Role of Interleukin-1 Signaling in a Mouse Model of Kawasaki Disease–Associated Abdominal Aortic Aneurysm


Objective—Kawasaki disease (KD) is the most common cause of acquired cardiac disease in US children. In addition to coronary artery abnormalities and aneurysms, it can be associated with systemic arterial aneurysms. We evaluated the development of systemic arterial dilatation and aneurysms, including abdominal aortic aneurysm (AAA) in the Lactobacillus casei cell-wall extract (LCWE)–induced KD vasculitis mouse model.

Methods and Results—We discovered that in addition to aortitis, coronary arteritis and myocarditis, the LCWE-induced KD mouse model is also associated with abdominal aorta dilatation and AAA, as well as renal and iliac artery aneurysms. AAA induced in KD mice was exclusively infrarenal, both fusiform and saccular, with intimal proliferation, myofibroblastic proliferation, break in the elastin layer, vascular smooth muscle cell loss, and inflammatory cell accumulation in the media and adventitia. Il1ra−/−, Il1rα−/−, and Il1b−/− mice were protected from KD associated AAA. Infiltrating CD11c+ macrophages produced active caspase-1, and caspase-1 or NLRP3 deficiency inhibited AAA formation. Treatment with interleukin (IL)-1R antagonist (Anakinra), anti–IL-1α, or anti–IL-1β mAb blocked LCWE-induced AAA formation.

Conclusions—Similar to clinical KD, the LCWE-induced KD vasculitis mouse model can also be accompanied by AAA formation. Both IL-1α and IL-1β play a key role, and use of an IL-1R blocking agent that inhibits both pathways may be a promising therapeutic target not only for KD coronary arteritis, but also for the other systemic arterial aneurysms including AAA that maybe seen in severe cases of KD. The LCWE-induced vasculitis model may also represent an alternative model for AAA disease. (Arterioscler Thromb Vasc Biol. 2016;36:886-897. DOI: 10.1161/ATVBHA.115.307072D.)

Key Words: abdominal aortic aneurysm □ aortitis □ caspase-1 □ dilatation □ vasculitis

Kawasaki disease (KD) is an acute febrile illness characterized by systemic vasculitis of unknown cause that may lead to acquired heart disease that predominantly affects children <5 years of age, with a male:female ratio of 1.5:1.1-10 KD, if untreated leads to coronary artery abnormalities in ≤30% of children and is the leading cause of acquired heart disease in children in the United States. The pathogenesis of KD remains unknown despite 40 years of intensive studies, although the current paradigm is that KD is triggered by an infectious agent that elicits an inflammatory response directed at cardiovascular tissues in genetically susceptible hosts.2,3,4,11,12

Treatment with high dose of intravenous immunoglobulin (IVIG) resolves inflammation and reduces the occurrence of coronary abnormalities, predominantly coronary artery aneurysms from 25% to 30% down to 5% to 7%.13-15 Currently, studies from the United States report an aneurysm rate of ≈5%, despite IVIG therapy.10 Importantly, up to 20% of patients with KD are IVIG resistant and do not respond to the initial IVIG dose and thus have a particularly high risk of developing coronary artery aneurysm.13 Coronary artery abnormalities in KD are characterized histologically by inflammatory cell infiltration and focal destruction of the arterial media, especially elastic tissue in the media, with resultant coronary artery aneurysm formation. Subsequent thrombosis or, less commonly, rupture of diseased coronary or other systemic vessels may occasionally be fatal.16 KD vasculitis, once thought of as an acute self-limiting disease, is now being increasingly recognized to induce long-term vascular changes and remodeling such as luminal myofibroblast proliferation, leading to coronary artery stenosis with both cardiovascular and myocardial...
Importantly, a well-described and well-accepted mouse model of KD vasculitis and coronary arteritis closely mimics the important histological as well as immune-pathological features of the cardiovascular lesions (ie, coronary arteritis, aortitis, myocarditis, aneurysms, including luminal myofibroblast proliferation and scarring and stenosis in the coronary arteries). This mouse model (Lactobacillus casei cell-wall extract [LCWE]–induced KD vasculitis) reliably predicts efficacy of treatment options in children with KD. Although no animal model can fully mimic human disease, the LCWE-induced KD mouse model, has been accepted by many in the Kawasaki research community, as a reliable experimental model with the goal to provide novel insights that can be tested in children. The translational value of this animal model has recently been shown again when discovery for the role of IL-1 signaling in the pathogenesis of KD vasculitis, coronary artery lesions, and myocarditis in mice. In this study, we now show that in addition to aortitis, coronary arteritis, and myocarditis, the LCWE-induced KD model also induces the development of abdominal aorta dilatation and AAA, which are exclusively infrarenal, as well as renal and iliac artery aneurysms, mostly in male mice. We also demonstrate the critical pathophysiological role of nucleotide binding domain and leucine-rich repeat pyrin domain containing 3 (NLRP3)-inflammasome–dependent activation of IL-1/IL-1R signaling, and the role of IL-1α and IL-1β in the development of systemic arteritis, abdominal aorta dilatation, and AAA and provide detailed histopathologic data of the aortic vessel wall.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Development of Abdominal Aorta Aneurysms and Dilatation in LCWE-Induced KD Vasculitis Mouse Model

