Interleukin-1β Inhibition Attenuates Vasculitis in a Mouse Model of Kawasaki Disease

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Background: Kawasaki disease (KD), a systemic vasculitis, is suspected to be related to abnormalities in innate immunity. Based on the important role of IL-1 signaling in innate immunity, we investigated the effects of an anti-IL-1β antibody using a Candida albicans water-soluble fraction (CAWS)-induced mouse model of KD.

Methods: CAWS (0.5 mg/mouse) was injected intraperitoneally into 5-week-old DBA/2 mice on five consecutive days. An anti-Murine IL-1β antibody (01BSUR) was administered at various doses (2.5, 5.0, and 10.0 mg/kg) and time points (2 days before, same day, and 2, 5, 7, and 14 days after CAWS administration). After 4 weeks, vasculitis in the aortic root was investigated histologically. Cytokines including IL-1β, -6, -10, and TNF-α were also measured.

Results: Groups administered 01BSUR at all doses showed a significant reduction in the area of vasculitis. In addition, 01BSUR inhibited vasculitis until 7 days after CAWS administration. In the analysis of various time points, the level of IL-6 was lower in all groups compared to the CAWS only group, but the levels of IL-1β, TNFα, and IL-10 were lower when 01BSUR was administered before CAWS. On the other hand, TNFα and IL-10 levels were restored when 01BSUR was administered after CAWS, suggesting that 01BSUR may have additional effects beyond blocking IL-1β signaling.

Conclusions: The anti-IL-1β antibody significantly attenuated CAWS-induced vasculitis. The mechanism of inhibiting vasculitis is thought to include inhibition of the IL-1β pathway and additional effects beyond blocking IL-1β signaling. (J Nippon Med Sch 2019; 86: 108–116)

Key words: Kawasaki disease, interleukin-1beta, Kawasaki disease model mouse, cytokine profile, interleukin-10

Introduction

Kawasaki disease (KD), a systemic vasculitis of unknown etiology, is the most common cause of childhood-acquired heart disease in developed countries. Severe inflammation of the coronary arteries results in coronary sequelae such as coronary artery aneurysms and stenosis in about 3% of all patients with KD. Murata et al. reported that the alkaline component of Candida albicans in the stools of patients with KD causes systemic vasculitis. Ohno et al. reported that the C. albicans water-soluble fraction (CAWS) causes strong systemic vasculitis in the mouse aortic root. Resembling the phenotype of vasculitis in KD, CAWS-induced vasculitis is a major mouse model for studies of KD and has been used to investigate the mechanisms underlying vasculitis and potential treatments.

KD is characterized by hypercytokinemia and suspected abnormalities of innate immunity at onset. The inhibition of IL-1 signaling is an established strategy for preventing tissue damage and inflammation in innate im-
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A: Various 01BSUR dosage experiment

CAWS 0.5mg ip

The second day after CAWS stated
01BSUR: 2.5, 5.0, 10.0 mg/kg (n=6)

Day 28 Sacrifice

B: Various timing 01BSUR administration experiment

CAWS 0.5mg ip

01BSUR 10.0 mg/kg (n=6) once at various time point, two days before, the same day, two days after, 5 days after, 7 days after and 14 days

Day 28 Sacrifice

Fig. 1 Experimental procedure

KD-like vasculitis was induced by the peritoneal injection of CAWS (0.5 mg for five consecutive days) in 5-week-old male DBA/2 mice. The anti-mouse IL-1β antibody 01BSUR was administered to mice at various doses and various time points. For the evaluation of various doses, 01BSUR was administered at 2.5, 50, and 10.0 mg/kg on the second day after the initial CAWS injection (A). For the evaluation of 01BSUR various timings, 01BSUR at 10.0 mg/kg was administered 2 days before, the same day, 2 days after, 5 days after, 7 days after, and 14 days after the initial CAWS injection (B).

