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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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 interleukin (IL)-1β signaling are required for the Lactobacillus casei cell wall extract–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 and macrophages, and the relative contribution of hematopoietic and stromal cells for IL-1-responsive cells, as well as the MyD88 signaling, in Lactobacillus casei cell wall extract–induced KD mouse model of vasculitis.

Approach and Results—Using mouse knockout models and antibody depletion, we found that both IL-1α and IL-1β were required for Lactobacillus casei cell wall extract–induced KD. Both dendritic cells and macrophages were necessary, and we found that MyD88 signaling was required in both hematopoietic and stromal cells. However, IL-1 response and signaling were critically required in nonendothelial stromal cells, but not in hematopoietic cells.

Conclusions—Our results suggest that IL-1α and IL-1β, as well as CD11c+ dendritic cells 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, whereas IL-1 signaling and response are required only in stromal cells, but not in endothelial cells. Determining the role of 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. (Arterioscler Thromb Vasc Biol. 2015;35:2605-2616. DOI: 10.1161/ATVBAHA.115.306475.)

Key Words: endothelial cells • dendritic cells • interleukin-1 • mucocutaneous lymph node syndrome • MyD88 protein

Kawasaki disease (KD) is an acute febrile illness and systemic vasculitis1-4 that predominantly afflicts children <5 years of age. The cause 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 and 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 Although intravenous IgG (IVIG) treatment within the first 10 days of illness have reduced the cardiovascular complications of KD (CAA) from 25% down to 5%,15 ≤20% to 25% of patients with KD 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 CAA formation.19 The limited availability of tissue samples from

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patients with KD has significantly impeded our progress in understanding the cause 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 and immune pathological features of the cardiovascular lesions (ie, coronary arteritis, aortitis, myocardiitis, aneurysms, including abdominal aorta aneurysms 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 interleukin (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 myocardiitis 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 2 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. Both IL-1α and IL-1β bind to and activate the IL-1R, although their regulation and activity differ. Because the IL-1R antagonist blocks both 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 are also unknown.

We previously reported that dendritic cells (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 patients with KD.\(^{24,27}\) Although DCs are thought 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 seem 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 (BM) chimera experiments show that IL-1 signaling is required only in stromal cells, but not in endothelial cells (ECs).

### 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 are most commonly found in macrophages. Recent literature has indicated the involvement of CD11c\(^+\) macrophages in various proinflammatory disorders.\(^{22-24}\) However, which cells are producing IL-1 and which type of inflammasome is required are not known in this mouse model of KD. 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 fluorescent labeled inhibitor of caspases assay (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 nucleotide binding domain and leucine-rich repeat pyrin domain containing 3 (NLRP3) inflammasome is required for caspase-1 activity and IL-1β production in BM-derived macrophages in vitro.\(^{29}\) However, although 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 wild-type (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 myocardiitis compared with WT mice (Figure 2A–2C). 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 it in this model because Aim2\(^{−/−}\) mice were not protected and developed severe KD vasculitis with similar intensity to WT mice (data not shown).
CD11c⁺ DCs 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 (Figure 1A), indicating a large number of DCs in the lesion as we have previously reported. 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 DTx receptor under the CD11c promoter. The animals were euthanized on day 7, and their hearts were harvested. CD11c⁺ 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 with 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 with controls (Figure 2E–2G). Furthermore, interferon-γ production by splenocytes was significantly reduced after LCWE restimulation, whereas it was unaffected after anti-CD3 stimulation (Figure 2H). We next treated mice with clodronate liposomes to more specifically deplete macrophages during LCWE-induced KD, 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 (Figure 2A–2E in the online-only Data Supplement). Taken together, these data demonstrate that CD11c⁺ DCs and 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. 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–3D). 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. We therefore wished to confirm the data obtained with IL-1 knockout mice by depleting the specific cytokines with anti–IL-1α and anti–IL-1β monoclonal antibodies (mAbs) alone or in combination with

