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Journal

Arteriosclerosis Thrombosis and Vascular Biology, 35(12)

ISSN

1079-5642

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Publication Date

2015-12-01

DOI

10.1161/atvbaha.115.306475

Peer reviewed

HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2016 December 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2015 December ; 35(12): 2605–2616. doi:10.1161/ATVBAHA.115.306475.

IL-1 signaling is critically required in stromal cells in Kawasaki Disease Vasculitis Mouse Model. Role of both IL-1α and IL-1β

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Abstract

Objective—Kawasaki disease (KD) is the most common cause of acute vasculitis and acquired cardiac disease among US children. We have previously shown that both TLR2/MyD88 and IL-1β signaling are required for the *Lactobacillus casei* cell wall extract (LCWE)-induced KD vasculitis mouse model. The objectives of this study were to investigate the cellular origins of IL-1 production, the role of CD11c+ Dendritic Cells (DCs) and macrophages and the relative contribution of hematopoietic and stromal cells for IL-1 responsive cells, as well the MyD88 signaling in LCWE-induced KD mouse model of vasculitis.

Approach and Results—Using mouse knockout models as well as antibody depletion, we found that both IL-1α and IL-1β were required for LCWE-induced KD. Both DCs and macrophages were necessary and we found that MyD88 signaling was required in both hematopoietic and stromal cells. However, IL-1 response and signaling was critically required in non-endothelial stromal cells, but not hematopoietic cells.

Conclusions—Our results suggest that IL-1α and IL-1β as well as CD11c+ DCs and macrophages are essential for the development of KD vasculitis and coronary arteritis in this mouse model. Bone marrow chimera experiments suggest that MyD88 signaling is important in both hematopoietic and stromal cells, while IL-1 signaling and response is required only in

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Disclosures

None.

stromal cells, but not in endothelial cells. Determining the role IL-1α and IL-1β and of specific cell types in the KD vasculitis mouse model may have important implications for the design of more targeted therapies and understanding of the molecular mechanisms of KD immunopathologies.

Keywords

Kawasaki disease; coronary artery vasculitis; dendritic cells; IL-1; MyD88

Introduction

Kawasaki disease (KD) is an acute febrile illness and systemic vasculitis[1–5] that predominantly afflicts children <5 yrs of age. The etiology of KD remains unknown, although the current paradigm is that KD is triggered by an infectious agent (with a conventional Ag) that elicits an inflammatory response directed at cardiovascular tissues in genetically susceptible hosts[6, 7]. It often causes acute coronary as well as systemic arteritis with coronary artery aneurysms (CAA), and can lead to ischemic heart disease, myocardial infarction, and even death[8–11]. KD vasculitis, once thought of as an acute self-limiting disease, is now known to result in long term complications with ongoing vascular remodeling and myocardial fibrosis[12]. Indeed, long term cardiovascular complications among survivors of childhood KD are reported with increasing frequency.[13–15] KD represents the leading cause of acquired heart disease among children in developed countries.[9–11] While intravenous IgG (IVIG) treatment within the first 10 days of illness have reduced the cardiovascular complications of KD (CAA) from 25% down to 5%[16], up to 20–25% of KD patients are IVIG-resistant and at even higher risk for developing CAA[17]. Therefore, discovery of more effective treatments for KD is one of the highest priorities in pediatric research[18].

KD involves systemic inflammation with a distinct predilection for the coronary arteries. The resulting coronary arteritis in KD is characterized histologically by inflammatory cell infiltration and destruction of extracellular matrix, especially elastic tissue in vascular media, with resultant coronary artery aneurysm formation.[19] The 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 widely used 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 abdominal aorta aneurysms (AAA) seen in KD)[20–23]. This mouse model (*Lactobacillus casei*-cell wall extract (LCWE)-induced KD vasculitis) also predicts efficacy of treatment in children with KD[21, 23]. 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 model to provide novel insights that can be tested in children.

Although both the trigger and the precise pathogenic mechanism of KD are unclear, there are strong indications that the pathology is immune mediated.[24–27] We previously

demonstrated that Caspase-1 activation and IL-1β are critically required for LCWE-induced KD vasculitis[28, 29]. We showed that an IL-1R antagonist (Anakinra) significantly blocked the coronary arteritis, aortitis and myocarditis associated with the LCWE-induced KD vasculitis model[29]. This experimental mouse study, together with several case reports of successful use of Anakinra in IVIG-unresponsive KD patients[30, 31], lead to two recent clinical trials using this IL-1R antagonist for patients with IVIG-resistant KD. However, the exact mechanism by which IL-1 plays a role in KD pathogenesis is still unknown. IL-1α and IL-1β both bind to and activate the IL-1R, although their regulation and activity differ. Since the IL-1R antagonist blocks both of these cytokines, the role that IL-1α may play in this model is unknown. Most importantly, the host target cell(s) that respond to IL-1 in this experimental KD vasculitis model is also unknown.

We previously reported that DCs and macrophages are localized in the coronary lesions of LCWE-induced KD in mice[24], similar to what has been reported in coronary lesions from KD patients.[24, 32] While DCs are believed to mainly play a role as an antigen presenting cell, macrophages can provide wide ranging innate immune responses, including IL-1 secretion after inflammasome activation.

In this study we determined that CD11c+ DCs and macrophages are absolutely required for the LCWE-induced KD vasculitis model and these macrophages appear to be the cellular source of IL-1β production in the lesions. We also found that both IL-1α and IL-1β significantly contribute to LCWE-induced KD vasculitis model, and bone marrow chimera experiments show that IL-1 signaling is required only in stromal cells, but not in endothelial cells.

Materials and Methods

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

Results

CD11c+ macrophages in KD lesions have active Caspase-1 activity

IL-1 signaling and Caspase-1 are both required for LCWE-induced KD[29]. We previously reported that macrophages are present in LCWE-induced KD vasculitis and coronary arteritis lesions[22]. We now stained the coronary arteritis lesions from LCWE-induced KD mice with anti-F4/80 and anti-CD11c and observed the presence of F4/80 and CD11c+ double positive stained macrophages in the lesion site (Figure 1A). Inflammasome activity and IL-1β secretion is most commonly found in macrophages. Recent literature has indicated the involvement of CD11c+ macrophages in various proinflammatory disorders.[33–35] However, which cells are producing IL-1, as well as which type of inflammasome is required is not known in this mouse model of KD. In order to determine which cells in the KD coronary lesions produce IL-1β, we assessed the expression of caspase-1 activity in heart sections of KD mice using FLICA, which identifies caspase-1 activity and can be used as a surrogate marker for IL-1β production. FLICA+ cells were readily visible in the aortitis and coronary arteritis lesions 7 days after LCWE injection but not in naive animals (Figure 1B). FLICA+ cells also expressed both F4/80 and CD11c,

indicating that these cells were macrophages, and not DCs (Figure 1C). Thus it is likely that CD11c+ macrophages are the main source of IL-1β production in the LCWE-induced KD vasculitis lesions.

