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ORIGINAL RESEARCH

Intestinal Microbiota Contributes to the Development of Cardiovascular Inflammation and Vasculitis in Mice

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BACKGROUND: Alterations in the intestinal microbiota contribute to the pathogenesis of various cardiovascular disorders, but how they affect the development of Kawasaki disease (KD) an acute pediatric vasculitis, remains unclear.

METHODS: We used the *Lactobacillus casei* cell wall extract (LCWE) murine model of KD vasculitis to assess the contribution of the intestinal microbiota to the development of vascular inflammation. We evaluated the severity of vasculitis in microbiota-depleted mice. 16S rRNA gene sequencing was used to characterize the fecal microbiome composition of LCWE-injected mice. Some groups of mice were orally treated with selected live or pasteurized bacteria, short-chain fatty acids, or Amuc_1100, the Toll-like receptor 2 signaling outer membrane protein from *Akkermansia muciniphila*, and their impact on vasculitis development was assessed.

RESULTS: We report that depleting the gut microbiota reduces the development of cardiovascular inflammation in a murine model mimicking KD vasculitis. The development of cardiovascular lesions was associated with alterations in the intestinal microbiota composition and, notably, a decreased abundance of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*. Oral supplementation with either of these live or pasteurized individual bacteria or with short-chain fatty acids produced by them attenuated cardiovascular inflammation, as reflected by decreased local immune cell infiltrations. Treatment with Amuc_1100 also reduced the severity of vascular inflammation.

CONCLUSIONS: This study reveals an underappreciated gut microbiota-cardiovascular inflammation axis in KD vasculitis pathogenesis and identifies specific intestinal commensals that regulate vasculitis in mice by producing metabolites or via extracellular proteins capable of enhancing and supporting gut barrier function.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: coronary aneurysm ■ coronary vessels ■ gastrointestinal microbiome ■ interleukin-1 ■ vasculitis

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Kawasaki disease (KD) is an acute febrile pediatric vasculitis that leads to coronary artery (CA) aneurysms in up to 25% of untreated children and is the primary cause of acquired heart disease in children in the United States.[1] Timely treatment with intravenous immunoglobulin G (IVIG) significantly reduces the incidence of CA aneurysm development.[1] However, up to 20% of patients with KD are resistant to this treatment, have a higher risk of developing cardiac complications, and require additional therapies.[1] Although the KD

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Novelty and Significance

What Is Known?

- Kawasaki disease (KD) is an acute febrile pediatric vasculitis affecting small- to medium-sized vessels and the leading cause of acquired heart disease in children in the United States. Gastrointestinal symptoms are often reported during the acute phase of the disease.
- The gastrointestinal tract is home to trillions of microorganisms, collectively known as the intestinal microbiota. This microbiota contributes to various host biological processes locally and at distant organs. Alterations in the intestinal microbiota composition have been linked to the onset of inflammatory disorders and cardiovascular diseases.
- Changes in the composition of fecal bacterial communities, characterized by the blooming of pathogenic bacteria and decreased relative abundance of short-chain fatty acid (SCFA)–producing bacteria, have been reported during the KD acute phase. However, the functional implications of these alterations remain unknown.

What New Information Does This Article Contribute?

- The absence or depletion of the microbiota decreases the severity of vasculitis in the *Lactobacillus casei* cell wall extract murine model, which mimics human KD immunopathological features.
- Alterations in the intestinal microbiota composition are present in mice developing vasculitis, characterized by the increased relative abundance of *Bilophila wadsworthia* and *Bacteroides fragilis* and reduced relative abundance of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*.
- Supplementation with *B wadsworthia* and *B fragilis* tended to exacerbate murine *Lactobacillus casei* cell wall extract–induced KD vasculitis.
- Oral supplementation with live or pasteurized *A muciniphila* and *F prausnitzii*, or with SCFAs known to be produced by these bacteria, or Amuc_1100, the TLR-2 (toll-like receptor 2) signaling outer membrane protein of *A muciniphila*, was sufficient to alleviate the development of cardiovascular lesions and was associated with decreased immune cell infiltrates in vascular tissues along with improved expression of markers commonly linked with dysfunctional intestinal barrier.

The intestinal microbiota affects a wide range of host biological processes, including maintaining intestinal barrier function and regulating immune responses. Intestinal dysbiosis, or changes in the composition of intestinal bacteria communities, has been associated with the development of inflammatory, metabolic, and cardiovascular disorders. KD is the leading cause of acquired heart disease in children in the United States, and if untreated, it can lead to the development of coronary artery lesions. Gastrointestinal manifestations, such as diarrhea, vomiting, and abdominal pain, are present during the acute phase of the disease. Furthermore, recent studies report alterations in the fecal microbiota composition of patients with KD, often characterized by decreased presence of SCFA-producing bacteria. However, the functional contribution of these alterations to KD immunopathology remains unknown. In this study, we aimed to address this question using a murine model that mimics the main features of human KD. We observed that the absence of microbiota in germ-free mice or antibiotic treatment of mice decreased the severity of vasculitis. We also report the presence of alterations in the fecal bacterial composition of mice developing vasculitis, and the modulation of the gut microbiota impacts disease outcome. The relative abundance of the SCFA producers *A muciniphila* and *F prausnitzii* was decreased in mice developing cardiovascular lesions, and oral supplementation with either one of these bacteria in a live or pasteurized form significantly improved disease outcome. Furthermore, supplementation with the SCFA butyrate, acetate or propionate, or Amuc_1100, an outer membrane protein from *A muciniphila* acting via TLR-2, also decreased the severity of cardiovascular lesions in this murine model of KD. These studies highlight a gut-vascular axis in the context of KD, identify mechanisms by which alterations in the gut microbiota contribute to the development of cardiovascular lesions in a mouse model of KD vasculitis, and suggest that modulation of the gut microbiota composition or supplementation with specific bacteria-derived products can improve disease outcomes.

etiological agent(s) remains unidentified, the disease epidemiology, clinical presentation, and associated immune response indicate an infectious origin with a mucosal portal of entry, possibly through airway epithelial cells or the intestinal barrier.[2–4] KD is associated with systemic inflammation in several organs, including the liver and gastrointestinal tract, and aneurysm development in medium-sized peripheral arteries.[1] Infiltration of innate immune cells have been reported in the inflamed CA of patients with KD, which co-occurs with elevated circulating levels of proinflammatory cytokines.[5–8] Evidence from both human studies and murine models that mimic KD strongly supports the significant role of IL (interleukin)-1 in driving its pathology (reviewed in the studies by Burns et al[9] and Atici et al[10]). Indeed, a phase II open-label study indicates that anakinra, an IL-1 receptor antagonist, is well tolerated and may contribute to the reduction of fever, inflammation parameters, and CA dilations

Nonstandard Abbreviations and Acronyms

BMDM	bone marrow–derived macrophage
CA	coronary artery
FLICA	fluorochrome-labeled inhibitors of caspases
GF	germ-free
HDAC	histone deacetylase
IL	interleukin
IVIG	intravenous immunoglobulin G
KD	Kawasaki disease
LCWE	*Lactobacillus casei* cell wall extract
SCFA	short-chain fatty acid
SPF	specific pathogen-free
TJ	tight junction
WT	wild type

in patients with IVIG-resistant KD[11] and is currently being tested in a randomized phase III clinical trial.[12]

The gut microbiota modulates homeostasis and immune responses in the gastrointestinal tract and distant organs by producing pathogen-associated molecular patterns and metabolites, such as short-chain fatty acids (SCFAs).[13] While gastrointestinal symptoms are not considered for KD diagnosis, abdominal pain, diarrhea, and vomiting are often reported at KD onset.[1,14] These gastrointestinal manifestations frequently complicate diagnosis, delay adequate treatment, and correlate with treatment resistance and the development of severe CA aneurysms.[14,15] Significant efforts have been made to determine whether patients with KD exhibit altered intestinal microbiota composition and to identify signature microbiota features associated with this disease (reviewed in the study by Jena et al[16]). Initial culture-based studies from fecal samples and jejunal biopsies of patients with KD indicated the loss of the *Lactobacillus* genus and the presence of Streptococci and Staphylococci.[17,18] A metagenomic study analyzing feces from patients with KD revealed alterations in the intestinal microbiota composition during the acute phase of the disease and showed that the KD convalescence phase was associated with a higher relative abundance of SCFA-producing bacteria.[19] However, the reported alterations may reflect an antibiotic-mediated effect, as most enrolled patients were treated with antibiotic pre-KD diagnosis.[19] A 16S rRNA gene sequencing–based longitudinal profiling of fecal microbiota communities of nonantibiotic-treated patients with KD also demonstrated that the relative abundance of bacteria belonging to the *Enterococcus*, *Acinetobacter*, *Helicobacter*, *Lactococcus*, *Staphylococcus*, and *Butyricimonas* genera was increased during acute KD compared with age-matched healthy controls.[20] Interestingly, several genera known to be SCFA producers were enriched in healthy controls and convalescent IVIG-treated patients with KD compared with patients with acute KD.[20] However, studies investigating whether an altered microbiota composition is linked to KD development and pathogenesis are constrained by several limitations, such as the lack of a febrile control group, few of them account for potential antibiotic treatment preceding KD diagnosis, and most have small sample sizes per group.[16] Thus, while perturbations of the intestinal microbiota composition and function have been associated with inflammatory, autoimmune, metabolic, and cardiovascular disorders, the functional contributions of microbiota alterations to KD pathogenesis remain unknown.

Due to the overlapping clinical symptoms between KD and other pediatric infectious diseases, patients with KD often receive antibiotic treatment prediagnosis.[21–24] This treatment is ineffective and has even been associated with an increased risk of IVIG resistance and higher rates of CA development.[21,24,25] Antibiotics persistently impact the composition and function of the intestinal microbiota.[26] Thus, these clinical findings support the hypothesis that microbiota composition may be a key determinant of KD pathogenesis.

Here, we examined the functional impact of the intestinal microbiota on KD using the *Lactobacillus casei* cell wall extract (LCWE)-induced KD vasculitis mouse model, which recapitulates key immunopathologic features of human KD, including coronary arteritis, aortitis, and luminal myofibroblast proliferation in the CA lumen.[3,27–31] LCWE-induced KD is an immune-mediated vasculitis characterized by the rapid influx of innate and adaptive immune cells into the inflamed vascular tissues.[29–32] Monocytes, macrophages, and dendritic cells surrounding the inflamed CA and infiltrating the dilated abdominal aorta of LCWE-injected mice produce IL-1β.[29–31] In this model, vascular smooth muscle cells, which coexpress *Il1r1*, *Il1rap*, and *Myd88*, respond to IL-1β by phenotypically switching to a synthetic phenotype, expressing fibroblast-related transcripts, matrix metalloproteinase, chemokines, and cytokines involved in the recruitment of immune cells into the tissues.[29]

Here, we found that depletion of the gut microbiota in mice by antibiotic treatment, or absence of microbiota in germ-free (GF) mice, attenuated the severity of LCWE-induced KD cardiovascular lesions. We observed alterations in the intestinal microbiota composition of mice after LCWE injection and evaluated the causal roles of these changes in promoting or decreasing the severity of vasculitis. We report a reduced relative abundance of the SCFA-producing species *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, which correlate with the severity of LCWE-induced vasculitis. Supplementing either live or pasteurized *A muciniphila* or *F prausnitzii*, or acetate, propionate, butyrate, or Amuc_1100, a TLR-2 signaling protein present in the outer membrane of *A*

muciniphila, attenuated the severity of LCWE-induced KD cardiovascular inflammation, which correlated with improvements in biomarkers of intestinal barrier function. Together, these results highlight the critical role of the gut microbiota in modulating cardiovascular inflammation in the LCWE model and suggest potential therapeutic strategies for KD.

