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The Role of IL-1 signaling in a mouse model of Kawasaki Disease-associated Abdominal Aortic Aneurysm

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Abstract

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.

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Disclosures

None.

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. *Il1r*$^{-/-}$*, Il1a*$^{-/-}$*, and Il1a*$^{-/-}$ 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 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 that 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.

Keywords

Kawasaki Disease; abdominal aortic aneurysm; vasculitis; IL-1β

Introduction

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 under 5 years of age, with a male:female ratio of 1.5:1[1–10]. KD, if untreated leads to coronary artery abnormalities in up to 30% of children and is the leading cause of acquired heart disease in children in the US[8]. The etiology 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, 11].[3, 4, 12]

Treatment with high dose of intravenous immunoglobulin (IVIG) resolves inflammation and reduces the occurrence of coronary abnormalities, predominantly coronary artery aneurysms (CAA) from 25–30% down to 5–7%[13–15]. Currently, studies from the U.S. report an aneurysm rate of approximately 5% despite IVIG therapy[10]. Importantly, up to 20% of KD patients are IVIG resistant and do not respond to the initial IVIG dose and thus have a particularly high risk of developing CAA[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 CAA 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 complications[17]. While IVIG reduces the rate of CAA and the morbidity and mortality associated with KD, lack of a specific etiologic 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 very limited availability of tissue samples from patients with KD has significantly impeded our progress in understanding the etiology and pathogenesis of the disease, making the availability of a relevant animal model extremely valuable.

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 (i.e. coronary arteritis, aortitis, myocarditis, aneurysms, including luminal myofibroblast proliferation and scarring and stenosis in the coronary arteries)[18–22]. This mouse model (*Lactobacillus casei*-cell wall extract (LCWE)-induced KD vasculitis), reliably predicts efficacy of treatment options in children with KD[19, 2118, 23, 24]. A single i.p. injection of LCWE reproducibly induces aortitis and proximal coronary arteritis (including epicardial coronary arteritis) that are histopathologically very similar to the coronary arteritis observed in human KD[18]. While 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 development of the coronary arteritis/stenosis and myocarditis associated with this KD mouse model[25] has led to successful treatment of several IVIG-resistant KD cases with the IL-1 R antagonist (Anakinra)[26, 27] and most importantly to the initiation of two Phase II clinical trials with Anakinra in KD patients.

KD not only causes vessel inflammation in small and medium size arteries with a distinct predilection for the coronary arteries, but is also associated with dilation and aneurysms in almost any systemic arteries including, axillary, subclavian, brachial, renal and iliac arteries as well as the abdominal aorta (AAA)[28–31]. The presence of systemic aortic aneurysms frequently indicates acute severe vasculitis and increases the likelihood of severe cardiac sequelae[32]. However, although coronary artery abnormalities, including aneurysms, has been well evaluated, investigations into inflammation at other systemic arterial sites in KD patients are relatively limited. In addition, it is still unknown whether these aortic dilatations and aneurysms are caused by a process similar to the coronary arteritis in KD patients. Progression to excessive remodeling and vasculopathies, cardiovascular diseases, and early death among survivors of childhood KD has been reported with increasing frequency[33–35]. It is now accepted that systemic vascular inflammation in KD patients in childhood may persist beyond the acute stages and lead to an increased risk of subsequent cardiovascular diseases.

IL-1β plays a critical role in broad spectrum of diseases, including chronic inflammatory diseases such as rheumatoid arthritis, metabolic syndrome, diabetes, atherosclerosis and more recently was linked to Kawasaki Disease vasculitis[25, 36–41]. IL-1α also plays a critical role in chronic inflammation and studies suggest that IL-1α can regulate IL-1β secretion[37, 42–44]. We have shown the key role of TLR2 and IL-1R via MyD88 signaling in coronary arteritis and aortic root vasculitis in this KD mouse model[25, 45]. IL-1 receptor antagonist (Anakinra) given either prophylactically or up to three days after LCWE injection efficiently prevents the development of KD vasculitis, coronary artery lesions and

myocarditis in mice[25]. In the current 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 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 histopathological 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-induce KD mouse model. At 0, 1, 2, 5, and 8 weeks following LCWE injection into male mice, the diameter of abdominal aorta was measured. Over 80% of the mice developed significant dilation of abdominal aorta (Fig. 1A and 1B). LCWE-injected mice showed obvious dilatation and aneurysms exclusively below the right renal artery but not in the suprarenal aorta (Supplemental Fig. I A and 1B), similar to what is found in KD patients[28, 29]. We observed localized eccentric dilatation indicative of a saccular aneurysm, and a long cylindrical dilatation indicative of a fusiform aneurysm (Supplemental Fig. I C). Aneurysm formation was also detected in the renal and iliac arteries (Fig. 1C). These results indicate that LCWE injection induces site specific vascular remodeling (i.e. below the right renal artery). Moreover, MRI 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 (Fig 1D–F, Supplemental Figure II A–C). In human patients, KD is 1.5 times more likely in males compared with females[7, 10]. Male mice showed a significantly greater increase in abdominal aortic diameter compared with female mice (Fig. 1G and H). Indeed, female mice did not develop a statistically significant increase in abdominal aortic diameter upon 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 naïve mice and LCWE-injected mice at 14 days. H&E 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 (Fig. 2A). In addition, the AAA lesions showed loss of smooth muscle cells in the media, which is a typical of AAA histology (Fig. 2A lower panels). The lesions of iliac and renal arteries also showed similar inflammation (Supplemental Fig. III). 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 dilatation of the abdominal aorta (Fig. 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, while fewer $CD45^+$ cells were detected in the media and intima of AAA lesions (Fig. 3A, B and Supplemental Figure IV). Upon 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 (Fig. 3B). Moreover, co-staining with antibodies against CD11c and F4/80 revealed the presence of $CD11c^+$ $F4/80^+$ double positive macrophages in the adventitia lesions (Fig. 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 SMC architecture and marked inflammatory cell accumulation (Fig. 2 and Fig. 3). In order 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 naïve mice, and then compared these data with the gene expression changes between the aortas of healthy donors and AAA patients[46]. We found up or down regulation of 6,046 genes and 1,643 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 AAA patients 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 orientation of change (579 up-regulated and 445 down-regulated genes) between KD mouse and AAA patients, with the genes of top fold changes indicated (Fig. 4B). The up-regulated genes included inflammatory cytokine IL-1β and some chemokines, which lead to accumulation of immune cells and inflammatory responses. Additionally, the gene expression level of matrix metalloproteinase 12 (MMP12), also known as macrophage elastase, was increased 270 times in KD mouse AAA lesion compared with control normal mouse abdominal aorta (Fig. 4B). Consistently, most of the correlations between human adult AAA disease and mouse KD vasculitis associated AAA involved immune pathways (Fig. 4C).