We first investigated the development of abdominal aortic dilatation and aneurysms in the LCWE-induced KD mouse model. At 0, 1, 2, 5, and 8 weeks after LCWE injection into male mice, the diameter of abdominal aorta was measured. Over 80% of the mice developed significant dilatation of abdominal aorta (Figure 1A and 1B). LCWE-injected mice showed obvious dilatation and aneurysms exclusively below the right renal artery but not in the suprarenal aorta (Figure 1A and 1B in the online-only Data Supplement). We observed localized eccentric dilatation indicative of a saccular aneurysm and a long cylindrical dilatation indicative of a fusiform aneurysm (Figure IC in the online-only Data Supplement). Aneurysm formation was also detected in the renal and iliac arteries (Figure 1C), similar to what is found in patients with KD. These results indicate that LCWE injection induces site-specific vascular remodeling (ie, below the right renal artery). Moreover, magnetic resonance imaging analysis showed that both the lumen and the overall area of abdominal aorta in KD mice significantly increased compared with control mice, indicating that LCWE injection leads to luminal dilatation of abdominal aorta (Figure 1D–1F; Figure IIA–IIC in the online-only Data Supplement). In human patients, KD is 1.5× more complications. Although IVIG reduces the rate of coronary artery aneurysm and the morbidity and mortality associated with KD, lack of a specific pathogenic agent and incomplete understanding of the molecular mechanisms mediating the cardiovascular pathology of KD have hampered the development of targeted and more effective treatment options. The limited availability of tissue samples from patients with KD has significantly impeded our progress in understanding the pathogenesis of the disease, making the availability of a relevant animal model extremely valuable.

In this study, we now show that in addition to aortitis, coronary arteritis, and myocarditis, the LCWE-induced KD model also induces the development of abdominal aorta dilatation and AAA, which are exclusively infrarenal, as well as renal and iliac artery aneurysms, mostly in male mice. We also demonstrate the critical pathophysiological role of nucleotide binding domain and leucine-rich repeat pyrin domain containing 3 (NLRP3)-inflammasome–dependent activation of IL-1/IL-1R signaling, and the role of IL-1α and IL-1β in the development of systemic arteritis, abdominal aorta dilatation, and AAA and provide detailed histopathologic data of the aortic vessel wall.

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| Nonstandard Abbreviations and Acronyms |
|-----------------------------|-----------------------------|
| AAA | abdominal aortic aneurysm |
| IL | interleukin |
| IVIG | intravenous immunoglobulin |
| KD | Kawasaki disease |
| LCWE | Lactobacillus casei cell-wall extract |
| MMP | matrix metalloproteinase |

IL-1β plays a critical role in broad spectrum of diseases, including chronic inflammatory diseases such as rheumatoid arthritis, metabolic syndrome, diabetes mellitus, atherosclerosis, and more recently it was linked to KD vasculitis. IL-1α also plays a critical role in chronic inflammation and studies suggest that IL-1α can regulate IL-1β secretion. We have shown the key role of Toll-like receptor 2 and IL-1R via MyD88 signaling in coronary arteritis and aortic root vasculitis in this KD mouse model. IL-1 receptor antagonist (Anakinra) given either prophylactically or ≤3 days after LCWE injection efficiently prevents the development of KD vasculitis, coronary artery lesions, and myocarditis in mice. In this study, we now show that in addition to aortitis, coronary arteritis, and myocarditis, the LCWE-induced KD model also induces the development of abdominal aorta dilatation and AAA, which are exclusively infrarenal, as well as renal and iliac artery aneurysms, mostly in male mice. We also demonstrate the critical pathophysiological role of nucleotide binding domain and leucine-rich repeat pyrin domain containing 3 (NLRP3)-inflammasome–dependent activation of IL-1/IL-1R signaling, and the role of IL-1α and IL-1β in the development of systemic arteritis, abdominal aorta dilatation, and AAA and provide detailed histopathologic data of the aortic vessel wall.
likely in males compared with females.7,10 Male mice showed a significantly greater increase in abdominal aortic diameter compared with female mice (Figure 1G and 1H). Indeed, female mice did not develop a statistically significant increase in abdominal aortic diameter on LCWE injection.

Abdominal Aorta in KD Mice Show Intense Vessel Wall Inflammation

We next performed a histological analysis of the abdominal aortic lesions. Abdominal aortic tissue was dissected from naive mice and LCWE-injected mice at 14 days. Hematoxylin and
eosin and elastin/collagen staining of abdominal aortic sections showed significant intimal proliferation and myofibroblastic proliferation that penetrated and broke the elastin layer with significant inflammatory cell accumulation in media and adventitia (Figure 2A). In addition, the AAA lesions showed loss of smooth muscle cells in the media, which is a typical of AAA histology (Figure 2A, bottom). The lesions of iliac and renal arteries also showed similar inflammation (Figure III in the online-only Data Supplement). We also collected the heart from these same animals to compare the lesion intensity of the coronary and aortic root with the AAA lesions. The intensity of inflammation in the aortic sinus/coronary artery significantly correlated with dilation of the abdominal aorta (Figure 2B and 2C), suggesting that heart/coronary lesion progression and AAA were linked.

