Inhibition of NF-κB activation by a novel IKK inhibitor reduces the severity of experimental autoimmune myocarditis via suppression of T-cell activation

Ryo Watanabe,1 Ryoko Wakizono Azuma,2 Jun-ichi Suzuki,3 Masahito Ogawa,3 Akiko Itai,4 Yasunobu Hirata,2 Issie Komuro,5 and Mitsuaki Isobe1
1Department of Cardiovascular Medicine, Tokyo Medical and Dental University, Yushima, Bunkyo, Tokyo, Japan; 2Department of Clinical Laboratory, Tokyo Medical and Dental University, Yushima, Bunkyo, Tokyo, Japan; 3Department of Advanced Clinical Science and Therapeutics, University of Tokyo, Hongo, Bunkyo, Tokyo, Japan; 4Institute of Medicinal Molecular Design, Hongo, Bunkyo-ku, Tokyo, Japan; and 5Department of Cardiovascular Medicine, University of Tokyo, Hongo, Bunkyo, Tokyo, Japan

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Watanabe R, Azuma RW, Suzuki J, Ogawa M, Itai A, Hirata Y, Komuro I, Isobe M. Inhibition of NF-κB activation by a novel IKK inhibitor reduces the severity of experimental autoimmune myocarditis via suppression of T-cell activation. Am J Physiol Heart Circ Physiol 305: H1761–H1771, 2013. First published October 4, 2013; doi:10.1152/ajpheart.00159.2013.—NF-κB, which is activated by the inhibitor of NF-κB kinase (IKK), is involved in the progression of inflammatory disease. However, the effect of IKK inhibition on the progression of myocarditis is unknown. We examined the effect of IKK inhibition on the progression of myocarditis. Lewis rats were immunized with porcine cardiac myosin to induce experimental autoimmune myocarditis (EAM). We administered the IKK inhibitor (IMD-0354; 15 mg·kg−1·day−1) or vehicle to EAM rats daily. Hearts were harvested 21 days after immunization. Although the untreated EAM group showed increased heart weight-to-body weight ratio, and severe myocardial damage, these changes were attenuated in the IKK inhibitor-treated group. Moreover, IKK inhibitor administration significantly reduced NF-κB activation and mRNA expression of IFN-γ, IL-2, and monocyte chemoattractant protein-1 in myocardium compared with vehicle administration. In vitro study showed that the IKK inhibitor treatment inhibited T-cell proliferation and Th1 cytokines production induced by myosin stimulation. The IKK inhibitor ameliorated EAM by suppressing inflammatory reactions via suppression of T-cell activation.

myocarditis; inflammation; NF-κB kinase

ACUTE MYOCARDITIS IS A SERIOUS disease in humans; patients with myocarditis may suffer from rapidly progressive heart failure, shock, or severe arrhythmia. Although acute myocardial inflammation is an essential etiology for its progression, no effective treatment has been elucidated (3, 13, 30, 49). Because autoimmunity is important in myocarditis, a reaction to cardiac myosin may contribute to the development (14). In the pathology of myocarditis, T cells activated by antigen infiltrate into the myocardium, which may lead to myocardial damage by inflammatory reaction to myosin (23). Subsequently, the infiltration of CD4-positive T cells and macrophages into the myocardium promotes inflammatory reactions in the myocardium by releasing Th1 cytokines (e.g., IFN-γ and IL-2) (7) and chemokines [e.g., monocyte chemoattractant protein (MCP)-1] (11). NF-κB, which is regulated by NF-κB kinase (IKK), induces expression of genes that participate in the progression of inflammation (29, 41). Previously, we examined the effect of decoy oligonucleotide against NF-κB on ventricular remodeling after myocarditis (47). This report showed that NF-κB activity increased markedly in experimental autoimmune myocarditis (EAM) rat myocardium and that NF-κB is a key regulator in the progression of EAM. Activation of NF-κB induces gene programs that lead to transactivation of factors including Th1 cytokines and chemokines, promoting the inflammatory status involved in myocarditis (12, 16, 20, 38). Because NF-κB is the main factor in the development of inflammation, inhibition of its activation may be an effective therapy for myocarditis from the standpoint of preventing inflammation.