Materials and Methods

The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23, revised 1996). The study protocol was approved by the Animal Care and Use Committee of Nippon Medical School (27-150).

Animals

Five-week-old male DBA/2 mice were purchased from Sankyo Labo Service Co., Ltd. (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions according to the Guidelines for Animal Care of the National Institute of Infectious Diseases in Tokyo (NIID).

CAWS and Anti-Murine IL-1β Antibody Preparation

CAWS was prepared from C. albicans strain NBRC1385 in accordance with a previously reported method. Briefly, 5 L of C-limiting medium was maintained in a glass incubator for 2 days at 27°C while air was supplied at a rate of 5 L/min and the mixture was swirled at 400 rpm. Following culture, an equal volume of ethanol was added. After allowing the mixture to stand overnight, the precipitate was collected. The precipitate was dissolved in 250 mL of distilled water, and ethanol was added. The mixture was then allowed to stand overnight. The precipitate was collected and dried with acetone to obtain CAWS.

An anti-Murine IL-1β antibody (01BSUR) identified as a neutralizing antibody for mouse IL-1β[2,13], was provided by Novartis Pharma K.K. Co., Ltd. (Tokyo, Japan).

Induction of Vasculitis and Administration of 01BSUR

As summarized in Figure 1, CAWS (0.5 mg/mouse) was injected intra-peritoneally in 5-week-old DBA/2 mice on five consecutive days. 01BSUR was administered to mice at various doses and at various time points. Six mice were assigned to each group. As a control, in-
stead of CAWS, mice were administered saline.

On the experiment at 01BSUR various doses, 01BSUR was administered intraperitoneally at doses of 2.5, 5.0, and 10.0 mg/kg on the second day after CAWS administration started. On the experiment at 01BSUR various timing, 01BSUR at 10.0 mg/kg was injected 2 days before, on the same day, 2 days after, 5 days after, 7 days after, and 14 days after the administration of CAWS.

At 4 weeks, the mice were killed with an over-dose administration of pentobarbital. The blood and heart were obtained for subsequent analyses.

**Histological Evaluation**

The heart tissues were fixed in formalin and embedded in paraffin. Serial sections were made at a thickness of 4 μm and stained by hematoxylin and eosin (H&E). Sections showing both the aortic root and the most severe inflammatory cell invasion were used. The area (mm²) of inflammatory cell infiltration was measured using the hybrid cell count system (KEYENCE) and KEYENCE BZ-X analyzer (Osaka, Japan). The ratio of the inflammatory area to the total tissue area on the aortic root was calculated.

**Serologic Evaluation**

Serum samples collected were stored at −20°C until analysis. The Bio-Plex multiplex system with Bio-PLEX Pro™ Mouse Cytokine Th17 Panel A 6-Plex (BIO-RAD, Hercules, CA, USA) was used for cytokine profiling according to the manufacturer’s protocol. IL-1β, IL-6, IL-10, IL-17, IFN-γ, and TNF-α were measured.

**Statistical Analysis**

Data are expressed as means ± SD. ANOVA was used to analyze statistical differences among groups. When significance was detected, the Wilcoxon test was used as a post-hoc test to compare the two groups. A value of p < 0.05 was considered statistically significant.

**Results**

There was no incidence of death in the mice used for the analysis of various 01BUSR dosages. In the 01BUSR various timing study, one mouse died in the group injected with 01BUSR 2 days before the CAWS injection.

**Histological Evaluation**

In the analysis of the various 01BUSR dosages, the total inflammatory area was significantly lower in each 01BSUR dosage group than in the CAWS group (CAWS group: 2.60 ± 0.55 mm², 01BSUR 2.5 mg/kg group: 0.30 ± 0.07 mm² (p = 0.0051), 01BUSR 5 mg/kg group: 0.19 ± 0.03 mm² (p = 0.0051), and 01BUSR 10 mg/kg group: 0.12 ± 0.03 mm² (p = 0.0051)) (Fig. 2). The ratio of the inflammatory area to the total aortic root tissue area was also significantly lower in each 01BSUR group than in the CAWS group (CAWS group: 50.0 ± 5.1%, 01BSUR 2.5 mg/kg group: 15.3 ± 1.8% (p = 0.0050), 01BUSR 5 mg/kg group: 7.5 ± 1.5% (p = 0.0050), and 01BUSR 10 mg/kg group: 8.2 ± 1.7% (p = 0.0050)) (Fig. 3A).