**Innate Inflammatory Cells Infiltrate into the Vessel Wall in Aorta Lesions**

To evaluate which types of immune cells are infiltrating into AAA lesions in KD mouse model, AAA sections were stained with antibodies against several cell type–specific markers. We found that large numbers of CD45+ immune cells infiltrated into the adventitia, whereas fewer CD45+ cells were detected in the media and intima of AAA lesions (Figure 3A and 3B; Figure IV in the online-only Data Supplement). On closer examination, we found the accumulation of large numbers of Ly6G+ cells (neutrophils), as well as CD11c+ or F4/80+ cells (dendritic cells and macrophages) with smaller populations of CD3+ T cells. We did not find any CD19+ (B cells) or NK1.1+ (NK and NKT) cells in the lesions (Figure 3B). Moreover, costaining with antibodies against CD11c and F4/80 revealed the presence of CD11c+ F4/80+ double-positive macrophages in the adventitia lesions (Figure 3C).

**Correlation of Gene Expression Profiles of Human Adult AAA Disease With That of AAA Seen in the KD Vasculitis Mouse Model**

Abdominal aortic lesions in LCWE-induced KD mouse showed the typical histology of human AAA, such as destruction of vascular smooth muscle cell architecture and marked inflammatory cell accumulation (Figures 2 and 3). To investigate the relevance of the mouse KD AAA lesions to the human (non-KD) AAA that is seen in adults, we evaluated the gene expression profile changes in abdominal aortic lesions of LCWE-injected mice compared with naive mice, and then compared these data with the gene expression changes between the aortas of healthy donors and patients with AAA.46 We found upregulation or downregulation of 6046 genes and 1643 genes in KD mouse model and AAA patient data, respectively. As shown in the heat map of Figure 4A, the gene expression profiles of KD mouse and patients with AAA were remarkably correlated. Intriguingly, gene expression profile in AAA of KD mice showed marked similarity with adult human AAA disease, including increased expression of IL-1β. Among the 1133 genes that are in common, 1024 genes are in the same
IL-1 Signaling and the NLRP3 Inflammasome Are Required for LCWE-Induced Aortic Dilation and Aneurysm Formation in the KD Vasculitis Mouse Model

Among the commonly upregulated genes in human and mouse, AAA lesions were IL-1–related genes. We previously reported the important role of IL-1β in heart vessel inflammation in KD mouse model. To investigate the potential role for IL-1/IL-1R signaling in LCWE-induced abdominal aorta dilation/aneurysm, we injected LCWE into wild-type and Il1r−/− mice. Similar to LCWE-induced KD coronary lesions, Il1r−/− mice were completely protected from AAA development (Figure 5A and 5B). Both IL-1α and IL-1β bind to same IL-1R, and bone marrow–derived macrophages produced both IL-1α and IL-1β in response to LCWE in vitro (Figure V in the online-only Data Supplement). We then addressed which IL-1 cytokine was important in AAA development in KD mouse model by injecting LCWE into wild-type, Il1α−/−, and Il1β−/− mice. Both Il1α−/− and Il1β−/− mice were completely protected from abdominal aorta dilatation compared with wild-type mice, indicating that both IL-1α and IL-1β play an important nonredundant role in AAA development (Figure 5A and 5B).

Inflammasome activation of caspase-1 lead to the production of inflammatory cytokines, including both IL-1β and IL-1α, although to a lesser degree for IL-1α. The NLRP3 inflammasome can be activated by many different danger signals and in vitro data indicated that LCWE activated the inflammasome in macrophages via this pathway. To investigate the role of NLRP3 inflammasome/caspase-1 pathway in formation of LCWE-induced AAA, Casp1−/− and Nlrp3−/− mice were injected with LCWE and AAA development was monitored. Both Casp1−/− and Nlrp3−/− mice were significantly protected from AAA formation in LCWE-injected mice (Figure 5C and 5D). However, the Aim2 inflammasome, which detects intracellular DNA, was not involved in LCWE-induced AAA formation (Figure 5C and 5D).

CD11c+ Macrophages in LCWE-Induced AAA Lesions Have Caspase-1 Activity and Are Critical for AAA Formation in the KD Mouse Model

Macrophages, as well as neutrophils are well known producers of IL-1β. Our data indicated large numbers of both these cell types in AAA lesions. To determine which of these cells could be producing IL-1 at the lesions, we visualized caspase-1 activity in the lesions using the fluorescent-labeled inhibitor of caspases assay. Caspase-1 activity was readily detectable at the lesion site, with no activity seen in naive animals (Figure 6A). Further investigation found that caspase-1 activity was confined to CD11c+ F4/80+ macrophages (Figure 6B), with no caspase-1 activity found in Ly6G+ neutrophils. Because IL-1 and NLRP3 inflammasome were critical for LCWE-induced AAA formation, and this activity was confined to macrophages at the lesion site, we next depleted macrophages using clodronate liposomes (clodrosomes). Mice given clodrosomes were completely protected from AAA and dilatation (Figure 6C and 6D), suggesting that macrophages were a key player in AAA development, potentially through their production of IL-1.