Recently, we developed IMD-0354, a novel inhibitor of IKK (19, 34, 41). This drug is a selective IKK-β inhibitor, blocks IkBα phosphorylation, and prevents NF-κB p65 nuclear translocation. Some previous studies reported the beneficial effects of IMD-0354 and its prodrg on inflammation-related cardiovascular diseases (15, 34, 35, 42). Therefore, regulation of NF-κB activation by the IKK inhibitor might have a potent effect on the treatment of myocarditis. However, the effect of IKK inhibitor treatment on the progression of myocarditis is unknown. Thus we assessed the hypothesis that IKK inhibitor treatment attenuates cardiac inflammation in the progression of myocarditis. EAM in a rat model is characterized by severe myocardial damage and multinucleated giant cell infiltration. This has been used as a disease model for human acute giant cell myocarditis (8). We examined the effect of IKK inhibition on EAM.

METHODS

Induction of EAM. Male Lewis rats (6-wk-old; body weights 150 to 200 g) were purchased from Charles River Laboratories Japan. They were fed a standard diet and water and were maintained in compliance with animal welfare guidelines of the Institute of Experimental Animals, Tokyo Medical and Dental University. Also, protocols were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University. Purified porcine cardiac myosin (Sigma Chemical, St. Louis, MO) was emulsified with an equal volume of complete Freund’s adjuvant (Difco, Sparks, MD) supplemented with Mycobacterium tuberculosis H37RA (Disco) at a concentration of 10 mg/ml. On day 0, rats were injected in the footpads subcutaneously with 0.2 ml of emulsion, yielding an immunizing dose of 1.0 mg/body of cardiac myosin per rat (9) anesthetized by intra-peritoneal administration of pentobarbital sodium (25 mg/kg; Dainihon Chemical, Osaka, Japan). We also used unimmunized (normal)
rats parallel with the diseased protocol. Unimmunized rats were injected saline instead of emulsion of myosin on day 0.

IKK inhibitor administration. The IKK inhibitor IMD-0354 was provided by the Institute of Medicinal Molecular Design. The drug was dissolved in 0.5% carboxy methyl cellulose (CMC) solution immediately before use. In the EAM phase, cardiac inflammation starts on approximately day 14, and the peak of inflammation is expected to occur on day 21 (25). For this reason, we administered the IKK inhibitor either from day 1 or from day 14, and harvested hearts and spleens on day 21. Rats were assigned randomly to five groups: 1) daily CMC intraperitoneal injection to normal rats from day 1 to day 20 [normal + vehicle group; n = 6], 2) daily IMD-0354 intraperitoneal injection (15 mg·kg⁻¹·day⁻¹) to normal rats from day 1 to day 20 [normal + IKKi(1–20); n = 5], 3) daily CMC intraperitoneal injection to EAM rats from day 1 to day 20 [EAM + vehicle; n = 18], 4) daily IMD-0354 intraperitoneal injection (15 mg·kg⁻¹·day⁻¹) to EAM rats from day 1 to day 20 [EAM + IKKi(1–20); n = 13], and 5) daily IMD-0354 intraperitoneal injection (15 mg·kg⁻¹·day⁻¹) to EAM rats from day 14 to day 20 [EAM + IKKi(14–20); n = 10]. Administration dose of the IKK inhibitor was calculated from that of previous articles (17, 19, 31, 34, 35).