In the analysis of the 01BUSR various timing study, the total inflammatory area for each group, i.e., CAWS, 2 days before, on the same day, 2 days after, 5 days after, 7 days after, and 14 days after CAWS administration, were 0.55 ± 0.11 mm², 0.24 ± 0.06 mm², 0.19 ± 0.06 mm², 0.19 ± 0.09 mm², 0.19 ± 0.05 mm², 0.18 ± 0.06 mm², and 0.53 ± 0.11 mm², respectively (Fig. 4). Compared with the CAWS group, the inflammatory area was significantly smaller in the same day group, 2 days after group, 5 days after group, and 7 days after group, but not in the 14 days after group (p = 0.0268, p = 0.0184, p = 0.0184, and p = 1.0000, respectively). The 2 days before group tended to exhibit suppression (p = 0.0513). The ratios of inflammatory area to total aortic root tissue area in the 2 days before, same day, 2 days after, 5 days after, 7 days after, and 14 days after groups were 44.3 ± 4.14%, 14.2 ± 0.86%, 10.7 ± 1.3%, 12.7 ± 2.0%, 13.2 ± 2.6%, 10.0 ± 1.6%, and 39.3 ± 4.0%, respectively. Compared with the CAWS group, the ratios were significantly lower in the 2 days before group, same day group, 2 days after group, 5 days after group, and 7 days after group, but not in the 14 days after group (p = 0.0056, p = 0.0033, p = 0.0033, p = 0.0033, and p = 0.2221, respectively) (Fig. 3B).

**Serum Cytokine Profiling**

Cytokine profiles for various 01BUSR dosages:

The levels of IL-1β in the serum in the control (saline administration, instead of CAWS), CAWS, and 01BUSR 2.5, 5.0, and 10.0 mg/kg groups were 99.4 ± 21.2 pg/mL, 74.0 ± 4.41 pg/mL, 25.8 ± 2.8 pg/mL, 61.5 ± 10.0 pg/mL, and 71.6 ± 11.1 pg/mL, respectively (Fig. 5A). There were no significant differences between the CAWS and other groups, except for the 2.5 mg group (p = 0.0051). The level of IL-6 serum concentrations in the control, CAWS, and 01BUSR 2.5, 5.0, and 10.0 mg/kg groups were 9.8 ± 0.4 pg/mL, 58.4 ± 13.0 pg/mL, 23.6 ± 6.5 pg/mL, 17.5 ± 2.9 pg/mL, and 16.5 ± 1.8 pg/mL, respectively (Fig. 5B). Compared with the CAWS group, the levels of IL-6 in the serum were significantly lower in the control (p = 0.0051), 5 mg/kg (p = 0.0453), and 10 mg/kg groups (p = 0.0306). IL-6 tended to be lower in the 2.5 mg/kg group than in the CAWS group (p = 0.0656).
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The levels of TNF-α in the serum in the control, CAWS, and 01BUSR 2.5, 5.0, and 10.0 mg/kg groups were 258.2 ± 13.6 pg/mL, 559.0 ± 124.2 pg/mL, 376.3 ± 31.9 pg/mL, 278.5 ± 36.4 pg/mL, and 366.8 ± 30.9 pg/mL, respectively (Fig. 5C). Compared with the CAWS group, the levels of TNFα tended to be lower in each group (control: p = 0.0656, 01BSUR 2.5 mg/kg group: p = 0.4712, 5.0 mg/kg group: p = 0.1735, and 10 mg/kg group: p = 0.5752).