Pharmacological Blockade of IL-1/IL-1R Signaling Inhibits AAA Formation in KD Vasculitis Mouse Model

We had previously found that IL-1R antagonist (Anakinra) could inhibit LCWE-induced coronary lesions and myocarditis. To
investigate whether Anakinra could also inhibit AAA formation in the KD mouse model, we injected Anakinra into LCWE-injected KD mouse. Anakinra-treated mice showed a significant reduction of maximal aorta diameter and inflammatory histology compared with control mice (Figure 7A and 7B). As a second strategy for treatment, we used neutralizing antibody against either IL-1α or IL-1β to block IL-1 function in vivo. Consistent with the results in gene-deficient mice, both anti–IL-1α and anti–IL-1β mAb administration completely protected the mice from AAA formation after LCWE injection (Figure 7C–7F).

**Discussion**

This study demonstrates that the LCWE-induced KD vasculitis mouse model not only exhibits coronary arteritis, aortitis, myocarditis, but it can also trigger robust systemic artery inflammation involving the abdominal aorta, iliac, and renal arteries, with dilatation and aneurysms (AAA), myofibroblast proliferation, disruption of smooth muscle cell architecture, and massive accumulation of immune cells, which are all hallmark of human KD as well as human AAA histology. Both IL-1α and IL-1β played a key role in the formation of the
abdominal aorta dilation and AAA. Notably, inhibition of the inflammasome and IL-1/IL-1R signaling cascade by gene deletion or pharmacological blockade inhibited AAA formation in the KD vasculitis mouse model, suggesting a crucial role of inflammasome-related IL-1 activation in abdominal aorta dilatation and AAA development.

Although aortography analysis indicated a generally low incidence of systemic artery aneurysms (1.4%) in patients with KD, pathological analysis of autopsy samples of patients with KD showed abnormalities in systemic arteries, including in the abdominal aorta, iliac, and renal arteries with much higher incidence (>75%).28,47 Among patients with KD and systemic artery aneurysms, the brachial and internal iliac arteries are most commonly affected.48–50 Interestingly, most systemic artery aneurysms reported in patients with KD were symmetrical and multiple. Abdominal aorta aneurysms were detected mostly in infants during the first 8 months of life.51 Kato et al47 showed that KD patients with systemic aortic aneurysms also developed multiple giant coronary aneurysms. The outcomes of systemic arterial aneurysms resemble those of coronary artery lesions.52 Although some aneurysms regress and others persist, larger aneurysms often lead to stenosis. Similar to coronary arteries, the fate of systemic aortic aneurysms also depends on their diameter in the acute stage of the illness,53,54 but the progression of localized stenosis is slower in systemic aortic aneurysms than in the coronary arteries.55 Progression to excessive remodeling and vasculopathies, cardiovascular diseases, and early death among survivors of childhood KD has been reported with increasing frequency.53–35 It is now accepted that systemic vascular inflammation in patients with KD may persist beyond the acute stages and lead to increased risks of subsequent cardiovascular diseases.

In this study, by using the LCWE-induced KD vasculitis model, we observed that the intensity of inflammation in coronary arteries correlate with maximal abdominal aorta diameter, dilatation, and formation of AAA. Of interest, a recent study using the *Candida albicans* extract-induced KD vasculitis mouse model also reported the development of systemic artery lesions in addition to coronary lesions.55 Together, these observations suggest that in children with KD the incidence of AAA and dilation may be higher than currently appreciated, particularly in infants <1 year of age with severe KD cases. Our findings also demonstrate that blockade of IL-1/IL-1R signaling, by blocking both IL-1α and IL-1β, may be a promising therapeutic target not only for KD coronary arteritis and myocarditis but also for systemic aortic aneurysms, including abdominal aorta dilatation and AAA that maybe associated with severe KD cases.

Although systemic artery abnormalities other than coronary do not always influence outcome in patients with KD, systemic arteritis is frequently associated with intense systemic vasculitis and can cause severe cardiac sequelae. Progression to excessive remodeling and KD vasculopathies, myocardial dysfunctions, and early death among survivors of childhood
KD are being reported with increasing frequency. Indeed, recent reports showed progression of systemic arteritis into stenotic lesions in patients with KD, and these coronary artery sequelae have led to ischemic heart disease in young adults.

