Original Article

Marked Acceleration of Atherosclerosis After \textit{Lactobacillus casei}–Induced Coronary Arteritis in a Mouse Model of Kawasaki Disease


Objective—The purpose of this study was to investigate whether \textit{Lactobacillus casei} cell wall extract–induced Kawasaki disease (KD) accelerates atherosclerosis in hypercholesterolemic mice.

Method and Results—Apolipoprotein E knockout or low-density lipoprotein receptor knockout mice were injected with \textit{Lactobacillus casei} cell wall extract (KD mice) or PBS, fed high-fat diet for 8 weeks, and atherosclerotic lesions in aortic sinuses, arch (AC), and whole aorta were assessed. KD mice had larger, more complex aortic lesions with abundant collagen, and both extracellular and intracellular lipid and foam cells, compared with lesions in control mice despite similar cholesterol levels. Both apolipoprotein E knockout KD and low-density lipoprotein receptor knockout KD mice showed dramatic acceleration in atherosclerosis versus controls, with increases in en face aortic atherosclerosis and plaque size in both the aortic sinuses and AC plaques. Accelerated atherosclerosis was associated with increased circulating interleukin-12p40, interferon-γ, tumor necrosis factor-α, and increased macrophage, dendritic cell, and T-cell recruitment in lesions. Furthermore, daily injections of the interleukin-1Ra, which inhibits \textit{Lactobacillus casei} cell wall extract–induced KD vasculitis, prevented the acceleration of atherosclerosis.

Conclusion—Our results suggest an important pathophysioligic link between coronary arteritis/vasculitis in the KD mouse model and subsequent atherosclerotic acceleration, supporting the concept that a similar relation may also be present in KD patients. These results also suggest that KD in childhood may predispose to accelerated and early atherosclerosis as adults. (\textit{Arterioscler Thromb Vasc Biol.} 2012;32:0-0.)

Key Words: atherosclerosis • coronary disease • interleukin 1 beta • IL-1 receptor antagonist • Kawasaki disease • mouse model of Kawasaki • vasculitis

Kawasaki disease (KD) is a multisystem inflammatory disease with unknown etiology that results in an acute febrile syndrome, most common among children younger than 5 years.\textsuperscript{1} KD represents the leading cause of acquired heart disease among children.\textsuperscript{2} The disease brings about its most detrimental effects via acute coronary arteritis, often accompanied by the development of coronary artery aneurysms in $\approx$25% of untreated patients. The vasculitis and coronary arteritis are characterized histologically by inflammatory cell infiltration and destruction of extracellular matrix, especially elastic tissue in vascular media, with resultant coronary artery aneurysm formation.\textsuperscript{3} Long-term cardiovascular complications among survivors of childhood KD are reported with increasing frequency.\textsuperscript{4-6} There are data suggesting that premature atherosclerosis and cardiovascular disease occur with increased frequency among survivors of childhood KD.\textsuperscript{5,6}

Atherosclerosis is a lipid-driven, chronic inflammatory disease of the vessel wall in which both innate and adaptive immune responses play a role.\textsuperscript{10} Immune cells and their mediators directly cause the chronic arterial inflammation that is a hallmark of atherosclerosis. It is clinically and experimentally reported that postinflammatory vascular remodeling induces the development of arteriosclerosis or early onset of atherosclerosis.\textsuperscript{11} There is evidence that clinical or subclinical vasculitis that occurs in KD may be the precipitating factor in lasting sequelae of the disease, namely atherosclerosis of the coronary and systemic arteries.\textsuperscript{12,13}
the first cohort of patients diagnosed with KD are reaching middle age, epidemiological evidence is mounting that shows greater incidence of cardiac events among adults with a history of KD. In a scientific statement from the American Heart Association’s expert panels, KD was listed among the 8 pediatric diseases that are associated with high risk for accelerated atherosclerosis in children. Children with coronary aneurysms, and even those in whom coronary dilatation was never detected after KD, appear to be at increased risk for future atherosclerotic coronary artery disease. Recent reports further suggest that KD patients may be at increased risk for accelerated atherosclerosis. However, there are conflicting clinical studies on this association, and whether KD is a risk factor for accelerated atherosclerosis still remains controversial.