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 up-regulated 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[25]. In order to investigate the potential role for IL-1/IL-1R signaling in LCWE-induced abdominal aorta dilation/aneurysm, we injected LCWE into

WT and *Il1r*$^{-/-}$ mice. Similar to LCWE-induced KD coronary lesions, *Il1r*$^{-/-}$ mice were completely protected from AAA development (Fig. 5A, B). 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 (Supplemental Fig. V). We then addressed which IL-1 cytokine was important in AAA development in KD mouse model by injected LCWE into WT, *Il1α*$^{-/-}$, and *Il1β*$^{-/-}$ mice. Both *Il1α*$^{-/-}$ and *Il1β*$^{-/-}$ mice were completely protected from abdominal aorta dilatation compared with WT mice, indicating that both IL-1α and IL-1β play an important non-redundant role in AAA development (Fig. 5A, B). 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[25]. To investigate the role of NLRP3 inflammasome/casapse-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 (Fig. 5C, D). However, the Aim2 inflammasome, which detects intracellular DNA, was not involved in LCWE-induced AAA formation (Fig. 5C, D).

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. In order to determine which of these cells could be producing IL-1 at the lesions, we visualized Casapse-1 activity in the lesions using the FLICA assay. Caspase-1 activity was readily detectable at the lesion site, with no activity seen in naive animals (Fig. 6A). Further investigation found that Caspase-1 activity was confined to CD11c^{+} F4/80^{+} macrophages (Fig. 6B), with no Caspase-1 activity found in Ly6G^{+} neutrophils. Since IL-1 and the 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 (Fig. 6C, D), 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[25]. 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 (Fig. 7A, B). 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 (Fig. 7C–F).

Discussion

The present 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 SMC 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 KD patients, pathological analysis of autopsy samples of KD patients 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 KD patients were bilateral, symmetrical, and multiple. This includes abdominal aorta aneurysms that were detected mostly in infants during the first 8 months of life[51]. Kato H. et al showed that KD patients with systemic aortic aneurysms also developed multiple giant coronary aneurysms[47]. The outcomes of systemic aortic aneurysms resemble those of coronary artery lesions[52]. While 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[49]. Progression to excessive remodeling and vasculopathies, cardiovascular diseases, and early death among survivors of childhood KD has been reported with increasing frequency.[33–35] It is now accepted that systemic vascular inflammation in KD patients may persist beyond the acute stages and lead to increased risks of subsequent cardiovascular diseases.

In the current 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 less than one year of age with very severe KD vasculitis and coronary aneurysms. 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 KD patients, 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[33–35]. Indeed, recent reports showed progression of systemic arteritis into stenotic lesions in KD patients.[29] and these coronary artery sequelae have led to ischemic heart disease in young adults[56].

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[25, 57]. In the current study, we found that IL-1/IL-1R signaling and both IL-1 α 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 NLRP3 inflammasome is activated is through oxidative mitochondrial DNA in macrophages[58]. It is intriguing that another recent study found that mitochondrial oxidative stress in macrophages can also lead to activation of NLRP3 activation and to the development of AAA in the angiotensin II-induced murine aneurysm model[59]. Although the precise mechanism of NLRP3 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.

Similarly 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 non-overlapping roles for IL-1α and IL-1β in LCWE-induced coronary arteritis lesions[57]. 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[60], further suggesting a role of IL-1α in human AAA formation as well. In this study, $Il1b^{-/-}$ or WT 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[61]. It is also possible that these isoforms are released sequentially, as IL-1α is often thought of as an alarmin and is produced early during acute inflammation, while IL-1β is more associated with the chronic phase of inflammation[62]. Only *IL-1β* mRNA was up-regulated in AAA of both human and KD mouse models. The novel observation that IL-1α and IL-1β may have non-overlapping 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 Kawasaki Disease, 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 to control mouse abdominal aorta. MMP12 haplotypes have been implicated in aneurysm formation in KD patients[63], and the expression of MMP12 has been reported in human AAA lesions[64]. 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[65]. MMP12 deficiency also attenuated AAA development in the $CaCl_2$ model of mouse AAA as well as in the Ang II-induced AAA model[66,67]. Interestingly TNF-α and IL-1 can synergistically induce MMP12[68]. In addition to degrading extracellular matrix proteins, MMP12 may also promote macrophage recruitment to the vessel wall by activating TNF-α or by modulating levels of proinflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1)[69].

Using available online databases we were able to compare human AAA up and down regulated genes with those from our LCWE-induced AAA mice. While the number of genes that were up or down regulated 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 very 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[70, 71]. Male gender has been consistently identified as a non-modifiable risk factor for AAA. Evaluation of the AAA histology and pathophysiology in the LCWE-KD vasculitis mouse model revealed the following marked similarities with human AAA disease; (1) Histologic characteristics (SMC 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), (4) gene expression profiles. Consistent with our experimental findings, the IL-1β gene expression and protein levels are markedly elevated in human AAA[72–74]. Johnston et al. demonstrated that genetic disruption of IL-1β as well as Anakinra treatment inhibits AAA development and progression in the elastase-induced AAA mouse model[75]. It is very 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 most 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 is also associated with increased male susceptibility[76]. In the Angiotensin II model, only 20 to 40% of animals develop AAA, which universally occur in left suprarenal location (unlike the human counterpart where AAA are always located infrarenally). On the other hand, 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, and area that will need to be further investigated.

