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Phocaeicola dorei and *Phocaeicola vulgatus* Protect against Atherosclerosis by Regulating Gut Immunity

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Aim: Arteriosclerosis is a condition that leads to coronary artery disease (CAD) and stroke. Basic and clinical studies have suggested a link between the gut microbiota and various diseases, including atherosclerosis. Therefore, we focused on gut microbiota and aimed to develop a probiotic-based treatment for atherosclerosis.

Method: From 6 to 14 weeks of age, *apoE*-deficient mice, a mouse model of atherosclerosis, were orally administered with *Phocaeicola dorei* and *Phocaeicola vulgatus* or saline five times/week. The diet was changed to a western diet at eight weeks of age. Finally, the mice were sacrificed at 14 weeks of age, and the atherosclerotic lesion area was evaluated.

Result: Previous studies have shown that atherosclerosis is suppressed by the administration of live type strains (TS) of *P. dorei* and *P. vulgatus* in *apoE*-deficient mice. In this study, we isolated *P. vulgatus* AF299, which highly expresses commensal colonization factors. Oral administration of *P. dorei* TS and AF299 to model mice further suppressed atherosclerosis compared to the administration of *P. dorei* TS and *P. vulgatus* TS. Genetic analysis of lesion tissues showed that the expression levels of genes associated with inflammatory responses were significantly reduced in mice treated with *P. dorei* TS and AF299. Moreover, gene expression related to immune response and IgA secretion was increased in the ileum.

Conclusion: Our results suggest that the bacteria-induced immune response in the gut leads to the suppression of the inflammatory response in atherosclerotic lesions. These results indicate the potential for the development of prophyllactic drugs for atherosclerosis.

Key words: Arteriosclerosis, Gut microbiota, Gut immunity, *Phocaeicola* spp.

Introduction

The gut microbiota, composed of more than 50 trillion, plays an important physiological role in regulating host nutrition, metabolism, and immunity,

thus gaining increased attention of all of the researchers in the world¹⁾. Aside from obesity and inflammatory bowel disease, cardiovascular diseases are also deeply influenced by the gut microbiota. Hazen *et al.* reported that trimethylamine-N-oxide

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(TMAO), produced from dietary choline, carnitine, or betaine metabolized by the gut microbiota, can predict the risk of developing cardiovascular diseases in a large, independent clinical cohort^{2, 3}). Another group demonstrated that administration of a Western diet reduced the abundance of *Akkermansia muciniphila* in the murine gut, resulting in a compromised gut barrier and increased endotoxemia, exacerbating vascular inflammation. Because gut microbiota-dependent immune systems are suspected to have critical impacts on the formation of atherosclerosis in humans, we attempted to reveal the composition of the gut microbiota in humans and clarify its molecular function and mechanism in mice.

For the first time, we revealed via terminal-restriction fragment length polymorphism analysis that the abundance of the genus *Bacteroides* is lower in patients with coronary artery disease (CAD) than in non-CAD patients with coronary risk factors or in healthy volunteers^{4, 5}.

The genus *Bacteroides* is the predominant gram-negative bacterium in the human gut and plays an important role in maintaining a healthy gut ecosystem. In line with our observations, the abundance of bacteria of the genus *Bacteroides* was decreased in patients with atherosclerotic ischemic stroke and transient ischemic attack. Moreover, enterotype 3, which is characterized by low levels of *Bacteroides*⁶, tends to have a higher incidence of symptomatic atherosclerosis than enterotype 1 and enterotype 2^{7, 8}. Phylogenetic and genomic analyses of *Bacteroides dorei* and *B. vulgatus* have revealed genetic characteristics markedly different from those of the *Bacteroides* reference species, *B. fragilis*⁹. As a result, a reclassification was carried out in 2020, and the genus name was changed. Hereafter, *B. dorei* and *B. vulgatus* are referred to as *Phocaeicola dorei* and *P. vulgatus*, respectively.

Using 16S rRNA gene sequencing, we demonstrated that the abundances of *P. dorei* and *P. vulgatus* were lower in the gut microbiota of patients with CAD. In atherosclerosis-prone *apoE*-deficient mice, oral gavage with live *P. dorei* and *P. vulgatus* can decrease the fecal and plasma lipopolysaccharide (LPS) concentrations and protect mice against atherosclerosis¹⁰. Although the genus *Phocaeicola* (formerly *Bacteroides*) comprises gram-negative bacteria containing LPS, *E. coli* LPS has a hexa-acylated lipid A moiety strongly induced endotoxemia. Meanwhile, *Phocaeicola* LPS has a penta- or tetra-acylated lipid A moiety that inhibits innate immune signaling and endotoxin tolerance¹¹. To determine the functional difference between *E. coli* LPS and *Phocaeicola* LPS and their involvement in

atherosclerosis, we tested the effect of injecting *E. coli* LPS and *Phocaeicola* LPS in *apoE*-deficient mice. We discovered that the injection of *E. coli* LPS induced neutrophil accumulation and subsequent neutrophil extracellular trap formation and IL-1 β production in plaque lesions, leading to exacerbated atherosclerosis; this was not observed upon injection with *Phocaeicola* LPS¹².

Experiments were also conducted using representative strains of *P. dorei* and *P. vulgatus* (JCM 13471 and JCM 5826, respectively). However, it is imperative to understand the function of bacteria at the strain level due to functional variations across strains, even within the same species¹³. For example, the fitness of *Phocaeicola* in the human gut is associated with Acyl-CoA transferase expression regulated by nucleotide polymorphisms of *Phocaeicola* strains, suggesting a dynamic strain-specific range of fitness in the human gut¹⁴. Therefore, we searched for strains that were effective against atherosclerosis.

Furthermore, the mechanism by which mammals assemble and maintain *Phocaeicola* in the gut is explained by the commensal colonization factor (CCF) system. Deleting the *ccf* genes in the model symbiont *Phocaeicola* resulted in colonization defects in mice and reduced horizontal transmission¹⁵. In another report, CCF-producing *Bacteroidetes* have been shown to fortify the gut immune barrier and macrophages to prevent the gut colonization of *Klebsiella pneumoniae*¹⁶. Taken together, these reports suggest that CCFs are key molecules for colonization in the gut and are important for the clinical development of bacterial reagents.

Aim

In this study, we isolated new strains with a focus on identifying a more effective strain to explore the link between immunity and inflammation in *P. dorei* and *P. vulgatus*. Using the isolated strains of *P. vulgatus*, we aimed to evaluate its ability to inhibit the formation of atherosclerotic plaques, and assess the underlying mechanisms in mice. The findings of this study aim to inform future clinical applications of *P. dorei* and *P. vulgatus*-based treatment strategies.

Methods

Mice

ApoE-deficient mice were on C57BL/6 background. All mice were housed in a specific pathogen-free animal facility at the Kobe University Institute. Under a strict 12 hours light/dark cycle, mice were fed ad libitum on regular feed (CE-2,

CLEA, Japan) and water, and 6-week-old mice were randomly divided into two groups: the control group received saline, and the live *Bacteroides* group received 200 μ L of live *P. dorei* and *P. vulgatus* orally or rectally five times per week. At 8 weeks of age, the diet was changed to a western diet (CE-2 82.7%, cocoa butter 17%, cholesterol 0.2%, CLEA, Japan) and the dosing continued until 14 weeks of age. And the mice were euthanized by cervical dislocation under anesthesia. 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.

Isolation of Bacteria from Human Feces

Collected human feces from healthy individuals were suspended in phosphate buffer, diluted 10^5 - 10^6 times, and smeared on BL agar medium (No. 05430 Shimadzu Diagnostics, Japan) with the addition of horse de-fibred blood. After several days, single colonies growing on the plate were picked and cultured in Difco-reinforced clostridial medium (RCM; No. 218081, BD Bioscience, USA).

Culture of *Phocaeicola dorei* and *Phocaeicola vulgatus* and Preparation of Dosing Solution

The strains of *P. dorei* and *P. vulgatus* used in this experiment were isolated from the feces of healthy volunteers. The type strains used as the reference strains (JCM 13471 and JCM 5826) were provided by RIKEN BRC. *P. dorei* and *P. vulgatus* were cultured anaerobically about 24 hours in RCM at 37°C. At this time, agar contained in the RCM was removed using a filtration filter (10.0 μ m, ADVANTEC, Japan). An anaerobic chamber (HIRASAWA, Japan) containing 10% CO₂, 10% H₂, and 80% N₂ was used for all anaerobic microbiology steps. Anaerobic cultures of *P. dorei* and *P. vulgatus* were grown at 37°C for approximately 24 hours and centrifuged at 15,000 rpm for 10 minutes. Then the culture supernatant was then discarded, and the bacteria were suspended in physiological saline solution to make the administered bacterial solution. The number of bacteria in the solution was prepared to be 1×10^8 - 10^9 .

Histology

Arterial root was embedded in OCT compound (No. 45833, Sakura Finetek Japan, Japan), frozen in liquid-nitrogen-cooled isopentane, and cryosectioned (10 μ m-thick). We used antibodies to identify neutrophil (Ly6G, 1:100; BD Biosciences), macrophages (CD68, 1:100; BioLegend, USA) and nucleus (DAPI, 1:500; BioLegend). Arteriosclerotic

areas were also evaluated using Oil Red O staining. Oil Red O powder (No. 154-02072, FujiFilm-Wako, Japan) was dissolved in isopropyl alcohol and mixed with distilled water to make an ORO solution. The sections and aortic arch were fixed in 10% formalin, and then Oil Red O staining was performed using the prepared ORO solution. Stained sections were digitally captured using an all-in-one fluorescence microscope (BZ-8100; Keyence, Japan), and the stained area was calculated. Stained lesions were analyzed in each mouse, and the average values were used for statistical analysis.