We have previously shown that the caspase-1/IL-1α and IL-1β pathways are important for the development of coronary arteritis and myocarditis in the LCWE-induced KD murine model. In this study, we found that IL-1/IL-1R signaling and both IL-1α and IL-1β are critical for the formation of AAA, suggesting that the development of cardiovascular lesions associated with KD in both coronary artery and systemic arteries, including for AAA, may share a similar pathophysiology through the NLPR3 inflammasome and the IL-1 signaling pathway. The selective activation of caspase-1 in F4/80+ CD11c+ macrophages present in AAA lesions of KD mice, suggests that these cells are responsible for IL-1β production. We previously showed that the mechanism by which the NLPR3 inflammasome is activated is through oxidative mitochondrial DNA in macrophages. It is intriguing that another recent study found that mitochondrial oxidative stress in macrophages can also lead to activation of NLPR3 and to the development of AAA in the angiotensin II–induced murine aneurysm model. Although the precise mechanism of NLPR3 inflammasome activation in LCWE-induced AAA remains unknown, our findings highlight the importance of IL-1/IL-1R signaling in both coronary arteritis and systemic artery inflammation with AAA formation in this experimental KD vasculitis model.

Similar to IL-1β, IL-1α can bind and activate the downstream signaling cascade of IL-1R. However, the role of IL-1α in KD vasculitis and AAA development remains largely unknown. For the first time, we demonstrate that in addition to Il1b−/−, disruption of Il1a or the use of IL-1α neutralizing antibody significantly inhibited LCWE-induced AAA formation, highlighting the critical role played by IL-1α, in addition to IL-1β in AAA formation in this vasculitis model. Indeed, we have also recently shown the nonoverlapping roles for IL-1α and IL-1β in LCWE-induced coronary arteritis lesions. Supporting these experimental findings, serum IL-1α levels are significantly correlated with AAA severity in human patients and surgical endovascular repair decreased the IL-1α levels, further suggesting a role of IL-1α in human AAA formation as well. In this study, Il1b−/− or wild-type mice treated with anti–IL-1β mAb were completely protected from AAA formation. The fact that IL-1α and IL-1β may recruit neutrophils and macrophages, respectively, and regulate the different phases of the inflammatory response support this finding. It is also possible that these isoforms are released sequentially, because IL-1α is often thought of as an alarmin and is produced early during acute inflammation, whereas IL-1β is more associated with the chronic phase of inflammation. Only IL-1β mRNA was upregulated in AAA of both human and KD mouse models. The novel observation that IL-1α and IL-1β
may have nonoverlapping roles in LCWE-induced cardiovascular lesions could have important implications both for our understanding of the pathogenesis of aneurysms and for the use of IL-1R antagonist (Anakinra) versus anti–IL-1β antibodies in the various treatment trials of KD, other vasculitis disorders and even AAA disease. Taken together, IL-1α and IL-1β may work in different ways at systemic or local levels, respectively, for KD vasculitis, and AAA and may influence each other.

Of interest, we observed that MMP12 gene expression was significantly increased in LCWE-induced murine AAA lesions compared with control mouse abdominal aorta. MMP12 haplotypes have been implicated in aneurysm formation in patients with KD,6 and the expression of MMP12 has been reported in human AAA lesions.65 Indeed, a recent proteomics study suggests that MMP12 is one of the most abundant metalloproteinase in AAA tissue, underscoring its role in the pathology of human AAA.64 MMP12 deficiency also attenuated AAA development in the CaCl2 model of mouse AAA as well as in the angiotensin II–induced AAA model.65,66 Interestingly tumor necrosis factor-α and IL-1 can synergistically induce MMP12.67 In addition to degrading extracellular matrix proteins, MMP12 may also promote macrophage recruitment to the vessel wall by activating tumor necrosis factor-α or by modulating levels of proinflammatory cytokines, such as monocyte chemotactic protein-1.68

Using available online databases, we were able to compare human AAA upregulated and downregulated genes with those from our LCWE-induced AAA mice. Although the number of genes that were upregulated or downregulated and the fold of upregulation were much greater in mice than in humans, this is not surprising as the experimental model is an induced acute phase model with large lesions developing versus human AAA development, which would be expected to have greater variance than a mouse model. Nevertheless, these findings suggest that the pathways involved in LCWE-induced AAA development may share a similar pathogenesis with human AAA disease and that this KD vasculitis mouse model.

The incidence and severity of abdominal aortic dilations are greater in males than females.69,70 Male sex has been consistently identified as a nonmodifiable risk factor for AAA. Evaluation of the AAA histology and pathophysiology in the LCWE-KD vasculitis mouse model revealed the following