METHODS

Data Availability

Data supporting this study's findings are available from the corresponding authors upon reasonable request. An expanded Materials and Methods section can be found in the Supplemental Material.

RESULTS

The Gut Microbiota Promotes LCWE-Induced KD Vasculitis

We used the LCWE-induced KD vasculitis mouse model to assess the potential contributions of the gut microbiota to vasculitis development. LCWE-injected specific pathogen-free (SPF) wild-type (WT) mice developed heart vessel inflammation characterized by aortitis, coronary arteritis, and abdominal aorta dilations (Figure 1A and 1B), as previously described.[27–33] To gain insight into whether the gut microbiota modulates vascular inflammation in this model, we assessed the severity of LCWE-induced KD vasculitis in microbiota-depleted SPF mice treated with antibiotics (Figure 1C and 1D; Figure S1A). SPF mice were provided an antibiotic cocktail in the drinking water either as a prophylactic treatment, given 1 week before LCWE injection, or as a sustained antibiotic treatment, starting 3 days before LCWE injection until the experimental end point (Figure S1A). Both prophylactic and sustained antibiotic treatments resulted in a significant depletion of intestinal bacteria in the feces of treated animals (Figure S1B). Whereas LCWE-injected SPF mice developed severe heart vasculitis and abdominal aorta dilation and aneurysms, prophylactic and sustained antibiotic treatments were both associated with decreased severity of LCWE-induced heart vessel inflammation and development of abdominal aorta dilations (Figure 1C and 1D).

Antibiotic treatment during pregnancy limits microbiota diversity and its transfer from pregnant dams to their offspring. Therefore, in another set of experiments, we treated pregnant SPF dams with antibiotics, and their offspring were kept under the same treatment until LCWE injection at 5 weeks of age (Figure S1C). This resulted in the depletion of intestinal bacteria in the feces of both antibiotic-treated pregnant dams and their offspring (Figure S1D). Furthermore, the offspring born from antibiotic-treated dams also exhibited decreased severity of LCWE-induced vasculitis, characterized by reduced heart inflammation and development of abdominal aorta dilations (Figure S1E and S1F). These results indicate that microbiota depletion decreases the severity of LCWE-induced KD vasculitis.

To further determine whether mice reared in the absence of microbiota (GF) were also protected, we next assessed the development of LCWE-induced cardiovascular inflammation in GF mice (Figure 1E and 1F). Indeed, the severity of cardiovascular lesions was significantly reduced in LCWE-injected GF mice compared with SPF mice (Figure 1E and 1F). Because mucosal and systemic immune responses are underdeveloped in GF mice,[34] we next tested the effect of LCWE injection in GF mice colonized with the altered Schaedler flora, a community consisting of 8 murine bacteria known to support typical gut structure and immune system development in mice.[35] In this model, the severity of LCWE-induced KD vasculitis was significantly decreased compared with SPF mice (Figure S1G and S1H). These results reveal a crucial role of the intestinal microbiota in promoting the development of cardiovascular lesions in the LCWE-induced KD vasculitis in mice.

LCWE-Induced KD Vasculitis Is Associated With Alterations in Gut Microbiota Composition

We next used 16S rRNA gene sequencing to determine changes in fecal microbiota composition associated with LCWE-induced KD vasculitis. Principal coordinate analysis, as well as linear discriminant analysis effect size, indicated changes in the gut microbiota composition at 2 weeks post-LCWE injection compared with baseline (day 0; Figure 2A; Figure S2A). LCWE-injected mice also showed an increased number of bacterial species richness (alpha diversity) and a higher number of observed operational taxonomic units than baseline mice, which may indicate some bacteria blooming (Figure 2B). Indeed, at the phylum level, we observed an increased relative abundance of Firmicutes and Proteobacteria and a decreased relative abundance of Actinobacteria and Verrucomicrobia in the feces of LCWE-injected mice developing robust lesions (Figure 2C). To determine whether either Firmicutes, mainly composed of Gram-positive bacteria, or Proteobacteria, mainly Gram-negative bacteria, contribute to LCWE-induced KD vasculitis, SPF mice received either colistin or vancomycin in the drinking water to deplete Gram-negative or Gram-positive bacteria, respectively (Figure S2B and S2C). Both colistin and vancomycin reduced the severity of LCWE-induced heart inflammation, indicating that Gram-positive and Gram-negative bacteria equally contribute to the development of LCWE-induced cardiovascular lesions (Figure S2B and S2C). The relative abundance of several bacterial families and genera was significantly altered in the LCWE-injected group (Figure 2D through 2F; Figure S2D).

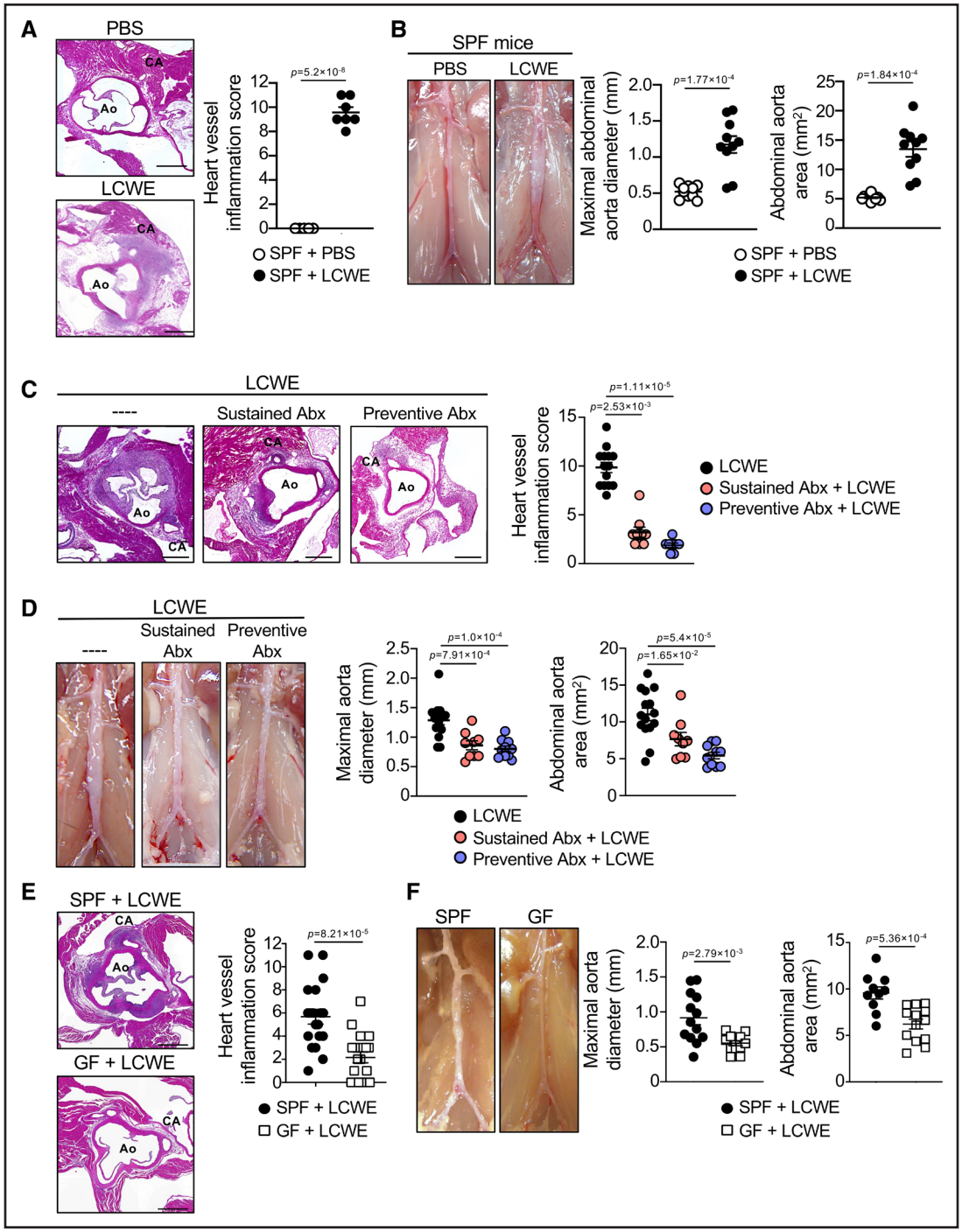

Figure 1. The intestinal microbiota promotes *Lactobacillus casei* cell wall extract (LCWE)–induced Kawasaki disease (KD) vasculitis.

A, Representative hematoxylin and eosin (H&E)–stained heart sections and heart vessel inflammation score of specific pathogen-free (SPF) mice injected with either PBS or LCWE (n=5–7/group). Scale bars, 500 μm. **B**, Representative pictures of the abdominal aorta area, maximal abdominal aorta diameter, and abdominal aorta area measurements of SPF wild-type (WT) mice injected with either PBS or LCWE (n=10/group). **C**, Representative H&E-stained heart sections and heart vessel inflammation score of LCWE-injected SPF mice and LCWE-injected SPF mice with preventative or sustained antibiotic (Abx) treatment (n=8–14/group). Scale bars, 500 μm. **D**, Pictures of the abdominal aorta area, maximal abdominal aorta diameter, and abdominal aorta area measurements of LCWE-injected SPF mice and LCWE-injected SPF mice with preventative or sustained Abx treatment (n=9–15/group). **E**, Representative H&E-stained heart sections and heart vessel inflammation score of LCWE-injected SPF and germ-free (GF) mice (n=19/group). Scale bars, 500 μm. **F**, Representative pictures of the abdominal aorta area, maximal abdominal aorta diameter, and abdominal aorta area measurements of SPF and GF mice injected with LCWE (n=10–13/group). Data are presented as mean±SEM and representative of at least 3 independent experiments (**A** and **B**) or compiled from 2 to 3 independent experiments (**C**–**F**). Each symbol represents an individual mouse. *P* values obtained by the unpaired *t* test with the Welch's correction (**A**, **B**, and **E**), the unpaired *t* test (**F**), the One-way ANOVA with the Kruskal-Wallis test with Dunn's multiple comparison tests (**C**), and One-way ANOVA with Tukey's multiple comparison tests (**D**). Ao indicates aorta in H&E-stained heart sections; and CA, coronary artery.