NLRP3 is required for LCWE-induced KD vasculitis and coronary lesions

We previously reported that the NLRP3 inflammasome is required for Caspase-1 activity and IL-1β production in bone marrow derived macrophages in-vitro[29]. However, while we showed that IL-1 signaling and Caspase-1 are absolutely required *in vivo* for the LCWE-induced KD vasculitis[29], we did not directly investigate the role of NLRP3 in KD lesion induction. We therefore injected *Nlrp3*$^{-/-}$ and WT mice with LCWE and harvested the hearts 14 days later. We observed that *Nlrp3*$^{-/-}$ mice were protected and developed significantly reduced vasculitis lesions and myocarditis compared with WT mice (Figure 2A–C). These data confirmed the involvement of the NLRP3 inflammasome in LCWE-induced KD vasculitis. We also assessed the role of the AIM2 inflammasome, and did not find a role for AIM2 inflammasome in this model as *Aim2*$^{-/-}$ mice were not protected and develop severe KD-vasculitis with similar intensity to WT mice (data not shown).

CD11c+ dendritic cells and macrophages are required for the development of LCWE-induced KD vasculitis and coronary arteritis—In addition to CD11c+ macrophages, there were also many CD11c single positive cells (Fig. 1A), indicating a large number of DCs in the lesion as we have previously reported[22]. To investigate the requirement of DCs or macrophages in the LCWE-induced KD model, we treated the CD11c-DTR transgenic mice with diphtheria toxin (DTx) on days −1 and 1 relative to LCWE injection (day 0) to deplete CD11c+ cells. CD11c is a cell surface marker for DCs and some peripheral macrophages and these mice express the human Diphtheria toxin receptor under the CD11c promoter. The animals were sacrificed on day 7 and their hearts were harvested. CD11c positive cells were depleted after DTx injection as confirmed by flow cytometry analysis (data not shown). Mice depleted of CD11c+ cells by DTx developed significantly less KD vasculitis and coronary arteritis lesions compared to PBS injected control mice, and DTx itself had no effect on naïve CD11c-DTR mice (Figure 2D). CD11c+ cells depletion also resulted in a significant reduction in incidence as well as vascular inflammation and myocardial inflammation severity scores when compared to controls (Figure 2E–G). Furthermore, IFN-γ production by splenocytes was significantly reduced after LCWE restimulation, while it was unaffected after anti-CD3 stimulation (Fig. 2H). We next treated mice with clodronate liposomes to more specifically deplete macrophages during LCWE-induced KD[34], although some DCs can also be targeted this way. Similar to the DTR model, we also found that macrophages were required for LCWE-induced KD vasculitis and coronary lesions (Supplemental Figure IA–E). Taken together, these data demonstrate that CD11c+ DCs and or macrophages play a critical role in LCWE-induced KD vasculitis, coronary arteritis and myocarditis.

Both IL-1α and IL-1β are required for LCWE-induced vasculitis—Our data revealed that macrophages are likely critically required for LCWE-induced KD in mice. We also found that CD11c+ macrophages had inflammasome activity at the lesions, thus making them the likely source of IL-1β. We previously reported that *Il1r1*$^{-/-}$ mice were completely

protected and that IL-1Ra treatment can prevent LCWE-induced KD vasculitis and coronary arteritis.[29] However, both IL-1α and IL-1β can bind to and activate the same IL-1 receptor and IL-1Ra treatment would block both cytokines. To test the role of IL-1α and IL-1β in LCWE-induced KD vasculitis and coronary arteritis, we injected *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ mice with LCWE and examined their hearts at day 14. We observed that both *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ mice were protected from KD vasculitis and showed significantly diminished vascular inflammatory lesions, coronary arteritis and myocarditis compared with WT mice (Figure 3A–D). These results demonstrate that both IL-1α and IL-1β play critical roles in LCWE-induced KD vasculitis and myocarditis.

Recent reports have suggested that *Il1a*$^{-/-}$ mice may have diminished NLRP3 inflammasome activation and IL-1β secretion.[36] We therefore wished to confirm the data obtained with IL1 KO mice by depleting the specific cytokines with anti-IL-1α and anti-IL-1β monoclonal antibodies alone or in combination in LCWE-induced KD vasculitis. Monoclonal anti-IL-1α and/or anti-IL-1β mAb were injected on days -1, 2, 5 from LCWE injection. Mice treated with anti-IL-1α or anti-IL-1β mAb were protected as they developed significantly less KD vasculitis, coronary arteritis, as well as myocarditis compared with mice injected with isotype control antibody (Figure 3E–H). Interestingly, while mice that received either anti-IL-1α or anti-IL-1β mAb still displayed some small residual vasculitic lesions, when both antibodies were given together, the mice were completely protected (Figure 3E–H), similar to IL-1R antagonist (Anakinra)-treated mice[29] These results indicate that either anti-IL-1α or IL-1β mAb alone can significantly protect against LCWE-induced KD vasculitis and myocarditis, but the protection is not complete unless the two mAbs are given together or an IL-1R antagonist such as Anakinra, which blocks both IL-1α and IL-1β, is used.

MyD88 in CD11c+ cells is not sufficient for the development of LCWE-induced KD vasculitis—We previously reported that MyD88 signaling is important in the development of LCWE-induced KD vasculitis, as MyD88-deficient mice were completely protected.[29] MyD88 signaling is required for most TLR signaling, as well as IL-1R1 signaling and we have already shown that LCWE-induced KD model requires both TLR2 and IL-1R1 signaling.[28, 29] Since our data in Figure 1 suggested that both DCs and macrophages may be critically required, we reasoned that MyD88 signaling in DCs and or macrophages may be important but we wished to investigate if that would be sufficient to induce the vasculitis lesions in this experimental model. To test this, we injected MyD88-deficient mice that express transgenic MyD88 only in CD11c+ cells (*Cd11c-Myd88-TG/Myd88*$^{-/-}$)[37] with LCWE. We observed that MyD88 expression alone in CD11c+ cells was not sufficient to restore LCWE-induced KD vasculitis, as both full *Myd88*$^{-/-}$ and *Cd11c-Myd88-TG/Myd88*$^{-/-}$ mice were protected from KD vasculitis development (Figure 4A–D). These observations suggest that while CD11c+ DCs and macrophages are required for LCWE-induced KD vasculitis, MyD88 signaling is also required in cells other than or in addition to CD11c+ DCs, most likely in IL-1 responsive stromal cells.

MyD88 signaling is required in both hematopoietic and stromal cells for the development of LCWE-induced KD vasculitis mouse model—In previous studies

we found that LCWE signals via TLR2 and MyD88, that *Myd88*$^{-/-}$ bone marrow derived macrophage (BMDM) do not express IL-6 and TNF-α *in vitro* in response to LCWE[28], and that IL-1 signaling is critically important in LCWE-induced KD vasculitis.[29] To further define in which cellular compartment MyD88 is important for LCWE-induced KD vasculitis development, we next generated *Myd88*$^{-/-}$ bone marrow chimeric mice. Chimerism was typically greater than 90% (Supplemental Figure II). Irradiated WT mice reconstituted with WT BM transplantation (control mice) developed KD vasculitis lesions as expected (Figure 5A–D), while irradiated WT mice reconstituted with *Myd88*$^{-/-}$ BM were completely protected form developing KD vasculitis (Figure 5A–D). Unexpectedly, irradiated recipient *Myd88*$^{-/-}$ mice transplanted with WT BM were also completely protected, indicating a critical requirement for MyD88 in not only hematopoietic cells, but also in stromal cells. These data would also explain why the CD11c-MyD88-TG mice were unable to develop LCWE-induced KD vasculitis and coronary arteritis (Figure 4). Since LCWE-signals via TLR2/MyD88 pathway[28] and IL-1 signaling also require MyD88 for signaling, MyD88 would be required both for initial IL-1 production following the LCWE injection as well as subsequent IL-1R signaling in the target cells.