Echocardiogram. Transthoracic echocardiography was performed on animals anesthetized by intraperitoneal administration of pentobarbital sodium (25 mg/kg) on day 21. An echocardiography machine with a 7.5-MHz transducer (Nemio; Toshiba, Tokyo, Japan) was used for M-mode left ventricular (LV) echocardiographic recording. A two-dimensional targeted M-mode echocardiogram was obtained along the short-axis view of the LV papillary muscles (40). LV posterior wall thickness at diastole (LVPWd), interventricular septal thickness at diastole (IVSTd), and percent ejection fraction (EF) were calculated from the M-mode recordings.

Histopathological examination. Hearts and spleens were harvested immediately after all rats were killed by the cutting of the abdominal aorta under anesthesia after the echocardiographic examination on day 21. After heart weights were measured, hearts were divided into apex, midventricular, and basal level slices. Apex level slice was frozen by liquid nitrogen, and it was stored at -80°C until before use. This slice was used as the sample for Western blotting or real-time RT-PCR. Histopathological examination was performed by a computer-assisted analyzer (Scion Image beta 4.0.2) as expected to occur on day 14 (17, 19, 31, 34, 35).

Immunohistochemistry. Immunohistochemistry was performed to detect CD4-, CD8-positive T cells, macrophages, NF-κB p65, and phospho-NF-κB p65 in the heart or the spleen on day 21. Paraffin or frozen sections were incubated with primary antibodies against CD4 (10B5; Abcam, Cambridge, UK), CD8 (OX8; BD PharMingen California), CD68 (as a marker of macrophage; ED1; Santa Cruz Bio-technology, Santa Cruz, CA), and NF-κB p65 (Abcam) for 8 h at 4°C and washed in PBS followed by biotinylated secondary antibodies (Nichirei, Tokyo, Japan) at 5 mg/ml for 30 min at room temperature. Finally, each section was reacted with AEC (aminomethylcarbazole complex) solution (Nichirei) for 5 to 30 min. Sections were counterstained with hematoxylin solution. We counted the number of positive cells against CD4, CD8, and CD68 in randomly selected five random fields (original magnification, ×200) per sample section, and averaged it (10). Immunofluorescence double staining was performed to identify colocalization of CD4 positive cells and phospho-NF-κB p65 in the heart and spleen. Antibodies against CD4 (OX35; mouse monoclonal; Abcam) and phospho-NF-κB p65 (Ser536; 93H1; rabbit monoclonal; Cell Signaling Technology) were co-incubated and detected with Alexa 488 anti-mouse antibody (Life Technologies Japan) and Alexa 568 anti-rabbit antibody (Life Technologies, respectively). Nuclei were stained with 4',6-diamidino-2-phenylindole.

Extraction of proteins from hearts. To extract tissue protein, frozen cardiac tissues from an apex level heart slice was homogenized in lysis buffer of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 10 mM EDTA, 100 mM NaF containing protease inhibitor cocktail tablets (Roche Diagnostic, Basel, Switzerland) and phosphatase inhibitor cocktail tablets (Roche Diagnostic). Nuclear and cytosolic proteins were isolated from frozen cardiac tissues from the same apex level heart slice using Nuclear Extraction Kit (Epi genteK Group). Protein concentrations were measured with a BCA protein assay (Bio-Rad, Milan, Italy) to equalize the protein concentrations of all samples. Protein samples were stored at -80°C until they were used.

Western blotting. Equal amounts of proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated overnight with antibodies to IxB (Abcam), phospho-IxB (Cell Signaling Technology), GAPDH (Cell Signaling Technology), NF-κB p65 (Abcam), phospho-NF-κB p65 (Ser536; Cell Signaling Technology), and Lamin A/C (Cell Signaling Technology) at 4°C. The membranes were incubated with a secondary antibody (Amersham Biosciences, Piscataway, NJ) for 2 h and developed with ECL reagent (Amersham Biosciences). Enhanced chemiluminescence was detected with an LAS-1000 (Fujifilm, Tokyo, Japan). The value was calculated using ImageJ [National Institutes of Health (NIH)].