Dalla Pozza et al found that there were indeed significant controversy. KD is a risk factor for accelerated atherosclerosis still remains controversial. McCrindle et al concluded that vessels in post-KD teenage patients were not significantly altered and thus posed no increased cardiovascular risk. In contrast, Dalla Pozza et al found that there were indeed significant changes in vascular profile, specifically an increase in carotid artery intima-media thickness. These and other recent studies addressing the association between KD and atherosclerosis have come to opposing conclusions.

To address these conflicting results and explore the possibility that vasculitis observed during KD predisposes to accelerated development of atherosclerosis, we took advantage of a well-established mouse model of *Lactobacillus casei* cell wall extract (LCWE)–induced coronary arteritis and KD, which mimics histopathologically the coronary lesions observed in KD patients. We evaluated the effects of KD vasculitis on progression of atherosclerotic changes in mice genetically predisposed to develop atherosclerosis on high-fat diet, including apolipoprotein E knockout (ApoE−/−) or low-density lipoprotein receptor knockout (Ldlr−/−) models of atherosclerosis. Here, we show that mice with LCWE-induced coronary arteritis (KD group) in hypercholesterolemic atherosclerosis models develop a dramatic acceleration in atherosclerosis compared with non-KD control group, despite similar serum cholesterol levels. We also observed that prevention of coronary arteritis and vasculitis with interleukin (IL)-1Ra treatment in the KD mouse model significantly inhibited the acceleration of atherosclerosis in hypercholesterolemic mouse models of atherosclerosis.

### Methods

#### Mice

ApoE−/−, Ldlr−/− mice (all on C57BL/6 background) were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were housed under specific pathogen-free conditions at the animal center of the Cedars-Sinai Medical Center. Experiments were conducted under approved Institutional Animal Care and Use Committee protocols. Each experimental group had 12 mice unless noted otherwise.

#### Reagents

Recombinant human IL-1 receptor antagonist (IL-1Ra) (Anakinra, Amgen), recombinant mouse IL-1p (Sigma, St. Louis, MO) and IL-1Ra were used at 25 mg/kg or 500 μg/mouse given IP. The dose was based on our published study showing almost complete protection from coronary lesions.

### Atherosclerosis Development in LCCWE-Induced Coronary Arteritis Model

Group B *L. casei* (American Type Culture Collection 11578) cell wall extract was prepared, as previously described. Five-week-old ApoE−/− or Ldlr−/− mice were injected IP with 250 μg of LCWE in PBS to induce KD or PBS alone (controls), as previously described. Five mice from each group were euthanized 14 days later to confirm the coronary arteritis, hearts were removed, and coronary arteries were identified in serial sections (6 μm) and stained with hematoxylin-eosin as described in our early publication. Other mice from each group were fed a high-fat diet containing 0.15% cholesterol starting at 14 days after LCWE or PBS injection. After 8 weeks of high-fat diet, mice were euthanized, heart and aorta were harvested, and the aortic root and aorta en face preparations were examined. To prevent any gender effect, we used only male ApoE−/− or Ldlr−/− mice in both groups.

### Assessment of Atherosclerotic Lesions in the Aorta and Aortic Sinus

Mice were anesthetized and aortas were excised from the aortic arch to the iliac bifurcation. Whole aortas en face and aortic sinus were prepared and stained with Oil red O as previously described. Lesion areas were quantified with Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Image analysis was performed by a trained observer who was blinded to the genotypes of mice as previously described. The lesion area and lipid-stained areas in the aortic sinus were measured. Lipid content in aortic root plaques was expressed as aortic sinus lesion area or as percent of plaque area. The lesion area in the aorta en face preparations was expressed as a percent of the aortic surface area as previously reported.

### Assessment of DCs, Macrophages, and T Cells in the Coronary Artery and Aortic Sinus

Heart sections were immunohistochemically analyzed for the presence of myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs), macrophages, and T-cell expression. For this purpose, we used the following rat antimouse antibodies (Ab): anti-MIDC-8 Ab (Serotec) specific for mature mDCs, anti-PDCA-1 Ab specific for pDCs, anti-E480 Ab (Serotec), a specific marker for macrophages, and anti-CD3 Ab for T cells. For negative control, a mixture of different isotype antibodies (IgG2a and IgG2b) was used (Serotec). Immunostainings of serial cross-sections were performed using the catalyzed signal amplification kit according to manufacturer’s instructions (CSA System, DakoCytomation, Hamburg, Germany) as described earlier. Brown staining was obtained by incubation with 3, 3′-diaminobenzidine tetrahydrochloride.