In summary, we found that the LCWE-induced KD vasculitis mouse model also exhibits robust systemic artery inflammation involving the abdominal aorta, iliac, and renal arteries, with dilatation and aneurysms, in addition to coronary arteritis, aortitis, and myocarditis. We also show inhibition of IL-1α, IL-1β or IL-1R leads to marked reduction of AAA formation, suggesting that these IL-1-related molecules are potential therapeutic target for KD vasculitis and AAA patients. The LCWE-induced KD vasculitis mouse model may not only be useful in providing novel mechanistic clues and therapeutic approaches for Kawasaki Disease but potentially also for AAA disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

KD	Kawasaki Disease
AAA	abdominal aortic aneurysm
LCWE	*Lactobacillus casei* cell wall extract
IVIG	intravenous immunogolubulin
CAA	coronary artery aneurysm

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Significance

Similar to clinical Kawasaki Disease (KD), the LCWE-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 AAA with luminal dilation, intimal proliferation, breaks in elastin layer, vascular smooth muscle cell loss, and inflammatory cell accumulation. Our findings demonstrate non-overlapping roles for 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 AAA 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 KD patients as well as potentially for AAA disease. The LCWE-induced KD vasculitis model may be a valuable alternative model to investigate AAA disease.

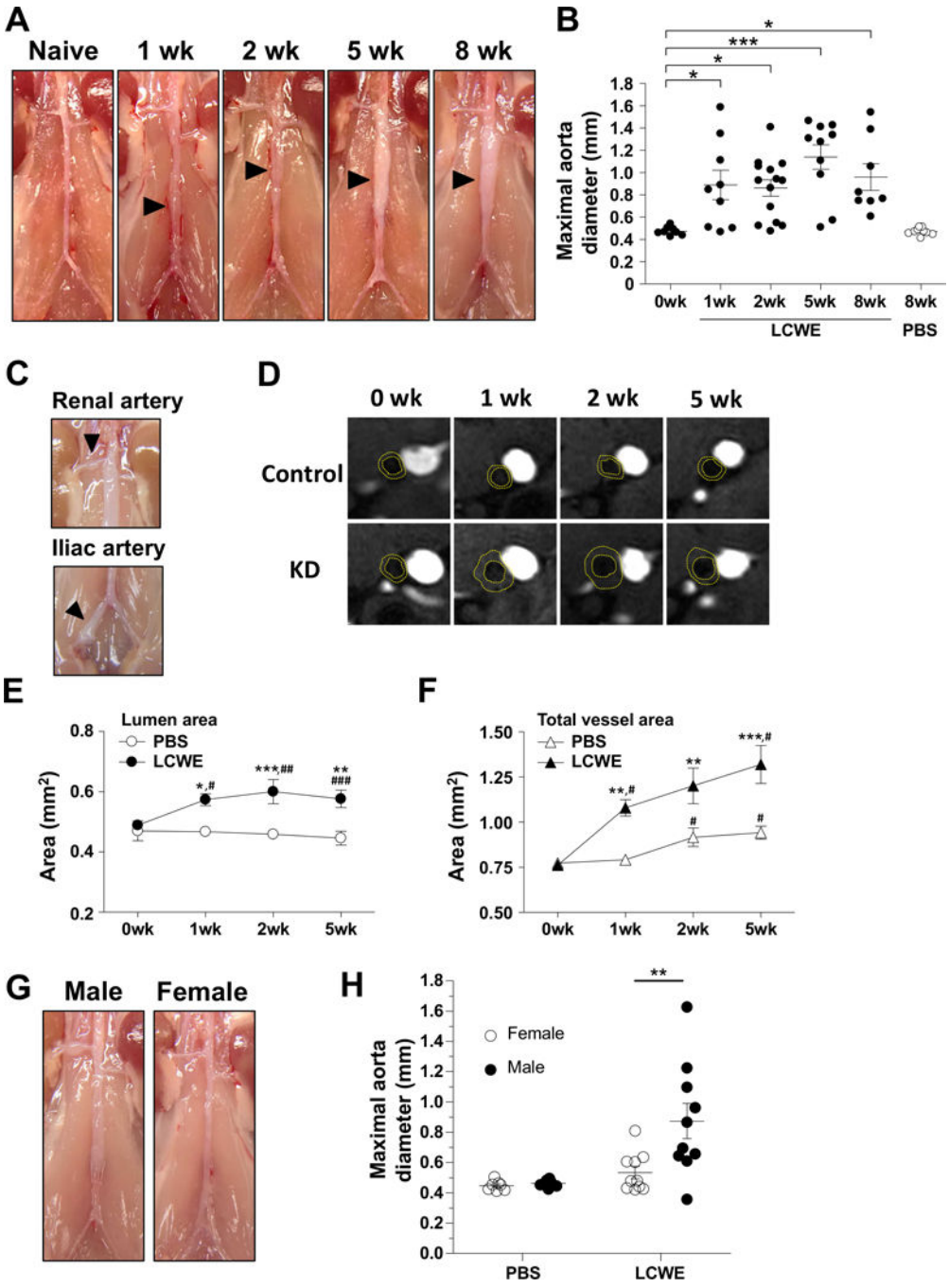


Figure 1. LCWE-induced Kawasaki Disease mouse model induces abdominal aorta dilatation and aneurysms (AAA)

Male mice were injected with LCWE (400 μg) i.p. and the abdominal aorta was dissected at the indicated time points. (**A**) Representative gross photographs of abdominal aorta. (**B**) Maximal diameter of abdominal aorta. Data were analyzed by One-way ANOVA with Tukey's post test. (*; $p<0.05$, **; $p<0.01$) (**C**) Representative gross photographs of renal and iliac arteries of the mice 2 weeks after LCWE injection are shown. The arrows indicate lesions. (**D–F**) The inner area of abdominal aorta in naïve and LCWE-injected mice were

analyzed by MRI. (**D**) Representative pictures are shown. (**E, F**) Inner area (E) and external diameter area (F) of abdominal aorta (mean ± SEM). Data was analyzed by 2-way repeated measures ANOVA with the Bonferroni post hoc test for PBS vs LCWE (*; $p<0.05$, **; $p<0.01$, ***; $p<0.001$), and by one-way ANOVA for 0 wk vs different time points in each group (#; $p<0.05$, ##; $p<0.01$, ###; $p<0.001$). (**G–H**) The abdominal aortas of male and female mice were dissected at 2 weeks after LCWE injection. (**G**) Representative gross photographs of abdominal aortae are shown. (**H**) The maximal diameter (mean ± SEM) of AAA in male (n=10) and female (n=10) are shown. Data were analyzed by 2-way ANOVA with the Bonferroni post hoc test for male vs female (**; $p<0.01$). Reanalysis for control vs LCWE did not find any significance in females for AAA lesion formation (data not shown).