Lox-Index Measurement

Lox-index was calculated as LOX1 $\times$ ApoB. LOX1 and ApoB were measured in mouse plasma, respectively, with ELISA Kit for Lectin Like Oxidized Low Density Lipoprotein Receptor 1 (LOX1) (SEB859Mu, Cloud-Clone Corp., USA) and Mouse ApoB ELISA Kit (ab230932, Abcam, UK).

Flow Cytometry

Antibodies used for flow cytometric analyses are provided in [Supplemental Table 1](#) in the Supplemental Material. Data were acquired on an LSR Fortessa X-20 flow cytometer (BD Biosciences, Franklin Lakes) and analyzed with FlowJo v8.8.6 (Tree Star, Inc., USA). Cells were treated with FcBlock (BD Biosciences) for 15 minutes before incubation with the antibody cocktail for 30 minutes. Samples were fixed before flow analysis.

DNA Extraction from Gut Microbiota

Genomic DNA was extracted using NucleoBond HMW DNA (No. 740160.20, TaKaRa Bio. Inc., Japan) and Genomic-tips 20/G (No. 10223, QIAGEN, Netherlands) according to the manufacturer's protocol.

Genome Sequencing of *Phocaeicola* Species

The whole genomes of strains *P. vulgatus* TS and AF299 were sequenced as follows. SMRTbell libraries were constructed using a SMRTbell Express Template Prep Kit 2.0 (Pacific Bioscience, CA, USA) according to manufacturer's protocol. Sequencing libraries were size-selected using the BluePippin system (Saga Science, MA, USA) with a minimum fragment length cutoff of 20 kbp. Each sequencing library was run on the PacBio Sequel system using one SMRT cell (1M v3 LR) with a Binding Kit3.0/Sequencing Kit3.0 and 20-hour movie collections. PacBio reads were assembled using the canu v2.1.1¹⁷⁾, followed by polishing with PacBio SMRT Link v9.0 (Allow software). In addition, genomic DNAs were

fragmented to an average size of 600 bp with the DNA Shearing System M220 (Covaris Inc., Woburn, MA, USA). Paired-end libraries were constructed with a TruSeq DNA PCR-Free Library Prep kit (Illumina, San Diego, CA, USA) and size-selected on agarose gels using a Zymoclean Large Fragment DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The final libraries were sequenced on the Illumina HiSeq 2500 sequencer with a read length of 250 bp. The Illumina short reads were used to correct PacBio assembly by Pilon v.1.23¹⁸⁾. Genome sequence analysis was performed using the DDBJ Fast Annotation and Submission Tool (DFAST; <https://dfast.nig.ac.jp/>)¹⁹⁾. Protein-coding genes of the genomes were automatically annotated by sequence similarity search against the KEGG Prokaryotes Protein database using DIAMOND v2.0.13.151 with parameters (very-sensitive mode, E-value < 1e-5, bit score ≥ 70, identity ≥ 40%, and query and reference coverage ≥ 50%)²⁰⁾. Genome sequence of AF299 was submitted to DDBJ BioProject ID PRJDB19001.

Isolation of the Lamina Propria

The ileum was excised and thoroughly washed with PBS. The ileum tissue was then shaken at room temperature in a solution of DTT (0.45 mM) in Hanks' balanced salt solution for 5 minutes. After decantation, this step was repeated once again. Then, the ileum tissue was incubated in a solution of DTT (0.45 mM) + EDTA (2 mM) in Hanks' solution at 37°C for 20 minutes with shaking. After decantation, the tissue was shaken in RPMI medium containing FCS (5%) at 37°C for 15 minutes, a process that was repeated twice. Subsequently, collagenase digestion was conducted using Collagenase Yakult in a CO₂ incubator for 1 hour. After centrifugation, the cells were washed with RPMI (5% FCS), and the lamina propria was isolated using Percoll.

Mouse Aortic Sections and Ileum RNA Sequencing and Functional Analysis

Total RNA was extracted from the tissues using NucleoSpin RNA (No. 740955.50, TaKaRa Bio. Inc.). RNA extraction was performed according to the manufacturer's protocol. Then, RNA sequencing was outsourced to Rhelixa (Tokyo, Japan). The samples were processed according to the company's standard protocols. After data filtering, we performed a comprehensive gene expression analysis. For differential expression analysis, we normalized the data to identify differentially expressed genes. In the TS/AF299 group, the top 20 genes with the highest expression level of Z score were designated as Differential Expressed Genes. GO enrichment and

KEGG pathway analyses were performed.

Quantitative-PCR Analysis for Gut Microbiota

16S rDNA was extracted from *P. vulgatus* TS and AF299 using Nucleospin Microbial DNA (No. U0235B, TaKaRa Bio. Inc.) (Forward; ACTCCTACGGGAGGCAGCAGT, Reverse; ATTACCGCGGCTGCTGGC). Quantitative PCR was performed using TB Green Premix Ex Taq II (No. RR820A, TaKaRa Bio. Inc.) and a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

Real-Time Reverse Transcription PCR Analysis

Total RNA was prepared as described above. For reverse transcription (RT), a PrimeScript RT reagent kit (RR037A, TaKaRa Bio. Inc.) was used. Quantitative RT-PCR was performed using TB Green Premix Ex Taq II (No. RR820A, TaKaRa Bio. Inc.) 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 *ccf* gene expression, primers containing GAPDH were used for standardization. The primers used in this experiment were as follows. (*ccf* Forward; CTGGTGTAGTAGCCATGAGC, *ccf* Reverse; CGCTCAATACCATCAACTAACACC, 16S rRNA universal Forward; ACTCCTACGGGAGGCAGCAGT, 16S rRNA universal Reverse; ATTACCGCGGCTGCTGGC)

CYBERSORTx

Gene expression data from single-cell RNA sequencing and bulk RNA sequencing were prepared for CIBERSORTx analysis. For the single-cell RNA sequencing data, single-cell count data from *apoE*-deficient mice were utilized (GSE155513, ²¹⁾). Low-quality cells were filtered out, and the remaining single-cell count data were converted to TPM (Transcripts Per Million). Clustering was performed using Seurat (v4.1.0) in R, with each cell as input. Cells were grouped into 12 clusters based on their gene expression profiles, and cell types were annotated according to marker gene expression levels. A signature matrix, representing the gene expression profile specific to each cell type, was generated from the processed single-cell data using CIBERSORTx. For the bulk RNA sequencing data, samples from four TS/AF299 cases and four control cases were used. Following preprocessing with fastp (v0.20.1), gene expression profiles were generated by quantifying gene expression levels using kallisto (v0.46.2). Finally, the

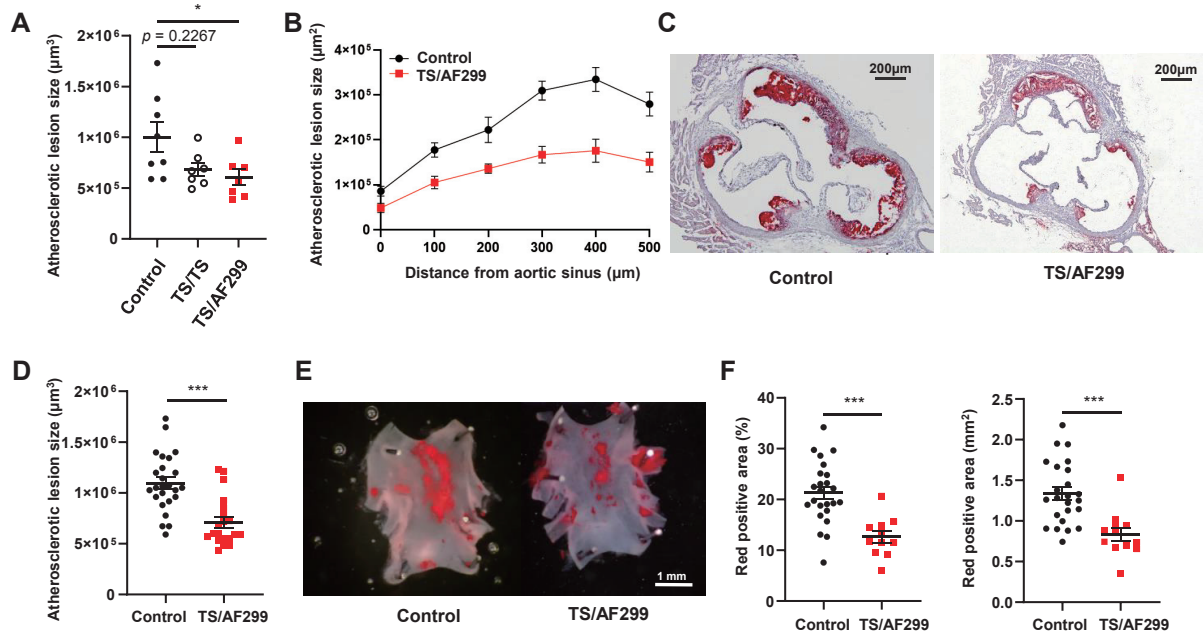


Fig. 1. *Phocaicola dorei* (*P. dorei*) type strain (TS) and *Phocaicola vulgatus* (*P. vulgatus*) AF299 suppressed atherosclerosis progression

Six-week-old *apoE*-deficient C57BL/6J mice fed a normal diet were treated with *P. dorei* and *P. vulgatus* or saline (control) and euthanized at 14 weeks of age. Loading with a western diet was initiated at 8 weeks of age. TS/TS indicates the administration of *P. dorei* TS and *P. vulgatus* TS. TS/AF299 indicates the administration of *P. dorei* TS and *P. vulgatus* AF299.