### Computer-Assisted Image Analysis

Digital images were taken at a magnification of ×200 with a charge-coupled device camera (Nikon DXM 1200) of representative areas of coronary lesions, aortic root, and myocardium. mDCs, pDCs, macrophages, and T cells were quantified in different areas (0.2 mm²) by computer-assisted histomorphometry (Image-J) as described before.

### Serum Levels of Cytokines

IL-12p40, tumor necrosis factor-α, and interferon-γ concentrations in the sera of mice were measured by ELISAs according to the manufacturer’s instructions (BD Biosciences).

### Statistical Analysis

Results are reported as mean±SEM. All data were analyzed using Prism 4.03 Statistical Program. A probability value of <0.05 was considered statistically significant. We used the 2-tailed Student t test (at 95% confidence interval) to compare unpaired samples between experimental groups or 1-way ANOVA with Tukey post hoc test for multiple comparison. All data analyzed were normally distributed (*P<0.05, **P<0.01, ***P<0.001).
Results

LCCWE Injection Accelerates Atherosclerotic Plaque Development in Apoe−/− or Ldlr−/− Mice Fed High-Fat Diet

To directly investigate whether induction of vasculitis and coronary arteritis in the KD mouse model accelerates the development of atherosclerosis in the presence of high-fat diet, we injected 5-week-old Apoe−/− mice with either 250 μg LCCWE or PBS intraperitoneally. Two weeks later, 5 mice from each group were euthanized to confirm the development of vasculitis and coronary arteritis. One hundred percent of the mice that received LCCWE injection demonstrated coronary arteritis as expected (Figure I in the online-only Data Supplement). Another 15 mice from each group were fed a high-cholesterol diet, starting 2 weeks after the LCCWE injection, and continued for 8 weeks before killing (Figure 1A). At that time, the heart, aortic arch, great vessels, and aorta were harvested revealing to the naked eye considerable atherosclerotic plaques as whitish patches along the arteries, particularly in the proximal regions in KD group, but not in the control group (Figure 1B). Aortic sinus, aortic arch, and en face aorta were stained for lipids with Oil Red O. KD mouse developed significantly increased atherosclerotic lesions in the en face aorta compared with control mouse group (P<0.001, Figure 1C). After morphometric studies, KD mouse had significantly increased total atherosclerotic lesion area and lipid accumulation in the aortic sinus (P<0.001; Figure 1D). In addition, the 3 branches of the aortic arch also showed significantly increased lesions as measured by total plaque area and lipid accumulation (P<0.01; Figure 1E). In 6 of 15 mice, the innominate artery (the first branch coming off the aortic arch) was nearly completely occluded (Figure 1E). Importantly, these differences were independent of serum cholesterol levels as the 2 groups had similar blood cholesterol levels (Table I in the online-only Data Supplement) and the same lipoprotein profiles (Table I in the online-only Data Supplement). These results strongly indicate that initial vascular insults, such as KD arteritis and vasculitis, lead to significantly accelerated atherosclerosis in this mouse model during subsequent high-fat feeding.

We next investigated the serum concentration levels of several proatherogenic cytokines to better understand the mechanisms by which LCWE-induced KD might lead to accelerated atherosclerosis. LCWE-injected Apoe−/− KD mice had significantly increased circulating concentrations of interferon-γ, IL-12p40, and tumor necrosis factor-α (Figure 1F) compared with PBS-injected mice placed on high-fat diet. These results appear most consistent with the interpretation that at least part of the acceleration of atherosclerosis observed in Apoe−/− KD mice may be mediated by a general increase in circulating levels of proatherogenic inflammatory cytokines.

Because Apoe−/− mice were reported to display certain immune defects,23,24 we repeated the above experiment using Ldlr−/− mice, another widely studied murine model of atherosclerosis. As in the Apoe−/− group, Ldlr−/− mice that first developed KD vasculitis before high-fat diet also developed significantly accelerated atherosclerosis (Figure 2B). Quantification of the lesion area of aortic sinus and aortic arch plaques revealed a significant increase in lesion size in KD Ldlr−/− mice compared with PBS littermate controls (P<0.01; Figure 2B and 2C). KD Ldlr−/− mice also developed significantly increased lipid accumulation in the aortic sinus plaque, aortic arch lesions (P<0.01; Figure 2B and 2C), and total lesion area in the en face aorta (P<0.01; Figure 2D) compared with non-KD, control Ldlr−/− mice. The serum cholesterol levels (Table I in the online-only Data Supplement) and serum lipoprotein profiles (Table I in the online-only Data Supplement) were equal between the KD and non-KD Ldlr−/− groups.