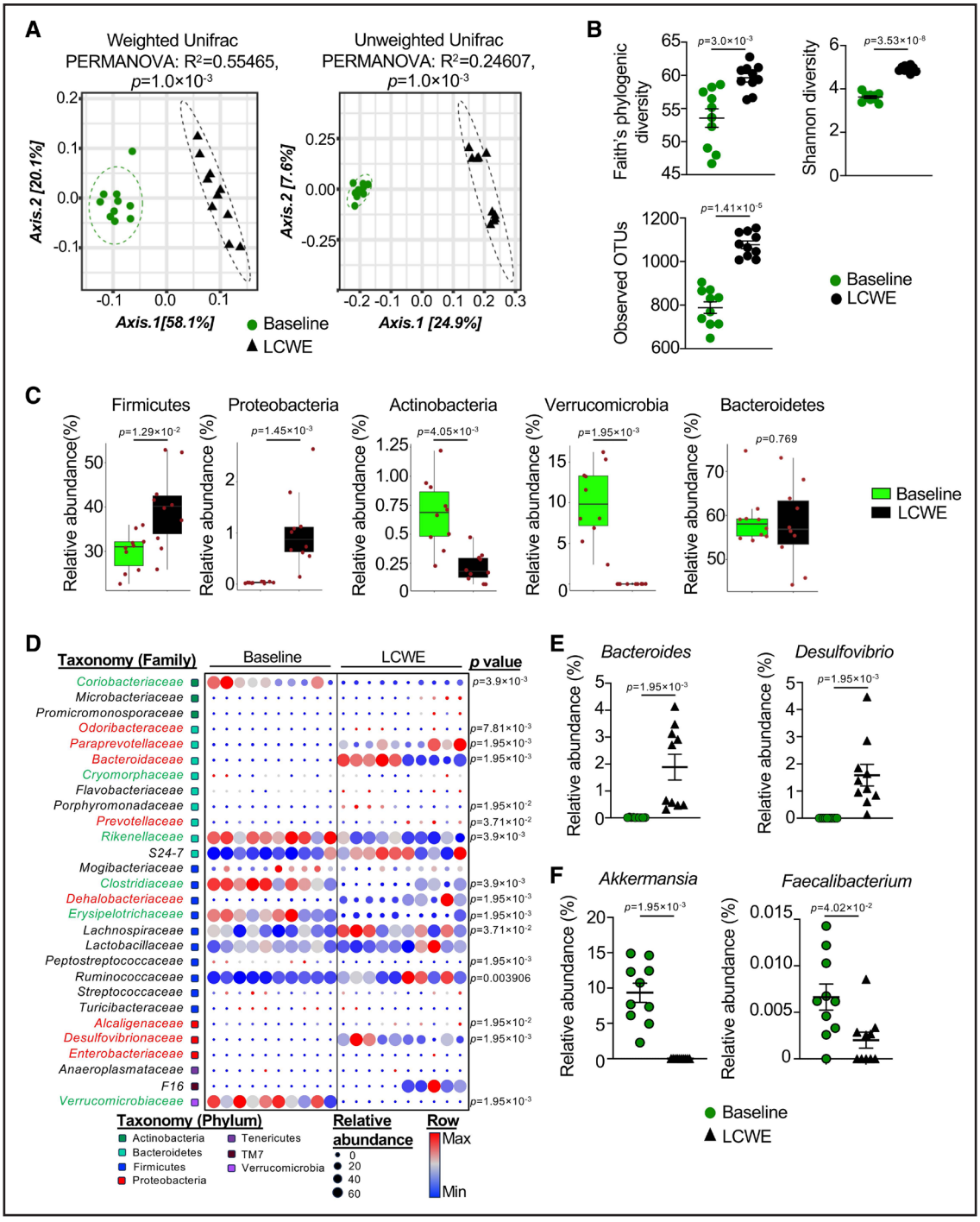

Figure 2. Intestinal microbiota composition changes associated with *Lactobacillus casei* cell wall extract (LCWE)–induced Kawasaki disease (KD) vasculitis.

A, Principal coordinates analysis (PCoA) plots of weighted (**left**) and unweighted (**right**) UniFrac distance based on 16S rRNA gene sequencing of feces from specific pathogen-free (SPF) mice before (baseline) and 2 weeks after LCWE injection (n=10/group). **B**, Alpha diversity index from 16S rRNA gene sequencing of feces from SPF mice before (baseline) and 2 weeks after LCWE injection. **C**, Relative abundances of the phyla Firmicutes, Proteobacteria, Actinobacteria, Verrucomicrobia, and Bacteroidetes in feces of SPF mice at baseline and 2 weeks after LCWE injection based on 16S sequencing results (n=10/group). **D**, Bubble plot showing bacterial relative abundance at the family level based on the 16S rRNA gene sequencing of feces of SPF mice at baseline and 2 weeks post-LCWE injection (n=10/group). The red-to-blue gradient indicates the maximum and minimum strengths of association between a bacterial family at either baseline or after LCWE injection. Green and red labeling of bacterial family names indicates significantly decreased or increased relative abundances, respectively. **E** and **F**, Relative abundances of genera *Bacteroides*, *Desulfovibrio*, *Akkermansia*, and *Faecalibacterium* in the fecal pellets from mice at baseline and 2 weeks after LCWE injection based on 16S sequencing results (n=10 mice/group). Data presented as mean±SEM (**B**, **E**, and **F**) and median±interquartile range (**C**). Each symbol represents an individual mouse. *P* values obtained by permutational multivariate analysis of variance (PERMANOVA) (**A**), paired *t* test (**B** and **C**), and Wilcoxon rank-sum test (**D**, **E**, and **F**).

Interestingly, we observed an increased relative abundance of members from the *Desulfovibrionaceae*, *Prevotellaceae*, and *Bacteroidaceae* families in LCWE-injected mice (Figure 2D). *Bilophila wadsworthia*, which belongs to the Proteobacteria phylum and *Desulfovibrionaceae* family, and *Bacteroides fragilis*, a member of the *Bacteroidaceae* family, are both opportunistic pathobionts capable of blooming in the intestinal tract and promoting intestinal inflammation and barrier dysfunctions.[36–38] The relative abundance of bacteria belonging to the *Bacteroides*, *Desulfovibrio*, and *Prevotella* genera was also increased in LCWE-injected mice (Figure 2E; Figure S2D). In contrast, the abundance of bacteria from the *Akkermansia* and *Faecalibacterium* genera was reduced (Figure 2F; Figure S2D). *A muciniphila* (*Akkermansia*) and *F prausnitzii* (*Faecalibacterium*) are known for their anti-inflammatory properties, specifically in the context of host metabolism and intestinal inflammation, respectively.[39–42]

Our 16S rRNA gene sequencing analysis did not include a longitudinal PBS control group, therefore, to determine whether the reported microbiota alterations we observed were specific to LCWE injection and not resulting from temporal changes, fecal pellets from another independent cohort of mice were collected at baseline (day 0) and 2 weeks post-PBS or LCWE injection, and bacterial DNA copy number of *B fragilis*, *B wadsworthia*, *A muciniphila*, and *F prausnitzii* was directly assessed by quantitative PCR analysis (Figure S3A). As expected, LCWE injection induced heart vessel inflammation and abdominal aorta dilations (Figure S3B and S3C). While the abundance of *B fragilis* and *B wadsworthia* increased in the fecal pellets of mice 14 days post-LCWE injection (Figure S3D), the abundance of *A muciniphila* and *F prausnitzii* was reduced in the fecal pellets of LCWE-injected mice (Figure S3E). These changes were unique to LCWE-injected mice developing vasculitis and were not detected in control mice at baseline or 2 weeks post-PBS injection (Figure S3D and S3E). Overall, these results indicate alterations in the intestinal microbiota composition specific to LCWE-induced KD, characterized by the enrichment of bacteria with proinflammatory capacities and the reduction of bacteria with anti-inflammatory effects.

B wadsworthia and *B fragilis* Promote the Severity of LCWE-Induced KD Vasculitis

To determine whether *B wadsworthia* and *B fragilis* functionally promote the development of LCWE-induced KD vasculitis, mice were treated with antibiotics for 1 week to deplete their microbiota and then orally administered every other day with a bacterial mix composed of *B wadsworthia* and *B fragilis*, starting 1 day before LCWE injection and continuing until the experimental end point (Figure S3F). Compared with LCWE-injected antibiotic-treated mice, relative abundances of *B wadsworthia* and *B fragilis* were increased in the feces of antibiotic-treated mice orally supplemented with the bacterial mix (Figure 3A). However, we noticed a bimodal distribution in the levels of fecal *B wadsworthia* and *B fragilis* in the supplemented mice. Indeed, of the 19 mice supplemented with the bacterial mix, 5 mice (blue dots) exhibited low levels of *B wadsworthia*, and among those, 3 mice also exhibited low enrichment in *B fragilis*, similar to the levels detected in antibiotic-treated LCWE-injected mice (Figure 3A). While oral coadministration of *B wadsworthia* and *B fragilis* exacerbated LCWE-induced heart vessel inflammation compared with antibiotic-treated mice (Figure 3B), it did not significantly change the development of abdominal aorta dilations and aneurysms (Figure 3C and 3D). However, when only highly colonized animals (red dots) were analyzed, we observed increased development of abdominal aorta dilations (P=0.0343 for maximal abdominal aorta dilations and P=0.062 for abdominal aorta area; Figure 3D). LCWE-induced KD vascular inflammation is characterized by intense and acute infiltration of immune cells, including neutrophils, monocytes, T cells, and F4/80$^+$ cells with caspase-1 activity (fluorochrome-labeled inhibitors of caspases [FLICA]$^+$), into cardiovascular lesions.[29–31] We next assessed the impact of *B wadsworthia* and *B fragilis* supplementation on the vascular inflammatory responses (Figure 3E through 3H; Figure S4A and S4B). Compared with antibiotic-treated LCWE-injected mice, antibiotic-treated LCWE-injected mice highly colonized with *B wadsworthia* and *B fragilis* (red dots) exhibited higher numbers of infiltrating T cells (CD3$^+$; P=8.17×10^{-5} for heart tissues [Figure 3G] and P=2.40×10^{-3} for abdominal aorta [Figure 3H]), neutrophils (Ly6G$^+$; P=2.05×10^{-3} for heart tissues [Figure 3G] and P=2.12×10^{-5} for abdominal aorta [Figure 3H]), and F4/80$^+$ FLICA$^+$ cells (P=4.41×10^{-4} for heart tissues [Figure S4A] and P=6.84×10^{-3} for abdominal aorta [Figure S4B]). Thus, reconstitution with these 2 species appears sufficient to exacerbate LCWE-induced vasculitis in antibiotic-treated mice.