A. Quantitative analysis of the atherosclerotic lesion areas in the aortic sinus during bacterial administration. Control, $n = 8$; TS/TS, $n = 7$; TS/AF299, $n = 7$; Kruskal–Wallis test with Dunn’s post-hoc analysis. B. AUC ratio of the atherosclerotic lesion size in the aortic sinus. Control, $n = 12$; TS/AF299, $n = 13$; Mann–Whitney U test. C. Representative photomicrographs of Oil Red O staining. The black bars represent 200 μm . D. Quantitative analysis of the atherosclerotic lesions in the aortic sinus. Control, $n = 25$; TS/AF299, $n = 20$; Mann–Whitney U test. E. Representative micrographs of the Oil Red O staining of atherosclerotic lesion sites in the aortic arch and the corresponding quantitative analyses. The white bars represent 1 mm. F. Quantitative analysis of the atherosclerotic lesion areas in the aortic arch. Left: Control, $n = 24$; TS/AF299, $n = 11$, Right: Control, $n = 24$; TS/AF299, $n = 12$; Mann–Whitney U test.

A, B, D, F: Error bars represent the standard error of the mean. * $P < 0.05$, *** $P < 0.001$

relative abundance of each cell type was estimated using CIBERSORTx by integrating the gene expression profiles from the signature matrix and bulk RNA sequencing data.

Statistical Analysis

Results are expressed as mean $\pm$ 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 were used to compare proportional data. $P \leq 0.05$ was considered to denote significance.

Results

Administration of *Phocaicola dorei* and *Phocaicola vulgatus* Inhibits Atherosclerosis in *ApoE*-Deficient Mice

We previously demonstrated that the oral administration of *P. dorei* TS and *P. vulgatus* TS

inhibited atherosclerosis in mice¹⁰. In this study, we first aimed to find a strain of *Bacteroides* spp. that is a potent inhibitor of atherosclerosis. It has been shown that *Bacteroides* spp. produces *cef* that promotes the ability of IgA to settle in the host by coating bacteria with IgA and regulating capsular polysaccharide expression²². Therefore, we focused on screening strains with high *cef* expression. As a result, we selected *P. vulgatus* AF299, which had the highest *cef* expression when compared to the reference strain. Genomic analysis of strain AF299 showed a transposon insertion in the *cef* gene compared to *P. vulgatus* TS (Supplemental Fig. 1A and 1B). Treatment with a combination of *P. dorei* TS and *P. vulgatus* AF299 (TS/AF299) inhibited atherosclerosis in mice (Fig. 1A). Moreover, the number of atherosclerotic lesions in the aortic sinus and aortic arch was significantly lower in the TS/AF299 group than in the control group (Fig. 1B to 1F). Despite seeing dramatic changes in atherosclerosis, there were no differences in body weight or plasma cholesterol

levels between the two groups (Supplemental Fig. 1C and 1D).

Effect of *Phocaeicola dorei* and *Phocaeicola vulgatus* on Vascular Inflammation

We also observed the immunological effects of TS/AF299 treatment on atherosclerosis. To observe immune cells in the aortic sinus, Ly6G and CD68 were stained using fluorescent immunostaining (Fig. 2A). Interestingly, the Ly6G-positive areas and their relative sizes to the atherosclerotic lesions were surprisingly reduced in the TS/AF299 group (Fig. 2B). Meanwhile, the CD68-positive area was significantly reduced in the TS/AF299 group, whereas there was no difference between the groups when normalized by atherosclerotic lesion size (Fig. 2B). Hence, the administration of TS/AF299 may have an inhibitory effect on the accumulation of neutrophils in atherosclerotic lesions.

Next, we performed a comprehensive gene expression analysis of the aortae. More genes were suppressed than activated in the TS/AF299 group versus the control group (data not shown). The expression of genes involved in inflammatory responses and immune reactions, such as *Olr1* (*Lox1*), *Mmp19*, *Cxcl1*, *Spp1*, and *Ccr5*, decreased in the TS/AF299 group compared to the control group. The reduction in *Cxcl1* expression might support the reduced accumulation of neutrophils in atherosclerotic lesions²³ (Fig. 2C and 2D). The gene ontology (GO) analysis results showed that the greatest expression reduction was observed in genes related to the defense response, including those involved in the development of atherosclerosis, such as *Olr1*, *Cxcl1*, *Ccr5*, and *Spp1*²⁴ (Fig. 2D and 2E). RNA-seq showed that the expression of *Olr1* (also known as *Lox1*) was dramatically reduced. Moreover, the LOX index (LOX1×ApoB) has been reported as a diagnostic marker of CAD²⁵; this value was significantly reduced in the TS/AF299 group (Fig. 2F).

To determine cell distribution in the aorta, we estimated the percentage of each cell type from bulk RNA-seq data based on publicly available single-cell RNA-seq data. The results showed an increase in the relative number of smooth muscle cells (SMCs) in the TS/AF299 group and an increase in *Tagln* expression, a marker of smooth muscle differentiation. Furthermore, the number of immune cells, such as monocytes, macrophages, and dendritic cells, was also reduced. In particular, *Flt3* expression in dendritic cells was reduced in the TS/AF299 group. *Flt3* is an immune response-regulating gene, and TS/AF299 treatment regulated this abnormal immune response (Supplemental Fig. 2). As the reference single-cell

RNA-seq dataset did not include neutrophils, we could not reproduce the data for neutrophils that exhibited the most dramatic changes in our immunostaining assay.

Phocaeicola dorei and *Phocaeicola vulgatus* Activated the Immune System in the Small Intestine

We investigated the effects of *P. dorei* and *P. vulgatus* on the gut immune system. Mesenteric lymph nodes (mLN) are important tissues responsible for the immune response against various non-self-antigens that invade the gut tract²⁶. The administration of TS/AF299 did not alter the number of neutrophils, monocytes, or T cells in the mLN (Fig. 3A). Although the number of T cells was not affected by TS/AF299 administration, the percentage of effector memory T cells significantly increased and the percentage of naïve T cells decreased (Fig. 3B). Interestingly, the expression of IL-17 in the ileum, as evaluated by qPCR, was drastically reduced by the Western diet compared to the normal diet. However, the administration of TS/AF299 restored the expression of IL-17 in the ileum (Fig. 3C). Administration of TS/AF299 also increased the percentage of Th17 cells and Foxp3+ CD4+ T cells among TCRb+ T cells, and the expression of MHC class II in dendritic cells (Fig. 3D). Thus, the administration of TS/AF299 enhanced gut immunity. However, because the power of the statistical test we used was dependent on sample size, the Mann–Whitney *U* test did not indicate a statistically significant difference at the significance level of $P = 0.05$ with the current sample size. In contrast, no significant changes were observed in the immune cells of tissues such as the spleen, blood, and bone marrow (Supplemental Fig. 3A to 3D).

Phocaeicola dorei and *Phocaeicola vulgatus* Increased the Expression of Immune Response-Related Genes in the Ileum

To investigate how TS/AF299 administration affects gut immunity, we conducted bulk RNA-seq of the ileum. Among the top 21 differentially expressed genes, all were primarily related to immune responses and upregulated by TS/AF299 administration (Fig. 4A). Of the top ten genes, seven (*H2-Aa*, *H2-DMb1*, *Cd74*, *H2-Ab1*, *H2-Eb1*, *Ciita*, and *H2-DMA*) were involved in MHC class II immune responses, with roles associated with the presentation of foreign antigens and the activation of CD4+ T cells. *Pigr* is associated with IgA-related immune responses and is involved in the regulation of mucosal immune responses (Fig. 4B). GO biological process analysis revealed that immune system processes and immune responses were enriched, supporting the observed