Figure 2. NLRP3 and CD11c+ cells are required for Lactobacillus casei cell wall extract (LCWE)–induced vasculitis. A–C, C57BL6/J wild-type (WT) or Nlrp3−/− mice were intraperitoneally injected with LCWE, and their hearts were harvested 14 days after injection. A, Hematoxylin and eosin (H&E) staining, (B) heart vessel inflammation score, and (C) myocardium inflammation. D–H, CD11c+ DTR transgenic mice were intraperitoneally injected with 8 ng/g body weight of diphtheria toxin (DTx) for depletion of CD11c+ cells on day −1 and day 1. LCWE was administered 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 Kawasaki disease (KD) lesions, and (G) myocardial inflammation score were evaluated as described in Materials and Methods section. H, Splenocytes were restimulated on day 14 from LCWE injection, and interferon (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 1-way ANOVA with Tukey post hoc test (E and G), and Fisher exact test for incidence of KD lesions (F). P≤0.05 was considered statistically significant. **P<0.01, ***P<0.001. The scale bar is 250 μm.
Figure 3. Critical role of interleukin (IL)-1α and IL-1β in Lactobacillus casei cell wall extract (LCWE)-induced vasculitis and coronary arteritis. C57BL/6J, Il1a−/−, and Il1b−/− mice were intraperitoneally injected with 250 μg of LCWE, and their heart was harvested and analyzed by hematoxylin and eosin (H&E) staining 14 days later. C57BL/6J mice were intraperitoneally administered with 80 μg of anti–IL-1α mAb and 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 Kawasaki disease (KD) lesions, and (D and H) myocardial inflammation score were evaluated as described in Materials and Methods section. Data shown are mean±SE and were compared by the normalized 1-way ANOVA with Tukey post hoc test (B and D) and Fisher exact test for incidence (C). P≤0.05 was considered statistically significant. *P<0.05, **P<0.01, ***P<0.001. The scale bar is 250 μm. WT indicates wild type.
LCWE-induced KD vasculitis. Anti–IL-1α and anti–IL-1β mAb were injected on days −1, 2, and 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, and myocarditis compared with mice injected with isotype control antibody (Figure 3E–3H). Interestingly, although mice that received either anti–IL-1α or anti–IL-1β mAb still displayed some small residual vasculitis lesions, when both antibodies were given together, the mice were completely protected (Figure 3E–3H), similar to IL-1R antagonist (Anakinra)–treated mice. 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 2 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. MyD88 signaling is required for most toll-like receptor (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, and we have already shown that LCWE-induced KD model requires both TLR2 and IL-1R1 signaling. Since our data in Figure 1 suggested that both DCs and macrophages may be critically required, we reasoned that MyD88 signaling in DCs and 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−/−) 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–4D). These observations suggest that although 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, Myd88−/− BM–derived macrophage do not express IL-6 and TNF-α (tumor necrosis factor alpha) in vitro in response to LCWE, and IL-1 signaling is critically important in LCWE-induced KD vasculitis. To further define in which cellular compartment MyD88 is important for LCWE-induced KD vasculitis development, we next generated Myd88−/− BM chimeric mice. Chimerism was typically >90% (Figure II in the online-only Data Supplement). Irradiated WT mice reconstituted with WT BM transplantation (control mice) developed KD vasculitis lesions as expected (Figure 5A–5D), whereas irradiated WT mice reconstituted with Myd88−/− BM were completely protected from developing KD vasculitis (Figure 5A–5D). Unexpectedly, irradiated recipient Myd88−/− mice transplanted with WT BM were also completely protected, indicating a critical requirement for MyD88 is not only in 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). Because LCWE signals via TLR2/Myd88 pathway and IL-1 signaling also require MyD88 for signaling, MyD88 would be required both for initial IL-1 production after the LCWE injection and subsequent IL-1R signaling in the target cells.

**Figure 4.** MyD88 in CD11c+ cells is not sufficient for *Lactobacillus casei* cell wall extract (LCWE)–induced Kawasaki disease (KD) vasculitis. C57BL/6J, Myd88−/−, and CD11c-Myd88 Tg mice were intraperitoneally injected with 250 μg of LCWE, and their heart was harvested 14 days later. A, Hearts were embedded in optimized cutting temperature compound and analyzed by hematoxylin and eosin staining. B, Heart vessel inflammation score, (C) incidence of KD lesions, and (D) myocardial inflammation score were assessed as described in Materials and Methods section. Data shown are mean±SE and were compared by the normalized 1-way ANOVA with Tukey post hoc test (B and D) and Fisher exact test for incidence (C). Ps≤0.05 was considered statistically significant. ***P<0.001. The scale bar is 250 μm (A). WT indicates wild type.
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. We here 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 BM 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–6D), but irradiated WT mice reconstituted with Il1r1−/− BM also developed normal KD vasculitis and coronary arteritis after LCWE injection (Figure 6A–6D). However, irradiated Il1r1−/− recipient mice that received WT BM did not develop LCWE-induced vasculitis and coronary lesions (Figure 6A–6D). These data suggest that stimulil IL-1