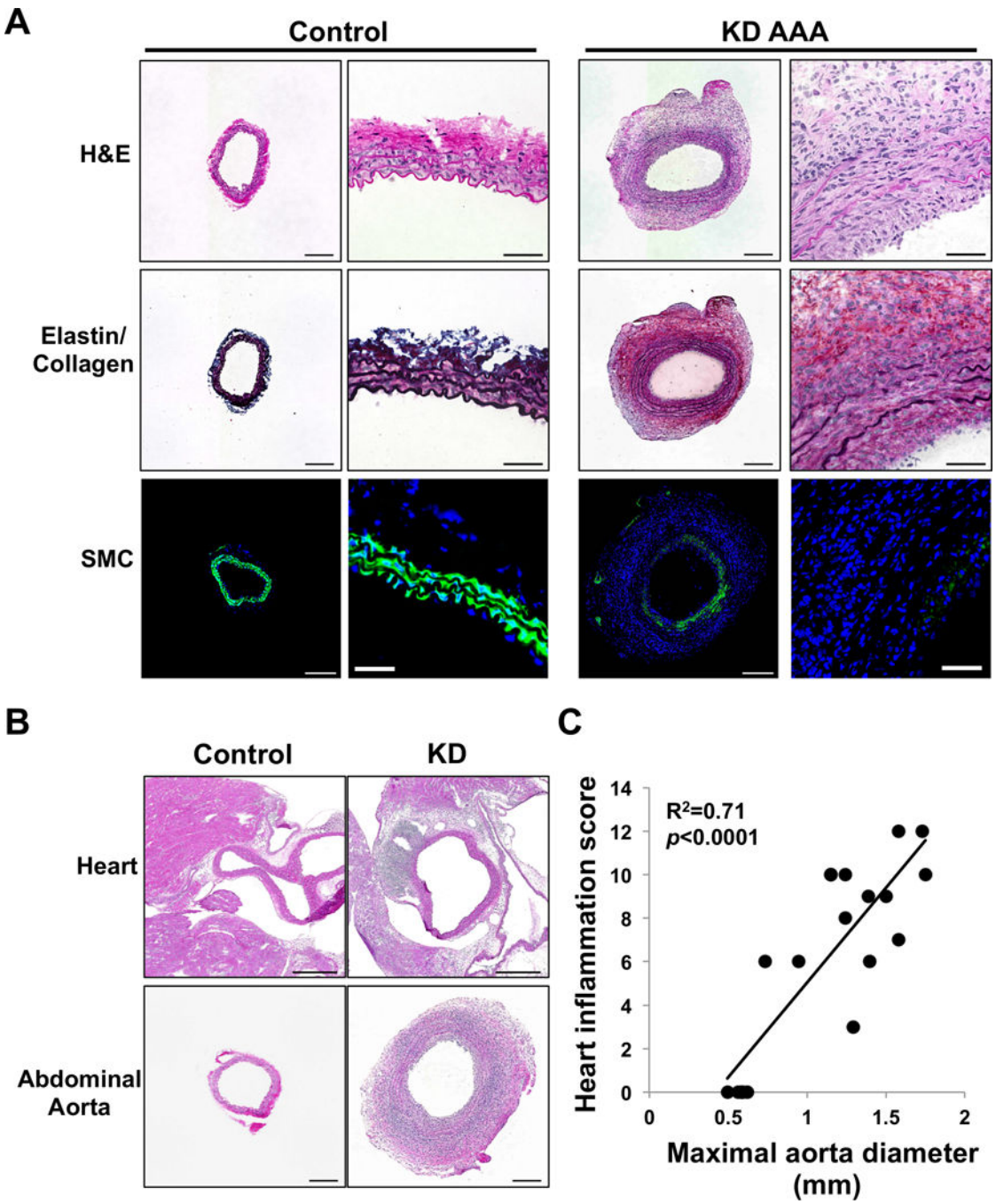


Figure 2. Intense inflammatory histology in LCWE-induced AAA

The heart and abdominal aorta tissues were collected 2 weeks after LCWE injection into male mice. (**A**) Abdominal aorta sections of naïve mice and LCWE-injected mice were stained with H&E, elastin/collagen staining, and anti-SM22 Ab. Photomicrographs show normal arterial morphology of naïve mice, while treated mice show aortae with transmural inflammation, neointimal hyperplasia, medial loss of smooth muscle cells, and fragmentation of medial elastic fibers. (**B**) The heart and abdominal aorta of naïve and LCWE-injected mice were stained with H&E (**C**) The maximal aorta diameter was

compared with inflammation score in heart vessels for each mouse. Data were analyzed by spearman's rank correlation test. The scale bar indicates 200 μm (left panel) and 50 μm (right panel) (**A**), 500 μm for heart and 200 μm for aorta (**B**).