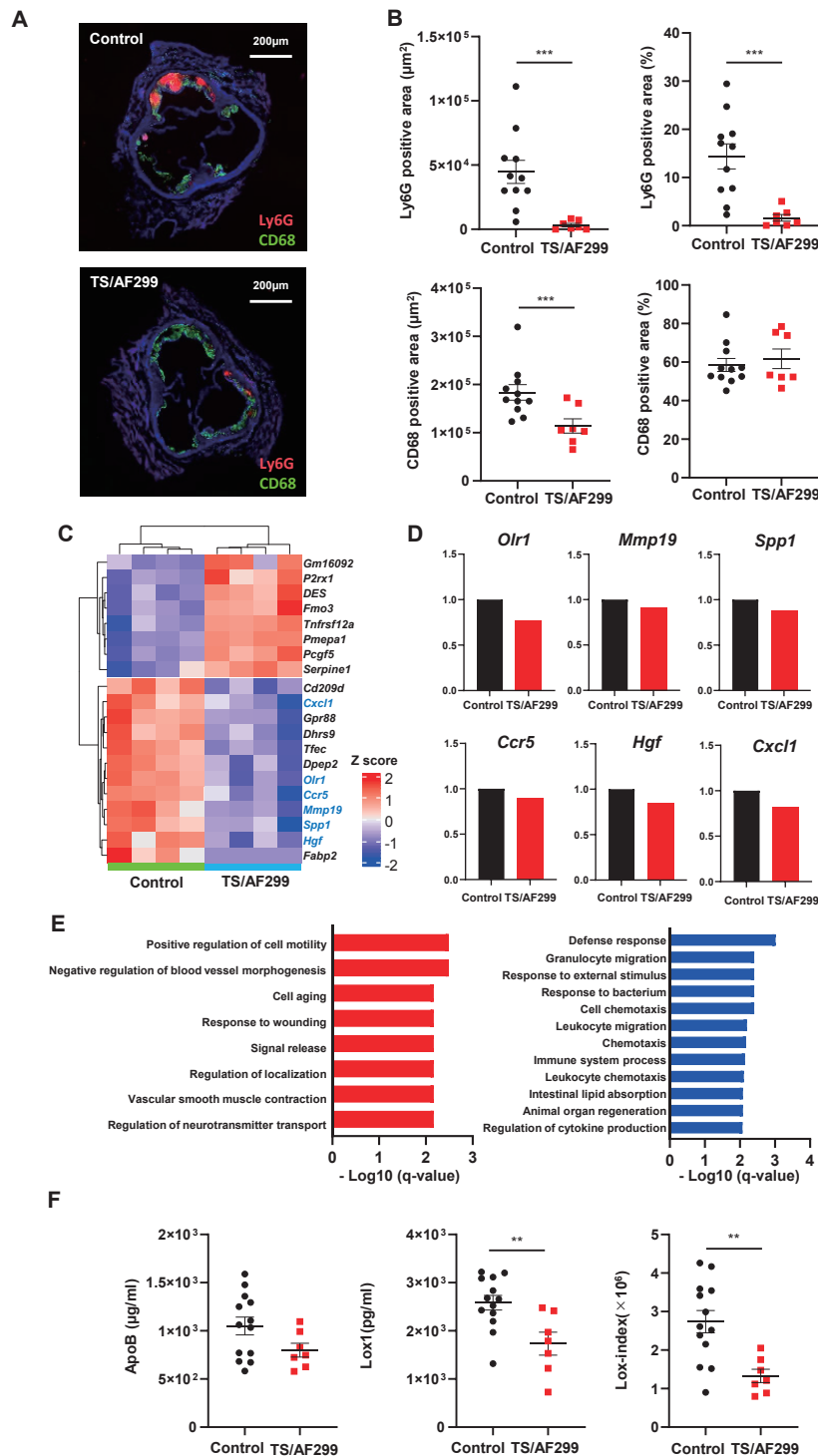


Fig. 2. *P. dorei* and *P. vulgatus* administration suppressed vascular inflammation caused by atherosclerosis

A. Fluorescence immunostaining of neutrophils (Ly6G; red), monocytes (CD68; green), and nuclei (DAPI; blue) in the aortic sinus. The white bar represents 200 µm. B. Quantification of Ly6G-positive and CD68-positive regions in the aortic sinus. Control, $n = 11$; TS/AF299, $n = 7$, Mann-Whitney U test. C. RNA-seq analysis of the aorta was performed, and the top genes related to inflammation and immunity were selected for further analysis. Relative expression levels are shown in a heat map ($q < 0.05$). D. Expression levels of the top 10 genes specifically repressed in the TS/AF299 group out of the 20 genes shown in the heat map. Comparisons were made relative to the control group, which was considered as 1. E. Representative coronary artery disease risk genes as determined via RNA-seq and analyzed via GO analysis. F. Concentration of LOX1 and ApoB in the plasma and LOX index values. Control, $n = 13$; TS/AF299, $n = 7$, Mann-Whitney U test. A-F: Error bars represent the standard error of the mean. ** $P < 0.01$, *** $P < 0.001$

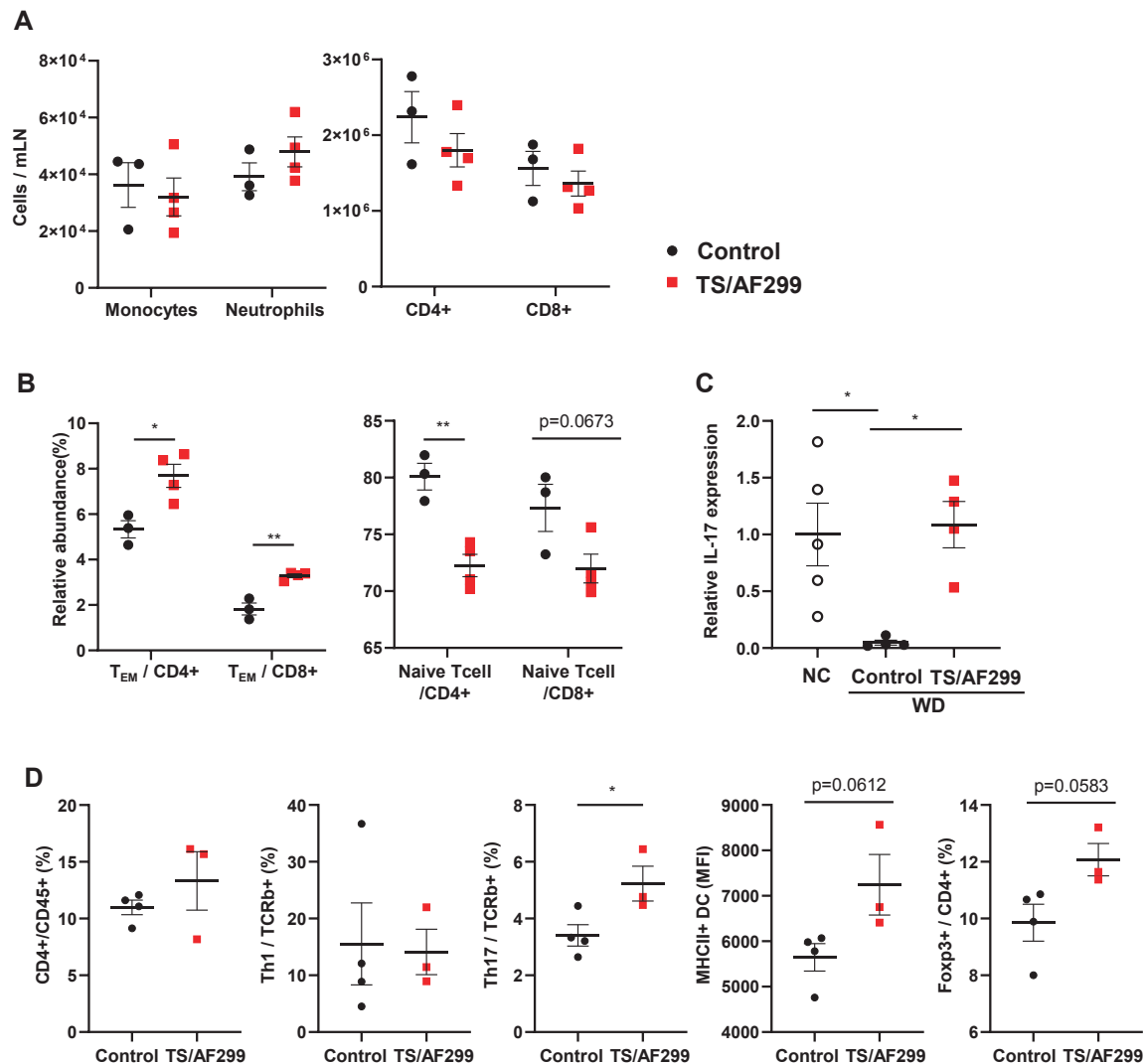


Fig. 3. *P. dorei* and *P. vulgatus* affected small intestine immunity

A, B. Quantification of neutrophils, monocytes, and T cells in the mLN. The proportions of CD68+ monocytes (A, left), CD11b+ Ly6G+ neutrophils (A, right), CD62L- effector memory T cells (B, left), and CD44+ naïve T cells (B, right) were analyzed via flow cytometry. Control, $n = 3$; TS/AF299, $n = 4$, Mann–Whitney U test. C. Quantification of IL-17 expression levels at the ileal site in mice administered with different diets. NC (normal chow), $n = 5$; Control (western diet, WD), $n = 4$; TS/AF299 (WD), $n = 5$, Kruskal–Wallis test with Dunn’s post-hoc analysis. D. Quantification of Th17 cells, MHC II+ dendritic cells, and Foxp3+ CD4-positive T cells in the mucosa specific layer of the ileum. Control, $n = 3$; TS/AF299, $n = 4$, Mann–Whitney U test. A–D: Error bars represent the standard error of the mean. * $P < 0.05$, ** $P < 0.01$.

alterations in the expression of individual genes, such as MHC class II-related genes (Fig. 4C). Similarly, in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, these gene expression changes were found to most significantly affect antigen processing and presentation (Fig. 4D). Visualization of the KEGG pathway for antigen processing and presentation revealed that a substantial portion of the pathway was enriched (Supplemental Fig. 4).

Rectal delivery of *Phocaeicola dorei* and *Phocaeicola vulgatus* via Did Not Inhibit Atherosclerosis in *ApoE*-Deficient Mice

The oral administration of TS/AF299 activated immune responses in the small intestine, consequently suppressing inflammatory responses in atherosclerotic lesions. Therefore, to determine whether these bacteria activate immunity while passing through the small intestine, we administered TS/AF299 rectally instead of orally and examined the effect of rectal treatment on atherosclerotic lesion formation and the number of

