IL-6-mediated Th17 differentiation through RORγt is essential for the initiation of experimental autoimmune myocarditis

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Received 22 March 2011; revised 30 April 2011; accepted 25 May 2011; online publish-ahead-of-print 27 May 2011

Time for primary review: 24 days

Aims
Interleukin (IL)-17-producing helper T (Th17) cells have been proposed to participate in the pathogenesis of chronic inflammation, such as autoimmune myocarditis. IL-6 gene ablation confers the resistance to experimental autoimmune myocarditis (EAM). In this study, we have addressed the pathological roles of IL-6 in the regulation of Th17 cells in EAM.

Methods and results
To induce EAM, mice were immunized twice with α-myosin heavy chain peptide. Three weeks after the first injection, the cardiac expression of the Th17-specific transcription factor, retinoic acid receptor-related orphan nuclear receptor (RORγt), was up-regulated. Consistently, Th17 cells were recruited into EAM hearts, as analysed by flow cytometry. Using the mice with enhanced green fluorescence protein (eGFP) gene knocked-in at RORγt locus (RORγt-eGFP mice), we observed Th17 cell infiltration into inflamed lesions. Pre-treatment with IL-6 receptor (IL-6R)-blocking antibody (anti-IL-6R Ab) inhibited EAM induction in terms of disease severity score (3.5 ± 0.8; IgG vs. 0.5 ± 0.8; anti-IL-6R Ab, n = 6, P < 0.01) and suppressed the myocardial expression of IL-17 and RORγt. In contrast, the administration of anti-IL-6R Ab 7 days after the first immunization failed to show the inhibitory effects, suggesting that IL-6 plays important roles in EAM initiation. Finally, by generating RORγt-eGFP homozygous mice, we revealed that RORγt gene ablation conferred the resistance to EAM induction.

Conclusion
IL-6-mediated induction of Th17 cells is critical for the onset of EAM, but not for its progression. IL-6/Th17 signalling could be a promising therapeutic target for the prevention of myocardial inflammation.

Keywords
Interleukin-6 • RORγt • Autoimmune myocarditis • Inflammation • Th17

1. Introduction
Myocarditis is one of the major causes of heart failure. Myocarditis is initiated by a wide range of stresses, such as microbial infection and cardiac toxins, leading to cardiac injury. Importantly, the autoimmune responses to myocardial components, which are induced by tissue damage, are believed to play important roles in the chronic progression of cardiac injury to heart failure. Experimental autoimmune myocarditis (EAM) is a rodent model of autoimmune responses after viral infection. The immunization of BALB/c mice with myosin heavy chain peptide leads to the infiltration of inflammatory cells into myocardium.¹ Recently, several lines of evidence have proposed that interleukin (IL-17)-producing helper T cells (Th17 cells) are involved in the pathogenesis of EAM,² because EAM severity

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is closely associated with the myocardial expression of IL-17 and the treatment of IL-17-blocking antibody attenuates the myocardial inflammation in EAM model.1 Moreover, EAM is worsened by the transplantation of Th17 cells that are purified from EAM hearts and amplified in vitro, proposing that Th17 cells are sufficient for the development of EAM. The differentiation of naïve T cells to Th17 cells is regulated by multiple mechanisms. Originally, it was reported that transforming growth factor-β and IL-6 co-ordinately induce Th17 differentiation4,5 through the induction of retinoic acid receptor-related orphan nuclear receptor (RORγt),6 a Th17 transcriptional factor, which is a downstream target of signal transducer and activator of transcription 3 (STAT3).7 IL-23 is also identified as an essential cytokine for Th17-mediated autoimmune diseases.8 Moreover, arylhydrocarbon receptor contributes to the Th17 differentiation through a cytokine-independent mechanism.9 Therefore, by making clear the molecular mechanisms that link between Th17 differentiation and autoimmune diseases, a novel therapeutic strategy would be provided.

Various kinds of cytokines positively or negatively regulate the development of EAM. Deletion of IL-6 or IL-23 gene demonstrated that these cytokines are essential for the pathogenesis of EAM.10,11 Granulocyte macrophage colony-stimulating factor promotes the expression of these cytokines, contributing to the progression of EAM.12 In contrast, interferon (IFN)-γ, a Th1 cytokine, is considered a negative regulator of EAM, because disruption of Th1 system by T-bet gene ablation enhances the severity of EAM.13 Thus, these findings indicate that cytokine network determines the progression or regression of EAM; however, spatiotemporal analyses on the significance of each cytokine have not been fully performed.

In this study, using IL-6 receptor (IL-6R)-blocking antibody, we examined the effects of IL-6 blockade on EAM, in light of Th17 cell differentiation and infiltration. Consequently, we have revealed that IL-6 is essential for the initiation of EAM, rather than its progression, through Th17 differentiation.

2. Methods

2.1 Animal experiments
Male BALB/c mice were obtained from Japan SLC and used at 6–8 weeks of age. All animal experiments were performed in compliance with the Osaka University animal care guideline. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All mice for the experiments were euthanized by inhalation of isoflurane in a euthanasia chamber. Death of the animals was confirmed by monitoring the absence of breath after removal of the carcasse from the euthanasia chamber.

2.2 Induction of EAM
EAM was induced as described previously.1,14 In brief, BALB/c mice were immunized with peptides derived from the sequence of the murine α-cardiac myosin heavy chain (Myhc-α, 164–634: Ac-SLKLMATLFSTYAS AD-OH). Myhc-α peptides were diluted in phosphate-buffered saline (PBS), and emulsified in complete Freund’s adjuvant with an equal volume. On Days 0 and 7, 100 μg in 200 μL of the emulsion was injected subcutaneously.