IL-1 signaling is required in stromal cells but not hematopoietic cells for LCWE-induced KD vasculitis—We previously reported that *Il1r1*$^{-/-}$ mice are completely protected and that IL-1Ra treatment can prevent LCWE-induced KD vasculitis and coronary lesions.[29] Here, we observed that MyD88 was required in both stromal and hematopoietic cells, consistent with requirement for MyD88 downstream of either TLR2 or IL-1R1 signaling. We therefore sought to determine next the specific cellular compartment where IL-1R1 signaling is required. We generated bone marrow chimeric mice between WT and *Il1r1*$^{-/-}$ mice to determine the IL-1 responsive cellular compartments. Irradiated WT mice reconstituted with WT BM transplantation (control mice) developed KD vasculitis lesions as expected (Figure 6A–D), but irradiated WT mice reconstituted with *Il1r1*$^{-/-}$ BM also developed normal KD vasculitis and coronary arteritis after LCWE injection (Figure 6A–D). However, irradiated *Il1r1*$^{-/-}$ recipient mice that received WT BM did not develop LCWE-induced vasculitis and coronary lesions (Figure 6A–D). These data suggest that stromal IL-1 signaling is indispensable to LCWE-induced vasculitis and coronary arteritis and that IL-1 signaling in hematopoietic cells is not required for the development of this cardiovascular pathology.

Endothelial MyD88 is not required in the development of LCWE-induced KD vasculitis—To begin to dissect which stromal cell is the IL-1/MyD88 responsive cell type i.e. vascular endothelial cells versus vascular smooth muscle cells (VSMC) or others, we first investigated the role of EC. Since IL-1 signaling requires MyD88, and we observed that MyD88 was also required in stromal cells, we sought to identify if MyD88 specifically in EC was required for LCWE-induced KD vasculitis. To this effect, we generated endothelial cell-specific MyD88-deficient mice. We crossed the *Myd88*$^{fl/fl}$ mice to *Tek*-Cre mice to create endothelial MyD88 conditional knockout mice (EC$^{Myd88-/-}$)[38]. *TEK* receptor tyrosine kinase is expressed almost exclusively in ECs[39]. These mice have normal MyD88 function except in their vascular ECs where exon 3 of MyD88 is removed by the cre recombinase, thus making them unresponsive to TLR/MyD88 signaling, as well as IL-1α/β

signaling (Supplemental Figure III).[40] We found that the endothelial MyD88 conditional knockout mice ($EC^{Myd88-/-}$) were not protected and developed KD vasculitis with no differences compared to WT mice (Fig. 7A–C). Control $Myd88^{fl/fl}$ mice (no cre) also developed lesions as expected. These results indicate that endothelial MyD88 signaling is not required for LCWE-induces KD vasculitis in mice, suggesting that the stromal cell responsive to IL-1/MyD88 is not endothelial cells, but some other stromal cell type, such as VSMC.

Discussion

KD is the leading cause of acquired heart disease in children in the United States and the developed world.[9–11] However, while the underlying etiology and mechanisms leading to vessel inflammation, coronary artery lesions, and aneurysms that are the hallmarks of KD remain largely unknown, various studies have characterized which immune cells infiltrate into the cardiovascular lesions seen in KD[24–27].

IL-1β plays a critical role in auto-inflammatory diseases as well as chronic inflammatory diseases such as atherosclerosis, diabetes[41–46], and more recently was also linked to KD vasculitis[29]. Serum level of IL-1β is significantly increased in KD patients compare to age matched healthy controls[47]. IL-1 related genes are upregulated in KD peripheral blood during acute phase of illness[48]. Previous studies have shown that IVIG influences the production and release of IL-1β in KD patients[49, 50] and genetic studies showed that IL-1β promoter polymorphisms with increased IL-1β production is associated with IVIG resistance[41]. In addition, IL-1α, often considered an alarmin, acting early during inflammatory responses and linked to many immunopathologies[51], also plays a critical role in acute and chronic inflammation, and recent studies suggest that IL-1α may even regulate IL-1β secretion[42, 52–54]. In our study, while both IL-1α and IL-1β are required for LCWE-induced vascultis, it is possible that IL-1α and IL-1β may play a role sequentialy in inflammaton, early and late respectively, similar to what other recent studies have reported[36, 55–57]. While specific function blocking mAbs of either IL-1α or IL-1β alone did lead to a significant reduction in KD vasculitis, blocking both cytokines at the same time proved to be more effective in completely preventing any KD lesion formation. Thus therapies that block both cytokines, such as anti IL-1R antagonists (Anakinra) may prove to be more efficacious than each individual specific mAbs.

While IVIG reduces the rate of coronary artery abnormalities (CAA), morbidity and mortality associated with KD, up to 20–25% of patients are resistant to IVIG and have a higher risk of CAA, and discovery of novel more effective treatment for KD is a priority in pediatric research[18]. Progress for more effective and targeted treatments have been hindered due to a lack of a specific etiologic agent and an incomplete understanding of the molecular mechanisms mediating the cardiovascular pathology of KD. Additionally, the severely limited availability of human tissue samples has significantly impeded any progress in our understanding of the etiology and pathology of KD, making the availability of a relevant animal model of KD vasculitis extremely valuable. Indeed, recent progress in KD pathogenesis was made with the advent of human genetic studies (GWAS) combined with relevant mouse models of KD, and these two exciting areas of research recently have

converged upon the importance of IL-1 pathway in KD pathogenesis[4, 29]. We have shown that IL-1β is critical in LCWE-induced KD vasculitis, coronary arteritis and myocarditis, and that an IL-1R antagonist (Anakinra) can effectively block these KD induced cardiovascular pathologies[29].

Recent GWAS studies have discovered that several genetic polymorphisms (SNPs) are associated with increased risk for KD and CAA[58, 59]. Among these SNPs that are associated with increased risk for KD as well as risk for CAA, one that has attracted a major interest is found in the inositol 1,4,5,-triphosphate 3 kinase (ITPKC) gene[4, 60–62]. ITPK3 acts as a negative regulator of intracellular Ca^{2+} influx[61, 62]. Ca^{2+} influx plays a critical in NLRP3 inflammasome activation[63], and the specific ITPKC SNPs associated with KD risk lead to sustained elevation of intracellular Ca^{2+}, and therefore increased NLRP3 inflammasome activation and IL-1α, IL-1β production, providing a possible mechanistic link between these SNPs and KD[64]. The convergence of emerging genetic data with our finding that IL-1 signaling plays a crucial role in this experimental mouse model of KD[29], now provide a very strong rational for further investigating the role of IL-1R antagonist therapies in KD patients. Following these studies, several case reports were published describing successful use and outcomes with Anakinra, in IVIG-non-responder KD patients[30, 31]. Importantly, all of these new data have led to two Phase II clinical trials for Anakinra in IVIG-resistant KD patients (NCT02179853 in UCSD and Univ. of Paris).