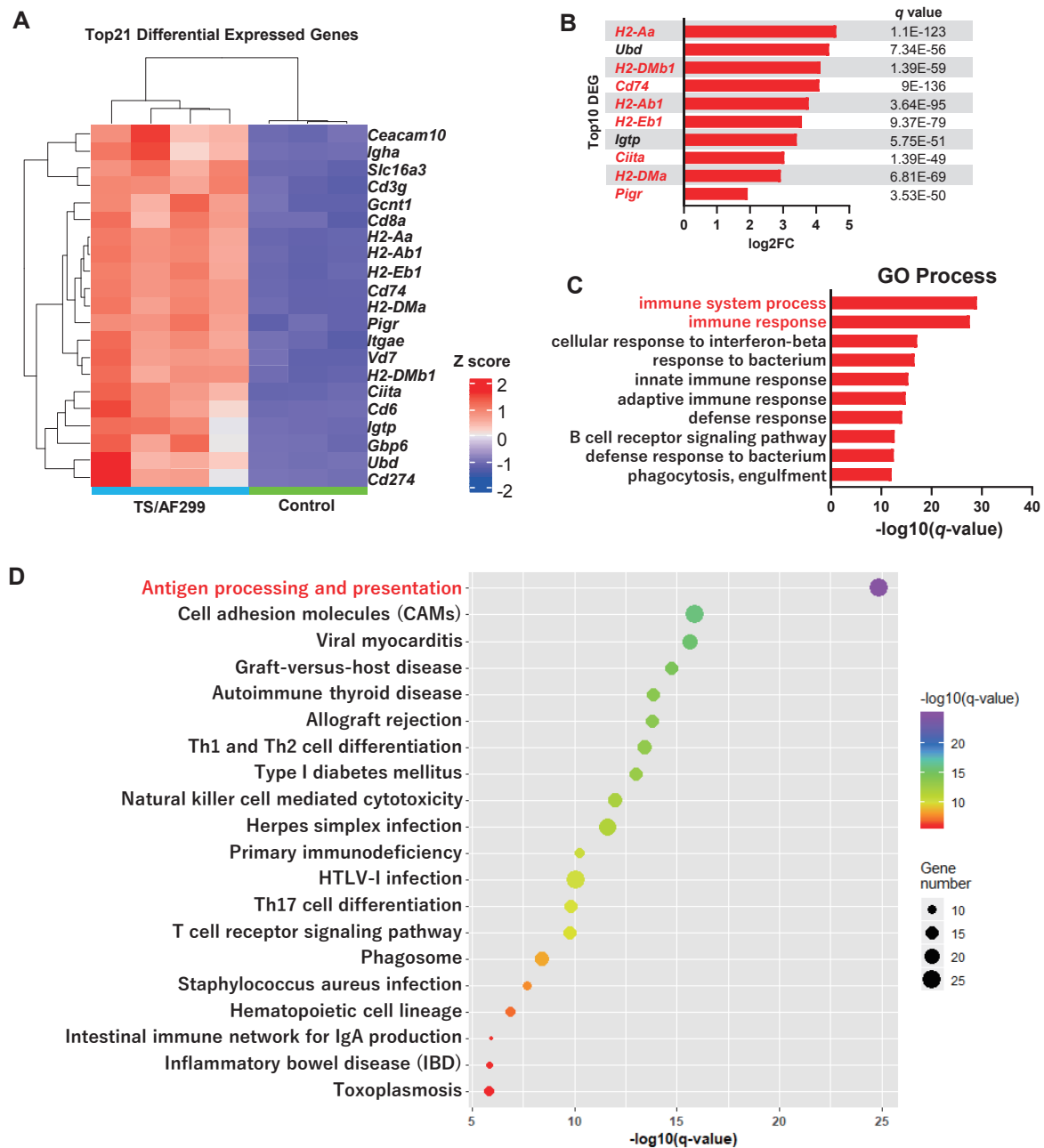


Fig. 4. Bulk RNA-seq of the ileum showed that the administration of *P. dorei* TS and *P. vulgatus* AF299 elicited a predominant enhancement of immune responses in the ileum and a suppression of inflammatory responses in the blood vessels

A. The top 21 genes with the highest gene expression in the TS/AF299 group were selected from bulk RNA-seq results of ileal tissues. B. Of the top genes with the highest expression in the TS/AF299 group, the top 10 genes were selected, and their expression levels were examined. C. Changes in gene expression using gene ontology (GO) process analysis. D. Changes in gene expression via KEGG pathway analysis. B–D: The areas of focus are indicated in red.

immune cells. In the case of rectal administration, even when the same volume of TS/AF299 was used as in oral administration, we confirmed that the bacterial solution did not extend beyond the ileocecal region (Fig. 5A). Rectal administration of TS/AF299 did not affect body weight change, while the atherosclerotic

lesions in the aortic sinus and plaque area in the aortic arch either significantly increased or showed no change (Fig. 5B to 5D). It was observed that, unlike oral administration, rectal administration of TS/AF299 did not inhibit atherosclerosis (Fig. 5C and 5D). Additionally, as expected, rectal administration

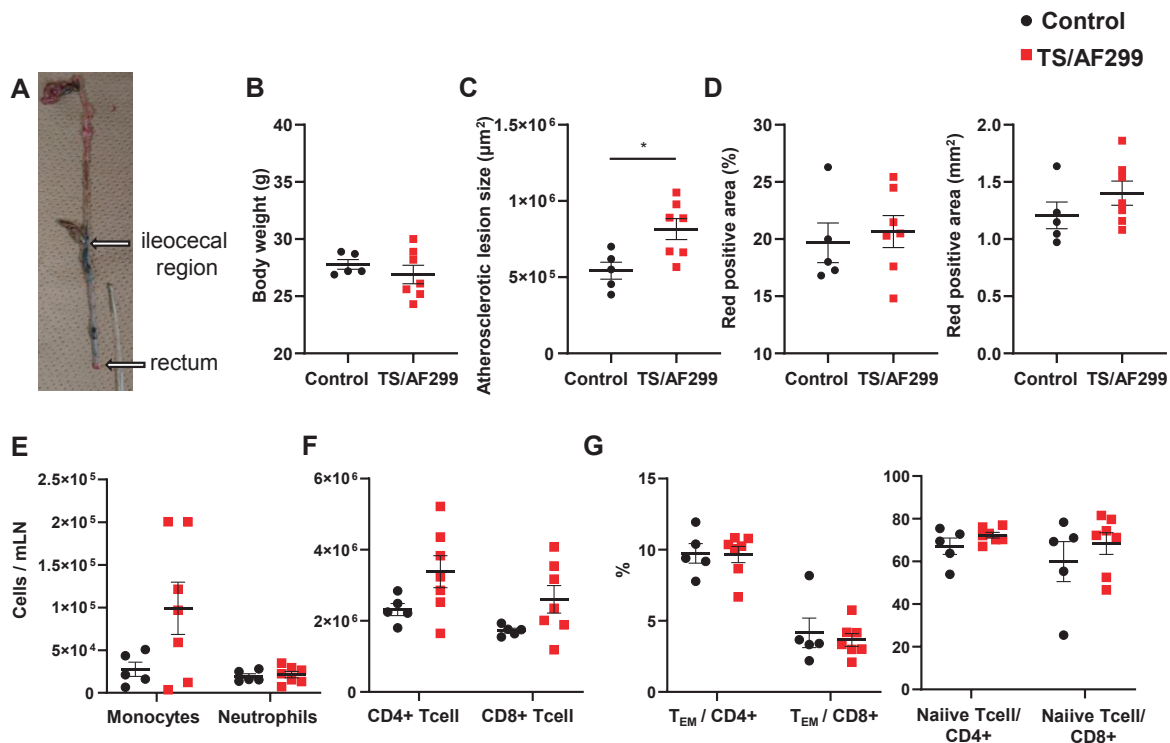


Fig. 5. Administration of *P. dorei* and *P. vulgatus* via rectal delivery did not inhibit atherosclerosis in *apoE*-deficient mice

A. Photograph of the ileum to rectum area when the ink (200 μL) was administered rectally. B. Body weight of the mice at 14 weeks of age. C. Quantitative analysis of the atherosclerotic lesion area in the aortic arch. D. Quantitative analysis of the atherosclerotic lesion area in the aortic arch. E–G: Quantification of immune cells (monocytes and neutrophils, CD4+ cells, CD8+ cells) in the mLN upon the rectal administration of *P. dorei* and *P. vulgatus*. B–G: Control, $n = 5$; TS/AF299, $n = 7$. Statistical analysis was performed using the Mann–Whitney U test. Error bars represent the standard error of the mean. * $P < 0.05$.

did not affect the number of immune cells in the mLN, and the proportions of effector memory and naïve T cells in the TS/AF299 group were comparable with those in the control group (Fig. 5E to 5G).

Discussion

Consistent with previous reports, our current study showed that the administration of *P. dorei* and *P. vulgatus* inhibited the progression of atherosclerosis, confirming the atheroprotective effects of these two bacterial species¹⁰. Notably, the administration of TS/AF299 resulted in a reduction in the number of neutrophils in atherosclerotic lesions. Additionally, mRNA expression analyses using next-generation sequencing revealed that these bacterial species suppress immune and inflammatory responses in atherosclerotic lesions. Furthermore, CYBERSORTx analysis of the TS/AF299 group revealed that inflammatory signaling in the tissues was inhibited via enhanced smooth muscle cell differentiation and a reduced number of immune cells, leading to overall inhibition of inflammation. Additionally, downregulated

Flt3 expression in dendritic cells may have contributed to the inhibition of adaptive immune response, playing an important role in tissue repair within inflamed areas and the regulation of abnormal immune system activity. This may indicate that TS/AF299 administration significantly inhibited the inflammatory response.

While the administration of TS/AF299 suppressed inflammation in atherosclerotic lesions in mice, it dramatically enhanced immune responses in the small intestine. Our results demonstrated that the gut microbiota could suppress inflammatory responses even in atherosclerotic lesions near the heart, a site distant from the digestive tract. These results provide valuable insights into the systemic effects of gut microbiota on inflammation. Moreover, the administration of TS/AF299 dramatically altered the immune cell composition of the mLN, with increased proportions of effector memory T cells and decreased proportions of naïve T cells.

Since the mLN plays a crucial role in immune responses in the small intestine^{27, 28}, we next investigated the gene expression changes in the ileum

following bacterial administration. The results confirmed that the administration of TS/AF299 markedly activated immune responses in the ileum. The most significant expression changes were observed in genes involved in immune responses related to MHC class II processes. MHC class II is crucial in gut immunity as it activates CD4⁺ T cells through antigen presentation, inducing IgA production^{29, 30}. Effector memory T cells contribute to rapid immune responses and enhance protection against reinfection in the gut. Thus, bacteria and related immune cells play essential roles in the systemic immune response via gut immunity.