Changes in intestinal microbiota composition can alter gut permeability and increase lipopolysaccharide dissemination to the systemic circulation, further promoting vascular inflammation, atherosclerosis, and thrombosis.[43] We have previously shown that LCWE-induced KD vasculitis is associated with a dysfunctional intestinal barrier and increased gut permeability.[28] To determine how the intestinal microbiome affects gut barrier integrity in LCWE-injected mice, we measured levels of circulating lipopolysaccharide. Antibiotic-treated mice showed lower levels of circulating lipopolysaccharide following LCWE injection than controls (Figure 3I). In contrast, supplementation of antibiotic-treated mice with *B wadsworthia* and *B fragilis* increased the levels of circulating lipopolysaccharide, supportive of decreased intestinal barrier functions (Figure 3I). Interestingly, we observed lower levels of circulating lipopolysaccharide and less severe

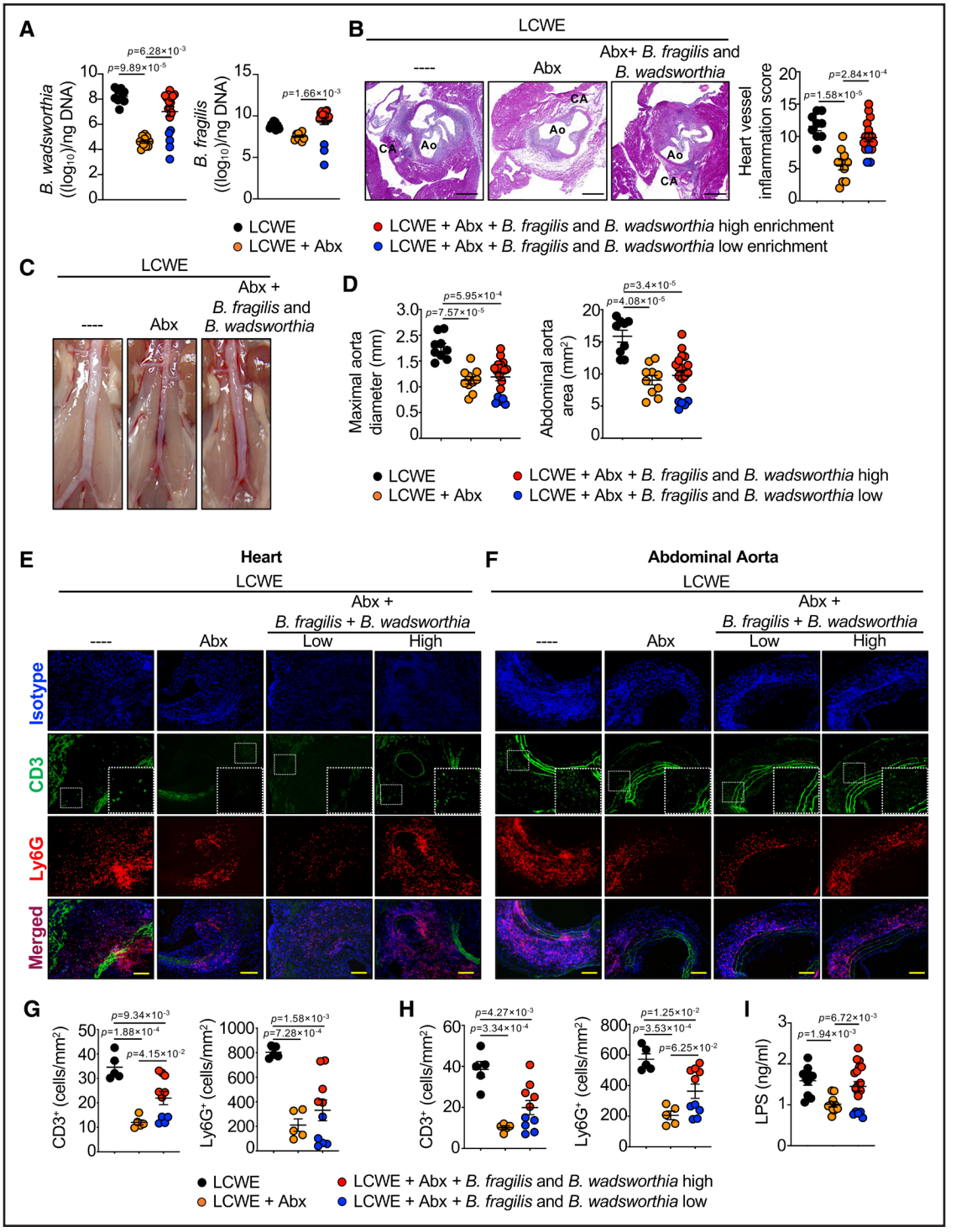

Figure 3. Oral supplementation with *B wadsworthia* and *B fragilis* exacerbates *Lactobacillus casei* cell wall extract (LCWE)–induced Kawasaki disease (KD) vasculitis in antibiotic (Abx)-treated mice.

A, Quantitative PCR (qPCR) quantification of *B wadsworthia* and *B fragilis* in fecal pellets from LCWE-injected control mice (n=9), Abx-treated mice (n=10), and Abx-treated mice orally supplemented with bacterial mix composed of *B wadsworthia* and *B fragilis* (n=19), at 2 weeks post-LCWE injection. Among the Abx-treated mice supplemented with the bacterial mix, subgroups of high (red dots) or low (blue dot) enrichment of *B wadsworthia* and *B fragilis* were observed. **B**, Representative hematoxylin and eosin (H&E)–stained heart sections and heart vessel inflammation score of LCWE-injected control mice (n=9), Abx-treated mice (n=10), and Abx-treated mice orally supplemented with *B wadsworthia* and *B fragilis* (n=17) at 2 weeks post-LCWE injection. Scale bars, 500 μm. **C** and **D**, Representative pictures of the abdominal aorta area (**C**), maximal abdominal aorta diameter, and abdominal aorta area measurements (**D**) of LCWE-injected control mice (n=9), Abx-treated mice (n=10), and Abx-treated mice orally supplemented with *B wadsworthia* and *B fragilis* (n=19) at 2 weeks post-LCWE injection. **E** and **F**, Representative immunofluorescent (IF) picture of isotype, CD3, and Ly6G staining in the heart (**E**) and abdominal aorta tissues (**F**) from LCWE-injected control mice (n=5), Abx-treated mice (n=5), and Abx-treated mice orally supplemented with *B wadsworthia* and *B fragilis* (n=10) at 2 weeks (*Continued*)

LCWE-induced vascular inflammation in the subset of antibiotic-treated, bacteria-supplemented mice that exhibited low bacterial enrichment (Figure 3I, blue dots). Overall, these results reveal that supplementation with *B wadsworthia* and *B fragilis* exacerbates LCWE-induced vasculitis.

F prausnitzii or *A muciniphila* Supplementation Protects From LCWE-Induced Cardiovascular Lesions

The 16S rRNA gene sequencing revealed a decreased relative abundance of *Akkermansia* and *Faecalibacterium* genera in fecal pellets of LCWE-injected mice, specific to LCWE injection and development of cardiovascular inflammation (Figure 2F; Figure S3). Supplementation with live *F prausnitzii* or *A muciniphila* has been shown to benefit the host in several disorders.[42,44–46] Furthermore, bacterial inactivation by pasteurization, a mild heat-mediated process, enhances their beneficial impact on the host, which may be mediated by increased stability and accessibility to bacterial proteins and components.[44,45] To assess the functional implications of these 2 species on the development of LCWE-induced KD vasculitis, SPF mice were supplemented orally with either live or pasteurized *F prausnitzii* daily, starting 1 week before LCWE injection until the experimental end point (Figure 4A). LCWE-injected mice supplemented with live *F prausnitzii* had an increased relative abundance of *F prausnitzii* in their feces (Figure S5A). As previously published, serum levels of IL-1β are increased in LCWE-injected mice,[29,33,47] and we found that circulating IL-1β was reduced by oral supplementation with either live *A muciniphila* or *F prausnitzii* of LCWE-injected mice (Figure S5B). Remarkably, supplementation with either live or pasteurized *F prausnitzii* reduced the severity of LCWE-induced heart inflammation and the development of abdominal aorta dilations (Figure 4B through 4D). Similar results were obtained when supplementing mice with either live or pasteurized *A muciniphila* (Figure 4E through 4H; Figure S5A and S5B). Furthermore, the relative abundance of either *F prausnitzii* or *A muciniphila* in the feces of LCWE-injected mice supplemented with the live bacteria negatively correlated with heart vessel inflammation (Figure S5C) and the development of abdominal aorta aneurysms (Figure S5D and S5E). We assessed the impact of live and pasteurized *F prausnitzii* or *A muciniphila* on the local inflammatory responses and observed that preventive oral supplementation with both live or pasteurized bacteria led to a significant reduction in the number of neutrophils ($Ly6G^+$) and T cells ($CD3^+$) infiltrating both the heart and abdominal aorta tissues of LCWE-injected mice (Figure S6A and S6B). Furthermore, this treatment also reduced the number of infiltrating $F4/80^+$ cells with caspase-1 activity ($FLICA^+$) in cardiovascular tissues (Figure S7A and S7B).

Next, we determined whether pasteurized *F prausnitzii* or *A muciniphila* reduced the severity of LCWE-induced KD vasculitis when provided in a therapeutic context and treated mice starting 1 day post-LCWE injection (Figure 4I). Treatment with pasteurized *F prausnitzii* or *A muciniphila* significantly reduced the severity of heart vessel inflammation in LCWE-injected mice and tended to decrease the development of abdominal aorta dilations (Figure 4J through 4L). These findings reveal that supplementation with either live or pasteurized *F prausnitzii* or *A muciniphila*, both of which show reduced relative abundance during LCWE-induced KD vasculitis, has a beneficial effect and decreases the severity of LCWE-induced KD cardiovascular lesion development.

SCFAs Decrease the Severity of LCWE-Induced KD Vasculitis

The beneficial effects of *F prausnitzii* and *A muciniphila* could be mediated by secreted metabolites from live bacteria, such as SCFAs, which are known for their anti-inflammatory properties and the enhancement of gut barrier function.[48] To address this possibility, we next assessed the levels of SCFAs in the feces of PBS and LCWE-injected mice. We observed a significant reduction in acetate and propionate and a trend toward reduced butyrate fecal levels after LCWE injection (Figure 5A). Acetate, propionate, and butyrate make up about 80% of all SCFAs produced.[49] Because *F prausnitzii* uses acetate to generate butyrate[50] and *A muciniphila* produces acetate and propionate,[39] we tested if preventively supplementing these SFCAs in the drinking water would alter the development of LCWE-induced KD vasculitis (Figure S8A). Indeed, prophylactic supplementation with each SCFA significantly reduced the development of LCWE-induced heart inflammation and abdominal aorta dilations (Figure 5B through 5D). SCFA prophylactic supplementation was also associated with a significant decrease in T cell and neutrophil infiltration in both heart and abdominal aorta tissues of LCWE-injected mice (Figure S8B and S8C). SCFA supplementation reduced the numbers of $F4/80^+$ cells

Figure 3 Continued. post-LCWE injection. **G** and **H**, Quantification of $CD3^+$ and $Ly6G^+$ cells in heart tissues (**G**) and abdominal aorta (**H**) from LCWE-injected control mice (n=5), Abx-treated mice (n=5), and Abx-treated mice orally supplemented with *B wadsworthia* and *B fragilis* (n=10) at 2 weeks post-LCWE injection. Scale bars, 50 µm. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). **I**, Serum lipopolysaccharide (LPS) concentrations of control mice that received PBS (n=10), Abx-treated mice (n=10), and Abx-treated orally supplemented with *B wadsworthia* and *B fragilis* (n=19) at 2 weeks post-LCWE injection. Data presented as mean±SEM and pooled from 2 experiments. Each symbol represents an individual mouse. *P* values obtained by Kruskal-Wallis with Dunn's multiple comparison tests (**A**) and One-way ANOVA with the Tukey's multiple comparison tests (**B**, **D**, **G**, **H**, and **I**). SPF indicates specific pathogen-free.

