbiota depleted mice. Depletion of gut microbiota-derived factors, such as Nod1 ligands, may contribute to fewer splenic reservoir monocytes, which in turn diminishes the number of monocytes mobilized into the blood by Ang II and suppresses the development of AAAs.

This study had several limitations. First, we were not able to use germ-free mice to deplete gut microbiota instead of AVNM because of the experimental complexities of this Apoe<sup>−/−</sup> murine model with Ang II pumps. Second, because depleting intestinal microbiota in humans by antibiotics is highly improbable because of side effects or toxicity, evaluating gut microbiota-derived important factors regulating reservoir monocytes such as Nod1 ligand in patients with AAAs is necessary. Thirdly, we did not clarify why Nod1 ligand affected only splenic monocytes. In the future, we will clarify the composition of the gut microbiota in patients with AAA and find a therapeutic option for AAA targeting the gut microbiota.

**PERSPECTIVE**

This study is first report that showed a potential contribution of gut microbiota to the pathophysiology of Abdominal Aortic Aneurysms. The relationship between microbiota and development of AAAs are very complex and still unclear. We revealed that depletion of gut microbiota suppressed the formation of AAAs by altering monocyte-macrophage dynamics, suggesting that the gut microbiota could be a therapeutic target, although we need to find another safer method than a cocktail of antibiotics.

**ARTICLE INFORMATION**

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Disclosure
The authors declare no known conflict of interest.

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