Real-time RT-PCR. Total RNA was isolated from frozen apex level heart slice tissues using TRIzole (Bioline, London, UK), and cDNA was prepared with the high capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR in a Step One real-time PCR system (Applied Biosystems) was used to determine the mRNA expression of IFN-γ (Assay ID: Rn099999014_m1), IL-2 (Assay ID: Rn00587673_m1), and MCP-1 (Assay ID: Rn00580555_m1) and 18S rRNA (Assay ID: Hs99999901_s1) as a control. cDNA was run in duplicates, quantitative data were calculated using the comparative

Table 1. Echocardiographic parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>LV Diameter at Diastole, mm</th>
<th>LV Diameter at Systole, mm</th>
<th>Ejection Fraction, %</th>
<th>Interventricular Septal Thickness at Diastole, mm</th>
<th>LV Posterior Wall Thickness at Diastole, mm</th>
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<tbody>
<tr>
<td>Normal + vehicle</td>
<td>6</td>
<td>6.3 ± 0.26</td>
<td>3.0 ± 0.17</td>
<td>89 ± 0.5</td>
<td>2.2 ± 0.26</td>
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<tr>
<td>Normal + IKKi(1–20)</td>
<td>5</td>
<td>6.5 ± 0.21</td>
<td>3.4 ± 0.09</td>
<td>85 ± 1.4</td>
<td>2.1 ± 0.35</td>
</tr>
<tr>
<td>EAM + vehicle</td>
<td>11</td>
<td>6.0 ± 0.28</td>
<td>3.6 ± 0.33</td>
<td>75 ± 4.7</td>
<td>2.5 ± 0.20</td>
</tr>
<tr>
<td>EAM + IKKi(1–20)</td>
<td>12</td>
<td>6.6 ± 0.15</td>
<td>4.0 ± 0.27</td>
<td>76 ± 3.9</td>
<td>1.7 ± 0.11</td>
</tr>
<tr>
<td>EAM + IKKi(14–20)</td>
<td>7</td>
<td>6.1 ± 0.11</td>
<td>3.8 ± 0.16</td>
<td>74 ± 2.2</td>
<td>1.6 ± 0.09*</td>
</tr>
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Values are means ± SE. Early IKK inhibitor treatment [experimental autoimmune myocarditis (EAM) + IKKi(1–20)] suppressed an increase of interventricular septal thickness at diastole and left ventricular (LV) posterior wall thickness at diastole values, whereas late IKK inhibitor treatment [EAM + IKKi(14–20)] only suppressed an increase of interventricular septal thickness at diastole value compared with vehicle treatment [EAM + vehicle] on day 21. #P < 0.05 vs. normal + vehicle; *P < 0.05 vs. EAM + vehicle.
The IKK inhibitor reduced myocardial damage characterized by cell infiltration and fibrosis in EAM. Histopathologically, the heart of the EAM + vehicle group showed severe myocarditis lesions that were composed of extensive inflammatory cell infiltration and myocardial fibrosis on day 21. However, the cell infiltration area ratios in the EAM + IKKi(1–20) group (12.9 ± 3.6%; P < 0.05) and the EAM + IKKi(14–20) group (22.5 ± 2.9%; P < 0.05) were significantly fewer than those in the EAM + vehicle group (36.5 ± 3.4%) (Fig. 3, A–C). Similarly, the fibrosis area ratios in the EAM + IKKi(1–20) group (15.8 ± 4.0%; P < 0.05) and the EAM + IKKi(14–20) group (21.4 ± 0.1 s (horizontal).

CT (ΔΔC_T) method, and the mRNA expression was normalized by normal rat hearts (28, 32).

T-cell proliferation assay. Cells were isolated from spleen in the EAM + vehicle group on day 21. Cells (5 × 10^6/well) were cultured in 96-well culture plates with 50 μg/ml purified porcine heart myosin. The IKK inhibitor was added to each well at various concentrations. In vitro dosage of the IKK inhibitor was determined according to that in previous articles (34, 35). Cultures were incubated at 37°C under 5% CO_2 for 3 days. Cells were centrifuged at 1,200 rpm for 5 min, and the supernatants were stored at −80°C until before use. T-cell proliferation was assessed with the Cell Counting Kit-8 (Dojindo, Tokyo, Japan) with the use of cells of the precipitation. Cell proliferation was expressed as the optical density (9).