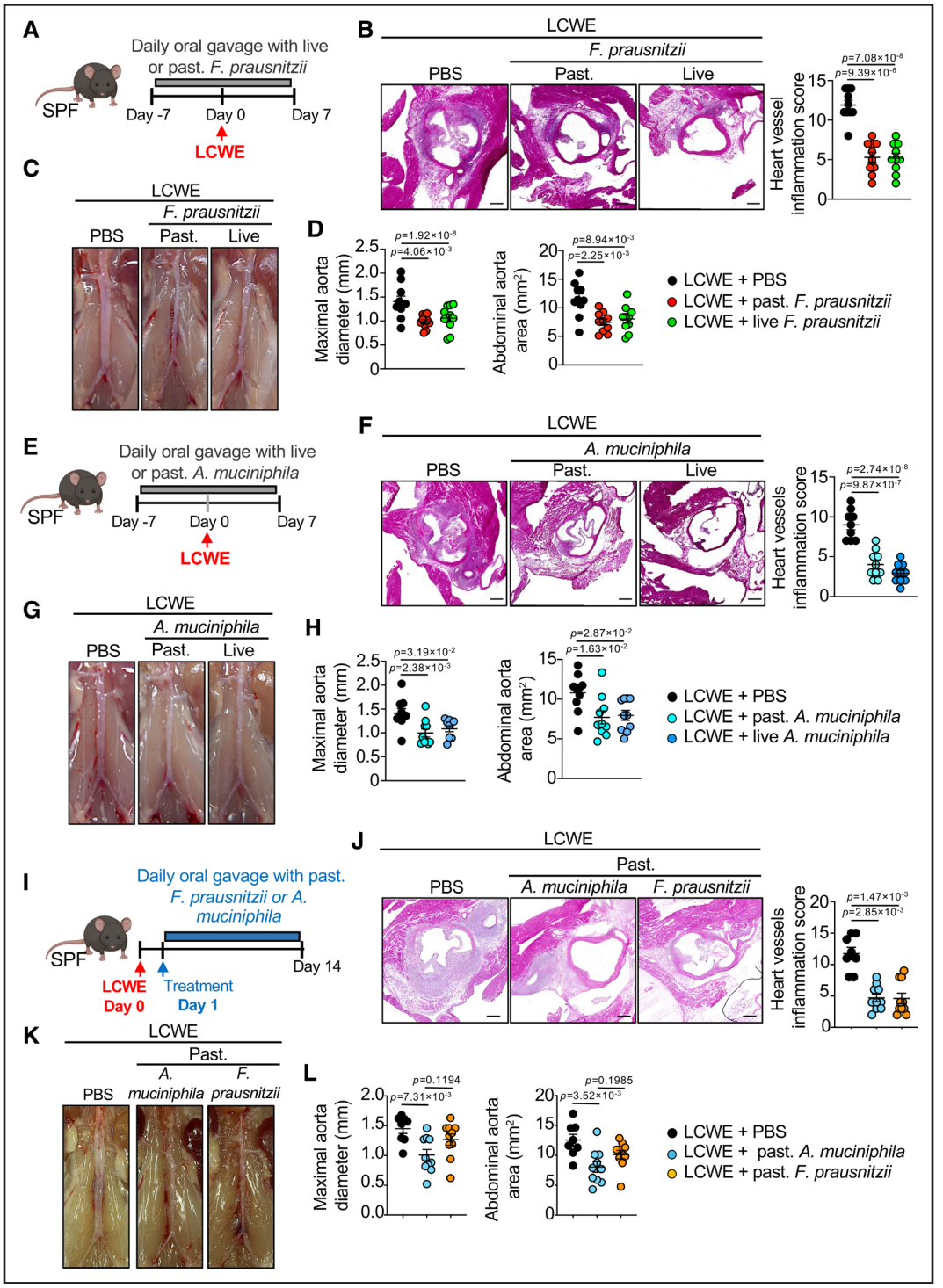

Figure 4. Live and pasteurized (past.) *F prausnitzii* or *A muciniphila* improve *Lactobacillus casei* cell wall extract (LCWE)–induced Kawasaki disease (KD) vasculitis.

A, Schematic of the experimental design. Mice received by daily oral gavage live or past. *F prausnitzii* starting 1 week before LCWE injection and continuing until the experimental end point, at day 7 post-LCWE. **B**, Representative hematoxylin and eosin (H&E)–stained heart sections and heart vessel inflammation score of LCWE-injected mice orally supplemented with either PBS, or live or past. *F prausnitzii* starting 1 week before LCWE injection until the experimental end point, at day 7 post-LCWE (n=10/group). Scale bars, 500 µm. **C** and **D**, Representative pictures of the abdominal aorta area (**C**), maximal abdominal aorta diameter, and abdominal aorta area measurements (**D**) of LCWE-injected mice untreated or orally supplemented with either PBS, or live or past. *F prausnitzii*, starting 1 week before LCWE injection until the experimental end point, at day 7 post-LCWE (n=10/group). **E**, Schematic of the experimental design. Mice received by daily oral gavage live or past. *A muciniphila* starting 1 week before LCWE injection and continuing until the experimental end point, at day 7 post-LCWE. **F**, Representative H&E-stained heart sections and heart vessel inflammation score of LCWE-injected mice orally supplemented with either PBS, or live or past. *A muciniphila*, starting 1 week before LCWE injection until the experimental end point, at day 7 post-LCWE (n=9–10/group). Scale bars, 500 µm. **G** and **H**, Representative (*Continued*)

with active caspase-1 (F4/80$^+$ FLICA$^+$) in heart tissue sections of LCWE-injected mice, with acetate having the most pronounced effect (Figure S9). Compared with untreated LCWE-injected mice, SCFA supplementation appeared to improve gut barrier integrity and led to reduced systemic levels of circulating lipopolysaccharide and D-lactate (Figure 5E). SCFA supplementation also increased the number of intestinal goblet cells and the expression of several intestinal tight junctions (TJs) in the gut tissues of LCWE-injected mice (Figure 5F through 5I). On the other hand, the expression of *Cldn2*, a TJ associated with a leaky intestinal barrier,[51,52] was decreased by SCFA supplementation (Figure 5H). SCFA supplementation increased the expression in intestinal tissues of the SCFA transporters MCT1, encoded by *Slc16a1*, and SMCT1, encoded by *Slc5a8* (Figure 5I). In a separate series of experiments, we evaluated the effects of tributyrin, a triglyceride analog form of butyrate that, unlike sodium butyrate, is absorbed more slowly in the small intestine. When degraded in the large intestine, tributyrin elevates luminal butyrate concentrations.[53,54] Supplementation of LCWE-injected mice with tributyrin significantly decreased the development of LCWE-induced heart vessel inflammation and abdominal aorta dilations (Figure S10A through S10D).

Because prophylactic supplementation with SCFAs reduced the severity of LCWE-induced KD vasculitis, we next asked if providing SCFAs therapeutically after LCWE injection would also impact the development of cardiovascular lesions. Mice were first injected with LCWE and then supplemented with a mix of SCFAs in the drinking water starting either on day 1, day 2, day 3, or day 5 post-LCWE injection (Figure S11A). We observed a therapeutic effect and significant reduction in heart inflammation and development of abdominal aorta dilations when SCFAs were provided in the drinking water on day 1 post-LCWE (Figure S11B through S11D). The therapeutic effect of SCFAs at day 1 post-LCWE injection was associated with increased intestinal mucus thickness and goblet cell numbers (Figure S11E and S11F). Delaying SCFA treatment to later time points had no significant impact on heart vessel inflammation and abdominal aorta dilations (Figure S11B through S11D). These data indicate that supplementing SCFAs either prophylactically or therapeutically decreases the severity of LCWE-induced KD vasculitis, potentially by acting on the intestinal barrier.

SCFAs and Amuc_1100 Promote Intestinal Barrier Integrity

LCWE-induced KD vasculitis depends on NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) activation and IL-1β production by monocytes and macrophages.[27,29–31] Indeed, targeting the NLRP3-IL-1β axis using *Nlrp3*$^{-/-}$ and *Il1r1*$^{-/-}$ mice completely prevents the development of LCWE-induced KD vasculitis.[27,30,31] Furthermore, our previous findings indicate that LCWE increases intestinal permeability through an IL-1β-mediated mechanism.[28] Therefore, we next assessed in vitro the impact of SCFAs on the production of IL-1β by bone marrow–derived macrophages (BMDMs). BMDMs were pretreated with either acetate, propionate, or butyrate for 8 hours and activated with either lipopolysaccharide and nigericin or LCWE (Figure S12A through S12D). While acetate and butyrate pretreatment of BMDMs decreased IL-1β production by both lipopolysaccharide/nigericin and LCWE-stimulated BMDMs, propionate only reduced IL-1β production by LCWE-stimulated BMDMs (Figure S12B and S12D).

We next examined the impact of oral supplementation of SCFAs on IL-1β production in vivo. As previously published,[29] LCWE injections resulted in increased levels of IL-1β in the peritoneal cavity at 24 hours post-injection, which were significantly reduced when the mice were orally administered a mix of SCFAs composed of butyrate, propionate, and acetate (Figure S12E). Levels of circulating lipopolysaccharide and D-lactate were increased at 24 hours post-LCWE injection and reduced by SCFA preventive supplementation (Figure S12F). Overall, these results indicate that the beneficial effects of SCFA during LCWE-induced KD are likely mediated by targeting the IL-1β pathway and by promoting the intestinal barrier.

Next, we further explored the connection between IL-1β, the microbiota, SCFAs, and intestinal barrier integrity in vitro using intestinal organoid cultures composed of a polarized epithelium surrounding a central lumen, generated by culturing intestinal stem cells isolated from naive WT or *Il1r1*$^{-/-}$ mice (Figure 6A). Cultured intestinal organoids generated from naive WT mice and stimulated

Figure 4 Continued. pictures of the abdominal aorta area (**G**), maximal abdominal aorta diameter, and abdominal aorta area measurements (**H**) of LCWE-injected mice orally supplemented with either PBS, or live or past. *A muciniphila* starting 1 week before LCWE injection until the experimental end point, at day 7 post-LCWE (n=10/group). **I**, Schematic of the therapeutic experimental design. Mice were injected with LCWE first and, 1 day later, were treated by oral gavage with either PBS or past. *F prausnitzii* or *A muciniphila* daily until day 14 post-LCWE injection. **J**, Representative H&E-stained heart sections and heart vessel inflammation score of LCWE-injected mice untreated or orally treated with past. *F prausnitzii* or *A muciniphila* (n=8–10/group). Scale bars, 500 µm. **K** and **L**, Representative pictures of the abdominal aorta area (**K**), maximal abdominal aorta diameter, and abdominal aorta area measurements (**L**) of LCWE-injected mice orally supplemented with either PBS, past. *F prausnitzii*, or *A muciniphila* starting at day 1 post-LCWE injection (n=8–10/group). Data were compiled from 2 independent experiments (**A**–**L**) and presented as mean±SEM. Each symbol represents an individual mouse. *P* values were obtained by One-way ANOVA with the Tukey's multiple comparison tests (**B**, **D**, **F**, **H right**, and **L**) or Kruskal-Wallis with the Dunn's multiple comparison tests (**H left** and **J**). cfu indicates colony forming unit; and SPF, specific pathogen-free.