Figure 5. MyD88 is important in both hematopoietic and stromal cells in Lactobacillus casei cell wall extract (LCWE)-induced Kawasaki disease (KD) coronary vasculitis. C57BL/6J or Myd88−/− were irradiated (7.5Gy) followed by bone marrow transplantation to create chimeric mice (WTWT, Myd88−/−WT, WTMyd88−/−). After 8 weeks of recovery, LCWE was injected, and 2 weeks later the mice were euthanized and their hearts were analyzed by hematoxylin and eosi staining. A, Representative histology, (B) heart vessel inflammation score, (C) incidence of KD lesions, and (D) myocardial inflammation score were evaluated as described in Materials and Methods section. Data shown are mean±SE and were compared by the normalized 1-way ANOVA with Tukey post hoc test (B and D) and Fisher exact test for incidence (C). P≤0.05 was considered statistically significant. **P<0.01, ***P<0.001. The scale bar is 250 μm. WT indicates wild type.

Figure 6. Interleukin (IL)-1 signaling is required in stromal cells for Lactobacillus casei cell wall extract (LCWE)-induced coronary vasculitis. CD45.1 wild-type (WT) (Ly5.1) or Il1r1−/− were irradiated (7.5Gy) followed by bone marrow transplantation to create chimeric mice (WTWT, Il1r1−/−WT, and WTIl1r1−/−). Eight weeks after irradiation and transplantation, the mice were intraperitoneally injected with LCWE. Two weeks later, the mice were euthanized, and the hearts were analyzed by hematoxylin and eosi staining. A, Representative histology, (B) heart vessel inflammation score, (C) incidence of Kawasaki disease lesions, and (D) myocardial inflammation score were evaluated as described in Materials and Methods section. Data shown are mean±SE and were compared by the normalized 1-way ANOVA with Tukey post hoc test (B and D) and Fisher exact test for incidence (C). P≤0.05 was considered statistically significant. ***P<0.001. The scale bar is 250 μm.
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, that is, vascular ECs versus vascular smooth muscle cells or others, we first investigated the role of ECs. Because IL-1 signaling requires MyD88, and we observed that MyD88 was also required in stromal cells, we sought to identify whether MyD88 specifically in ECs was required for LCWE-induced KD vasculitis. To this effect, we generated EC-specific MyD88-deficient mice. We crossed the Myd88<sup>−/−</sup> mice to Tek-Cre mice to create endothelial Myd88 conditional knockout mice (EC<sup>Myd88<sup>−/−</sup></sup>).<sup>37</sup> TEK receptor tyrosine kinase is expressed almost exclusively in ECs.<sup>38</sup> 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 and IL-1α/β signaling (Figure III in the online-only Data Supplement).<sup>39</sup> We found that the endothelial MyD88 conditional knockout mice (EC<sup>Myd88<sup>−/−</sup></sup>) were not protected and developed KD vasculitis with no differences compared with WT mice (Figure 7A–7C). Control Myd88<sup>−/−</sup> mice (no cre) also developed lesions as expected. These results indicate that endothelial MyD88 signaling is not required for LCWE-induced KD vasculitis in mice, suggesting that the stromal cell responsive to IL-1/MyD88 is not ECs, but some other stromal cell type, such as vascular smooth muscle cells.