Examination of hematoxylin-eosin and trichrome/elastin-stained histologic sections of the aortic root showed marked differences between the Apoe−/− KD and Apoe−/− non-KD groups. Apoe−/− KD mice developed larger, more complex aortic lesions with abundant collagen, and extracellular as well as intracellular lipid (Figure 3) compared with Apoe−/− non-KD control group. The aortic lesions in the control group were smaller and composed primarily of intracellular lipids in foam cells (Figure 3). In the Apoe−/− KD group, there were coronary lesions that resembled those of the aorta with variable degrees of luminal narrowing. For the most part, the coronary arteries in the control PBS group were normal or had minimal lesions.

LCWE-Induced Acceleration of Atherosclerosis Is Associated With Increased Numbers of Activated DCs, T Cells, and Macrophages in Aortic Sinus Plaques

Infiltration of immune cells into atherosclerotic lesions plays an important role in plaque development. Dendritic cells (DCs) directly control the innate and adaptive immune responses that occur during inflammatory diseases such as atherosclerosis25,26 and their functions in innate and adaptive immunity.27 DCs are present in normal arteries, but the numbers of activated DCs increase as atherosclerosis develops.28,29 Indeed, recent data indicate that both mDCs and pDCs are present in increased amounts in human plaques.30,31 We reasoned that in the Apoe−/− KD mice fed high-fat diet for 8 weeks, the number of infiltrating activated DC numbers in the aortic sinus plaques would increase further when compared with Apoe−/− non-KD mice. To test this hypothesis, we performed immunohistochemical staining using MIDC-8 Abs to quantitatively measure numbers of mature, activated mDCs, and PDCA-1 Ab for pDCs. As anticipated, Apoe−/− KD mice developed significantly increased numbers of activated mDCs and pDCs in the aortic sinus plaques compared with Apoe−/− non-KD mice (Figure 4A and 4B). In addition, we examined the coronary artery, as coronary arteritis is a key component of KD. Indeed, we also saw increased DCs and pDCs at the coronary artery (Figure 4A and 4B). These data suggest that acceleration of atherosclerosis induced by LCWE-induced KD vasculitis is accompanied by increased numbers of activated mDCs recruited into the plaques.

In addition to DCs, both macrophages (Mφ) and T cells participate in the development of atherosclerotic plaques,32 atherosclerosis, and coronary artery disease.33 Therefore, we also examined the extent of macrophage infiltration with F4/80 immunostaining and T-cell infiltration with CD3 immunostaining in the coronary lesions and aortic...
Figure 1. *Lactobacillus casei* cell wall extract (LCWE) injection induces acceleration of atherosclerosis in apolipoprotein E knockout (*Apoe<sup>-/-</sup>) mice with 8 weeks of high-fat diet. **A,** Schematic representation for LCWE-induced Kawasaki disease (KD) mouse model, and *Apoe<sup>-/-</sup> KD mouse model to study acceleration of atherosclerosis. **B,** Light photography showing meticulous dissection of the heart, aortic arch, great vessels, and aorta in an *Apoe<sup>-/-</sup> KD and *Apoe<sup>-/-</sup> non-KD mouse after 8 weeks of high-fat diet. Dramatic increase in atherosclerotic plaques can be seen by the naked eye as whitish patches all along the arteries in *Apoe<sup>-/-</sup> KD but not in *Apoe<sup>-/-</sup> non-KD mice after high-fat diet. **C,** Aorta en face lesion coverage and representative Oil Red O staining from *Apoe<sup>-/-</sup> KD and *Apoe<sup>-/-</sup> non-KD (PBS control) mice. **D,** Quantification of aortic sinus lesion size, lipid content in the aortic sinus lesions, and representative Oil Red O staining of aortic sinus plaque (original magnification ×40) from *Apoe<sup>-/-</sup> KD and *Apoe<sup>-/-</sup> non-KD (PBS control) mice (n=15/group). **E,** Quantification of aortic arch plaque size, lipid content in the aortic arch, and representative Oil Red O staining of aortic arch plaque (original magnification ×40). Data are presented as mean values±SEM. n=15/group. **F,** Serum concentrations of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-12p40 obtained at the end of high-fat diet for 8 weeks in *Apoe<sup>-/-</sup> KD and *Apoe<sup>-/-</sup> non-KD mice (n=10/group). *P*<0.05, **P*<0.005, ***P*<0.001.
Figure 2. *Lactobacillus casei* cell wall extract (LCWE) injection induces acceleration of atherosclerosis in low-density lipoprotein receptor knockout (*Ldlr*−/−) mice with 8 weeks of high-fat diet. A, Schematic representation for *Ldlr*−/− Kawasaki disease (KD) mouse model to investigate acceleration of atherosclerosis. B, Light photographs showing meticulous dissection of the heart, aortic arch, great vessels, and aorta in an *Ldlr*−/− KD and *Ldlr*−/− non-KD (PBS control) mice. Dramatic increase in atherosclerotic plaques seen by the naked eye as whitish patches all along the arteries in *Ldlr*−/− KD but not in *Ldlr*−/− non-KD (PBS control) mice. C, Quantification of aortic sinus lesion size, lipid content in the aortic sinus lesions, and representative Oil Red O staining of aortic sinus plaque (original magnification ×40) from *Ldlr*−/− KD and *Ldlr*−/− non-KD mice. D, Quantification of aortic arch plaque size, lipid content in the aortic lesions, and representative Oil Red O staining of aortic arch plaque (original magnification ×40) from *Ldlr*−/− KD and *Ldlr*−/− non-KD (PBS control) mice. E, Aorta en face lesion coverage and representative Oil Red O staining from *Ldlr*−/− KD and *Ldlr*−/− non-KD mice. Data are presented as mean value±SEM. n=10/group. *P<0.05, **P<0.005, ***P<0.001.
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sinus plaques. Apoe−/− KD mice had significantly increased T-cell numbers in coronary lesions and aortic sinus plaques (P<0.05; Figure 4C) as well as macrophage in coronary lesions when compared with Apoe−/− non-KD control mice (P<0.05, Figure 4D).