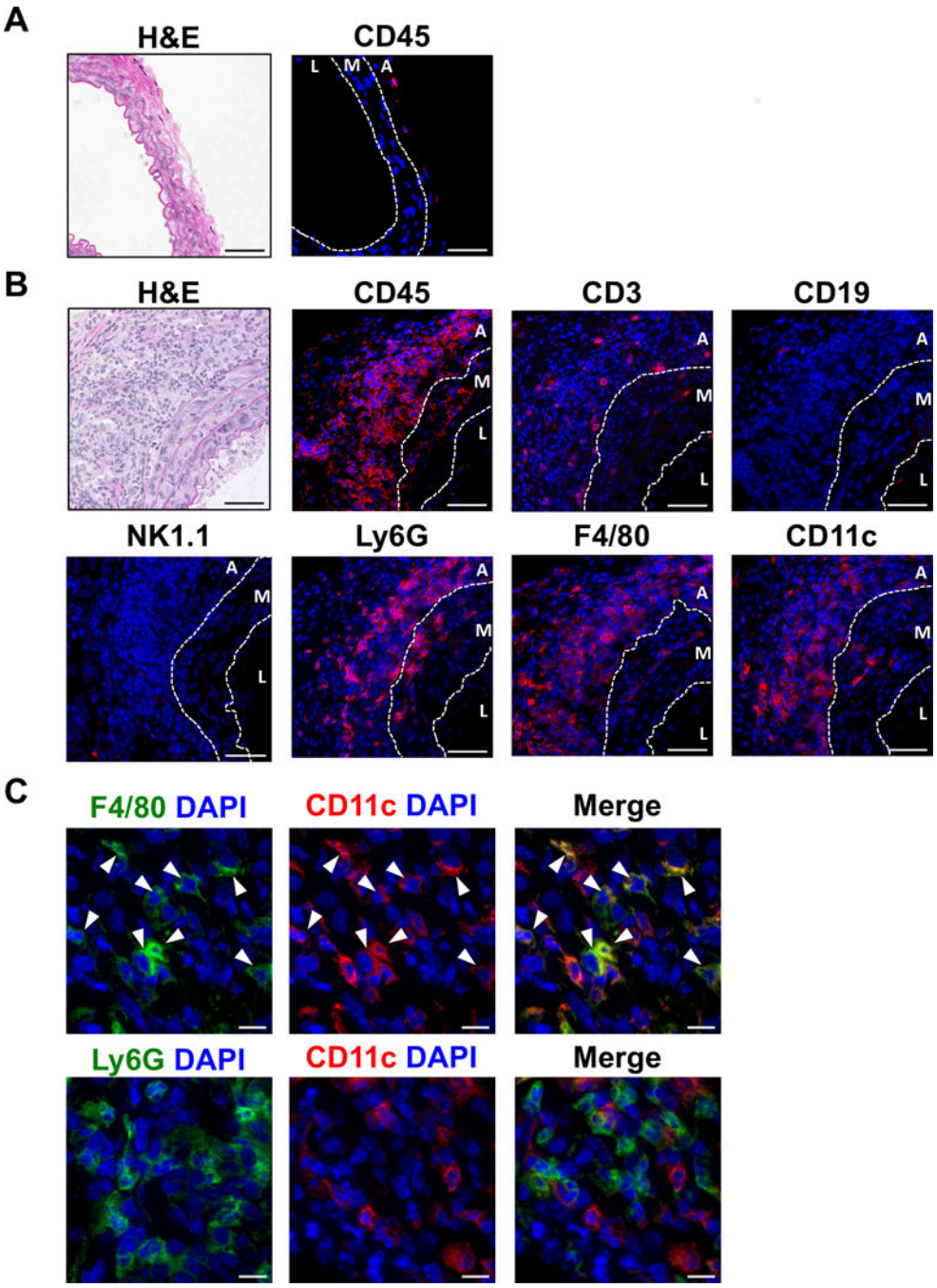
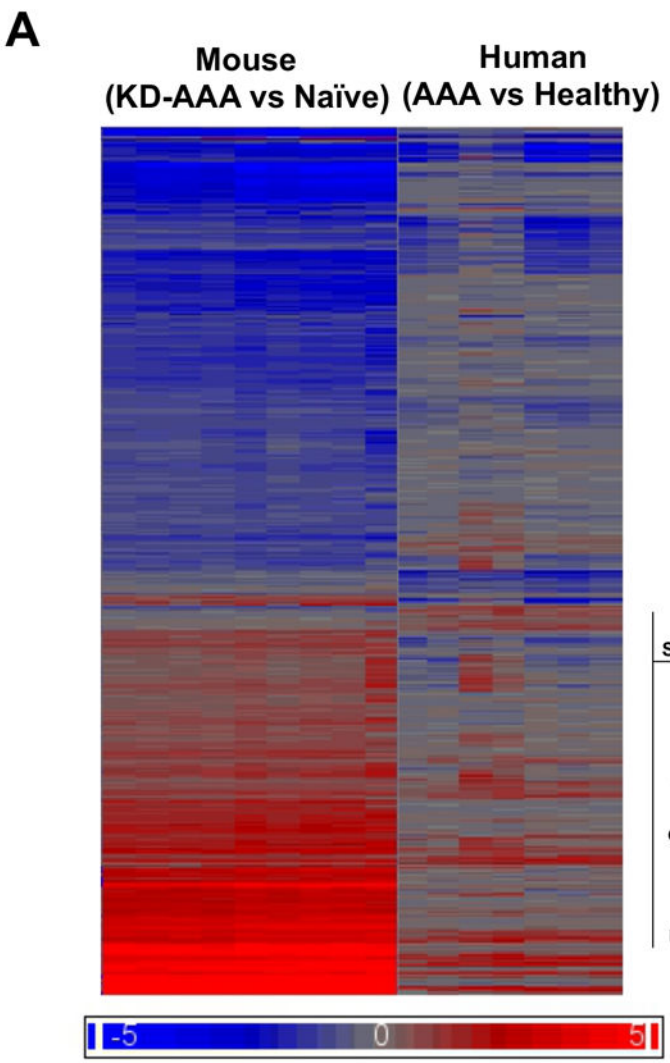
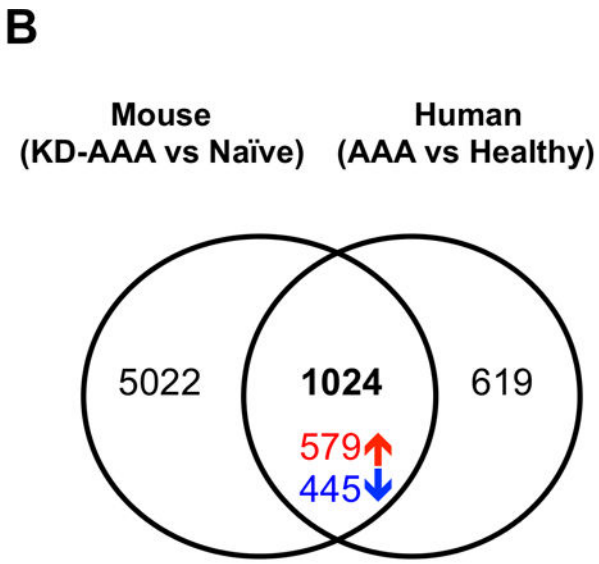