To confirm that the atheroprotective effects of TS/AF299 were mediated by gut immunity, we administered the bacteria rectally to ensure that they did not pass through the small intestine. As a result, gut immunity was not induced, and the progression of atherosclerosis was not suppressed. Although we observed a reversal in the atherosclerotic lesion size, we cannot provide a definitive explanation for this finding. However, this may have occurred by chance, due to statistical power limitations in detecting atherosclerotic lesion size variations caused by individual differences in mouse tissue size. Notably, the red-stained area in the aortic arch was nearly identical in the control group and rectally administered TS/AF299 group (Fig. 5D). As no significant intergroup differences were observed in atherosclerotic lesion in the aortic arch, either in absolute or relative terms, these results indicate that rectal administration of the bacteria did not exert an atheroprotective effect. Therefore, these results suggest that the atheroprotective effect of TS/AF299 is dependent on the induction of gut immunity, likely mediated by the activation of an appropriate immune response through the small intestine.

Gut microbiota-derived metabolites and gut microbiota-dependent immune systems have been shown to have critical effects on atherosclerosis. Reports of various inflammatory responses being suppressed by the gut microbiota often highlight the role of Foxp3⁺ regulatory T cells or Th17 cells³¹⁻³⁵. Specific bacteria, such as segmented filamentous bacteria (SFB), promote the development of gut immunity by inducing Th17 cells through their attachment to intestinal epithelial cells^{29, 34, 36}. A high-fat, high-sugar diet alters the gut environment, promoting the proliferation of certain bacterial groups through IL-22 secretion by innate lymphoid cells (ILC3). This ultimately leads to the elimination of Th17 cell-inducing bacteria such as SFB, the immune system in the gut collapses owing to a decrease in Th17 cells, and a diminished immune response³⁵. In

this study, TS/AF299 administration restored IL-17 levels comparable to those in mice fed with the standard chow diet, suggesting that *P. dorei* TS and *P. vulgatus* AF299 may be associated with Th17 cell and/or IL-17 induction. Given the close relationship between gut immunity and Th17, IL-17, and ILC3, further studies are needed to explore and understand the atheroprotective effects of *P. dorei* and *P. vulgatus*.

From another perspective of gut immune regulation, our study showed that TS/AF299 administration increased the proportion of Foxp3⁺ cells in mLN, suggesting a potential role in regulating gut immunity. Gut bacteria such as *Clostridia* have been shown to suppress inflammation through the induction of Treg cells^{34, 37}. Thus, the gut microbiota serves as a key regulator of gut immunity, orchestrating host immune responses and maintaining mucosal homeostasis, and our current study is consistent with these findings.

Furthermore, the effects of *Phocaeicola* spp. on immune cells in the small intestine are significantly related to MHC class II immune processes. Stimulating gut immunity can decrease the production of inflammatory substances by pathogenic bacteria and reduce bacterial translocation, where bacteria directly invade the body. This might explain why the inflammatory responses in cardiovascular tissues far from the gut were also suppressed. Additionally, the expression of *Pigr* increased, which likely led to the high IgA levels observed in this study. It has been reported that *B. fragilis* utilizes host IgA to adhere to the gut mucosa²². This may further enhance the interaction of the bacteria with the host, promoting the host immune response. However, the regulation of gut immunity by the gut microbiota is highly complex, likely involving multiple pathways. Although our study did not elucidate the mechanisms by which gut immunity is enhanced, our results suggest that the atheroprotective effects of *Phocaeicola* spp. are dependent on gut immunity.

The AF299 strain actively overexpresses CCFs^{15, 22}, which are necessary for the colonization of *Bacteroides* spp. and *Phocaeicola* spp. in the gut due to transposon insertion. CCFs are involved in capsular polysaccharide production and have been reported to significantly impact host immune responses²². The AF299 strain exhibited a greater atheroprotective effect than other strains. This may be attributed to the increased production of capsular polysaccharides due to the overexpression of the *ccf* gene, which may have increased its ability to colonize the intestinal mucosa more effectively than other bacteria. However, further research is needed to establish a link between mutations in the *ccf* gene and the inhibition of

atherosclerosis.

Reports that the gut microbiota is related to the progression of atherosclerosis are well known, particularly a study on TMAO by Hazen *et al.*³⁸⁾. They reported that TMAO produced from dietary choline, carnitine, or betaine by the gut microbiota could predict the risk of developing cardiovascular diseases in a large, independent clinical cohort. However, there is no strong correlation between the abundance of genes encoding TMAO-converting enzymes and the abundance of *Phocaeicola* spp. or *Bacteroides* spp., suggesting that *P. dorei* and *P. vulgatus* are not significantly involved in TMAO synthesis³⁹⁾. There are many other metabolites produced by gut microbiota that affect the organism in this way. Moreover, we also considered that gut microbiota metabolites produced by TS/AF299 administration may enhance gut immunity in living organisms.

Conclusion & Perspective

The mechanisms by which the gut microbiota inhibit atherosclerosis remain largely unknown. Our study suggests that the administration of *Phocaeicola* spp. may exert anti-inflammatory effects on blood vessels far from the gut by enhancing gut immunity. Furthermore, these findings indicate that the effects may vary among bacterial strains.

Collectively, this study presents the possibility of preventing atherosclerosis, which is related to heart failure and ischemic heart disease, through the microbiota and gut immunity. Although our study did not elucidate the mechanisms by which gut immunity is enhanced. To develop prophylactic agents for gut immune-mediated atherosclerosis, we need to identify substances that enhance gut immunity.

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Declaration of Interest

Hitomi Nakashima is an employee of Noster Inc.

References

- 1) Maynard CL, Elson CO, Hatton RD and Weaver CT: Reciprocal interactions of the intestinal microbiota and

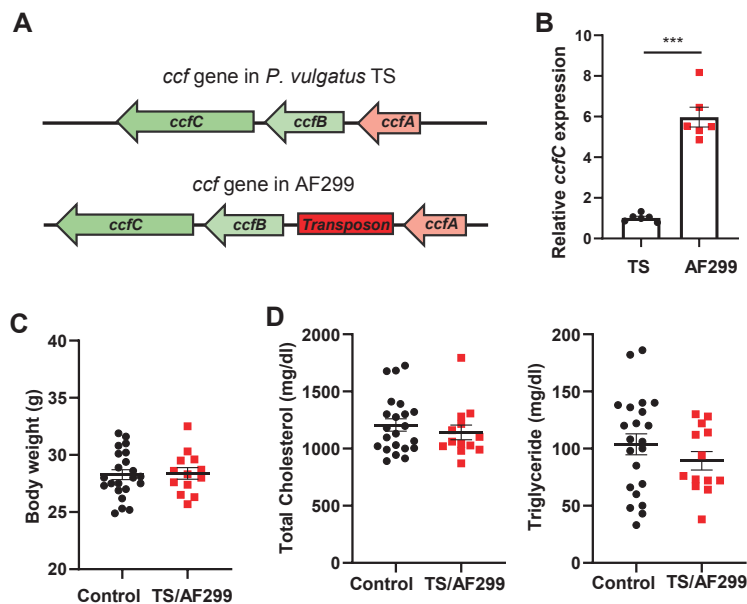
- immune system. *Nature*, 2012; 489: 231-241
- 2) Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, Wu Y, Schauer P, Smith JD, Allayee H, Tang WH, DiDonato JA, Lusis AJ and Hazen SL: Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*, 2011; 472: 57-63
- 3) Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, Smith JD, DiDonato JA, Chen J, Li H, Wu GD, Lewis JD, Warrier M, Brown JM, Krauss RM, Tang WH, Bushman FD, Lusis AJ and Hazen SL: Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med*, 2013; 19: 576-585
- 4) Emoto T, Yamashita T, Kobayashi T, Sasaki N, Hirota Y, Hayashi T, So A, Kasahara K, Yodoi K, Matsumoto T, Mizoguchi T, Ogawa W and Hirata KI: Characterization of gut microbiota profiles in coronary artery disease patients using data mining analysis of terminal restriction fragment length polymorphism: gut microbiota could be a diagnostic marker of coronary artery disease. *Heart Vessels*, 2017; 32: 39-46
- 5) Emoto T, Yamashita T, Sasaki N, Hirota Y, Hayashi T, So A, Kasahara K, Yodoi K, Matsumoto T, Mizoguchi T, Ogawa W and Hirata K: Analysis of Gut Microbiota in Coronary Artery Disease Patients: a Possible Link between Gut Microbiota and Coronary Artery Disease. *J Atheroscler Thromb*, 2016; 23: 908-921
- 6) Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Dore J, Meta HITC, Antolin M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariatz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Merieux A, Melo Minardi R, M'Rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD and Bork P: Enterotypes of the human gut microbiome. *Nature*, 2011; 473: 174-180
- 7) Jie Z, Xia H, Zhong SL, Feng Q, Li S, Liang S, Zhong H, Liu Z, Gao Y, Zhao H, Zhang D, Su Z, Fang Z, Lan Z, Li J, Xiao L, Li J, Li R, Li X, Li F, Ren H, Huang Y, Peng Y, Li G, Wen B, Dong B, Chen JY, Geng QS, Zhang ZW, Yang H, Wang J, Wang J, Zhang X, Madsen L, Brix S, Ning G, Xu X, Liu X, Hou Y, Jia H, He K and Kristiansen K: The gut microbiome in atherosclerotic cardiovascular disease. *Nat Commun*, 2017; 8: 845
- 8) Karlsson FH, Fak F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, Backhed F and Nielsen J: Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nat Commun*, 2012; 3: 1245
- 9) García-López M, Meier-Kolthoff JP, Tindall BJ, Gronow