Using BM chimera experiments, we found that IL-1 signaling is required in non-hematopoietic cells (i.e. stromal cells), but not endothelial cells (EC) in the LCWE-induced KD mouse model. The likely candidates among the stromal cells are smooth muscle cells (SMC), fibroblasts or pericytes, the former being the most likely candidate. It is intriguing that, IL-1 signaling drives proliferation of smooth muscle cells (SMC) and myofibroblast formation[65–67], a pathologic hallmark of subacute arteriopathy seen in KD (both patients and the animal model). Indeed, SMC-derived myofibroblast actively proliferate in an uncontrolled fashion in KD arterial wall leading to luminal myofibroblast proliferation (LMP), progressing to life-threating coronary artery stenosis and infarction, features that are present both in the KD patients and in the LCWE-induced KD mouse model[12, 68]. Although therapies are available to reduce risk of thrombosis in diseases of the coronary arteries, no therapies are available to reduce or prevent the LMP and coronary stenosis. Therefore, the pathophysiology of LMP must be better understood in order to develop novel targets to prevent or treat this detrimental ongoing vascular remodeling. Additionally, IL-1 induced SMC proliferation is driven by matrix metalloproteinases, including MMP3 and MMP9[65, 66, 69], both of which are implicated in human KD[5, 48, 67, 70–72]. Thus it is very intriguing that our data point towards non-endothelial cells stromal cells, such as VSMC as the key IL-1 responsive cells. The specific confirmation of this must await the generation of VSMC-specific IL-1R deficient mice (ongoing studies).

One possible caveat to our bone marrow chimera studies is the recent finding that many resident tissue macrophages originate from yolk-sac derived myeloid precursors[73]. While in some tissues the authors found that the macrophages can be replaced over time by bone marrow derived cells, no data exists regarding the heart. However, as our WT to WT chimeras developed LCWE-induced KD vasculitis normally, we do not think that they play

a critical role in this model and can be functionally substituted by bone marrow derived cells.

We previously observed that both DCs and macrophages are present in close contact to CD8 T cells in the LCWE-induced KD coronary arteritis lesions in mice[22], similar to what was described in human KD lesions[24]. Interestingly, we now observed that the majority of these macrophages were CD11c+, indicating a more specialized kind of macrophage[34, 74, 75]. Indeed, only CD11c+ macrophages were also FLICA positive (i.e. caspase 1 activity), suggesting that they may be the primary producers of IL-1β locally in the lesions. These cells are likely critical for the development of KD inflammation observed as mice depleted of CD11c+ cells were nearly completely protected from developing KD lesions. Additionally, mice depleted of phagocytic cells by clodronate liposomes were also completely protected. Recently, in another model of vascultis, CD11c+ macrophages were also found to be important for the induction of coronary arteritis.[34]

In our study FLICA activity was detected in CD11c+ macrophages 1 week after LCWE injection and it is possible that other cell types may have inflammasome activity earlier and or later in the progression of pathology. In contrast to our study, Chen et al found Caspase-1 activity localized to the coronary endothelium, and not in macrophages after LCWE injection.[76] Indeed, other studies have found that inflammasome activation can occur in endothelial cells.[77–79] However, in Chen et al's study, the coronary lesions generated after LCWE injection were dramatically smaller than in our study, thus making comparison between the two studies difficult.[76] Additionally, they find evidence for active caspase-1 in the coronary ECs even under naive conditions, which is unusual considering that inflammasome activation is tightly regulated.[80]

Our data strongly suggest the role of both IL-1α and IL-1β in LCWE-induced KD vasculitis model. These findings have important implications for the design of clinical studies to investigate the role of IL-1 in KD patients. The role of IL-1 was reported using this experimental mouse model, and these data has now led to clinical trials in children with KD with the IL-1R antagonist. Our new studies have placed IL-1R1 signaling as a critical step is KD cardiovascular lesion development, without which pathology is prevented. As anti-IL-1 therapeutics already exist for several chronic inflammatory diseases, IL-1 offers an attractive target for prevention and treatment of cardiovascular lesions seen in KD. Clinical trials to investigate the efficacy of anti-IL-1 modalities to prevent and treat KD vasculitis and aneurysm development should include agents that inhibit both IL-1α and IL-1β.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Wenxuan Zhang for technical support.

Funding Sources: Supported by grants from the National Institutes of Health AI072726 and AI070162 to M.A, American Heart Association 12POST9750014 to Y.L., and American Heart Association 15POST22810013 to D.W.

Non-standard Abbreviations and Acronyms

LCWE	*Lactobacillus casei* cell wall extract
EC	endothelial cell
KD	Kawasaki Disease

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Significance

This study highlights the critical requirement of IL-1α and IL-1β signaling, as well as CD11c+ DCs and macrophages in LCWE-induced vasculitis and coronary artery arteritis mouse model. IL-1 signaling is required only in stromal cells, most likely in VSMCs but not in endothelial cells.

These results further emphasize the importance of IL-1 and its downstream effects as they relate to the development of cardiovascular pathologies of KD and further strengthen the rational and need to design anti-IL-1 therapies for KD patients.