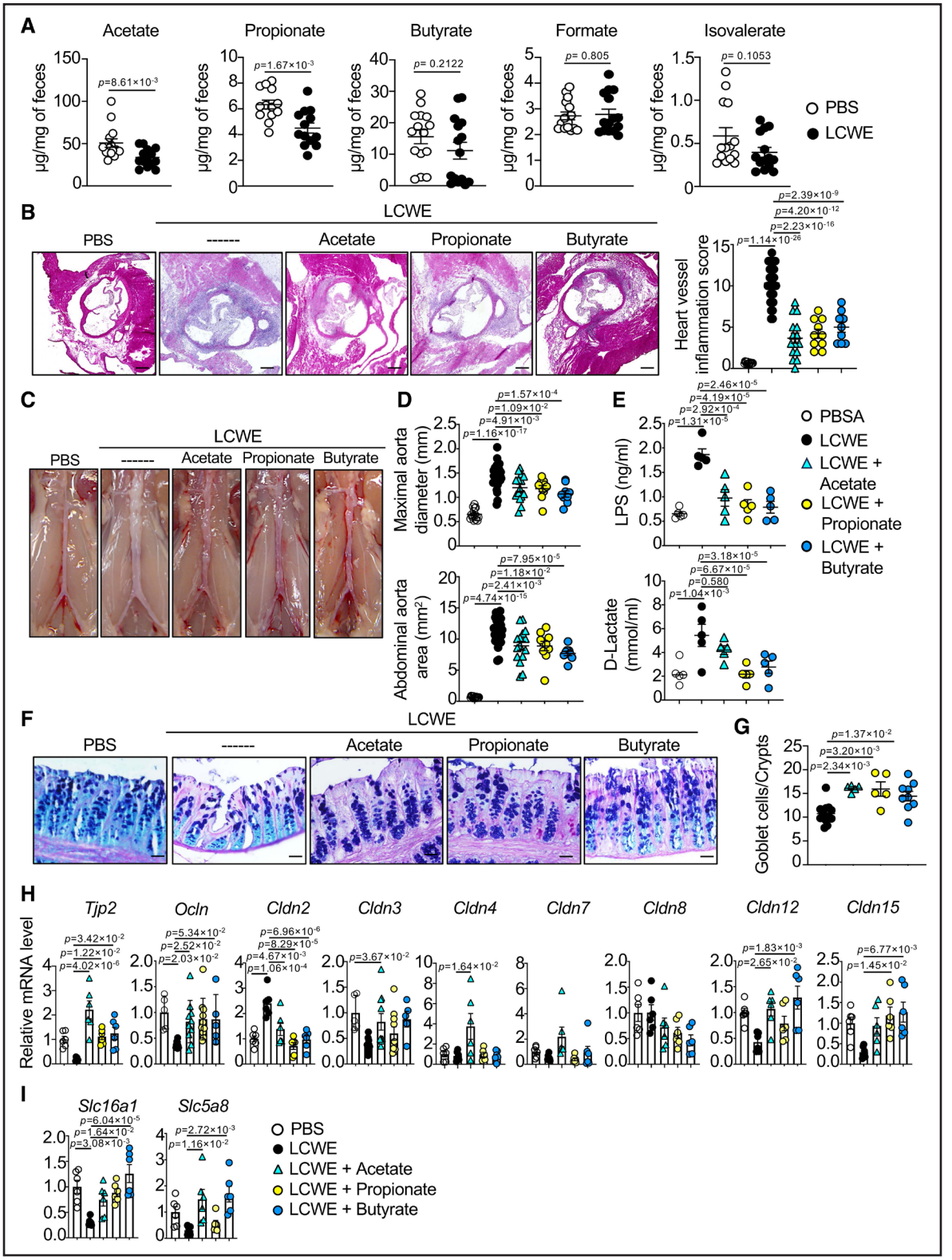

Figure 5. Short-chain fatty acids (SCFAs) oral supplementation reduces the severity of *Lactobacillus casei* cell wall extract (LCWE)–induced Kawasaki disease (KD) vasculitis.

A, Quantification of acetate, propionate, butyrate, formate, and isovalerate in the fecal pellets of PBS and LCWE-injected mice at 2 weeks post-injection (n=15/group). **B**, Representative hematoxylin and eosin (H&E)–stained heart sections and heart vessel inflammation score of untreated LCWE-injected mice and LCWE-injected mice orally supplemented with either acetate, propionate, or butyrate starting 3 days before LCWE injection until the experimental end point, at 1 week post-LCWE injection (n=9–32/group). Scale bars, 500 µm. **C** and **D**, Representative pictures of the abdominal aorta area (**C**), maximal abdominal aorta diameter, and abdominal aorta area measurements (**D**) of untreated LCWE-injected mice and LCWE-injected mice orally supplemented with either acetate, propionate, or butyrate, starting 3 days before LCWE and (*Continued*)

with recombinant (r)IL-1β exhibited reduced organoid formation efficiency and surface area (Figure 6B and 6C), a decreased in the expression of intestinal TJs *Ocln* and *Cldn3*, and increased *Il1b* and *Il6* mRNA levels (Figure 6D). IL-1R specifically mediated these effects, as these responses were not observed in rIL-1β-stimulated organoids generated from intestinal tissues of *Il1r1*$^{-/-}$ mice (Figure 6A through 6D). Altogether, these results further support the role of IL-1β in disrupting the intestinal barrier. Next, we asked whether SCFA oral supplementation, which reduces circulating levels of IL-1β, lipopolysaccharide, and D-lactate (Figure S12E and S12F) and decreases the severity of LCWE-induced KD in mice (Figure 5), could prevent the detrimental effects of IL-1β on intestinal organoids (Figure S13). Intestinal organoids were generated from WT mice and stimulated with rIL-1β in the presence of acetate, butyrate, or propionate (Figure S13A). While treatment with rIL-1β decreased organoid formation, surface area, and viability, as well as the expression of *Ocln* and *Cldn3* and increased levels of *Il1b* and *Il6* mRNA, all these parameters were improved by the addition of acetate and, to a lesser extent, by propionate (Figure S13B through S13D).

As noted above, given that supplementation with either live or pasteurized *A muciniphila* reduces the development of LCWE-induced cardiovascular lesions (Figure 4F thorugh 4L), the effect of this bacteria may be mediated by secreted molecules, such as SCFAs, or by bacterial proteins that remain stable after pasteurization.[39,44,55] Amuc_1100 is a 32-kDa protein abundantly expressed in the outer membrane of *A muciniphila*, which signals to the TLR-2 receptor. A His-tagged Amuc_1100 (Amuc_1100*) produced in *E coli* remains stable at 70 °C, the temperature used for pasteurization, and recapitulates the beneficial effect of *A muciniphila* on the gut barrier in the context of metabolic syndrome.[39,44] Therefore, we evaluated the impact of Amuc_1100* on IL-1β-stimulated intestinal organoid cultures (Figure 6E through 6H). Stimulation with rIL-1β decreased organoid growth, viability, and the expression of intestinal TJs *Ocln* and *Cldn3* and increased the expression of *Il1b* and *Il6*. However, Amuc_1100* addition reduced these IL-1β-mediated responses (Figure 6F through 6H). This suggests that Amuc_1100* modulates the expression of intestinal TJs and reduces proinflammatory mediators, such as IL-1β and IL-6.

Amuc_1100* Attenuates LCWE-Induced KD Vasculitis

To further explore whether Amuc_1100* may have a beneficial impact in vivo, we next compared the effects of supplementation with pasteurized *A muciniphila* and Amuc_1100* on the development of LCWE-induced KD. WT mice received daily by oral gavage either PBS with glycerol, pasteurized *A muciniphila*, or Amuc_1100*, starting 1 week before LCWE injection until the experimental end point (Figure 7A). Similar to the beneficial effect that we observed with pasteurized *A muciniphila* (Figure 4F through 4L), Amuc_1100* led to a significant reduction in the severity of LCWE-induced KD vasculitis, as demonstrated by decreased heart vessel inflammation and reduced development of abdominal aorta dilations (Figure 7B and 7C). Reduced development of LCWE-induced cardiovascular lesions in mice supplemented with Amuc_1100* or pasteurized *A muciniphila* was also associated with a significant decrease in T cell and neutrophil infiltration in both heart and abdominal aorta tissues (Figure S14A and S14B). Furthermore, supplementation with either Amuc_1100* or pasteurized *A muciniphila* reduced the numbers of F4/80$^+$ cells with caspase-1 activity (F4/80$^+$ FLICA$^+$) in heart tissues and abdominal aortas of LCWE-injected mice (Figure S15A and S15B).

Compared with untreated LCWE-injected mice, which exhibit a reduced colonic mucus layer thickness, Amuc_1100* preserved the mucus thickness at levels similar to those observed in PBS control mice or LCWE-injected mice that received pasteurized *A muciniphila* (Figure 7D). We next assessed the effect of Amuc_1100* on the expression of several TJ genes. As expected, LCWE injection increased the mRNA expression of *Cldn2*, associated with increased gut permeability,[51,52] and reduced the expression of *Tjp2*, *Ocln*, *Cldn3*, *Cldn12*, and *Cldn15*, but both Amuc_1100* and pasteurized *A muciniphila* significantly prevented these effects in vivo (Figure 7E). Immunofluorescence staining of intestinal tissues also indicated decreased expression of occludin and increased expression of CLDN2 at the protein levels in LCWE-injected mice,

Figure 5 Continued. until the experimental end point, 1 week post-LCWE injection (n=9–32/group). **E**, Serum lipopolysaccharide (LPS) and D-lactate concentrations of untreated LCWE-injected mice and LCWE-injected mice orally supplemented with either acetate, propionate, or butyrate starting 3 days before LCWE injection until the experimental end point, at 1 week post-LCWE injection (n=5/group). **F** and **G**, Representative photomicrograph showing Alcian blue periodic acid-Schiff (AB-PAS) staining of colon tissues (**F**) and goblet cell counts per crypts of LCWE (n=5–13/group) (**G**) from intestinal tissues of untreated LCWE-injected mice and LCWE-injected mice orally supplemented with either acetate, propionate, or butyrate starting 3 days before LCWE injection until the experimental end point, at 1 week post-LCWE injection (n=5–9/group). Scale bars, 500 µm. **H** and **I**, mRNA levels (normalized to *Rpl19*) of different tight junctions (TJs) (**H**) and SCFAs transporters (**I**) in colon tissues of LCWE-injected mice (n=10) and LCWE-injected mice treated with sodium acetate (n=10), sodium propionate (n=5), and sodium butyrate (n=10) at 1 week post-LCWE injection. Data are presented as mean±SEM and pooled from 2 to 3 independent experiments (**A**–**G**). Each symbol represents an individual mouse. *P* values obtained by the unpaired *t* test (**A**, propionate) and *t* the test with the Welch's correction (**A**), One-way ANOVA with the Tukey's multiple comparison test (**E**, **G**, and **H**), the Bonferroni multiple comparison tests (**B** and **D**), and Kruskal-Wallis with the Dunn's multiple comparison tests (**H** and **I**).