The levels of IL-10 in the serum in the control, CAWS, and 01BUSR 2.5, 5.0, and 10.0 mg/kg groups were 35.7 ± 3.4 pg/mL, 58.8 ± 13.0 pg/mL, 39.6 ± 6.4 pg/mL, 19.8 ± 7.02 pg/mL, and 45.3 ± 4.9 pg/mL, respectively (Fig. 5D). There were no significant differences between the CAWS and other groups (control: p = 0.1709, 2.5 mg/kg group: p = 0.2298, 10 mg/kg group: p = 0.3785), except for the 5.0 mg group (p = 0.0306).

Cytokine levels in groups 01BUSR various timing of injection

The levels of IL-1β in the serum in the CAWS, 2 days before, same day, 2 days after, 5 days after, and 14 days after groups were 52.4 ± 8.5 pg/mL, 4.0 ± 2.2 pg/mL, 3.2 ± 2.8 pg/mL, 27.1 ± 8.2 pg/mL, 46.0 ± 7.1 pg/mL, 23.5 ± 6.7 pg/mL, and 6.7 ± 6.3 pg/mL, respectively (Fig. 6A). Compared with the CAWS group, IL-1β levels were significantly lower in the 2 days before (p = 0.0053), same day (p = 0.0025), 7 days after (p = 0.0378), and 14 days after (p = 0.0099) groups. There was no significant difference between the CAWS group and the 2 days after (p = 0.0531) or 5 days after (p = 0.8299) groups.

The levels of IL-6 in the serum in the CAWS, 2 days before, same day, 2 days after, 5 days after, and 14 days after groups were 92.8 ± 12.6 pg/mL, 29.4 ± 12.8 pg/mL, 39.6 ± 4.7 pg/mL, 31.4 ± 9.1 pg/mL, 55.6 ± 7.6 pg/mL, 38.6 ± 15.2 pg/mL, and 30.0 ± 6.6 pg/mL (Fig. 6B). Compared with the CAWS group, IL-6 levels were significantly lower in all other groups (2 days before: p = 0.0149, same day: p = 0.0182, 2 days after: p = 0.0081, 7 days after: p = 0.0383, and 14 days after: p = 0.0082), except for the 5 day after group (p = 0.0538).

The levels of TNF-α in the serum in the CAWS, 2 days before, same day, 2 days after, 5 days after, and 14 days after groups were 244.4 ± 34.3 pg/mL, 27.2
The inflammatory cell invasion area was evaluated by the hybrid cell count system (KEYENCE) using the KEYENCE BZ-X analyzer (Osaka, Japan). The inflammation area is expressed as a ratio with respect to the total tissue area of the aortic root. A: On the 01BSUR various dosage experiments inflammation was significantly attenuated from the low dose of 01BSUR. B: On the 01BSUR various timing experiment, inflammation was significantly attenuated in all groups, but not in the 14 days after group. The number of mice in each group was six, except for the 2 days before group (n=5).

The number of mice in each group was six, except for the 2 days before group (n=5).

![Graph A](image1.png)

![Graph B](image2.png)

![Histological Findings](image3.png)

Fig. 3  Ratio of inflammatory area to total aortic root tissue area

Fig. 4  Histological findings of the aortic root for treatment with the anti-mouse IL-1β antibody at various time points

Representative images of each group as shown: 01BSUR injection (10.0 mg/kg) 2 days before (A), same day (B), 2 days after (C), 5 days after (D), 7 days after (E), and 14 days after (F) the administration of CAWS. (H&E stain, original magnification 4×)
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Fig. 5  Cytokine profiles for various doses of 01SUR

A: IL-1β levels were not significantly different between groups, except between the 01SUR 2.5 mg/kg group and the CAWS group. IL-1β in the 01SUR 2.5 mg/kg group was significantly lower than that in the CAWS group (p = 0.0051).