Enzyme-linked immunosorbent assay. We performed enzyme-linked immunosorbent assay (ELISA) to examine the production of Th1 cytokines from T cells using the supernatants of cell culture in vitro. Concentrations of IFN-γ and IL-2 in cell culture supernatant were determined with the Rat IL-2 quantikine ELISA kit (R & D Systems, Minneapolis, MN) and rat IFN-γ ELISA KIT (Gen-Probe, San Diego, CA) according to the manufacturer’s instructions.

Statistical analysis. All data are expressed as means ± SE. Statistical analyses were performed with statistical software (Stat View; SAS Institute). Student’s t-test was used to compare data between the two groups. Data differences between multiple groups were subjected to ANOVA followed by a Bonferroni-Dunn test. Differences were considered statistically significant at a value of P < 0.05.

RESULTS

Echocardiographic parameters. On day 21, the values of LV diameter at diastole, LV diameter at systole, and EF did not show any statistical difference among all groups. Regarding IVSd and LVPWd values, no significant difference was observed between the normal + vehicle group and the normal + IKKi(1–20) group. IVSd values significantly increased in the EAM + vehicle group compared with the normal + vehicle group. IVSd and LVPWd values in the EAM + IKKi(1–20) group were reduced compared with those of the EAM + vehicle group. Additionally, IVSd value was reduced in the EAM + IKKi(14–20) group compared with that of the EAM + vehicle group, although LVPWd value was not changed. There was no significant difference regarding IVSd and LVPWd values between the EAM + IKKi(14–20) group and the EAM + IKKi(1–20) group (Table 1 and Fig. 1).

The IKK inhibitor suppressed heart weight gain by cardiac inflammation. The normal + IKKi(1–20) group did not show any change in heart weight compared with the normal + vehicle group. The EAM + vehicle group hearts demonstrated an increase of the heart-to-body weight ratio compared with that of the normal + vehicle group. This ratio in the EAM + IKKi(1–20) group was significantly smaller than that of the EAM + vehicle group, whereas the EAM + IKKi(14–20) group was not statistically different compared with the EAM + vehicle group. There was no significant difference of this ratio between the EAM + IKKi(14–20) group and the EAM + IKKi(1–20) group (Fig. 2).

Fig. 2. Heart-to-body weight ratio. The experimental autoimmune myocarditis (EAM) + IKKi(1–20) group had a smaller the heart-to-body weight ratio than the EAM + vehicle group. The EAM + IKKi(14–20) group did not show the effect of the heart-to-body weight ratio compared with that of the EAM + vehicle group on day 21 [normal + vehicle, n = 5; normal + IKKi(1–20), n = 5; EAM + vehicle, n = 12; EAM + IKKi(1–20), n = 12; EAM + IKKi(14–20), n = 10]. *P < 0.05. NS, not significant.

Fig. 1. Representative M-mode echocardiograms on day 21. Arrows indicate anterior and posterior walls of left ventricle. Scale bars: 2 millimeter (vertical) and 0.1 s (horizontal).
Fig. 3. Histopathological findings. Representative low-power (magnification, ×10; scale bars = 1 millimeter; A) and high-power (magnification, ×400; scale bars = 100 μm; B) photomicrographs of hematoxylin and eosin-stained cross-sections are shown. C: quantitative data of cell infiltration area. IKK inhibitor treatment significantly suppressed cell infiltration into myocardium on day 21 [normal + vehicle, n = 5; normal + IKKi(1–20), n = 5; EAM + vehicle, n = 14; EAM + IKKi(1–20), n = 12; EAM + IKKi(14–20), n = 10]. *P < 0.05.