Figure 7. Inhibition of interleukin-1 (IL-1) pathway prevents Lactobacillus casei cell-wall extract (LCWE)–induced abdominal aortic aneurysm formation. A and B, Wild-type (WT) male mice were injected with LCWE at day 0 and treated with IL-1Ra (500 μg/mouse) or PBS everyday from days 1 to 6. The abdominal aorta tissues were collected on day 14. Control IgG mice show aortae with transmural inflammation. A, Gross photographs and hematoxylin and eosin (H&E) cross section. B, The maximal aorta diameter of the mice treated with PBS (n=8) and IL-1Ra (n=7) mice. Data were analyzed by Student t test (*P<0.05). C and D, WT mice were injected with LCWE at day 0 and treated with anti–IL-1α mAb (100 μg/mouse), control mouse IgG at days 1, 2, and 5. The abdominal aorta tissues were collected at day 7. C, Representative pictures of abdominal area and H&E cross section. D, The maximal diameter of the mice treated with anti–IL-1α mAb (n=8) and control mouse IgG (n=8). Data were analyzed by Student t test (*P<0.05). E and F, WT mice were injected with LCWE at day 0 and treated with anti–IL-1β mAb (200 μg/mouse), or control hamster IgG at days 1, 2, and 5. The abdominal aorta tissues were collected at day 7. E, Representative pictures of abdominal area and H&E cross section. F, The maximal diameter of the mice treated with anti–IL-1β mAb (n=8) and control hamster IgG (n=9) mice. Data were analyzed by Student t test with Welch correction (D and F; *P<0.05). The scale bar indicates 200 μm.
marked similarities with human AAA disease: (1) histological characteristics (smooth muscle cell loss, disruption of elastin layer, and marked inflammatory cell accumulation), (2) predominant susceptibility in male mouse, (3) development of aneurysmal lesions exclusively at infrarenal aorta (below right renal artery), and (4) gene expression profiles. Consistent with our experimental findings, the IL-1β gene expression and protein levels are markedly elevated in human AAA. 73–75 Johnston et al 8 demonstrated that genetic disruption of IL-1β as well as Anakinra treatment inhibits AAA development and progression in the elastase-induced AAA mouse model. It is intriguing that IL-1 blocking therapies are currently in phase II clinical trials for both children with KD and adults with AAA disease; Anakinra for KD (NCT02179853) and Canakinumab (anti–IL-1β mAb) for AAA disease (NCT02007252).

Finally, the commonly used experimental model of AAA is a murine model that requires 28 days of continuous infusion of angiotensin II via osmotic minipumps in Apoe−/− or Ldlr−/− mice, and it is also associated with increased male susceptibility. 75 In the angiotensin II model, only 20% to 40% of LCWE-induced KD vasculitis mouse model may not only be therapeutic target for KD vasculitis and AAA patients. The suggesting that these IL-1–related molecules are potential, or IL-1R leads to marked reduction of AAA formation, IL-1β aortitis, and myocarditis. We also show inhibition of IL-1β in the elastase-induced AAA mouse model. Thus, this model may provide some advantages and potentially may be a valuable alternative experimental model to investigate AAA disease, an area that will need to be further investigated.

In summary, we found that the LCWE-induced KD vasculitis model–associated AAA model requires only a single injection of LCWE, has a penetrance of 80% in mice, and produces exclusively infrarenal AAA lesions. Thus, this model may provide some advantages and potentially may be a valuable alternative experimental model to investigate AAA disease, an area that will need to be further investigated.

Acknowledgments
We thank Ganghua Huang for technical support.

Sources of Funding
This study was supported by grants from the National Institutes of Health AI072726 and AI097748 to M. Arditi, American Heart Association 12POST79750014 to Y. Lee, and American Heart Association 15POST22810013 to D. Wakita, and by the fund from Japan Society for the Promotion of Science for the Institutional Program for Young Researcher Overseas Visits to Y. Kurashima.

Disclosures
None.

References


IL-1 in AAA in Murine Kawasaki Disease Model


Significance

Similar to clinical Kawasaki disease (KD), the Lactobacillus casei cell-wall extract–induced KD vasculitis mouse model can also be accompanied by abdominal, renal, and iliac artery aneurysms, in addition to coronary arteritis, stenosis, and myocarditis. KD mice developed abdominal aorta dilation and infrarenal abdominal aortic aneurysm with luminal dilation, intimal proliferation, breaks in elastin layer, vascular smooth muscle cell loss, and inflammatory cell accumulation. Our findings demonstrate nonoverlapping roles for interleukin-1α (IL-1α) and IL-1β. The use of an IL-1R blocking agent that inhibits both these pathways may be a promising therapeutic target for KD coronary arteritis, as well as other systemic arterial aneurysms, including abdominal aortic aneurysm that may also develop in severe cases of KD. These results further strengthen the rational and need to design anti–IL-1 therapies that block both IL-1α and IL-1β for patients with KD as well as potentially for abdominal aortic aneurysm disease. The L casei cell-wall extract–induced KD vasculitis model may be a valuable alternative model to investigate abdominal aortic aneurysm disease.
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Arterioscler Thromb Vasc Biol. 2016;36:886-897; originally published online March 3, 2016; doi: 10.1161/ATVBAHA.115.307072

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Materials and Methods

Mice
Wild-type (WT) C57BL/6 and type I IL-1R (Il1r1)−/−, Aim2−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL1β−/− mice were provided from Dr. D. D. Chaplin (University of Alabama at Birmingham, Birmingham, Ala). Casp1−/− mice were obtained from Dr R.A. Flavell (Yale University, New Haven, CT). IL1α−/− were obtained from Dr Y. Iwakura (University of Tokyo, Tokyo, Japan). Nlrp3−/− mice were provided by Dr. K. A. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). 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.