B: IL-6 levels were significantly lower in the control, 01SUR 5 mg/kg, and 10 mg/kg groups. IL-1 tended to be lower in the 01SUR 2.5 mg/kg group (p = 0.0656) than in the CAWS group.

C: Compared to the CAWS group, TNF-α levels tended to be lower in each group.

D: IL-10 levels were not significantly different between groups, other than the 01SUR 5 mg/kg group (in which IL-10 was significantly lower than that in the CAWS group, p = 0.0306).

Control group: mice were injected with saline instead of CAWS. The number of mice in each group was six.

Discussion

KD is an acute systemic febrile illness of unknown etiology, predominantly affecting children under five years of age. It is the leading cause of acquired heart disease in children with the formation of coronary artery lesions (CALs), such as coronary aneurysms and coronary stenosis. The rate of CALs was about 25% prior to the establishment of intravenous immunoglobulin therapy (IVIG). IVIG attenuates KD vasculitis and lowers the rate of CAL occurrence. However, IVIG non-responders still account for about 20% of patients, and CAL formation is highly likely in this group\(^5\). Steroid therapy is used in addition to IVIG in high-risk cases where IVIG is not likely to be effective\(^6\). A combination of steroid and IVIG therapy reduces the IVIG non-responder rate as well as CAL occur-

The levels of IL-10 in the serum in the CAWS, 2 days before, same day, 2 days after, 5 days after, 7 days after, and 14 days after groups were 100.8 ± 21.2 pg/mL, 0.40 ± 0.0 pg/mL, 0.40 ± 0.0 pg/mL, 50.0 ± 24.2 pg/mL, 157.2 ± 27.0 pg/mL, 86.0 ± 28.4 pg/mL, and 23.2 ± 22.8 pg/mL, respectively (Fig. 6D). Compared with the CAWS group, IL-10 levels were lower in the 2 days before (p = 0.0042), same day (p = 0.0021), and 14 days after groups (p = 0.0153). However, there were no significant differences between the CAWS group and the 2 days after (p = 0.1004) or 7 days after group (p = 0.8303). IL-10 levels even tended to be higher in the 5 days after group than in the CAWS group (p = 0.1156).
Infliximab, a monoclonal antibody against human TNFα, is a potential strategy for refractory KD. A randomized trial of infliximab for initial IVIG non-responders showed a significantly greater defervescence rate as compared to that for repeated IVIG. However, the rate was 76.7% and there were still non-responders. To prevent CAL formation, patients with initial IVIG-refractory KD should be identified early and KD vasculitis should be controlled as early as possible, ideally by day 10 after onset. Anti-IL-1β therapy is a promising strategy for patients with severe refractory KD. Shafferman et al. reported the anakinra, an IL-1β receptor antagonist, is effective for severe neonatal KD. Our results confirmed that IL-1β inhibition attenuates CAWS inducing vasculitis. IL-1β is considered a gate keeper of innate immunity and excessive IL-1β signaling has been observed in many auto-inflammatory diseases. Auto-inflammation is related to gain-of-function mutations that contribute to the activation of caspase-1, a cysteine protease activating an IL-1β precursor, and the secretion of IL-1β. Blocking IL-1β signaling is a potential target for auto-inflammatory diseases, such as Familial Mediterranean Fever, Pyogenic Arthritis Pyoderma Gangrenosum and Acne, and Cryopyrinopathy-associated Periodic Syndrome. In addition, IL-1β blocking is also an excellent target for inflammatory diseases with high IL-1β, such as...
rheumatoid arthritis, systemic juvenile idiopathic arthritis, macrophage activating syndrome, and gouty arthritis. IL-1β is also elevated in KD, and abnormalities of the innate immune response are closely related to KD. DNA transcript profiling in acute-phase KD showed significantly higher transcript abundances for IL-1 pathway genes, suggesting that the IL-1 pathway is a reasonable target for KD therapy. Inhibiting IL-1β is particularly promising for KD therapy.