Figure 3. Identification of innate immune cells in abdominal aorta lesions

(**A–B**) Male mice were injected with LCWE, sacrificed at Day 14, and aortic cryosections were obtained. (**A**) Aortic cryosections were stained with H&E. (**B**) Sections were stained with mAbs against CD45 (Red) and nuclei were stained with DAPI (Blue). (**C**) The abdominal aorta sections of LCWE-injected mice were co-stained with anti-CD11c (Red), anti-F4/80 (Green), anti-Ly6G (Green) and DAPI. Representative pictures are shown indicating infiltrating CD11c+ macrophages (CD11c F4/80). DCs (CD11c only, and

neutrophils (Ly6G only)). The scale bar indicates 50 μm (**A**–**B**) and 10 μm (**C**). (L; lumen, M; media, A; adventitia).





Gene Symbol	Gene Description	Fold Increase Mouse	Fold Increase Human
Il1b	interleukin 1 beta	3829.41	7.49
Gpr84	G protein-coupled receptor 84	1465.73	1.54
Il1rn	interleukin 1 receptor antagonist	1287.95	6.13
Cxcl2	chemokine (C-X-C motif) ligand 2 (human homolog is CXCL3)	1163.78	6.07
Trem1	triggering receptor expressed on myeloid cells 1	1070.88	3.68
Ccl4	chemokine (C-C motif) ligand 4	703.83	9.63
Cd300lf	CD300 antigen like family member F	656.19	2.68
Fpr1	formyl peptide receptor 1	652.84	3.11
Osm	Oncostatin M	547.56	4.49
Ccl22	chemokine (C-C motif) ligand 22	290.44	3.33
MMP12	matrix metalloproteinase-12	236.2	5.3

C

Biogroups	P-Value	Number of genes
Genes involved in Adaptive Immune System	1.4E-31	67
TCR signaling in CD4+ T cells	2.3E-24	25
Cytokine-cytokine receptor interaction	2.7E-24	38

Figure 4. Gene expression profiles in AAA of KD mouse model and human AAA patients

The abdominal aortas were collected from LCWE-injected male mice at Day 14 and gene expression profiles were analyzed by RNA sequencing. The gene expression profiles of human AAA lesions were obtained from the public database of Gene Expression Omnibus under accession number GSE7084. (**A**) Heat map of union of differentially regulated genes in KD mouse (n=9) and human patients (n=7), relative to the average of naïve control mice and healthy patients, respectively. (**B**) Venn diagram comparing mouse and human AAA data. The gene numbers are shown in each area. Red and blue numbers indicate up- or down-regulated genes respectively in both KD mouse and human patients. The top up and

down regulated genes in common are listed. (**C**) The list of top ranking MSigDB pathways up-regulated in both KD mouse and human. P-values were calculated using NextBio software (451 El Camino Real, Suite 210, Santa Clara, CA 95050). The algorithm is based on a Running Fisher test as describes in NextBio User Manual.