Preparation of LCWE
LCWE (ATCC 11578) was prepared as previously described. In brief, L. casei were grown in Lactobacillus de Man, Rogosa, and Sharpe broth (Difco) for 48 hours, harvested, and washed with PBS. The harvested bacteria were disrupted by 2 packed volumes of 4% SDS/PBS overnight. Cell wall fragments were washed 8 times with PBS to remove any residual SDS. The SDS-treated cell wall fragment was sonicated for 2 hours with a 3/4-in horn and a garnet tip at maximum power. During sonication, the cell wall fragments were maintained by cooling in a dry ice/ethanol bath. After sonication, the cell wall fragments were spun for 20 minutes at 12 000 rpm and 4°C. The supernatant was centrifuged for 1 hour at 38 000 rpm and 4°C, and the pellet was discarded. The total rhamnose content of the cell wall extract was determined by a colorimetric phenol-sulfuric assay as described previously.

KD vasculitis mouse model
Male mice (unless otherwise stated) 4-6 weeks of age were injected intraperitoneally with 400 µg LCWE or PBS. Mice were euthanized at Day 7, 14, and 35 and perfused with PBS containing heparin. After dissection of aorta, the diameters of abdominal aorta were measured at 5 different parts (below the left renal artery) and average and maximal abdominal aorta diameters were calculated. Aortas and hearts were removed and embedded in optimal cutting temperature (OCT) compound for histological analysis. Serial cryosections (7 µm) were prepared from the tissues and stained with hematoxylin and eosin or elastin/collagen staining. Histopathological examination and inflammation severity scoring of the coronary arteries, aortic root vasculitis, and myocarditis were performed by an experienced cardiovascular pathologist blinded to the experimental groups (M.F.). KD lesions were assessed with the scoring system as described previously. In brief, Vessel inflammation score for coronary artery and aortic root: 0=no inflammation, 1=rare inflammatory cells, 2=scattered inflammatory cells, 3=diffuse infiltrate of inflammatory cells, and 4=dense cluster of inflammatory cells as previously described. KD penetrance in this model is typically 80%.

Magnetic resonance imaging (MRI) of abdominal aorta
MRI imaging was performed with a 9.4 T (94/20) Bruker BioSpec MRI scanner (Bruker, Billerica, MA) using 4 channel 1H mouse brain receiver coil (Bruker, Billerica, MA). A pilot scan consisting of fifteen 1 mm slices with an intra-slice distance 1.5 mm, five on each axis, were used to determine the abdominal aortic orientation using a rapid FLASH sequence available in IntraGate. A 1 mm 196 x 196 matrix scan with 100 µm in plane resolution was acquired with the IntraGate retrospective gating sequence with an echo time 2.83 ms and a repetition time of 8 ms; 100 repetitions. The gated reconstructed image was used to place a 1 mm perpendicular imaging slice and an IntraGate blackblood imaging sequence was then used to acquire 61 µm isotropic in-plane resolution images with a repetition time of 20 ms and an echo time of 2.04 ms; 5
minutes 25 second total acquisition time. From the images, the diastolic and systolic region of interests was calculated for the lumen area and total vessel area.

**RNA sequencing**
Sequenced reads in Illumina Hiseq2000 image files (BCL files) were converted to FASTQ format via Illumina Casava 1.8.2. Reads were decoded based on their barcodes and merged for each individual samples. Subsequently, reads in each sample were mapped against mouse transcriptome using an in-house pipeline based on Bowtie (ref http://bowtie-bio.sourceforge.net/index.shtml) with two allowed mismatches. For each gene, reads mapped to the sense-strand exons of the gene were identified and counted.

**Human AAA signature derivation and comparison with mouse signature**
Microarray gene profiling data of the abdominal aorta from seven adult (non KD) patients with AAA lesions and seven healthy controls were downloaded from GEO under accession number GSE7084. The significantly perturbed signatures in AAA patients relative to the health controls were obtained from NextBio Curated Studies (study id 2135, bioset id 16179). The human AAA signatures were summarized at gene level, resulting in total 1840 gene signatures with a fold change of at least 1.5 of up- or down-regulation and a p-value no greater than 0.05. The fold changes of multiple probes were summarized at gene level, resulting in total 2064 gene signatures in human AAA. When comparing the signatures of human AAA and the mouse model, we eliminated species-specific genes, resulting in 6046 mouse gene signatures and 1643 human AAA gene signatures.