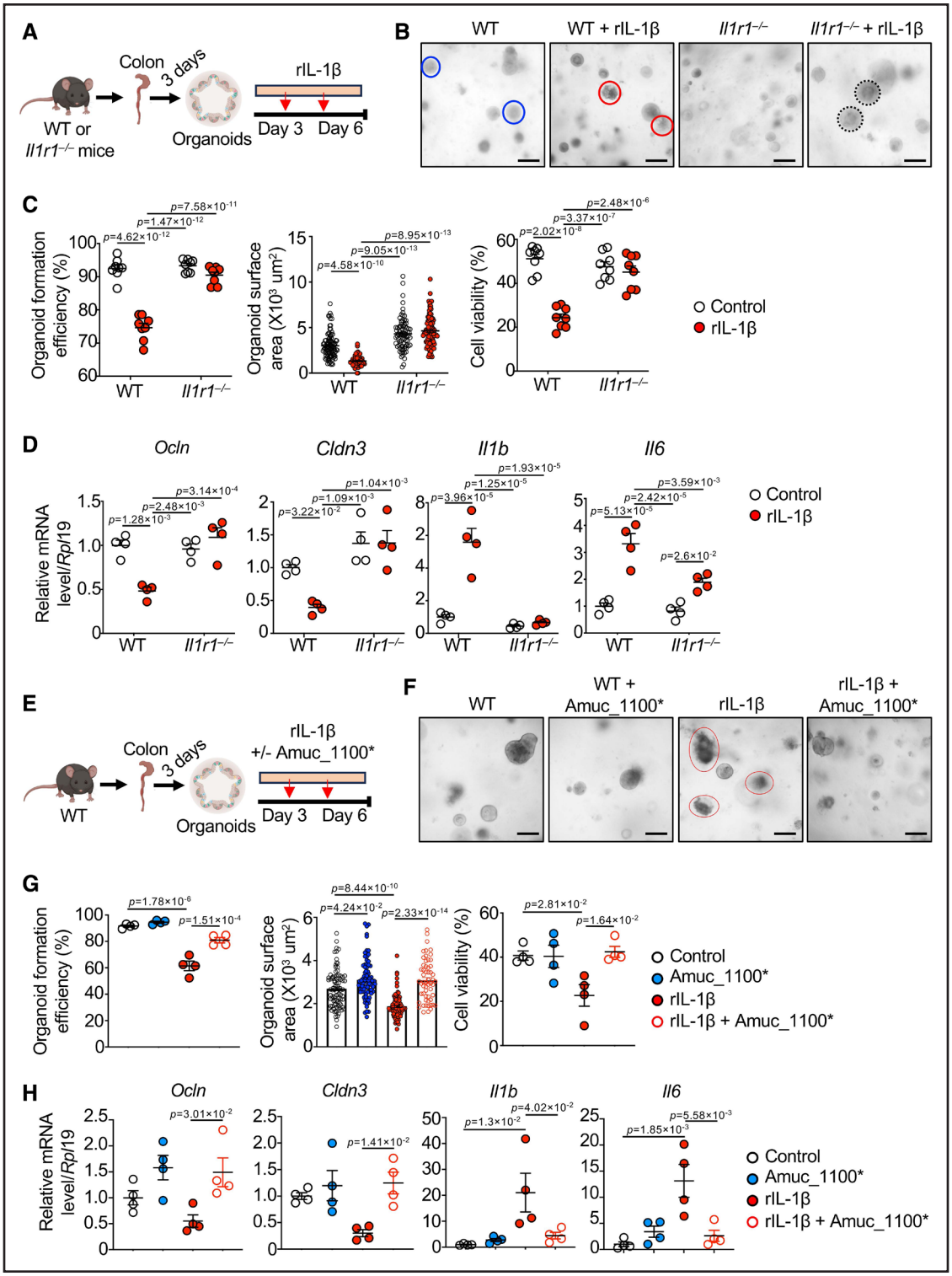

Figure 6. Amuc_1100* treatment counteracts IL (interleukin)-1β–mediated effects on intestinal organoids.
A, Schematic of the experimental design. Organoids were generated from the colons of naive wild-type (WT) and *Il1r1*$^{-/-}$ mice and treated with recombinant (r)IL-1β at the indicated days. **B**, Representative pictures of organoids generated from naive WT and *Il1r1*$^{-/-}$ mice, untreated or treated with rIL-1β at days 3 and 6 of the experiment. The black dotted line indicates the organoid surface area, and the blue and red circles indicate the live organoids and dead organoids, respectively. **C**, Formation efficiency, surface area, and viability of organoids generated from naive WT and *Il1r1*$^{-/-}$ mice, untreated or treated with rIL-1β at days 3 and 6 of the experiment (n=8/group). **D**, mRNA expression of intestinal tight junctions (TJs) *Ocln* and *Cldn3* and of *Il1b* and *Il6* in organoids generated from naive WT and *Il1r1*$^{-/-}$ mice, untreated or treated with rIL-1β at days 3 and 6 of the experiment (n=4/group). **E**, Schematic of the experimental design. Organoids were generated from the colons of naive WT mice treated with rIL-1β in the presence or absence of Amuc_1100*. **F**, Representative pictures of organoids generated from naive WT mice, treated with rIL-1β in the presence of Amuc_1100*. Red circles indicate dead organoids. **G**, Formation efficiency, surface area, and viability of (*Continued*)

which was reversed by both pasteurized *A muciniphila* or Amuc_1100* (Figure S16A and S16B). Finally, treatment with both Amuc_1100* and pasteurized *A muciniphila* restored the expression of the SCFA transporters MCT1, encoded by *Slc16a1*, and SMCT1, encoded by *Slc5a8*, to levels similar to those observed in PBS control mice (Figure 7F). Altogether, these data further support that LCWE-induced KD vasculitis is associated with a dysfunctional intestinal barrier characterized by decreased expression of intestinal TJs and that both Amuc_1100* and pasteurized *A muciniphila* decrease the development of LCWE-induced cardiovascular lesions potentially by promoting the intestinal barrier integrity.

DISCUSSION

Here, we show that the gut microbiota composition is altered in the setting of LCWE-induced vasculitis, a mouse model mimicking KD vasculitis. These changes could precipitate vasculitis development in mice or reflect a compensatory response of the gut microbiota to the intense host inflammatory response. We demonstrate that these alterations contribute to the development of cardiovascular lesions and show that specific manipulations of the intestinal microbiota either exacerbate or prevent the development of LCWE-induced vasculitis in mice. We demonstrate that in vivo supplementation with live or pasteurized *F prausnitzii* or *A muciniphila* decreases the severity of cardiovascular lesions in the LCWE model. This beneficial effect can be recapitulated by supplementing with SCFAs or with an outer membrane protein derived from *A muciniphila*, Amuc_1100, that is, known to signal to TLR-2, all of which promote intestinal barrier function and decrease the severity of vasculitis development.

KD affects mainly young children <5 years old, with the highest incidence in the first 2 years of life.[1,56] A retrospective study of an Italian cohort of patients with KD indicated that younger patients (<6 months) with abdominal and gastrointestinal symptoms are at the highest risk for developing CA aneurysms.[15] Furthermore, immunohistochemical analysis of intestinal biopsies showed increased counts of HLA-DR$^+$ cells and CD4$^+$ T cells in the lamina propria from patients with KD, indicating intestinal immune activation.[57] These observations and the frequent co-occurrence of gastrointestinal symptoms in patients with KD strongly point toward a gut-cardiovascular axis during KD vasculitis.

Antibiotic-mediated acute depletion or complete lack of intestinal microbiota in GF mice significantly reduced the severity of LCWE-induced cardiovascular lesions, implying the contribution of the gut microbiota to vascular inflammation in this experimental model. Because KD manifests in children with prolonged fever, increased levels of inflammatory markers, and clinical features highly similar to an infectious disease, patients are often treated with antibiotics during the acute phase of the disease before KD diagnosis.[21–24] Although intended to eliminate pathogenic organisms, the use of antibiotics dramatically impacts the gut microbiota composition and function by targeting other beneficial related bacterial members, disrupting the mucosal epithelial barrier integrity, thinning the intestinal mucus layer, and disturbing the expression of intestinal TJs.[58,59] In KD, antibiotic therapy is associated with an increased rate of IVIG resistance.[21,24,25] Therefore, it is possible that perturbations in the microbiota composition, such as decreased beneficial SCFA-producing bacteria, induced by antibiotics used pre-KD diagnosis, may further promote the proinflammatory response associated with KD vasculitis and, potentially, IVIG resistance and even higher risk of development of CA aneurysms.[21,24,25]

We identified *B wadsworthia* and *B fragilis* as enriched in the feces of LCWE-injected mice and showed that oral supplementation with these 2 bacteria further exacerbated the severity of heart vessel inflammation. Both bacteria are opportunistic pathobionts capable of promoting intestinal inflammation and barrier dysfunction.[36–38] In mice, *B wadsworthia* is associated with intestinal inflammation,[36] atherosclerosis,[60] and chronic metabolic conditions.[61] Increased relative abundance of *B wadsworthia* is also reported in the context of Behçet's disease,[62] a multisystem autoimmune disorder associated with whole-body vasculitis and juvenile idiopathic arthritis.[63] Higher relative abundance of *Bacteroidaceae* have been previously reported in the fecal microbiota of patients with IgA vasculitis, which commonly affects children from 4 to 7 years.[64,65] The LCWE-induced KD vasculitis mouse model is associated with IgA deposition in cardiovascular lesions and kidneys and increased intestinal permeability, a process that is IL-1β dependent.[28] Indeed, IL-1β increases intestinal permeability,[66,67] and a dysfunctional intestinal barrier may allow expanding luminal pathobionts and their metabolites to penetrate the lamina propria and trigger abnormal immune responses. In addition, heightened activation of the intestinal immune response has been reported during the acute phase of KD.[57] Therefore, we speculate that vascular diseases, such as IgA vasculitis and KD, may share a similar mechanistic pathway involving alterations in the intestinal microbiota composition and the blooming of pathobionts. However, the specific mechanisms and impact of expanding

Figure 6 Continued. organoids generated from naive WT mice, treated with rIL-1β in the presence of Amuc_1100* (n=4/group). **H**, mRNA expression (normalized to *Rpl19*) of intestinal TJs *Ocln* and *Cldn3* and of *Il1b* and *Il6* in organoids generated from naive WT mice, treated with recombinant IL-1β in the presence of Amuc_1100* (n=4/group). Data are presented as mean±SEM and combined/pooled from 2 independent experiments. Each symbol represents organoids generated from technical replicates. *P* values obtained by Two-way ANOVA with the Tukey's multiple comparison test (**C** and **D**), One-way ANOVA with the Tukey's multiple comparison test (**G** and **H**), or Kruskal-Wallis with the Dunn's multiple comparison tests (**G**).