LCWE Injection Accelerates Atherosclerotic Plaque Development in Apoe−/− Mice Even When Fed Regular Chow

As discussed above, LCWE injection induced acceleration of atherosclerosis in hypercholesterolemic Apoe−/− mice (Apoe−/− KD mice) after high-fat diet. To investigate whether KD vasculitis provide a strong stimulus for accelerated atherosclerosis even in the absence of high-fat diet, we repeated the above experiment in LCWE-injected Apoe−/− mice but fed them regular chow at day 14, after extract injection and kept for 8 weeks before killing. Quantification of the lesion area of aortic sinus plaques revealed a significant increase in atherosclerotic lesion size in Apoe−/− KD mice compared with Apoe−/− non-KD mice (P<0.01; Figure 5A). Apoe−/− KD mice had a significantly increased lipid accumulation in both the aortic sinus plaque lesions (P<0.01; Figure 5A and 5C) and total lesion area in the en face aorta (P<0.01; Figure 5D), as well as in the aortic arch compared with Apoe−/− non-KD mice (P<0.05; Figure 5B). Serum cholesterol concentrations (Table I in the online-only Data Supplement) and lipoprotein profiles (data not shown) were again similar in LCWE-injected and PBS control mice.

Treatment With IL-1 Receptor Antagonist (IL-1Ra) Significantly Inhibits KD Vasculitis-Induced Acceleration of Atherosclerosis in Apoe−/− Mice

We have recently shown that Caspase-1 and IL-1β signaling pathway is critical for the LCWE-induced KD mouse model and that IL-1Ra treatment effectively blocks LCWE-induced vasculitis, coronary arteritis, and myocarditis.17