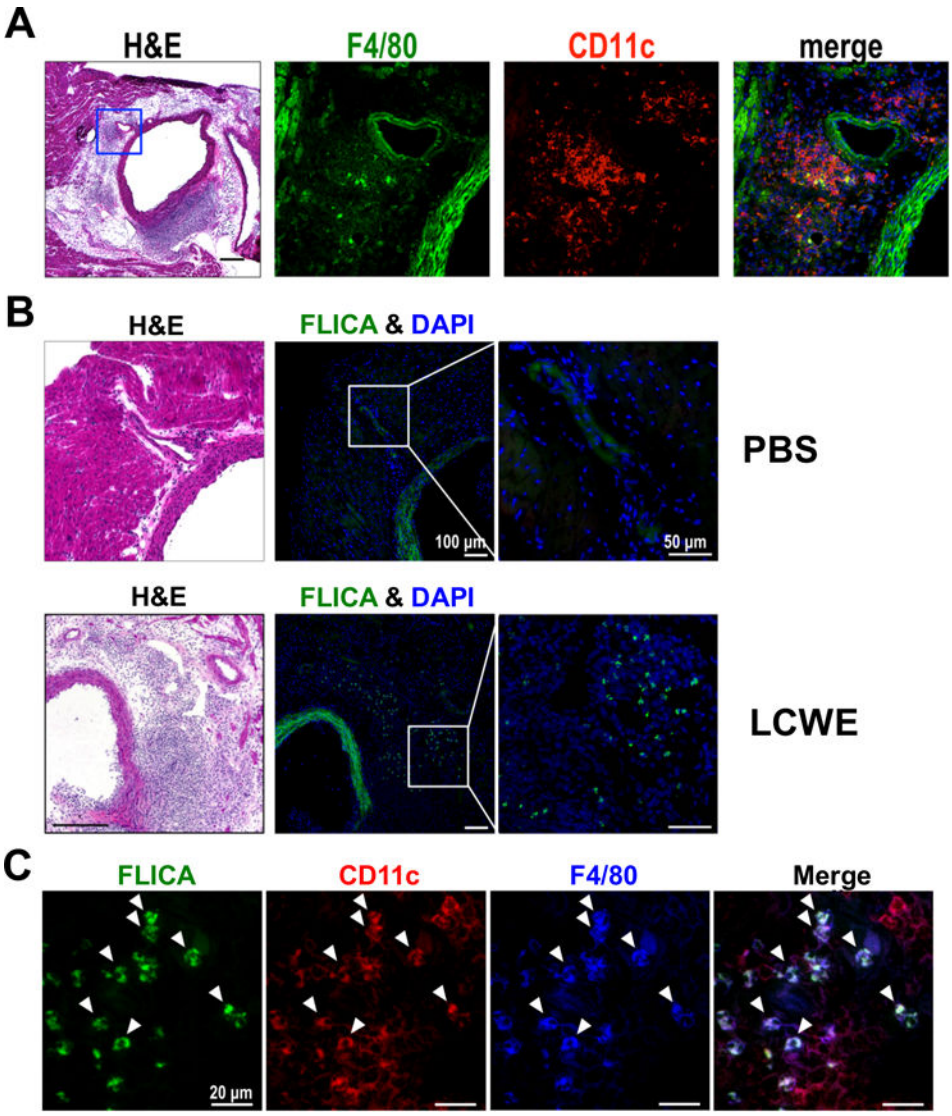

Figure 1. Caspase-1 activity is detected in CD11c and F4/80 positive cells in LCWE-induced vasculitis and coronary lesions

WT mice were i.p. injected with LCWE (250 ug) and heart tissues were collected 1 week after the injection. (A) Images were taken at the aortic root at the level of the 2nd branch of the coronary artery from aorta. F4/80 and CD11c single or double positive cells were detected in the coronary artery and aortitis lesions of LCWE-injected WT mice. (B) The heart sections of naïve and LCWE-injected mice were stained with H&E or FLICA (Green), specific for active caspase-1, and DAPI (Blue). (C) The serial heart sections of LCWE-injected mice were co-stained with FLICA (Green), and antibodies against CD11c (Red) or F4/80 (Blue). White arrows indicate the CD11c, F4/80 and FLICA positive cells. The scale bar is 250μm.

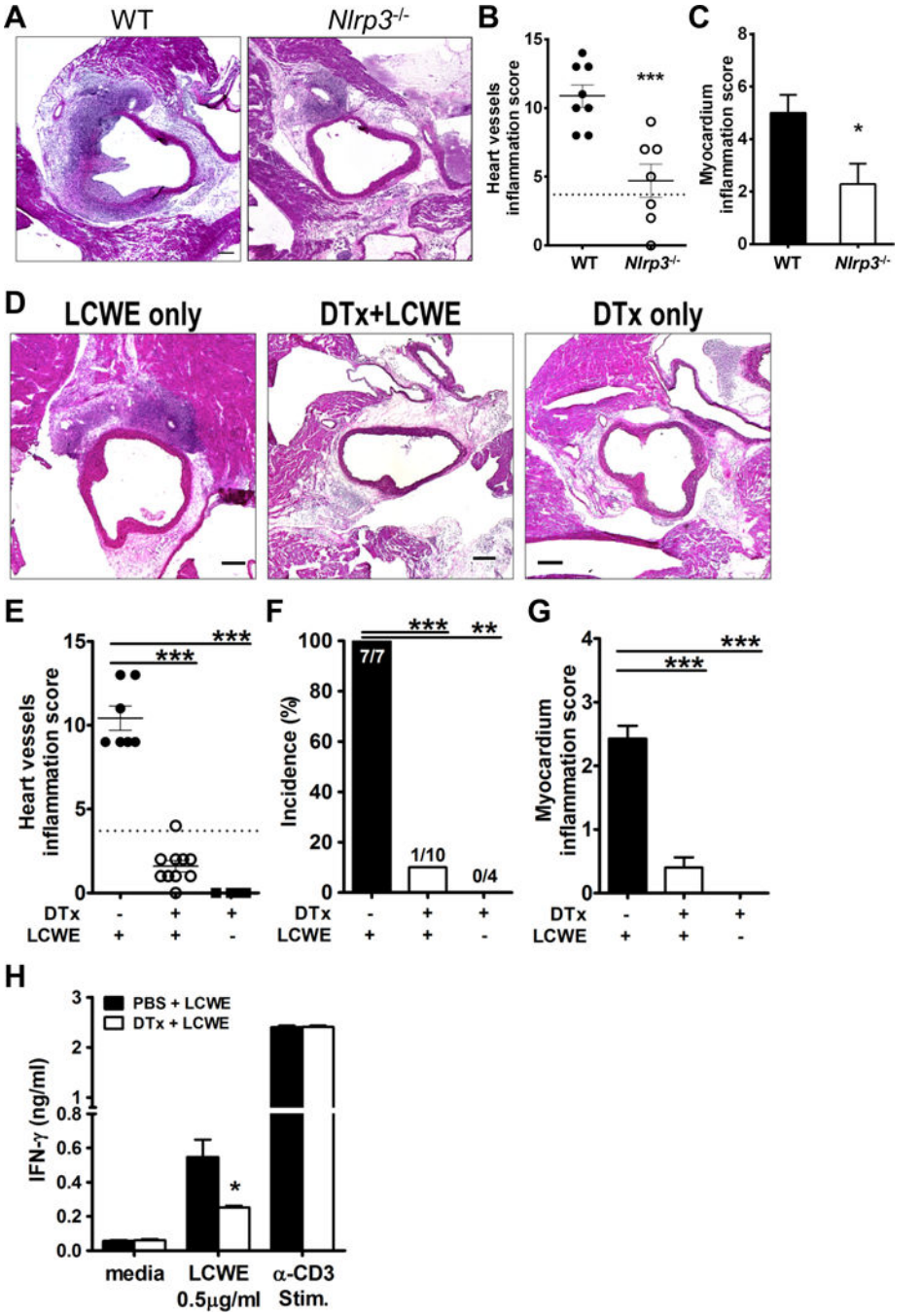

Figure 2. NLRP3 and CD11c+ cells are required for LCWE-induced vasculitis

(A–C) C57BL6/J WT or *Nlrp3*$^{-/-}$ mice were i.p. injected with LCWE and their hearts harvested 14 days after injection. (A) H&E staining, (B) Heart vessel inflammation score, and (C) Myocardium inflammation. (D-H) CD11c+ DTR transgenic mice were i.p. injected with 8 ng/g body weight of diphtheria toxin (DTx) for depletion of CD11c+ cells on day -1 and day 1. LCWE was administrated on day 0. Control mice were injected with PBS instead of toxin or LCWE. The hearts were harvested on day 7 and analyzed by H&E staining. (D) Representative H&E images of DTx treated mice, (E) Heart vessel inflammation score, (F)

incidence of KD lesions, (G) and myocardial inflammation score were evaluated as described in Methods. (H) Splenocytes were re-stimulated on day 14 from LCWE injection and IFN-γ in the supernatants was analyzed by ELISA. Data shown are mean±SE and were compared by the normalized unpaired Student *t* test with Mann-Whitney post test (B, C, and H), by the normalized One way ANOVA with Tukey's post hoc test (E and G), and Fisher exact test for incidence of KD lesions (F). A probability value of $P \leq 0.05$ was considered statistically significant. The scale bar is 250μm.