- S, Woyke T, Kyrpides NC, Hahnke RL and Göker M: Analysis of 1,000 Type-Strain Genomes Improves Taxonomic Classification of Bacteroidetes. *Front Microbiol*, 2019; 10: 2083
- 10) Yoshida N, Emoto T, Yamashita T, Watanabe H, Hayashi T, Tabata T, Hoshi N, Hatano N, Ozawa G, Sasaki N, Mizoguchi T, Amin HZ, Hirota Y, Ogawa W, Yamada T and Hirata KI: *Bacteroides vulgatus* and *Bacteroides dorei* Reduce Gut Microbial Lipopolysaccharide Production and Inhibit Atherosclerosis. *Circulation*, 2018; 138: 2486-2498
 - 11) Vatanen T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, Kolde R, Vlamakis H, Arthur TD, Hamalainen AM, Peet A, Tillmann V, Uibo R, Mokurov S, Dorshakova N, Ilonen J, Virtanen SM, Szabo SJ, Porter JA, Lahdesmaki H, Huttenhower C, Gevers D, Cullen TW, Knip M, Group DS and Xavier RJ: Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell*, 2016; 165: 842-853
 - 12) Saito Y, Yamashita T, Yoshida N, Emoto T, Takeda S, Tabata T, Shinohara M, Kishino S, Sugiyama Y, Kitamura N, Yamamoto H, Takaya T, Ogawa J and Hirata KI: Structural differences in bacterial lipopolysaccharides determine atherosclerotic plaque progression by regulating the accumulation of neutrophils. *Atherosclerosis*, 2022; 358: 1-11
 - 13) Schmidt TSB, Raes J and Bork P: The Human Gut Microbiome: From Association to Modulation. *Cell*, 2018; 172: 1198-1215
 - 14) Park SY, Rao C, Coyte KZ, Kuziel GA, Zhang Y, Huang W, Franzosa EA, Weng JK, Huttenhower C and Rakoff-Nahoum S: Strain-level fitness in the gut microbiome is an emergent property of glycans and a single metabolite. *Cell*, 2022; 185: 513-529 e521
 - 15) Lee SM, Donaldson GP, Mikulski Z, Boyajian S, Ley K and Mazmanian SK: Bacterial colonization factors control specificity and stability of the gut microbiota. *Nature*, 2013; 501: 426-429
 - 16) Sequeira RP, McDonald JAK, Marchesi JR and Clarke TB: Commensal *Bacteroidetes* protect against *Klebsiella pneumoniae* colonization and transmission through IL-36 signalling. *Nat Microbiol*, 2020; 5: 304-313
 - 17) Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH and Phillippy AM: Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Res*, 2017; 27: 722-736
 - 18) Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, Cuomo CA, Zeng Q, Wortman J, Young SK and Earl AM: Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One*, 2014; 9: e112963
 - 19) Tanizawa Y, Fujisawa T and Nakamura Y: DFAST: a flexible prokaryotic genome annotation pipeline for faster genome publication. *Bioinformatics*, 2017; 34: 1037-1039
 - 20) Buchfink B, Reuter K and Drost HG: Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nat Methods*, 2021; 18: 366-368
 - 21) Pan H, Xue C, Auerbach BJ, Fan J, Bashore AC, Cui J, Yang DY, Trignano SB, Liu W, Shi J, Ihuegbu CO, Bush EC, Worley J, Vlahos L, Laise P, Solomon RA, Connolly ES, Califano A, Sims PA, Zhang H, Li M and Reilly MP: Single-Cell Genomics Reveals a Novel Cell State During Smooth Muscle Cell Phenotypic Switching and Potential Therapeutic Targets for Atherosclerosis in Mouse and Human. *Circulation*, 2020; 142: 2060-2075
 - 22) Donaldson GP, Ladinsky MS, Yu KB, Sanders JG, Yoo BB, Chou WC, Conner ME, Earl AM, Knight R, Bjorkman PJ and Mazmanian SK: Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science*, 2018; 360: 795-800
 - 23) Wezel A, Lagrèauw HM, van der Velden D, de Jager SC, Quax PH, Kuiper J and Bot I: Mast cells mediate neutrophil recruitment during atherosclerotic plaque progression. *Atherosclerosis*, 2015; 241: 289-296
 - 24) Trabetti E, Biscuola M, Cavallari U, Malerba G, Girelli D, Olivieri O, Martinelli N, Corrocher R and Pignatti PF: On the association of the oxidised LDL receptor 1 (OLR1) gene in patients with acute myocardial infarction or coronary artery disease. *Eur J Hum Genet*, 2006; 14: 127-130
 - 25) Sawamura T, Wakabayashi I and Okamura T: LOX-1 in atherosclerotic disease. *Clin Chim Acta*, 2015; 440: 157-163
 - 26) Kawabe T, Suzuki N, Yamaki S, Sun SL, Asao A, Okuyama Y, So T, Iwakura Y and Ishii N: Mesenteric lymph nodes contribute to proinflammatory Th17-cell generation during inflammation of the small intestine in mice. *Eur J Immunol*, 2016; 46: 1119-1131
 - 27) Macpherson AJ and Smith K: Mesenteric lymph nodes at the center of immune anatomy. *J Exp Med*, 2006; 203: 497-500
 - 28) Mowat AM: Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol*, 2003; 3: 331-341
 - 29) Goto Y, Panea C, Nakato G, Cebula A, Lee C, Diez MG, Laufer TM, Ignatowicz L and Ivanov, II: Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity*, 2014; 40: 594-607
 - 30) Kato LM, Kawamoto S, Maruya M and Fagarasan S: The role of the adaptive immune system in regulation of gut microbiota. *Immunol Rev*, 2014; 260: 67-75
 - 31) Korn T, Bettelli E, Oukka M and Kuchroo VK: IL-17 and Th17 Cells. *Annu Rev Immunol*, 2009; 27: 485-517
 - 32) Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov, II, Umesaki Y, Itoh K and Honda K: Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science*, 2011; 331: 337-341
 - 33) Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, Glickman JN and Garrett WS: The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science*, 2013; 341: 569-573
 - 34) Atarashi K, Tanoue T, Ando M, Kamada N, Nagano Y, Narushima S, Suda W, Imaoka A, Setoyama H, Nagamori T, Ishikawa E, Shima T, Hara T, Kado S, Jinnohara T, Ohno H, Kondo T, Toyooka K, Watanabe E, Yokoyama S, Tokoro S, Mori H, Noguchi Y, Morita H, Ivanov, II, Sugiyama T, Nuñez G, Camp JG, Hattori M, Umesaki Y

- and Honda K: Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell*, 2015; 163: 367-380
- 35) Kawano Y, Edwards M, Huang Y, Bilate AM, Araujo LP, Tanoue T, Atarashi K, Ladinsky MS, Reiner SL, Wang HH, Mucida D, Honda K and Ivanov, II: Microbiota imbalance induced by dietary sugar disrupts immune-mediated protection from metabolic syndrome. *Cell*, 2022; 185: 3501-3519.e3520
 - 36) Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K and Littman DR: Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*, 2009; 139: 485-498
 - 37) Chiba T and Seno H: Indigenous clostridium species regulate systemic immune responses by induction of colonic regulatory T cells. *Gastroenterology*, 2011; 141: 1114-1116
 - 38) Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, Wu Y and Hazen SL: Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med*, 2013; 368: 1575-1584
 - 39) Rath S, Rud T, Pieper DH and Vital M: Potential TMA-Producing Bacteria Are Ubiquitously Found in Mammalia. *Front Microbiol*, 2019; 10: 2966