**Discussion**

KD is the leading cause of acquired heart disease in children in the United States and in the developed world.<sup>9–11</sup> However, although the underlying cause and mechanisms leading to vessel inflammation, coronary artery lesions, and aneurysms that are the hallmarks of KD remain largely unknown, various studies have characterized that which immune cells infiltrate into the cardiovascular lesions seen in KD.<sup>24–27</sup> IL-1β plays a critical role in autoinflammatory diseases, as well as chronic inflammatory diseases, such as atherosclerosis, diabetes mellitus,<sup>40–45</sup> and more recently was also linked to KD vasculitis.<sup>39</sup> Serum level of IL-1β is significantly increased in patients with KD compared with age-matched healthy controls.<sup>46</sup> IL-1-related genes are upregulated in KD peripheral blood during acute phase of illness.<sup>47</sup> Previous studies have shown that IVIG influences the production and release of IL-1β in patients with KD.<sup>48,49</sup> and genetic studies showed that IL-1β promoter polymorphisms with increased IL-1β production are associated with IVIG resistance.<sup>49</sup> In addition, IL-1α, often considered an alarmin, acting early during inflammatory responses and linked to many immunopathologies,<sup>50</sup> also plays a critical role in acute and chronic inflammation, and recent studies suggest that IL-1α may even regulate IL-1β secretion.<sup>51–53</sup> In our study, although both IL-1α and IL-1β were required for LCWE-induced vasculitis, it is possible that IL-1α and IL-1β may play a role sequentially in inflammation, early and late, respectively, similar to what other recent studies have reported.<sup>35,54–56</sup> Although 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.

Although IVIG reduces the rate of coronary artery abnormalities, morbidity, and mortality associated with KD, up to 20% to 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.<sup>48</sup> Progress for more effective and targeted treatments have been hindered because of a lack of a specific pathogenic agent and an incomplete understanding of the molecular mechanisms mediating the cardiovascular pathology of KD. In addition, the severely limited availability of human tissue samples has significantly impeded any progress in our understanding of the cause 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 (genome-wide association study) combined with relevant mouse models of KD, and these 2 exciting areas of research recently have converged on the importance of IL-1 pathway in KD pathogenesis.<sup>4,29</sup> 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.<sup>29</sup>

Recent genome-wide association studies have discovered that several genetic polymorphisms (single nucleotide polymorphisms) are associated with increased risk of KD and CAA.<sup>57,58</sup> Among these single nucleotide polymorphisms that are associated with increased risk of KD and CAA, one that has attracted a major interest is found in the inositol 1,4,5-triphosphate 3 kinase (ITPKC) gene.<sup>4,59–61</sup> ITPK3 acts as a negative regulator of intracellular Ca<sup>2+</sup> influx.<sup>60,61</sup> Ca<sup>2+</sup> influx plays a critical in NLRP3 inflammasome activation,<sup>62</sup> and the specific ITPKC single nucleotide polymorphisms associated with KD risk lead to sustained elevation of intracellular Ca<sup>2+</sup>, and therefore increased NLRP3 inflammasome activation and IL-1α, IL-1β production, providing a possible mechanistic link between these single nucleotide polymorphisms and KD.<sup>63</sup> The emergence of genetic data with our finding that IL-1 signaling plays a crucial role in this experimental mouse model of KD,<sup>29</sup> now provide a strong rational for further investigating the role of IL-1R antagonist therapies in patients with KD. Following these studies, several case reports were published describing successful use and outcomes with Anakinra in IVIG nonresponder KD patients.<sup>30,31</sup> Importantly, all of these new data have led to 2 phase II clinical trials for Anakinra in IVIG-resistant KD patients (NCT02179853 in UCSD and University of Paris).

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

One possible caveat to our BM chimera studies is the recent finding that many resident tissue macrophages originate from yolk sac–derived myeloid precursors. Although in some tissues the authors found that the macrophages can be replaced over time by BM-derived precursors, no data exist 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 BM-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, similar to what was described in human KD lesions. Interestingly, we now observed that the majority of these macrophages were CD11c⁺, indicating a more specialized kind of macrophage. Indeed, only CD11c⁺ macrophages were also FLICA positive (ie, 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. In addition, mice depleted of phagocytic cells by clodronate liposomes were also completely protected.

Recently, Chen et al found caspase-1 activity localized to the coronary endothelium and not in macrophages after LCWE injection. Indeed, other studies have found that inflammasome activation can occur in ECs. However, in the study of Chen et al, the coronary lesions generated after LCWE injection were dramatically smaller than in our study, thus making comparison between the 2 studies difficult. In addition, 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.

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 patients with KD. The role of IL-1 was reported using this experimental mouse model, and these data have 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 in 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.

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Disclosures

None.