**Immunofluorescence staining**
The frozen sections of abdominal aorta from naïve or LCWE-injected mice (2 wks) were stained with antibodies against SM22 (smooth muscle cells), CD45 (pan-leukocytes), CD3 (T cells), CD19 (B cells), Ly6G (neutrophils), CD11c (dendritic cells), or F4/80 (macrophages). After washing, the sections were stained with fluorescent-labeled secondary antibodies. Then the samples were mounted with DAPI. Isotype controls were used as negative controls (data not shown). To address the caspase-1 activity in the tissue, we used the FLICA Caspase-1 kit (ImmunoChemistry Technologies, MN USA) according to the manufactures protocol. Briefly, after fixation with cold acetone for 1 min, the sections were stained with anti-F4/80, anti-CD11c, anti-Ly6G mAb for 1hr at room temperature. The slides were washed and then incubated with FLICA probe and fluorescence-labeled secondary antibodies for 2 hrs at room temperature. All images were obtained using a Keyence BZ-9000 fluorescent microscope (Keyence Corporation Of America, IL USA).

**Depletion of macrophages**
C57BL/6 male mice were injected with LCWE (Day 0). The mice were injected with control liposome, clodronate-liposome (Encapsula NanoSciences LLC, TN USA) i.v. on Days -1, 2 and 5. The depletion of macrophages was confirmed by flow cytometry analysis.

**In vivo blockade of IL-1/IL-1R signaling**
C57BL/6 male mice were injected with LCWE (Day 0). The mice were given intraperitoneal injections of human IL-1Ra (Anakinra) (Swedish Orphan Biovitrum AB; 1 mg) every day from Day-1 to 5, or anti-IL-1α mAb (BioXCell; 100 µg), anti-IL-1β mAb (Novartis; 200 µg) at day-1, 2, 5. The abdominal aorta diameters were analyzed at Day14 for IL-1Ra treatment or Day 7 for antibody treatment.

**Statistics analysis**
Results were reported as mean ± SEM. All data were analyzed using Prism 4.03 statistical program. Statistical significance was evaluated by Student's *t* test (two-tailed) to compare unpaired samples between experimental groups. For some experiments where the variances were not equivalent, Welch’s correction was applied. In experiments where data was not normally distributed, the Mann-Whitney test was performed. For experiments involving 3 groups or more, we used the one-way ANOVA with Tukey’s post-hoc test. For experiments with two independent variables we used two-way ANOVA with Bonferroni post test. For experiments with repeated measurements we used the repeated measurement two-way ANOVA with Bonferroni post-hoc test. For analysis of correlation, we used spearman’s rank correlation test. A probability value of <0.05 was considered statistically significant.

For statistical analysis of differentially expressed genes in mouse, read counts summarized at gene level represented the raw gene expression measures. An empirical minimum read count of 10 was applied to flag the “absence” and “presence” of genes in each sample, assuming the quantitation of a gene with less than 10 mapped reads is not reliable. We normalized the raw gene expression in each sample by global scaling to match the median library size (i.e. the total number of mapped reads) as well as the 75% quartile of the gene-level read counts across all samples, as described in previous studies. For each comparison between two groups of samples, we first eliminated genes that were not flagged as “present” in all samples of the higher expressing group, resulting in on average about 13,000 out of total 35,161 genes for subsequent analysis. Next, fold changes associated with the comparison were calculated as the ratio between the arithmetic mean expressions in the two groups. The statistical significance (p-value) of the differential expressions was assessed under negative binomial distribution models using DESeq package (version 1.6). At the end, we selected genes with fold changes no less than 1.5 in either up or down directions with p-values of at least 0.05 as the significantly perturbed gene signatures.

Supplemental Figure I. LCWE-injected male mice have aortic aneurysms in abdominal, renal and iliac arteries but not thoracic aorta. The mice were injected with LCWE and aorta tissues from aortic root to iliac arteries were collected at Day 14. (A) Arrows indicated the abdominal aorta lesions. Note infrarenal dilatation of aortae indicative of aneurysm formation. (B) Representative photomicrographs of H&E stained cross-sections at aortic arch, thoracic aorta, and abdominal aorta. Note transmural inflammation at site of aneurysmal dilatation of abdominal aorta. (C) Example of localized eccentric dilatation indicative of a saccular aneurysm, and a long cylindrical dilatation indicative of a fusiform aneurysm. Scale bar indicates 200 μm.
Supplemental Figure II. The mice were injected with LCWE (400 µg) i.p. and the inner area of abdominal aorta in naïve and LCWE-injected mice were analyzed by MRI. (A) Representative pictures are shown. (B, C) Raw data of Lumen (B) and external diameter (C) (Area cm\(^2\)) were shown.
Supplemental Figure III. The iliac and renal artery sections of control mice or LCWE-injected mice at Day 14 were stained with H&E. Note dilatation and transmural inflammation at both sites in KD mice. The scale bar indicates 200 µm.
Supplemental Figure IV. Isotype controls for immunofluorescence staining. Representative images of isotype controls for CD45, CD3, CD19, F4/80-, Ly6G (Rat), CD11c (Hamster), and SM22 (mouse).
Supplemental Figure V. LCWE induces IL-1α and IL-1β production. BMDM were stimulated with LCWE for 24 hrs and cytokine production was determined by ELISA. Experiments were performed in triplicate. Data shown are Mean ± SEM and were compared by the use of One-way ANOVA with Tukey’s post test.