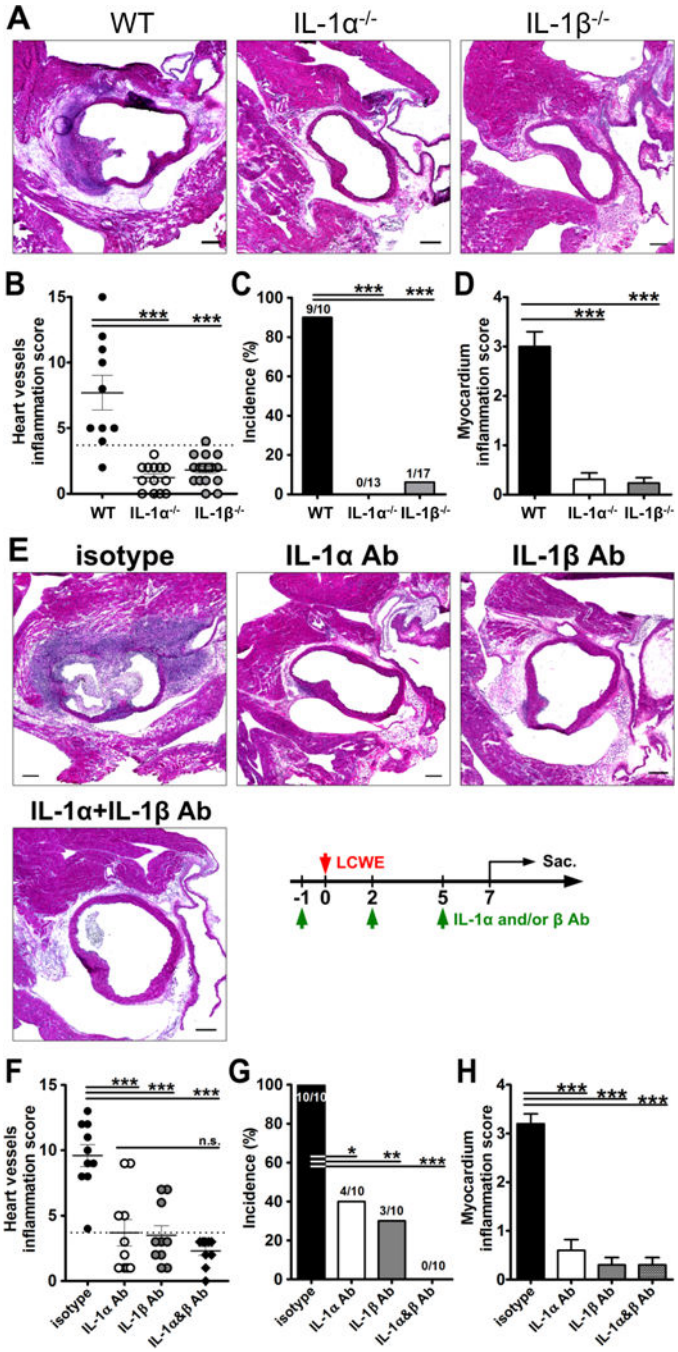

Figure 3. Critical Role of IL-1α and IL-1β in LCWE-induced vasculitis and coronary arteritis

C57BL/6J, *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ mice were i.p injected with 250 μg of LCWE and their heart was harvested and analyzed by H&E staining 14 days later. C57BL/6J mice were i.p administrated with 80 μg of anti-IL-1α mAb and/or 200 μg of anti-IL-1β mAb at day -1, 2, and 5 from 250 μg of LCWE injection. Their hearts were harvested on day 7 for H&E staining. (A and E) Representative histology, (B and F) Heart vessel inflammation score, (C and G) incidence of KD lesions and (D and H) myocardial inflammation score were evaluated as described in Methods. Data shown are mean ± SE and were compared by the

normalized One way ANOVA with Tukey's post hoc test (B and D) and Fisher exact test for incidence (C). A probability value of $P \leq 0.05$ was considered statistically significant. The scale bar is 250 μm.

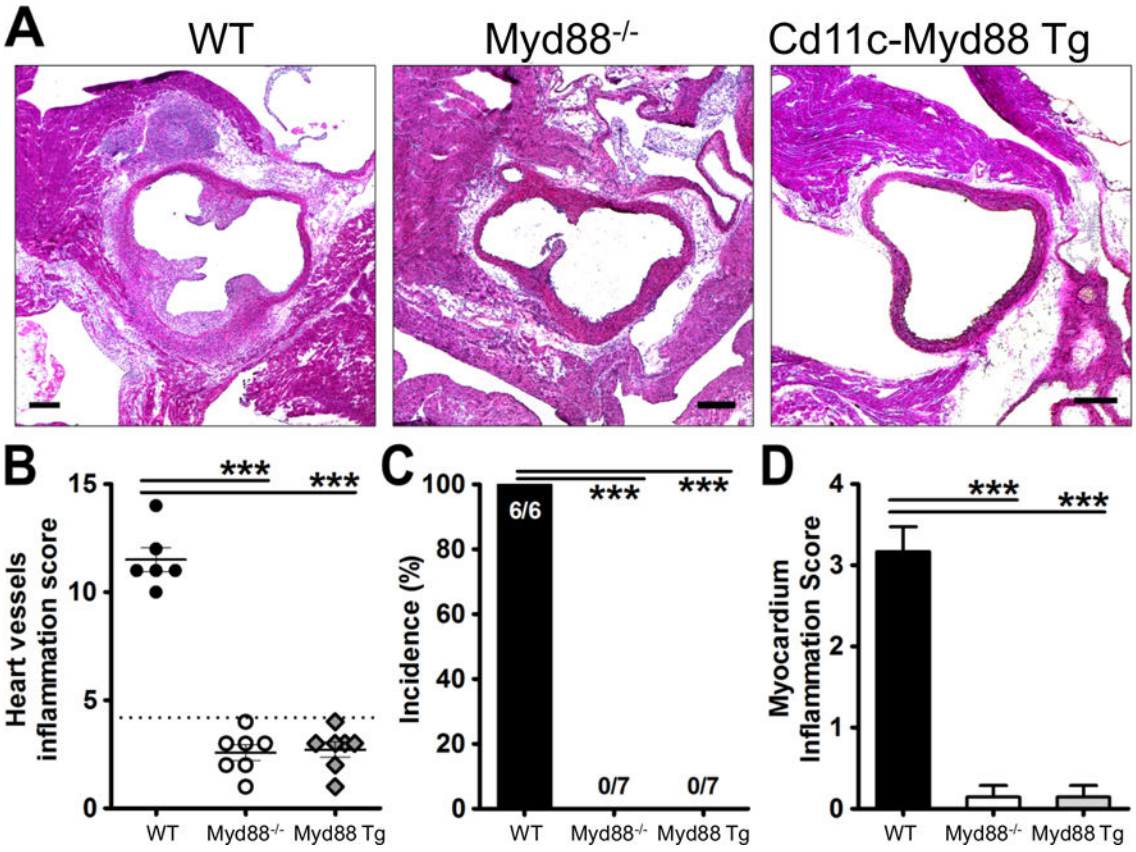

Figure 4. MyD88 in CD11c+ cells is not sufficient for LCWE-induced KD vasculitis

C57BL/6J, *Myd88*$^{-/-}$ and CD11c-*Myd88* Tg mice were i.p injected with 250 μg of LCWE and their heart was harvested 14 days later. (A) Hearts were embedded in OCT compound and analyzed by H&E staining. (B) Heart vessel inflammation score, (C) Incidence of KD lesions, and (D) myocardial inflammation score were assessed as described in Methods. Data shown are mean±SE and were compared by the normalized One-way ANOVA with Tukey's post hoc test (B and D) and Fisher exact test for incidence (C). A probability value of $P \leq 0.05$ was considered statistically significant. The scale bar is 250μm (A).