Figure 3. Trichrome/elastin stain of aortic sinus and coronary arteries of apolipoprotein E knockout (Apoe−/−) Kawasaki disease (KD) and Apoe−/− non-KD mice fed high-fat diet. With trichrome/elastin stain, the muscle tissue is red, collagen is blue, and foam cells are pink. Top show a cross-section of the aortic sinus from Apoe−/− KD and Apoe−/− non-KD (PBS control) mouse. Note that the lesions in Apoe−/− KD Lactobacillus casei cell wall extract (LCCWE) are larger and have more collagen (blue) than the lesion in the Apoe−/− non-KD (PBS control) mouse (arrows) (×12.5). The middle show lesions at a higher magnification (×100) showing the foam cells, collagen, and extracellular lipid in cholesterol clefts (arrow) in the Apoe−/− KD mouse, while the lesion in the Apoe−/− non-KD mouse is composed almost entirely of foam cells. The bottom show a coronary lesion in the and Apoe−/− KD mouse composed of collagen and foam cells, with marked narrowing of lumen (L), while the coronary arteries in the Apoe−/− non-KD group were mostly normal (L). Note that in this Apoe−/− non-KD (PBS control) mouse, there is a small cluster of foam cells (arrow) in the aorta (×200).
Therefore, we next investigated whether IL-1Ra given for prevention or treatment of the acute KD vasculitis can also inhibit or ameliorate the ensuing accelerated atherosclerosis that we observe in the Apoe<sup>−/−</sup> KD mice. We injected IL-1Ra (Kineret, Amgen) (500 μg) daily (IP) into Apoe<sup>−/−</sup> mice from 1 day before LCWE or PBS injection to day 5, as we recently described. 17 Five mice were euthanized on day 7 after extract injection, and their hearts were harvested for analysis to study the effect of IL-1Ra on LCWE-induced coronary lesions. As expected and reported, 17 the incidence of KD vasculitis was significantly decreased in IL-1Ra–treated mice compared with PBS-treated controls (Figure I in the online-only Data Supplement). Additional LCWE-injected 10 Apoe<sup>−/−</sup> mice from each group were either treated with IL1Ra or given PBS injections and fed a high-cholesterol diet for 8 weeks before killing. We observed that IL-1Ra–treated Apoe<sup>−/−</sup> KD group had significantly reduced acceleration of atherosclerosis compared with PBS-treated Apoe<sup>−/−</sup> KD group: IL-1Ra–treated Apoe<sup>−/−</sup> KD mice demonstrated a reduction in the atherosclerotic lesion development in both the aortic sinus and aortic arch, had less lipid accumulation in aortic sinus and aortic arch plaques, and had reduced size of atherosclerotic lesions in the aorta compared with the PBS-treated Apoe<sup>−/−</sup> non-KD mice (Figure 6A–6C). In addition, IL-1Ra treatment resulted in a reduction in the serum levels of tumor necrosis factor β compared with PBS-treated group (Figure 6D). Taken together, these data demonstrate that LCWE-induced KD vasculitis significantly accelerates atherosclerotic lesion development in Apoe<sup>−/−</sup> mice fed high-fat diet and that initial treatment of the KD vasculitis by IL-1Ra can prevent the accelerated atherogenesis seen in Apoe<sup>−/−</sup> KD mice.
Discussion
KD is the leading cause of pediatric acquired heart disease in the United States, and hospital admissions attributed to KD are increasing across the country. Although KD is considered to be an acute and self-limiting disease in the majority of cases, the coronary artery damages caused by KD and the diffuse vascular inflammation that is pathognomonic for this disease may have long-term sequelae. Multiple studies have shown that patients with KD and persistent coronary artery aneurysm after the acute phase of disease have various vascular abnormalities, generalized vascular disease, and enduring inflammation. The most prominent histological feature of coronary lesions after the acute phase of illness is intimal thickening, consisting of smooth muscle cells and extracellular matrix that is the result of cell migration through disrupted internal elastic intima. Even when coronary artery lesions regress to normal form on echocardiogram or angiogram, they are virtually always associated with intimal thickening in all

Figure 5. *Lactobacillus casei* cell wall extract (LCWE) injection induces acceleration of atherosclerosis in apolipoprotein E knockout (Apoe<sup>−/−</sup>) mice fed 8 weeks with regular chow. **A**, Schematic representation for and Apoe<sup>−/−</sup> Kawasaki disease (KD) on regular chow. **B**, Quantification of aortic sinus lesion size, lipid content in the aortic sinus lesions, and representative Oil Red O staining of aortic sinus plaque (original magnification ×40) from Apoe<sup>−/−</sup> KD and Apoe<sup>−/−</sup> non-KD mice. **C**, Quantification of aortic arch plaque size, lipid content in the aortic lesions, and representative Oil Red O staining of aortic arch plaque (original magnification ×40). Data are presented as mean values±SEM. n=15/group. **D**, Aorta en face lesion coverage and representative Oil Red O staining from Apoe<sup>−/−</sup> KD and Apoe<sup>−/−</sup> non-KD mice (n=10/group). *P<0.05, **P<0.005, ***P<0.001.
forms of lesion. Excessive intimal thickening has the potential to develop into stenosis or promote thrombus formation.