D: representative photomicrographs (magnification, ×200; scale bars = 100 μm) of Mallory-stained cross-sections. E: quantitative data of fibrosis area. IKK inhibitor treatment significantly suppressed cardiac fibrosis on day 21 [normal + vehicle, n = 5; normal + IKKi(1–20), n = 5; EAM + vehicle, n = 14; EAM + IKKi(1–20), n = 12; EAM + IKKi(14–20), n = 10]. *P < 0.05.
There was no significant difference regarding these histopathological changes between the EAM + IKKi(1–20) group and the EAM + IKKi(14–20) group. However, the EAM + vehicle group showed massive infiltrations of CD4-positive T cells and CD68-positive macrophages, and moderate infiltration of CD8-positive T cells on day 21. However, the EAM + IKKi(1–20) group showed significant reductions of CD4-, CD8-, and CD68-positive cell numbers compared with the EAM + vehicle group (Fig. 4, A–D).

**Localization of NF-κB activation in EAM.** To investigate NF-κB activity in EAM, we performed immunohistochemical analysis. On day 21, NF-κB p65 was expressed in the cytoplasm of cardiac tissues from the normal + vehicle group. In contrast, strong expression of NF-κB p65 was observed mainly in the nucleus of the infiltrating cells in the myocardium as shown by arrows in the hearts from the EAM + vehicle group (Fig. 5A). On this occasion, NF-κB p65 phosphorylation (as a marker of NF-κB activation) was detected in CD4-positive T cells that infiltrated the myocardium in the EAM + vehicle group (Fig. 5B). CD4-positive T cells showed massive infiltrations of CD-4 positive T cells and immune cells derived from the spleen migrate to the inflammatory affected area through the blood stream (37). Hence, we examined NF-κB activation in the spleen during the development of EAM. In the spleen obtained from the EAM + vehicle group, we observed enhanced colocalization of phospho-NF-κB p65 and CD4-positive T cells compared with that of the normal + vehicle group (Fig. 5C). NF-κB p65 was expressed in the cytoplasm (NF-κB protein expression was significantly reduced in the cytosol of cardiac tissues from the normal + vehicle group. However, nuclear NF-κB p65 protein expression was significantly reduced in the EAM + IKKi(1–20) group compared with the EAM + vehicle group on day 21 (Fig. 6, A and B). Phosphorylation level of NF-κB p65 (as shown by phospho-NF-κB-to-total NF-κB ratio) was reduced by IKK inhibitor administration (Fig. 6, C and D). Moreover, the level of cytosolic phospho-IκBα-to-IκB

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ratio in EAM hearts increased in the EAM + vehicle group compared with the normal + vehicle group. However, it decreased by IKK inhibitor administration (Fig. 6, E and F).

Altered inflammation-related gene expression by IKK inhibition in EAM hearts. Th1 cytokines and chemokines are known to promote myocardial inflammation during EAM (7, 11). Therefore, we examined the effect of IKK inhibitor treatment on mRNA expression of a chemokine and Th1 cytokines in hearts on day 21 using real-time RT-PCR. In the EAM + vehicle group, mRNA expressions of IFN-γ, IL-2, and MCP-1 were enhanced compared with the normal + vehicle group. However, mRNA expressions of these genes were significantly suppressed in the EAM +
IKKi(1–20) group compared with the EAM + vehicle group (Fig. 7, A–C).

Suppression of antigen-induced T-cell proliferation by IKK inhibitor treatment. Our present study revealed that NF-κB activation was observed in CD4-positive T cells in the heart and spleen during the development of EAM. Additionally, previous studies reported that EAM is transferable to other individuals via T cells derived from the spleen of EAM (21, 46). T cells activated by antigen mediate the development of EAM. Therefore, we performed a T-cell proliferation assay to examine the effect of IKK inhibitor on antigen-induced T-cell proliferation using splenocytes of EAM. As a result, T-cell proliferation was significantly increased by myosin restimulation. IKK inhibitor treatment suppressed myosin-induced T-cell proliferation in a dose-dependent manner (Fig. 8).