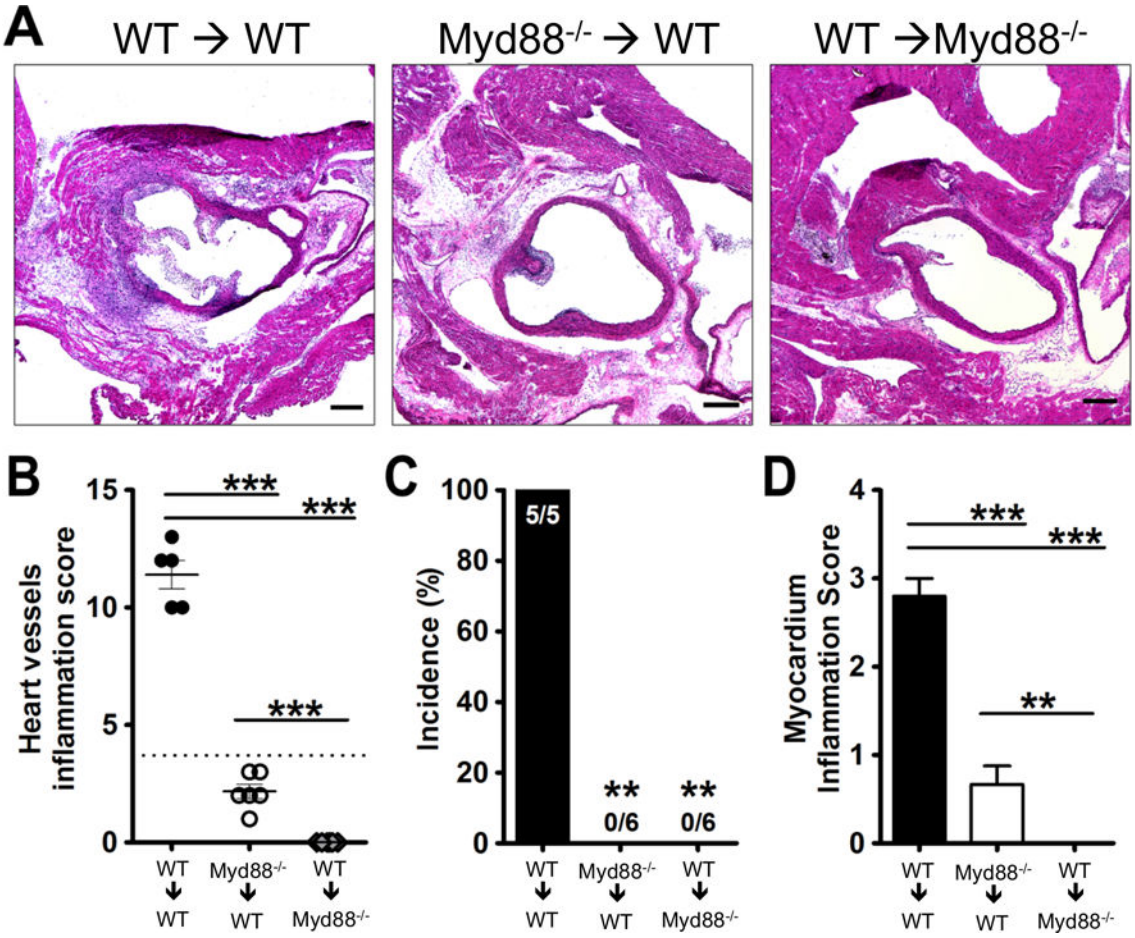

Figure 5. MyD88 is important in both hematopoietic and stromal cells in LCWE-induced KD coronary vasculitis

C57BL/6J or *Myd88*$^{-/-}$ were irradiated (7.5Gy) followed by bone marrow transplantation to create chimeric mice (WT→WT, *Myd88*$^{-/-}$→WT, WT→*Myd88*$^{-/-}$). Following 8 weeks of recovery, LCWE was injected and two weeks later the mice were sacrificed and their hearts analyzed by H&E staining. (A) Representative histology, (B) Heart vessel inflammation score, (C) Incidence of KD lesions, and (D) myocardial inflammation score were evaluated as described in Methods. Data shown are mean±SE and were compared by the normalized One-way ANOVA with Tukey's post hoc test (B and D) and Fisher exact test for incidence (C). A probability value of P≤0.05 was considered statistically significant. The scale bar is 250μm.