Three KD mouse models have been developed to date. KD-like vasculitis is induced by the administration of CAWS, water-soluble Lactobacillus casei cell extract (LCWE), or Nod-1 ligands. We have proven that cytokine profiles are also similar to those of KD in CAWS-induced vasculitis, and KD drugs, such as IVIG, methylprednisolone, cyclophosphamide, and TNF-α receptor inhibitors, attenuate CAWS-induced vasculitis. We found that the severity of vasculitis is highly correlated with the serum TNF-α concentration and the TNF-α receptor inhibitor etanercept significantly attenuates CAWS-induced vasculitis. TNFα inhibition is also effective for the reduction of LCWE-induced vasculitis. MyD88, an adapter protein for Toll-like receptors (TLR), and TLR2 are also required for inducing vasculitis in the LCWE model. In addition, IL-1β signaling is necessary for LCWE vasculitis. Lee et al. further showed that IL-1α is required for LCWE-induced vasculitis and that CD11c+ dendritic cells and macrophages are also necessary; these macrophages appear to be the cellular source of IL-1β production in the lesions. In CAWS vasculitis, IL-1β may be involved in the same way as in LCWE vasculitis.

Our results show that 01BSUR could inhibit CAWS vasculitis until 7 days after CAWS administration. Furthermore, IL-1β, TNFα, and IL-10 levels were low when 01BSUR was injected before CAWS. Intriguingly, these cytokines were back to the same level of the CAWS group when 01BSUR was injected after the CAWS injection, while IL-6, an indicator of inflammation, was low for all time points. We speculate that 01BSUR attenuates vasculitis by different mechanisms before and after CAWS administration. When 01BSUR is injected before CAWS administration, it inhibits vasculitis by depleting IL-1β signaling itself. When 01BSUR is injected after CAWS administration, it may attenuate vasculitis by not merely depleting IL-1β signaling. Because the level of proinflammatory cytokines IL-1β and TNF-a is almost the same as in the CAWS group, the effect of IL-1β signaling inhibition is not explained by simply attenuating proinflammatory cytokines. 01BSUR should have an inhibiting effect that attenuates inflammation even after proinflammatory cytokines are elevated. We did not identify any significant correlation between vascular inflammation area and IL-β level (data not shown). This may reflect the profound inflammation inhibitory mechanisms that 01 BSUR has. We speculate that one of the key mechanisms is IL-10. IL-10, a regulatory cytokine, was restored by 01 BSUR. The anti-inflammatory effect of IL-10 has also been reported in a CAWS vasculitis model. Miura et al. reported that CBA/J mice produce high levels of immunosuppressive IL-10 and are resistant to CAWS vasculitis. Additionally, IL-10 depletion facilitates inflammation via IL-1β activity in both mice and humans. We hypothesize that IL-1β inhibition by 01BSUR may restore IL-10, which attenuates vasculitis, even after CAWS administration when TNFα levels are already elevated. Further studies are necessary to evaluate this hypothesis.

**Conclusion**

01BSUR, a mouse anti-IL-1β monoclonal antibody, significantly attenuated CAWS-induced vasculitis. Its inhibitory effect can be observed even when administered 7 days after CAWS administration. The mechanism by which 01 BSUR inhibits vasculitis includes not only inhibition of the IL-1β pathway, but other mechanisms beyond blocking the IL-1β pathway. Inhibiting the IL-1β pathway is a promising strategy for KD therapy.

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**Conflict of Interest:** Ryuji Fukazawa was given the anti-IL-1β monoclonal antibody, significantly attenuated CAWS-induced vasculitis. Its inhibitory effect can be observed even when administered 7 days after CAWS administration. The mechanism by which 01 BSUR inhibits vasculitis includes not only inhibition of the IL-1β pathway, but other mechanisms beyond blocking the IL-1β pathway. Inhibiting the IL-1β pathway is a promising strategy for KD therapy.

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