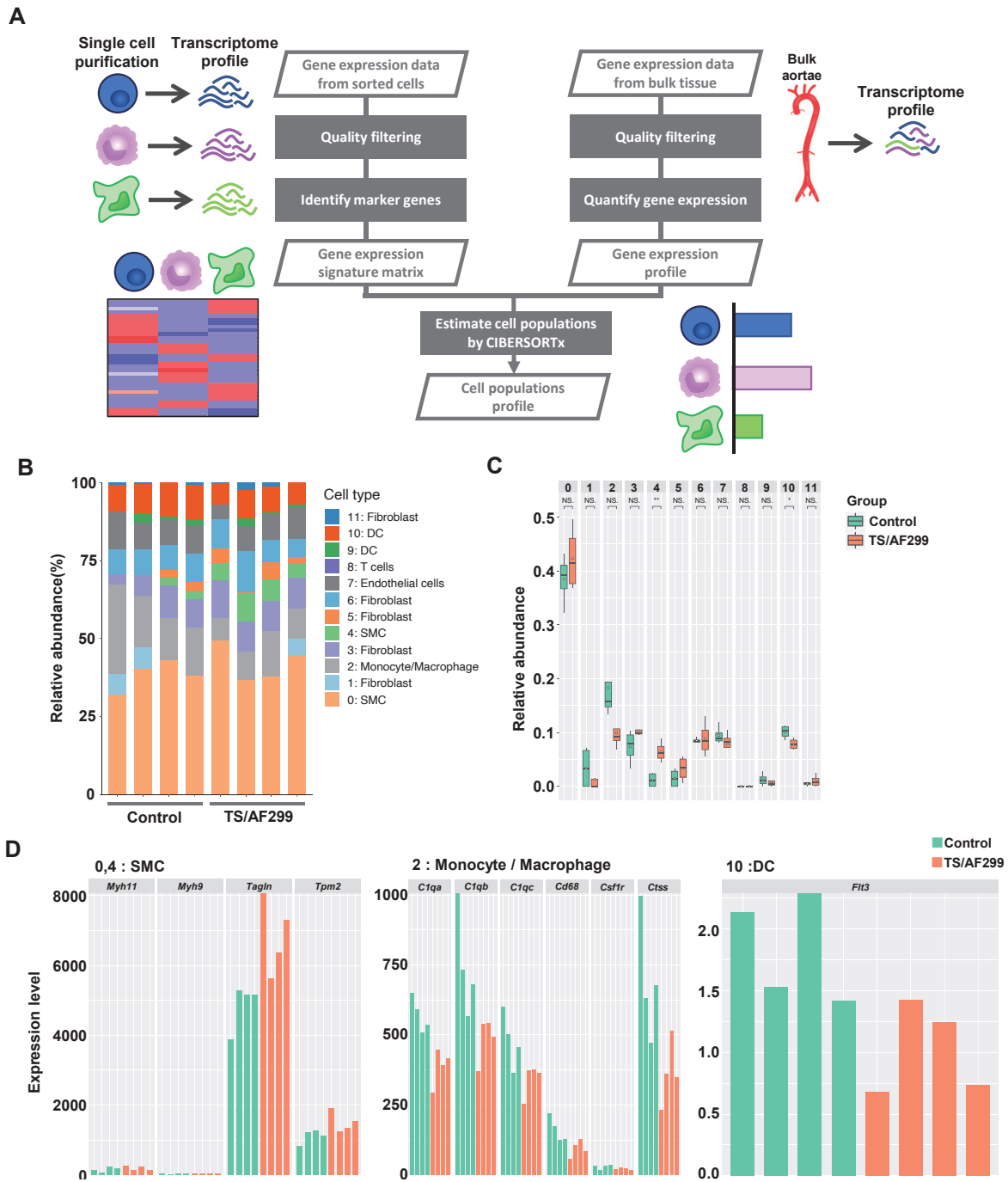
Supplemental Table 1. Flow Antibodies

Target	Fluorophore/Conjugate	Company	Catalogue Number
Ly6G	PE	BD Biosciences	551461
CD68	Alexa Fluor 647	BioLegend	137004
CD4	PE-Cy7	BD Biosciences	552775
CD8	FITC	BD Biosciences	553031
CD45	APC-Cy7	BD Biosciences	557659
CD44	PE	BD Biosciences	553134
CD62L	BV421	BioLegend	104436
CD11b	BV650	BD Biosciences	563402
IL-17	BV421	BioLegend	506925
Foxp3	APC	Invitrogen	17-5773-82
MHC	Alexa Fluor 647	BioLegend	107617
MHC Class II (I-A/I-E)	A700	BioLegend	107622
TCRb	APC-Cy7	BD Biosciences	560656
IFN γ	PE	Invitrogen	12-7311-82
Ly6C	Alexa Fluor 700	BioLegend	128024
Ly6C	PE/Cyanine7	BioLegend	128018

**Supplemental Fig. 1.** Genomic analysis of *P. vulgatus* TS and AF299 was performed, comparing genetic variations within the *ccf* gene region

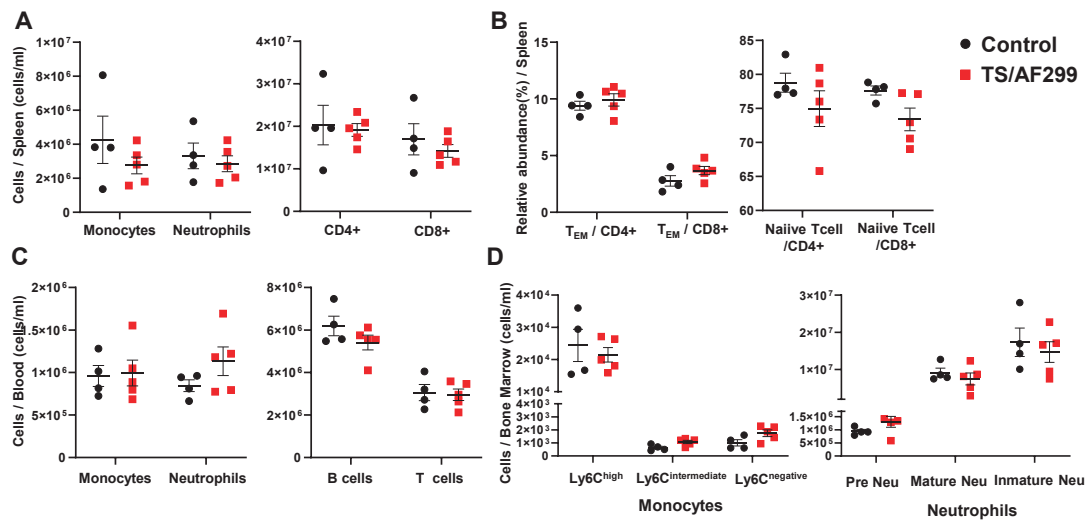
No predominant differences in body weight, total cholesterol, or triglyceride levels were observed after TS/AF299 administration.

A. Genomic organization of the *ccf* locus between *P. vulgatus* TS and AF299. B. Comparison of *ccf* gene expression between *P. vulgatus* TS and AF299. $n = 6$, Student's *t*-test. C. Body weight at 14 weeks of age. Control, $n = 22$; TS/AF299, $n = 13$; Mann–Whitney *U* test, control. D. Comparison of plasma lipid profiles (total cholesterol and triglyceride levels) at 14 weeks of age. Control, $n = 22$; TS/AF299, $n = 13$; Mann–Whitney *U* test. B–D: Error bars represent the standard error of the mean. *** $P < 0.001$



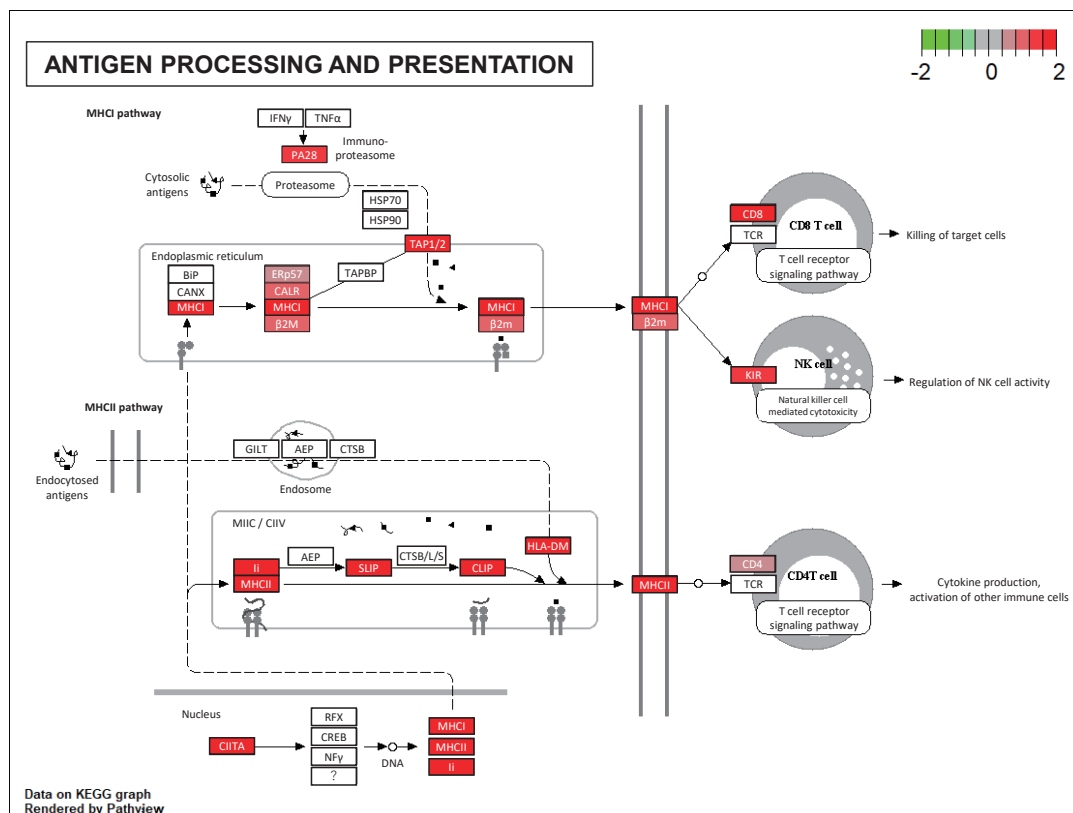
Supplemental Fig. 2. Alterations and corroboration of immune cells in atherosclerotic lesions via bulk RNA sequencing of mouse aortic sections

A. Cell population characterization was performed using single-cell and bulk RNA analyses of arteries with atherosclerotic foci. From these results, the estimates of the abundance of member cell types within the mixed cell population were calculated. B, C. Relative abundance of different cell types in the arterial region as determined via cell population characterization. Control, $n = 4$; TS/AF299, $n = 4$. D. Comparison of the gene expression levels in smooth muscle cells (SMCs), monocytes, macrophages, and dendritic cells (DCs).



Supplemental Fig. 3. No changes were observed in the number of immune cells in the spleen, blood, and bone marrow tissues after the administration of TS/AF299

A, B. Quantification of the number of monocytes and neutrophils in the spleen. The proportions of CD4⁺ and CD8⁺ cells in effector and naïve T cells were also determined. C. Quantitation of monocytes, neutrophils, and lymphocytes in the blood. D. Subsets of Ly6C-high, -intermediate, and -negative monocytes in the bone marrow monocytes and neutrophils. A–D: Control, *n* = 4; TS/AF299, *n* = 5; statistical analysis was performed using the Student's *t*-test. Error bars represent the standard error of the mean.



Supplemental Fig. 4. Visualization of the genes whose expression was significantly upregulated by TS/AF299 treatment in the antigen processing and presentation section of the KEGG pathway

Based on the results of the bulk RNA-seq analysis of the ileum of TS/AF299-treated mice, pathways predominantly associated with groups of genes and signaling molecules with variable expression were examined. The upper right bar shows the increase or decrease in the expression level of each gene relative to the control. The data represent logarithmic (log₂)-transformed fold changes.