References


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This study highlights the critical requirement of interleukin (IL)-1α and IL-1β signaling, as well as CD11c+ DCs and macrophages, in Lactobacillus casei cell wall extract–induced vasculitis and coronary arteritis mouse model. IL-1 signaling is required only in stromal cells, most likely in vascular smooth muscle cells 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 Kawasaki disease and further strengthen the rational and need to design anti–IL-1 therapies for patients with Kawasaki disease.
IL-1 Signaling Is Critically Required in Stromal Cells in Kawasaki Disease Vasculitis

Mouse Model: Role of Both IL-1α and IL-1β

Youngho Lee, Daiko Wakita, Jargalsaihan Dagvadorj, Kenichi Shimada, Shuang Chen, Ganghua Huang, Thomas J.A. Lehman, Michael C. Fishbein, Hal M. Hoffman, Timothy R. Crother and Moshe Arditi

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Materials and Methods

Mice: C57BL/6, CD45.1, Myd88+/−, Il1r1+/−, Aim2+/− and Cd11c-DTR transgenic mice were purchased from Jackson Laboratory (Bar Harbor, ME). CD11c–Myd88-TG/Myd88−/− (CD11c–MyD88-transgenic mouse on Myd88−/− background) were kindly provided by Dr. R. Medzhitov (Yale University, New Haven, CT). 1 Myd88fl/fl mice were obtained from Dr. A. L. DeFranco (University of California San Francisco, CA). Tek-Cre mice were obtained from Dr. R. A. Flavell (Yale University, New Haven, CT). Il1a−/− mice were provided by Dr. Y. Iwakura (Tokyo University of Science, Chiba, Japan). Il1b−/− mice were provided from Dr. D. D. Chaplin (University of Alabama at Birmingham, Birmingham, Alabama). Nlrp3−/− mice were provided by Dr. K. A. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). ECMyd88−/− mice were established by breeding Myd88fl/fl and Tek-Cre mice as we previously described. 2 All animals were housed under specific pathogen-free conditions at the animal center of the Cedars-Sinai Medical Center. Experiments were conducted under approved IACUC protocols. Number of animals used in various experiments ranged between 5-12 in each group as specified in the legends of each figure.

Reagents: Diphtheria toxin (DTx, Sigma, MO, US), anti-mouse IL-1α mAb (BioXcell, NH, US) and anti-mouse IL-1β mAb (Novartis, Basel, Switzerland) were used in these studies. Diphtheria toxin (DTx) was used at 8 ng/g body weight for CD11c depletion experiment using the CD11c–DTR tg mice. Anti-mouse IL-1α mAb was i.p injected at 80 µg/mouse and 200 µg/mouse for anti-mouse IL-1β mAb.

LCWE-induced Kawasaki Disease Vasculitis, Coronary arteritis, Aortitis and Myocarditis: L. casei (ATCC 11578) cell wall extract was prepared as previously described by Lehman 3 and us. 4 Mice aged 4-5 weeks old were i.p. injected with 250 µg of LCWE (total rhamnose amount as determined above) or PBS. Mice were sacrificed and hearts were removed at day 7 or 14 and embedded in OCT compound for histological examination. Following a cut through the aortic root, coronary artery lesions, aortic root vasculitic lesions (aortitis) and myocardial inflammation were identified in serial sections (7 µm) stained with hematoxylin and eosin and inflammation was scored as described previously. 5

Bone marrow transplantation: To create chimeric mice, 4 week old recipient mice were irradiated with 750 rads (Gammacell 40Cs γ-irradiation source) and injected with 10^7 BM cells from donors into the retro orbital vein as previously described. 2 The degree of chimerism was assessed by Flow cytometric analysis from blood leukocytes as CD45.1 and CD45.2 expression at 7 weeks after transplantation. After 8 weeks following BM transplantation, mice were injected with LCWE to induce KD vasculitis.

CD11c+ DCs and macrophages depletion in mice: CD11c+ DCs as well as certain tissue macrophages were depleted from mice by administration of DTx to Cd11c-DTr mice. 6 These mice express the human Diphtheria toxin receptor only in cells that express CD11c, which are mainly DCs. Briefly, we injected 8 ng/g body weight of DTx or PBS (control) into these mice at day -1 and day +1 for depletion of CD11c+ DCs as described earlier, 6 then followed with LCWE injection at day 0.
**Macrophage depletion:** For our Mφ depletion studies, WT mice were intravenously injected with 0.1 mL (5 mg/mL) clodronate (Cl2MDP: dichloromethylene diphosphonate) liposomes (Clodrosome, Encapsula NanoSciences) at day -1, 0, 2, and 4. LCWE was injected on day 0.