Another important sequelae of KD that is frequently discussed but is still controversial is the potential for accelerated development of atherosclerosis. Compounding the risk factor for accelerated atherosclerosis is the observation that KD is associated with altered lipid metabolism (in particular, lower high-density lipoprotein cholesterol) that persists beyond...
clinical resolution of disease.\textsuperscript{36} The observation of low-plasma high-density lipoprotein concentrations after KD is particularly important because the vasculitis in KD has a predilection for the coronary arteries at sites identical to those most often affected in atherosclerosis.\textsuperscript{37,38} For these children, intensive cardiovascular risk reduction is of critical importance. Frequently, awareness of the risk for premature atherosclerosis is often limited when the main focus is on timely diagnosis and acute medical care. Endothelial dysfunction is considered an initial event in the development of atherosclerotic plaques, as it promotes the migration of leukocytes and monocytes into the vessel wall, where macrophage interactions with T cells play an important pathogenic role.\textsuperscript{39} Other autoimmune vascular disorders such as systemic lupus erythematosus and Rheumatoid arthritis have also been associated with increased atherosclerosis leading to increased morbidity and mortality due to cardiovascular disease.\textsuperscript{40-44} The potential mechanisms whereby KD patients would be at increased risk for accelerated atherosclerosis include (1) arterial damage secondary to the acute disease process that alters the vascular structure itself, predisposing these vessels to development of atherosclerosis, and (2) enduring inflammation and vasculitis that promotes atherosclerotic processes.\textsuperscript{15} To study whether patients with a history of KD are at increased risk for atherosclerosis or other vascular abnormalities, researchers have turned to noninvasive techniques in assessing post-KD patients. One such technique is flow-mediated dilation, which measures nitric oxide–mediated vasodilatation of the brachial artery on ultrasonography. Decreased flow-mediated dilation occurs in children with a history of KD, as well as other conditions that predispose to the development of atherosclerosis such as diabetes mellitus and family history of premature coronary artery disease.\textsuperscript{8} Another technique to study vascular integrity is by measuring carotid intima-media thickness, where increased intima-media thickness, or thickening of vascular walls, correlates with development of atherosclerosis.\textsuperscript{5} Studies that use these tests and others to assess KD patients have been largely conflicting with regard to whether or not patients with a history of KD show evidence of long-term vascular damage. McCrindle et al\textsuperscript{16} found that children with a history of KD did not have significantly decreased flow-mediated dilation, whereas Dalla Pozza et al\textsuperscript{45} found that they had significantly increased intima-media thickness as did Noto et al.\textsuperscript{44} Many of these studies were hampered by small sample sizes, short duration of follow-up, and complicated by countless risk factors other than KD that influence vascular health, including dyslipidemia and diabetes mellitus. These discrepancies may also be related to differing KD characteristics during the acute phase of disease and subsequent treatment, and racial disparities.\textsuperscript{45} As KD was only described 40 years ago, there have yet to be any large-scale epidemiological studies that address these issues.

In view of conflicting clinical data, we wished to directly investigate whether KD vasculitis accelerates the development of atherosclerosis using the combination of the LCWE-induced KD mouse model and the hypercholesterolemic mouse models such as Apoe\textsuperscript{−/−} and Ldlr\textsuperscript{−/−} mice. The LCWE model of KD has been shown to be a valuable tool in the immunopathological studies of this disease, as it mimics the histopathology of the KD coronary arteritis, vasculitis, and myocarditis and even reliably predicts human intravenous immunoglobulin treatment responses.\textsuperscript{46-48} Therefore, the Apoe\textsuperscript{−/−} KD mouse model used in the present study has the potential to predict the acceleration of atherosclerosis in KD patients. In our study, acceleration of atherosclerosis was observed at the same sites where we saw the initial vasculitis, that is, coronary artery and aorta. It remains to be determined whether other vessels that have been reported to develop vasculitis also develop accelerated atherosclerosis. We found that in Apoe\textsuperscript{−/−} KD and Ldlr\textsuperscript{−/−} KD mice-fed high-fat diet developed significantly accelerated atherosclerosis as measured in their aortic sinus and aortic arch plaques compared with Apoe\textsuperscript{−/−} non-KD control mice. Apoe\textsuperscript{−/−} KD mice also had significantly increased lipid accumulation in the aortic sinus plaques, aortic arch lesions, and increased total atherosclerotic lesion area in aorta compared with Apoe\textsuperscript{−/−} non-KD mice despite similar level of serum cholesterol levels between the groups. While human KD has been associated with additional risk factors for atherosclerosis, such as increased lipid profiles, the KD mouse model provides compelling evidence that initial vascular injury predisposes to accelerated atherosclerosis, particularly in the presence of hypercholesterolemia.