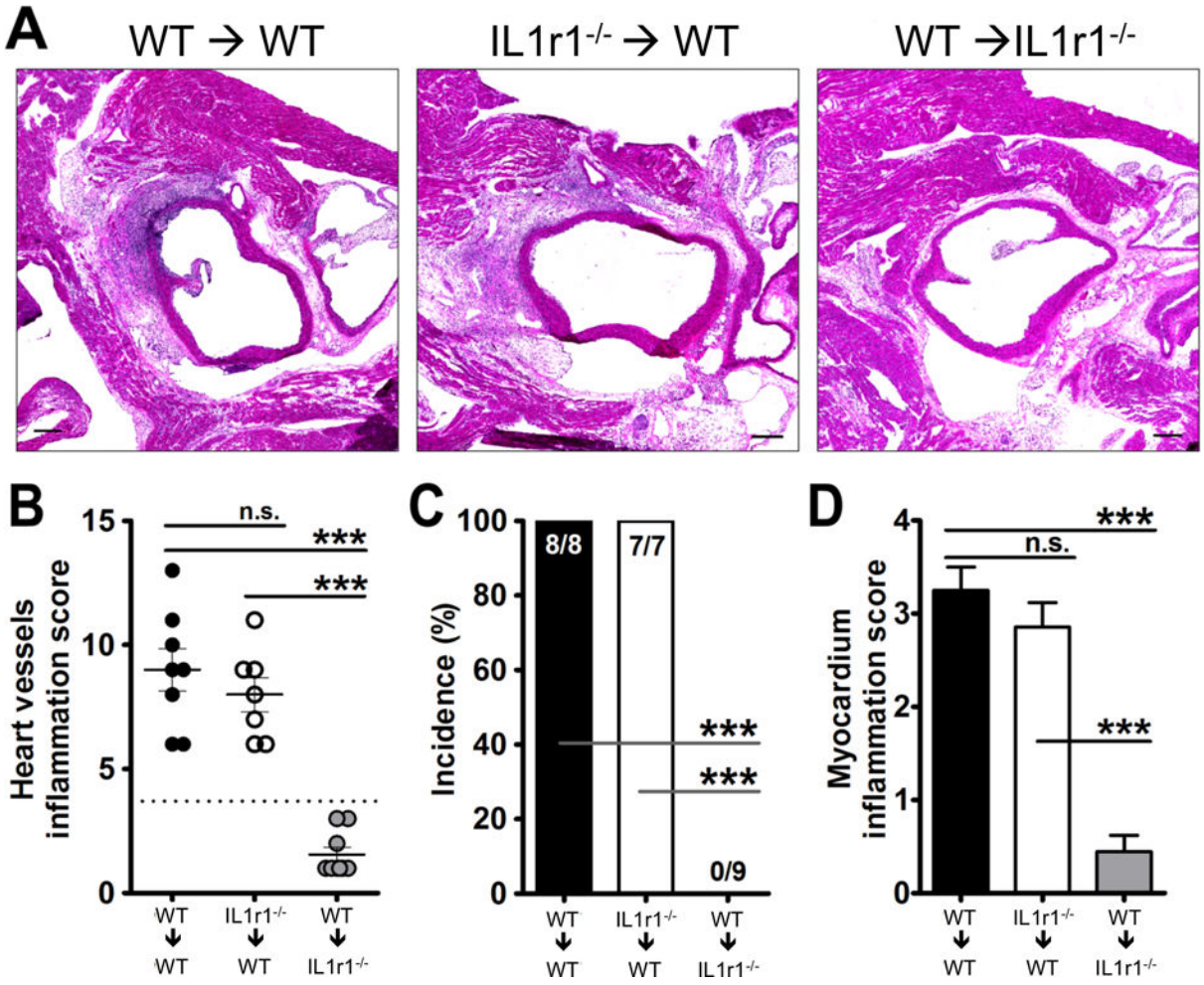

Figure 6. IL-1 signaling is required in stromal cells for LCWE-induced coronary vasculitis

CD45.1 WT (Ly5.1) or *Il1r1*$^{-/-}$ were irradiated (7.5Gy) followed by bone marrow transplantation to create chimeric mice (WT→WT, *Il1r1*$^{-/-}$→WT, and WT→*Il1r1*$^{-/-}$). 8 weeks after irradiation and transplantation the mice were i.p injected with LCWE. Two weeks later the mice were sacrificed and the hearts were analyzed by H&E. (A) Representative histology, (B) Heart vessel inflammation score, (C) incidence of KD lesions, and (D) and myocardial inflammation score were evaluated as described in Methods. Data shown are mean±SE and were compared by the normalized One way ANOVA with Tukey's post hoc test (B and D) and Fisher exact test for incidence (C). A probability value of $P \leq 0.05$ was considered statistically significant. The scale bar is 250μm.

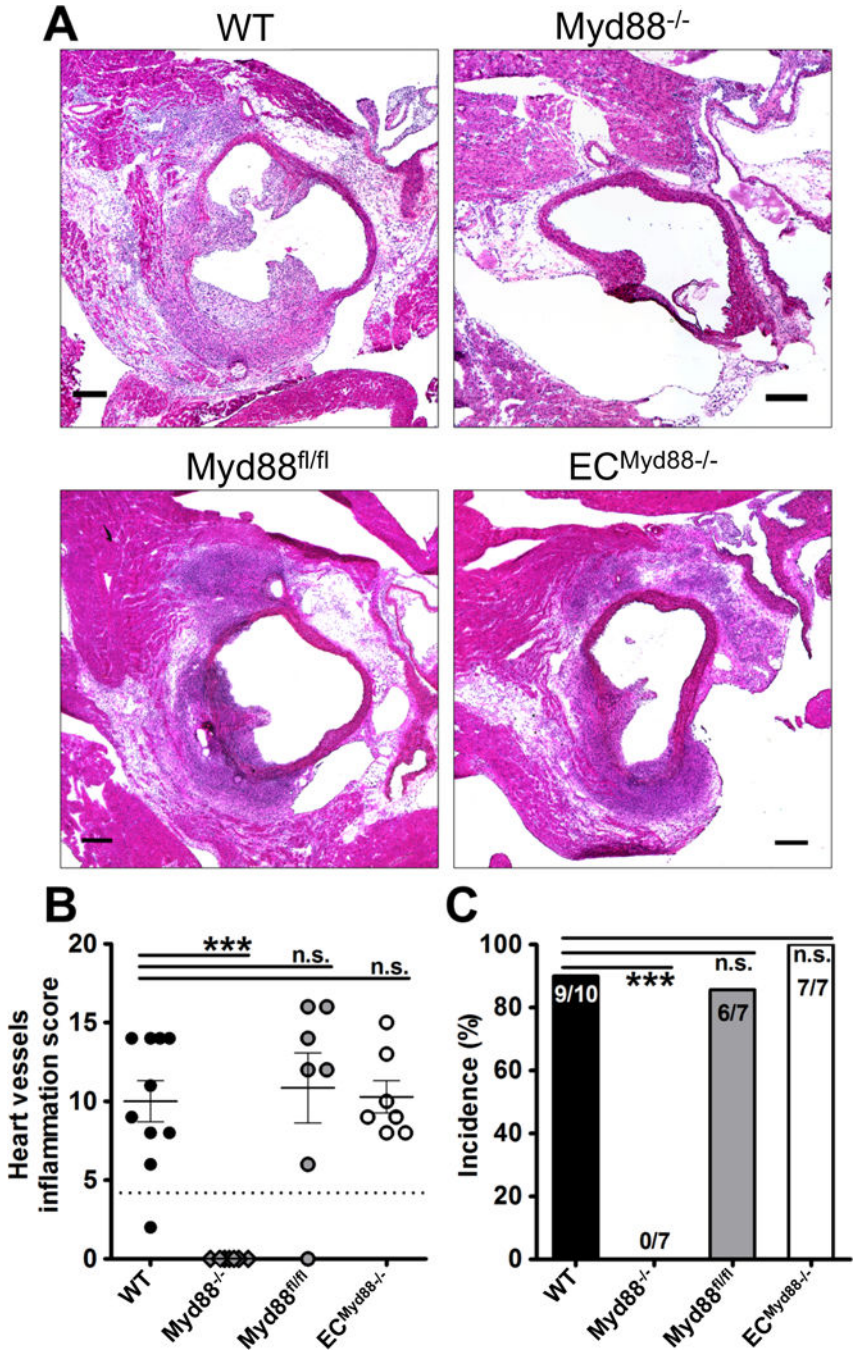

Figure 7. Endothelial MyD88 is not involved in pathogenesis of LCWE induced KD vasculitis and coronary arteritis

C57BL6/J WT, *Myd88*$^{-/-}$, and EC$^{MyD88-/-}$ conditional knock-out mice or their control mice (*Myd88*$^{fl/fl}$ mice) were i.p. injected with 250 μg LCWE and their hearts were harvested on day 14 for H&E staining. (A) Representative histology, (B) Heart vessel inflammation score, and (C) incidence of KD lesions was evaluated as described in Methods. Data shown are mean±SE and were compared by the normalized One-way ANOVA with Tukey's post

hoc test (B) and Fisher exact test for incidence (C). A probability value of $P \leq 0.05$ was considered statistically significant. The scale bar is 250μm.