**Spleen cells restimulation experiments from LCWE-induced KD mice:** Splenocytes from KD or control mice were harvested and restimulated with LCWE or plate bound anti-CD3. The supernatants were harvested at 24 and 72 hours. The IFN-γ concentration in culture supernatants was quantified by ELISA (eBioscience, CA, USA). The assays were performed as described in manufacturers’ protocols.

**Immunohistochemical Staining of the KD vasculitis and coronary arteritis lesions:** To investigate the type of cells infiltrating we performed immunofluorescence staining with rat anti-F4/80 (1:100, eBioscience, CA, US), hamster anti-CD11c (1:50, eBioscience, CA, US) for 1 hr at room temperature. After a wash, the sections were incubated with Alexa Fluor 594-conjugated anti-Hamster mAb and Alexa Fluor 488-conjugated anti-Rat mAb (1:500, Life Technologies) for 2 hrs. Protein block serum free (Dako co. CA, US) was used for blocking, and ProLong Gold Antifade Reagent with DAPI (Invitrogen, NY, US) for DAPI counter staining. For FLICA staining, each frozen section sample were fixed in acetone for 1 min and stained with rat anti-F4/80 mAb (1:100, eBioscience) or hamster anti-CD11c mAb (1:50, eBioscience) for 1 hr at room temperature. After a wash, the sections were incubated with FLICA probe (1:50, FAM FLICATM Caspase 1 Assay Kit, ImmunoChemistry Technologies), Alexa Fluor 594-conjugated anti-Hamster mAb and Alexa Fluor 350-conjugated anti-Rat mAb (1:500, Life Technologies) for 2 hrs. The data were obtained by fluorescent microscope (BZ-9000E, KEYENCE).

**Statistical Analysis:** Results are reported as mean ± SE. All data were analyzed using Prism 4.03 Statistical Program. To compare differences in serum cytokine levels, the two-tailed Student’s t-test (at 95% confidence interval) was used to compare unpaired samples between experimental groups. We used Fisher’s exact Test to compare incidence of coronary lesion formation. For experiments involving three groups we used one-way ANOVA with Tukey’s post-hoc test. When the data analyzed was not distributed normally, we used the Mann-Whitney test (to compare unpaired samples between experimental groups) or the Kruskal-Wallis with Dunn's post-hoc test (for experiments involving three groups). A probability value of p<0.05 was considered statistically significant. Asterisk marks means * : p<0.05, ** : p<0.01, *** : p<0.001.

**References**


Supplemental Figure I. Macrophage depletion prevents LCWE-induced KD vasculitis. C57BL6/J wt mice were i.v. injected with 0.1 ml of clodronate liposomes or control liposomes (Clodrosome, Encapsula NanoSciences, TN, US) at day -1, 0, 2 and 4. LCWE i.p was injected at day 0. (A) Spleens were analyzed at day 7, the endpoint of experiment by flow cytometer for macrophage depletion (F4/80 CD11b positive). (B) Representative H&E stained heart sections. (C) Heart vessel inflammation score, (D) incidence, and (E) Myocardial inflammation. Data shown are mean±SE and were compared by the normalized unpaired Student t test with Mann-Whitney post test (A, C and E) and Fisher exact test for incidence (D). A probability value of $P\leq0.05$ was considered statistically significant. The scale bar is 250µm.
Supplemental Figure II. BM chimerism is confirmed with PBMC by FACS. MyD88-/- mice were irradiated and transplanted (iv) with WT donor BM cells to MyD88-/- mice. After 7 weeks, peripheral blood was taken and analyzed by FACS. All the lived cells were gated and analyzed with Ly5.2-FITC and Ly5.1-PE. Each panel is individual mouse of experiment group. LCWE was injected each mouse after 1 more week, totally 8 weeks from BMT.
Supplemental Figure III. Aortic EC isolated from Myd88^{fl/fl} (WT) and EC^{Myd88/-} mice were stimulated with LPS (100ng/ml), TNF-α (20ng/ml) and IL-1α (15ng/ml) for 1h, and activation of p38 was assessed by Western blot. Total p38 was used as a loading control.