Furthermore, the accelerated atherosclerosis seen in the present study was associated with an increase in levels of cytokines interferon-γ, IL-12, p40, and tumor necrosis factor-α. As these cytokines have been associated with pathogenesis of atherosclerosis, we can conclude that this increase in cytokine levels caused by LCWE injection may in part contribute to the accelerated atherosclerosis observed. Immune cells and their mediators are critical players in atherogenesis and contribute to the chronic arterial inflammation that is a hallmark of the disease. The inflammatory response is mediated by components of the innate immune system, including macrophages and DCs,\textsuperscript{49,50} and by components of the adaptive immune system, including T lymphocytes.\textsuperscript{33,51} We observed an increase in DCs, macrophages, and T cells within the lesion areas, which is consistent with human data characterizing atherosclerotic lesions.\textsuperscript{7} Together these findings are consistent with the fact that atherosclerotic processes are accelerated in Apoe\textsuperscript{−/−} KD mice fed high-fat diet.

Secretion of IL-1β, a potent pyrogen that elicits a strong pro-inflammatory response,\textsuperscript{52} is tightly controlled by a diverse class of cytosolic complexes known as inflammasomes. It is well established that IL-1β plays a critical role in chronic inflammatory diseases such as atherosclerosis.\textsuperscript{54-56} IL-1β signaling is mediated through the type I IL-1 receptor (IL-1R1). In addition, the IL-1β receptor antagonist (IL-1Ra), an endogenous molecule, can bind the IL-1β receptor and prevent normal IL-1 signaling.\textsuperscript{57} Recombinant IL-1Ra (Anakinra) has been approved for the treatment of various inflammatory diseases, such as rheumatoid arthritis,\textsuperscript{58} and anti–IL-1β mAb is currently in phase III clinical trials for atherosclerosis. IL-1β has been associated with the pathogenesis of KD in our previous studies\textsuperscript{17} as well as by others, and in recent years its key role in vascular wall inflammation has been appreciated even further.\textsuperscript{59} Indeed, we have recently shown that blocking IL-1β in the LCWE-induced KD mouse by IL-1Ra can effectively block coronary arteritis, vasculitis, and myocarditis.\textsuperscript{17} In an attempt to modulate the KD vasculitis-mediated acceleration of atherosclerosis, we
treated LCWE-injected Apoe<sup>−/−</sup> mice with IL-1Ra and observed that mice treated with IL-1Ra developed significantly less atherosclerosis. This protection is most likely attributable to the IL-1Ra-mediated blocking of the initial KD vasculitis. It should be noted, however, that IL-1Ra was not completely protective for accelerated atherosclerosis in the Apoe<sup>−/−</sup> KD mice. This may be attributable to residual endothelial cell dysfunction despite treatment for the prevention of KD vasculitis. Recent studies suggest that statin treatment may also be beneficial in KD-induced coronary arteritis and in KD-induced acceleration of atherosclerosis as well. Studies suggest that novel anti-inflammatory therapies are needed not only for intravenous immunoglobulin–resistant KD patients but perhaps also to prevent potential acceleration of atherosclerosis and the resulting long-term cardiac complications in KD patients. The present study supports the possibility that KD patients may be at increased risk for developing accelerated atherosclerosis, and larger clinical studies with longer follow-up in these patients will be needed to prove this association clinically. Until then, our findings support the current AHA recommendations that children with a history of KD should be carefully monitored for known risk factors of atherosclerosis, and potentially treated for accelerated development of atherosclerosis. The observations from the current study, together with a recently published study, suggest that IL-1β signaling may play an important role in the development of LCWE-induced KD vasculitis as well as in the accelerated atherosclerosis that we observed in the Apoe<sup>−/−</sup> KD mice. These findings provide a justification for undertaking clinical studies to investigate whether Food and Drug Administration–approved anti–IL-1β agents may provide benefit in KD-induced coronary arteritis and in KD-induced acceleration of atherosclerosis as well.

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Disclosures
None.

References


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