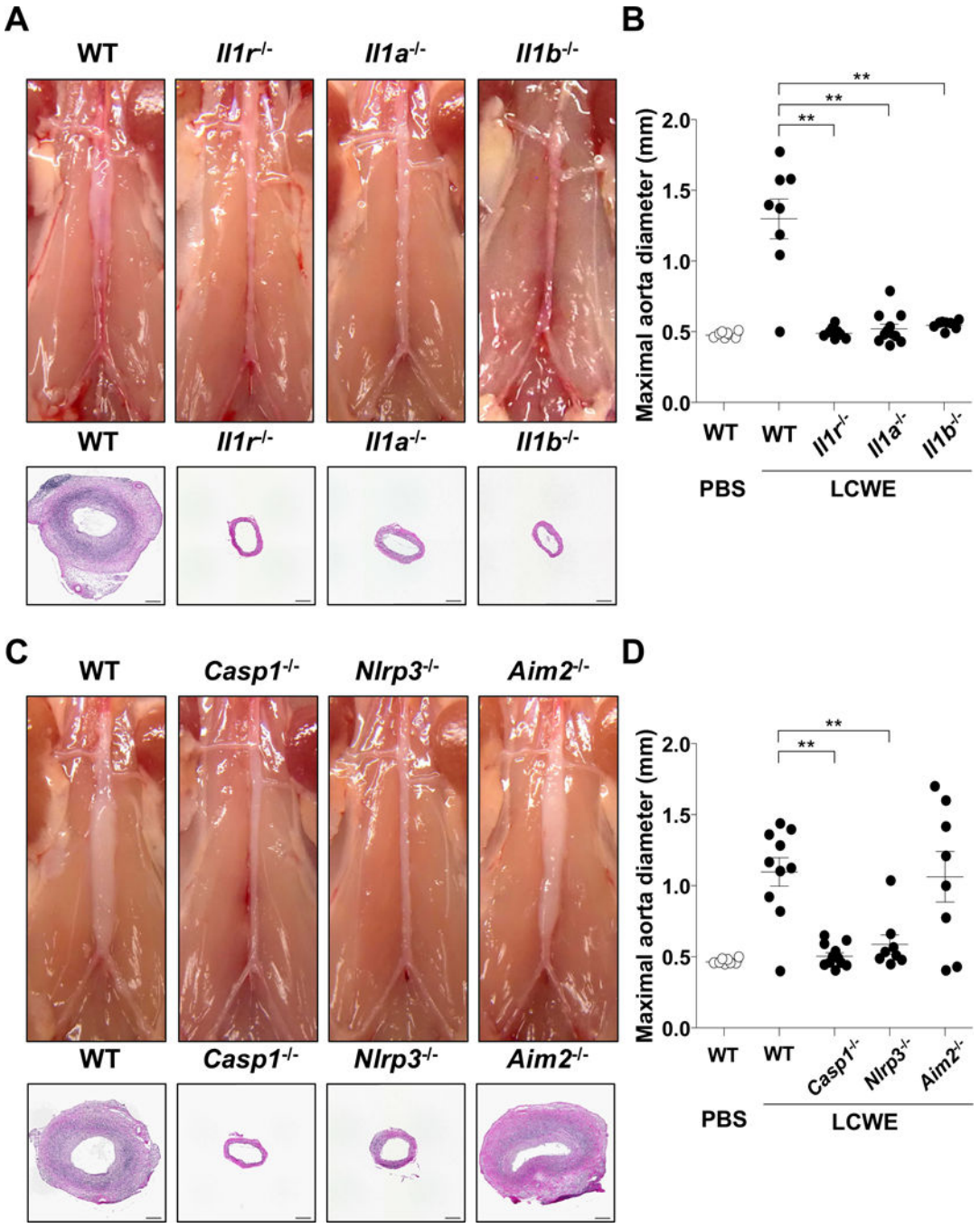


Figure 5. IL-1/IL-1R signaling and the NLRP3 inflammasome are required in development of LCWE-induced AAA

(**A–B**) WT, *Il1r*$^{-/-}$, *Il1a*$^{-/-}$, and *Il1b*$^{-/-}$ male mice were injected with LCWE and abdominal aorta tissues were collected at 2 weeks. (**A**) Representative pictures of abdominal area and H&E cross-section. (**B**) The maximal diameter of WT (n=8), *Il1r*$^{-/-}$ (n=8), *Il1a*$^{-/-}$ (n=11), and *Il1b*$^{-/-}$ (n=8) mice. Data were analyzed by One-way ANOVA (**; p<0.01). (**C-D**) WT, *Casp1*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice were injected with LCWE and abdominal aorta tissues were collected at 2 weeks. (**A**) and C show Representative photomicrographs with transmural

inflammation in WT and Aim2 aortae only. (**B**) The maximal diameter of WT (n=10), *Casp1*$^{-/-}$ (n=11), and *Nlrp3*$^{-/-}$ (n=8) mice. Data were analyzed by One-way ANOVA with Tukey's post test. (**; $p<0.01$). The scale bar indicates 200 μm.

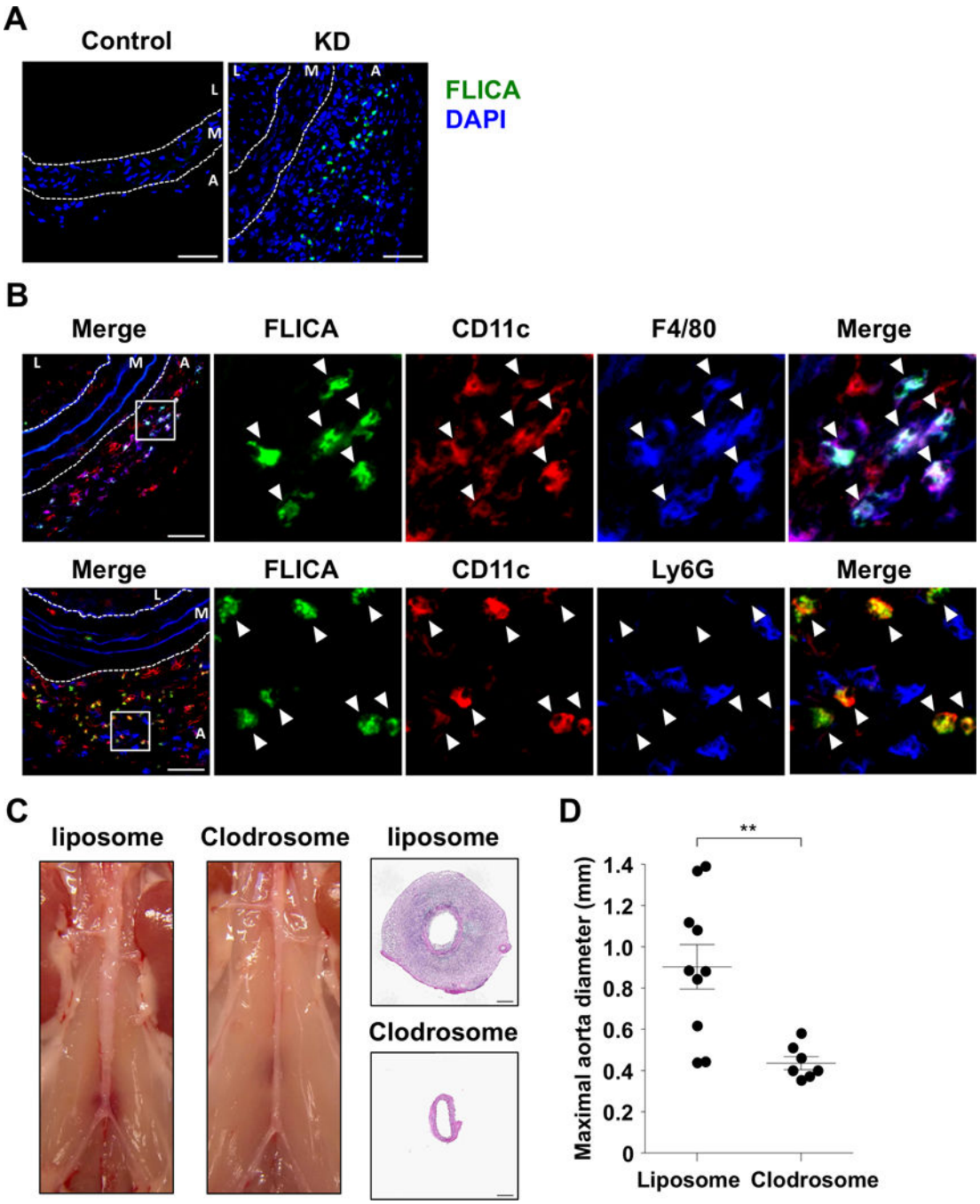


Figure 6. CD11c^{+} macrophages have active caspase-1 in adventitia of AAA and are required for LCWE-induced AAA