Effect of IKK inhibitor on the production of Th1 cytokines in vitro. It is known that Th1 cytokines released from CD4-positive T cells mediate the progression of EAM (33). Thus, to examine the effects of IKK inhibitor on the production of Th1 cytokines from T cells activated by antigen, we performed ELISA using supernatants collected from T-cell proliferation assays. In consequence, productions of IFN-γ and IL-2 were significantly increased by myosin restimulation. However, IKK inhibitor treatment suppressed the production of these Th1 cytokines in a dose-dependent manner (Fig. 9, A and B).

DISCUSSION

NF-κB is a key factor for the progression of inflammation (35, 47). Our previous articles showed that inhibition of NF-κB activation by the IKK inhibitor significantly suppressed pro-inflammatory mediators in myocardial ischemia model (34, 45). Inflammation is an essential pathological feature of acute myocarditis. Although the effectiveness of the IKK inhibitor on cardiac diseases associated with inflammation was shown, the effect of the IKK inhibitor on myocarditis remains unknown.

IKK inhibitor treatment did not change the value of EF compared with vehicle treatment on day 21. However, the EAM + IKKi(1–20) group exhibited an antihypertrophic effect, as shown by suppression of increases of LVPWd and IVSTd values. This effect was consistent with the result that IKK inhibitor treatment suppressed heart weight gain. The increased wall thickness is considered to be owing to interstitial edema associated with progression of myocardial inflammation. In contrast, the EAM + IKKi(14–20) group suppressed the increase of IVSTd value; however, it did not affect LVPWd value and heart weight gain. Earlier IKK inhibitor treatment may be more effective. The severity of histological myocardial damage characterized by cell infiltration and fibrosis was significantly reduced in both the EAM + IKKi(14–20) group and the EAM + IKKi(1–20) group compared with the EAM + vehicle group. Additionally, it should be noted that there was no significant difference regarding this effect between the EAM + IKKi(14–20) group and the EAM +
IKKi(1–20) group. We revealed that even when IKK inhibitor administration began from day 14, IKK inhibitor treatment suppressed myocardial damage and was not inferior to the early IKK inhibitor treatment group [EAM + IKKi(1–20)]. Thus, our results suggest that the treatment effect of IMD-0354 was due to the suppression of the inflammatory reaction that was caused around day 14. In a clinical setting, time lag occurs before patients are diagnosed with myocarditis and receive medical treatment. Therefore, IKK inhibitor treatment from day 14 to day 21 [EAM + IKKi(14–20)] was a trial to inspect utility as a therapeutic medicine of myocarditis. Meanwhile, there was no significant difference in echocardiographic parameters and histopathological findings between the normal + vehicle group and the normal + IKKi(1–20) group. These results suggest that the IKK inhibitor IMD-0354 does not affect normal hearts.

We observed massive infiltrations of CD4-positive T cells and CD68-positive macrophages, and moderate infiltration of CD8-positive T cells in the EAM + vehicle group hearts tissue. Infiltration cells into myocardium consist of mainly macrophages and CD4-positive T cells in EAM, and this result is consistent with those of previous studies (14, 22). These inflammatory cell infiltrations were suppressed by IKK inhibitor administration. NF-κB induces activation of inflammatory cells such as T cells and macrophages (2, 18). During the inflammatory phase of EAM, Th1 cytokines are enhanced and they mediate interaction between CD4-positive T cells and macrophages, which lead to cardiac damages (8, 14, 33). In particular, CD4-positive T cells proliferate oneself by releasing IL-2 and activate macrophages by releasing IFN-γ. Subsequently, activating macrophages damage myocardium (33). MCP-1 is known as a chemotactic factor and plays essential role in the inflammatory reaction involved in the progress of myocarditis by having an influence on the recruitment of inflammatory cells (11). Many inflammation-related genes including IFN-γ, IL-2, and MCP-1 are expressed by NF-κB (5, 43, 44), and its suppression prevents the development of EAM (11, 27, 36). Our in vivo studies revealed that mRNA expression of IFN-γ, IL-2, and MCP-1 were suppressed by IKK inhibitor treatment through inhibition of NF-κB activation in EAM heart tissues. Therefore, inhibition of NF-κB activation by IKK inhibitor treatment to suppress expression of inflammation-related genes may prevent harmful inflammatory cell infiltration in myocarditis.