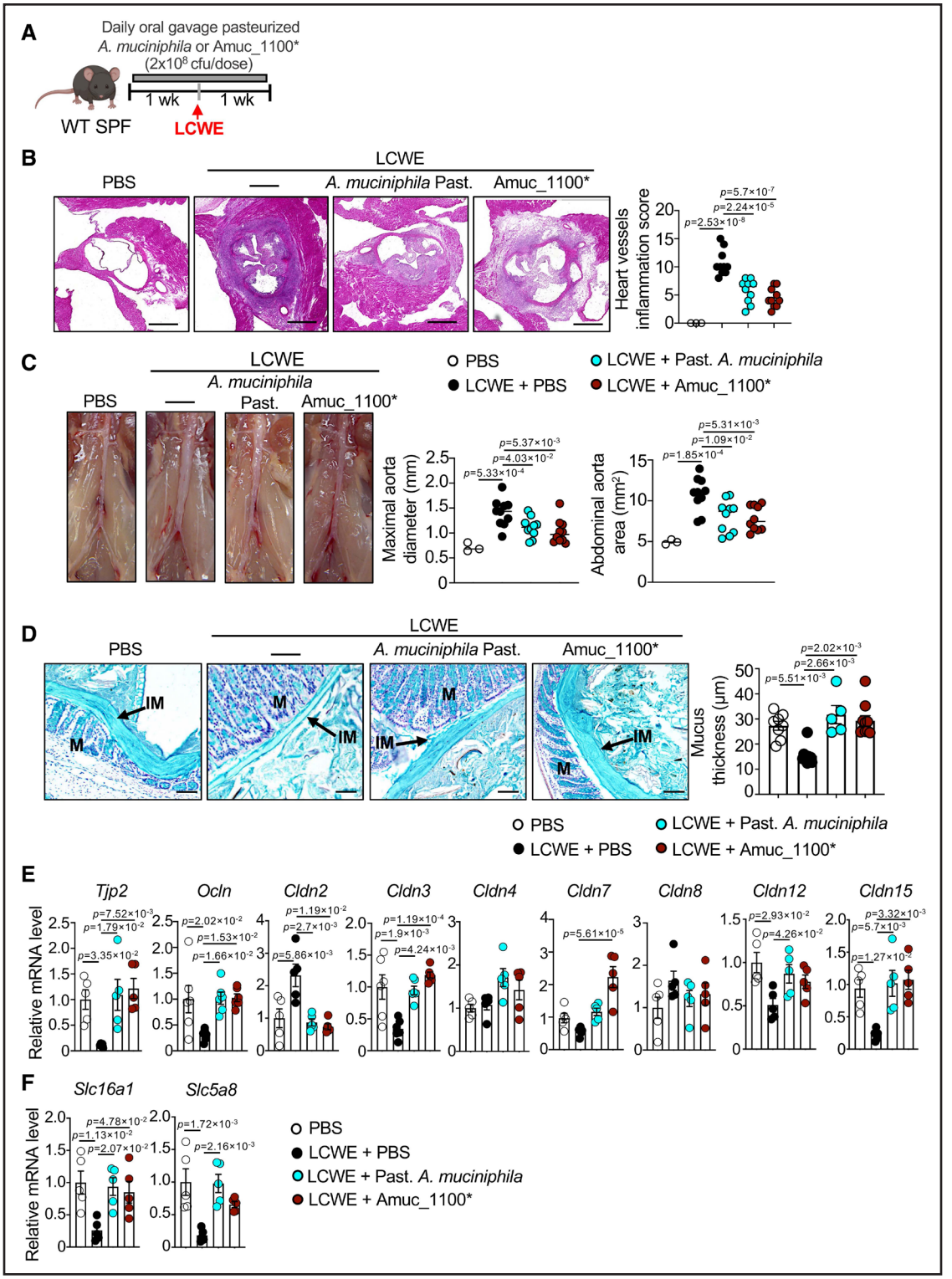

Figure 7. Pasteurized (past.) *A muciniphila* and Amuc_1100 oral administration prevents *Lactobacillus casei* cell wall extract (LCWE)–induced Kawasaki disease (KD) vasculitis.

A, Schematic of the experimental design. Wild-type (WT) mice were supplemented with past. *A muciniphila* or Amuc_1100* daily by oral gavage starting 1 week before LCWE injection and continuing until the end of the experiment at 1 week post-LCWE. **B**, Representative hematoxylin and eosin (H&E)–stained heart sections and heart vessel inflammation score of control mice and LCWE-injected mice orally supplemented with either PBS with 2.5% glycerol or past. *A muciniphila* or Amuc_1100*, starting 1 week before LCWE injection and continuing until the end of the experiment at 1 week post-LCWE injection (n=3–10/group). Scale bars, 500 μm. **C**, Representative pictures of the abdominal aorta area, maximal abdominal aorta diameter, and abdominal aorta area measurements of control mice and LCWE-injected mice orally supplemented with either PBS with 2.5% glycerol or past. *A muciniphila* or Amuc_1100*, starting 1 week before LCWE injection and continuing until the end of the experiment at 1 week post-LCWE injection (n=3–10/group). **D**, Representative Alcian blue staining of colon tissue sections showing mucus layer (*Continued*)

pathobionts on the host immune response will require further investigation.

In addition, we observed a decreased relative abundance of beneficial bacteria such as *F prausnitzii* and *A muciniphila* in the feces of LCWE-injected mice, which correlated with disease severity. The relative abundance of these 2 bacteria is often reported to be reduced in intestinal and extraintestinal disorders, including metabolic and cardiovascular diseases.[40,42,68,69] Our findings are in line with previous studies that reported decreased abundances of SCFA-producing bacteria in the feces of patients during the KD acute phase.[19,20,70] Furthermore, levels of SCFA butyrate were found to be significantly lower in the stool of patients with KD compared with controls.[71] Recently, Han et al[70] reported a decreased abundance of *A muciniphila* and *F prausnitzii* in pre-IVIG treatment patients with KD, which returned to control levels after IVIG treatment. Because of their robust positive systemic effects on host metabolism and anti-inflammatory properties, *Akkermansia* and *Faecalibacterium* are promising next-generation probiotics with high preventive and therapeutic potential.[39,72] We found that oral supplementation with *F prausnitzii* or *A muciniphila*, either prophylactically or therapeutically, significantly reduced LCWE-induced cardiovascular lesions. Mechanistically, this benefit may be mediated in part by metabolites, such as butyrate and microbial anti-inflammatory molecule (produced by *F prausnitzii*)[50,73,74] and acetate and propionate produced by *A muciniphila*,[75] which have anti-inflammatory and immune-modulatory properties and reinforce intestinal barriers.[39,72,74,75] Indeed, we showed that SCFA treatment prevented peritoneal production of IL-1β and decreased the number of neutrophils, T cells, and infiltrating F4/80$^+$ cells with caspase-1 activity in inflamed heart tissues. Butyrate decreases the production of proinflammatory cytokines by suppressing NF-κb activation,[76] and the butyrate treatment of activated BMDMs reduces the secretion of nitric oxide, IL-6, and IL-12p40 by inhibiting HDACs (histone deacetylases).[77] Similarly, acetate and propionate also decrease IL-1β production.[78,79] Here, we show that SCFA treatment also prevents IL1β production in vitro by LCWE-activated BMDMs. Altogether, these results suggest that a diminished relative abundance of SCFA-producing microbiota may play an important role in the development of cardiovascular lesions in the context of both experimental and human KDs, but further investigations with a larger number of patients are warranted.

Remarkably, providing *F prausnitzii* or *A muciniphila* in a pasteurized form reduced the severity of LCWE-induced KD vasculitis to a similar level as supplementation with live bacteria or SCFAs. This agrees with previous studies demonstrating the beneficial effects of pasteurized *F prausnitzii* and *A muciniphila* in several disorders.[44,45,55,68] Mice treated with pasteurized *A muciniphila* exhibit increased goblet cell numbers and a higher production of mucus,[39,44] and we observed similar results in LCWE-injected mice. The fact that pasteurized *F prausnitzii* and *A muciniphila* exhibit a similar effect to live bacteria in preventing LCWE-induced KD vasculitis is exciting from a treatment perspective, as both bacteria are anaerobic and sensitive to oxygen, so their administration to patients in their live form may be challenging.[39] Furthermore, the administration of live and pasteurized *A muciniphila* has been shown safe and tolerable in mice and in a proof-of-concept study involving patients.[39,44,68]

While pasteurization inactivates microorganisms and their production of SCFAs, this process also increases accessibility to extracellular bacterial proteins, which resist heat denaturation.[44] Amuc_1100 is an outer membrane protein specific to *A muciniphila*, which appears to remain stable at pasteurization temperature and recapitulates the beneficial effect of live or pasteurized *A muciniphila* in the context of metabolic disorders and colitis.[44,80–83] Purified Amuc_1100* is an efficient activator of the TLR-2 receptor.[39,44,84] We show that Amuc_1100 can prevent LCWE-induced cardiovascular inflammation and vasculitis. Furthermore, Amuc_1100 also counteracts the effects of IL-1β in vitro in intestinal organoids. Further studies are needed to determine whether Amuc_1100 could potentially be used in patients preventively or therapeutically. In addition, further studies are required to identify which pasteurization-resistant *F prausnitzii* proteins are involved in mediating its beneficial effect.

Because KD is the leading cause of acquired heart disease in children in the United States, and up to 20% of patients with KD are IVIG-refractory and at higher risk of developing cardiovascular lesions in childhood and long-term sequelae in adulthood, identifying novel intervention and therapeutic strategies is of critical interest. Overall, our study reveals a novel and important contribution of the gut microbiota in the development of cardiovascular lesions in a mouse model of KD vasculitis. Our results indicate that strategies aiming to modulate the production of microbial metabolites, or the microbiota composition, may be a preventive or adjunctive approach to current gold-standard therapy in treating cardiovascular disorders, including KD vasculitis. However, further studies are warranted to confirm

Figure 7 Continued. thickness in control PBS-injected mice and LCWE-injected mice orally supplemented with either PBS with 2.5% glycerol, past. *A muciniphila*, or Amuc_1100*, starting 1 week before LCWE injection and continuing until the end of the experiment 1 week post-LCWE injection (n=5–10/group). **E** and **F**, Colon mRNA levels (normalized to *Rpl19*) of intestinal tight junctions (TJs; **E**) and short-chain fatty acid (SCFA) receptors *Slc16a1* and *Slc5a8* (**F**) in tissues from control PBS-injected mice and LCWE-injected mice orally supplemented with either PBS with 2.5% glycerol, past. *A muciniphila*, or Amuc_1100*, starting 1 week before LCWE injection and continuing until the end of the experiment 1 week post-LCWE injection (n=5–6/group). Data are presented as mean±SEM and combined/pooled from 2 independent experiments. Each symbol represents an individual mouse. *P* values obtained by One-way ANOVA with the Tukey's multiple comparison tests (**B**, **C**, **E**, and **F**) and One-way ANOVA with the Kruskal-Wallis test with the Dunn's multiple comparison tests (**D** and **E**). IM indicates inner mucus; and M: mucosa.

our results in children with KD and to determine whether preventive or therapeutic strategies modulating microbiota-host interactions and promoting gut barrier functions can safely and effectively be applied to patients with KD.

ARTICLE INFORMATION

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Author Contributions

P.K. Jena, D. Wakita, M. Arditi, and M. Noval Rivas conceived the project and designed the experiments. P.K. Jena, D. Wakita, T.R. Crother, E. Aubuchon, Y. Takasato, Y. Kurashima, A.C. Gomez, A.E. Atici, M. Narayanan, and M. Noval Rivas performed experiments. M.C. Fishbein provided a pathological review. H. Kiyono, D.M. Underhill, S. Devkota, S. Chen, T.R. Crother, and K. Shimada helped conceptualize and discuss results and provided critical reagents. W.M. de Vos and P.D. Cani provided critical reagents, experimental design, and advice. P.K. Jena, M. Arditi, and M. Noval Rivas wrote the article with input from all authors.

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Disclosures

W.M. de Vos and P.D. Cani are inventors of patent applications dealing with the use of specific bacteria and components in the treatment of different diseases. W.M. de Vos and P.D. Cani have cofounded The Akkermansia Company SA, which is commercializing *Akkermansia muciniphila*, and have stocks in this start-up company. W.M. de Vos cofounded Caelus Health. P.D. Cani cofounded Enterosys. The other authors report no conflicts.

Supplemental Material

Supplemental Materials and Methods
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