(**A–B**) The abdominal aorta tissues were collected from naïve or LCWE-injected male mice at Day 14. (**A**) The abdominal aorta sections were stained with FLICA probe (Green) and DAPI (Blue). (**B**) The sections were co-stained with FLICA probe (Green), anti-CD11c (Red), anti-F4/80 (Blue), anti-Ly6G (Red) mAb. (**C–D**) LCWE-injected WT mice were injected with clodoronate-encapslated liposome (Clodrosome, n=10) or control-liposome (liposome, n=7) on Day -1, 2, and 5. Abdominal aorta tissues were collected at Day 14. (**C**)

Representative photomicrographs show transmural inflammaton in the liposome injected mice aorta. (**D**) Maximal aortic diameter (mean±SEM). Data was analyzed by Student's *t* test with Welch's correction (**; $p<0.01$). The scale bar indicates 50 μm (A–B) and 200 μm.

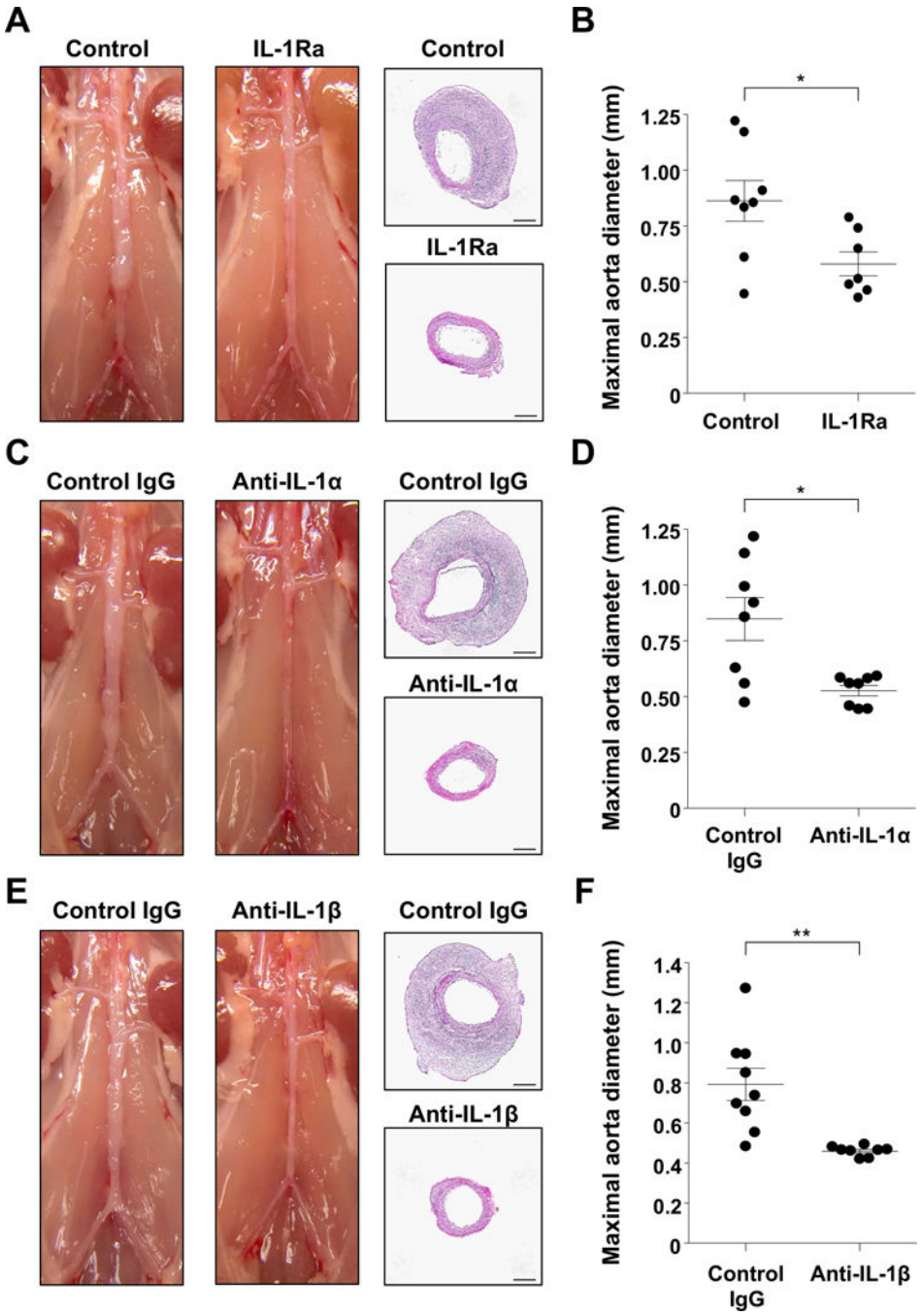


Figure 7. Inhibition of IL-1 pathway prevents LCWE-induced AAA formation

(**A–B**) WT male mice were injected with LCWE at Day 0 and treated with IL-1Ra (500 μg/mouse) or PBS everyday from Day -1 to Day 6. The abdominal aorta tissues were collected on Day 14. Control IgG mice show aortae with transmural inflammation. (**A**) Gross photographs and 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's *t* test (*; $p<0.05$). (**C–D**) WT mice were injected with LCWE at Day 0 and treated with anti-IL-1α mAb (100 μg/mouse), Control mouse IgG at Day -1, 2, 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's *t* test (*; $p<0.05$). (**E–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 Day -1, 2, 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's *t* test with Welch's correction (**D** and **F**) (*; $p<0.05$). The scale bar indicates 200 μm.