In the process of EAM, CD4-positive T cells were activated by cardiac myosin and then recruited to the target organ (heart) (39). It is known that this cell play a particularly significant role in the pathophysiology of EAM. We demonstrated that NF-κB activation was observed mainly in CD4-positive T cells infiltrating the myocardium in EAM. A major source of inflammatory cells, including CD4-positive T cells, is the spleen (37). Therefore, we examined NF-κB activity in CD4-positive T cells of the spleen during EAM. We also detected NF-κB activation of CD4-positive T cells in spleens in EAM. A recent study reported that immune cells derived from the spleen reach the myocardium via the blood stream and induced inflammation-related cardiac disease (24). Moreover, some previous studies reported that splenocytes play a role of the pathogen and transferred myocarditis (21, 26, 46). Therefore, we investigated the direct effects of the IKK inhibitor on T cells, which were activated by antigen and derived from the spleen. Our in vitro study revealed that IKK inhibitor treatment suppressed myosin induced T-cell proliferation and production of Th1 cytokines. NF-κB activation is necessary for T-cell activation, differentiation, and proliferation (4). Activated T cells secrete various cytokines and chemokines via NF-κB activation, which activate other inflammatory cells or cardiomyocytes in the myocardium and cause further recruitment of inflammatory cells (34, 48). Antigen-induced T-cell proliferation and subsequent production of Th1 cytokines from T cells are an essential response for the development of EAM (8). This evidence demonstrates that the IKK inhibitor suppressed the activation of T cells and attenuated the development of EAM. Therefore, our results suggested that IMD-0354 attenuated the severity of EAM by targeting NF-κB of T cells during the inflammatory phase in EAM.

The present study showed for the first time that the IKK inhibitor IMD-0354 reduces the severity of EAM. This effect is associated with the reduction of inflammatory-related gene expression by inhibition of NF-κB activation. Some previous studies reported that administration of pharmacological agents indirectly inhibited NF-κB activation and suppressed the progression of EAM (1, 14, 40). Therefore, the IKK inhibitor IMD-0354, which directly inhibits NF-κB activation, may be promising as a therapeutic drug for the treatment of myocarditis. It is noteworthy that the IKK inhibitor will be clinically available in the near future. IMD-1041, which is a prodrug of IMD-0354, also inhibits IKK-β in vivo and in vitro (6).
Because this compound is an investigational drug, it is not yet on the market. To prove the clinical effect of IMD-1041, we started an interventional, randomized, placebo control and double blind clinical trial. Thus the IKK inhibitor will be used to treat myocarditis and other cardiovascular diseases in clinical settings in the future. Our data clearly indicate that IKK is critical for EAM development and its inhibition has significant effects for treating myocardial inflammation. In conclusion, IKK regulation is promising in the treatment of clinical acute myocarditis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: R.W., R.W.A., J.-i.S., and A.I. conception and design of research; R.W. and R.W.A. performed experiments; R.W. and R.W.A. analyzed data; R.W., R.W.A., J.-i.S., M.O., and M.I. interpreted results of experiments; R.W. prepared figures; R.W. drafted manuscript; R.W., J.-i.S., M.O., and M.I. edited and revised manuscript; R.W., J.-i.S., M.O., A.I., Y.H., I.K., and M.I. approved final version of manuscript.

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