Anti-IL-6R mAb treatment was performed as described previously.15 One milligram of anti-IL-6R mAb (MR16-1, Chugai Pharmaceutical Co. Ltd, Tokyo, Japan) was intravenously injected into the immunized mice on Day 0 or 7. Purified rat IgG (Sigma) was used as a control. FTY720 (Cayman Chemical) was injected at the dose of 20 μg from Day 7 to 20 intra-peritoneally. The control group received PBS as a vehicle during the same period.

2.3 Histopathology
EAM severity was scored according to the previous report.3 Briefly, on Days 0, 7, 14, 21, 28 and 35, hearts were fixed in optimal cutting temperature compound. The frozen sections (5 μm thick) were prepared and stained with haematoxylin and eosin. EAM severity was scored by the researcher who was blinded to the assay condition, based on the percentage of the area of infiltrated myocardium as follows: 0 = no inflammatory infiltration, 1 = small foci of inflammatory cells between myocytes, 2 = large foci of >100 inflammatory cells, 3 = 10% of a cross section involved, and 4 = >30% of a cross section involved.

2.4 Quantitative RT–PCR
Total RNA was prepared from the hearts using the acid guanidinium thiocyanate-phenol-chloroform method.16 Total RNA (1 μg) was subjected for first strand cDNA synthesis by using the oligo (dT) first strand primer. After cDNA synthesis, the expression of RORγt, IL-6, IL-17, T-bet, IFN-γ, tumour necrosis factor (TNF)-α, IL-4, and GAPDH was estimated by real-time PCR using the SYBR Green kit (Applied Biosystems).

2.5 Immunofluorescent examination
Immunofluorescent microscopic analyses were performed as described previously.17 In brief, the frozen sections were prepared and fixed with 3.7% formaldehyde in PBS for 15 min and permeabilized with PBS containing 0.2% Triton X-100 for 3 min. The frozen sections were stained with FITC-conjugated anti-CD4 and anti-IL-17 antibodies. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) was used as secondary antibody. Nuclei were also stained with Hoechst 33258. The sections were examined with the fluorescent microscopy systems (Olympus, Japan).

2.6 Preparation of heart-infiltrating cells
Heart-infiltrating cells were isolated according to the previous reports18,19 with minor modification. Briefly, hearts, harvested from EAM mice, were perfused for 3 min with the perfusion buffer (120 mM NaCl, 5.4 mM KCl, 1.2 mM NaH2PO4, 20 mM NaHCO3, 5.6 mM glucose, 5 mM taurine, 1.6 mM MgCl2, and 10 mM 2,3-butanedione monoxime) at 37°C. The hearts were perfused for an additional 7 min with the perfusion buffer supplemented with 0.895 mg/mL collagenase type D (Roche) and 0.5 mg/mL protease type XIV (Sigma). Cells were filtered through 70 μm and 40 μm cell strainers, and suspended in PBS containing 3% foetal bovine serum (FBS). To separate CD3+ cells, cells were incubated with biotinylated anti-CD3+ antibody (BD Biosciences) for 15 min on ice and washed with IMag buffer (consisting of PBS with 0.5% bovine serum albumin and 2 mM EDTA) followed by incubation with streptavidin-conjugated particle for 30 min on ice. The labelled cells were resuspended in IMag buffer, and the CD3+ cells were separated from the cell suspension by using IMagnet (BD IMag Cell Separation System, BD Biosciences) according to the manufacturer’s protocol.

2.7 Cytokine measurement
Purified heart-infiltrating CD3+ cells (2 × 10⁶ cells/mL) were cultured in 24-well plate in RPMI 1640 medium with 10% FBS, penicillin/streptomycin, non-essential amino acids, 1 μM sodium pyruvate, 50 μM β-mercaptopoethanol, and 2 mM L-glutamine at 37°C and in 5% CO2. Heart-infiltrating CD3+ cells were stimulated with CD3 and CD28 (BD Biosciences) for 2 days. Cytokine levels of IL-17 were measured using ELISA kit (BioLedend).
2.8 Intracellular staining

T cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 750 ng/mL ionomycin for 4 h in the presence of 1 μg/mL GolgiPlug (BD Pharmergen). Cells were stained with the following fluorochrome-conjugated antibodies: APC-conjugated anti-CD4 and CD3 antibody, PE-labelled anti-IL-17 antibody, and FITC-labelled anti-IFN-γ antibody. For intracellular staining, the Cytofix/Cytoperp kit (BD Pharmergen) was used according to the manufacturer’s protocol. Flow cytometric analysis was performed with FACS Ariall and FACS Calibur (BD).

2.9 RORγt-enhanced green fluorescence protein transgenic mice

Rorc(γt)GFP+/− [RORγt-enhanced green fluorescence protein (eGFP)] mice, whose RORγt gene is ablated by knocking-in with eGFP cDNA, were purchased from the Jackson Laboratories. All mice were backcrossed to the BALB/c strain for more than six generations and were used at 6–8 weeks of age.

2.10 Immunoblot analyses

Cells were isolated from lymph node at the indicated time. After washed with ice-cold PBS twice, cell lysates were prepared by the addition of SDS–PAGE sample buffer and boiled for 5 min. Proteins were separated by SDS–PAGE and transferred onto polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 2% skim milk and incubated with anti-phospho-STAT3 antibody (Cell Signaling). The membranes were reprobed with anti-STAT3 antibody (Santa Cruz) to show equal amount of loading.

2.11 Statistical analysis

Comparisons between two groups were performed with the use of the Student t-test. The Mann–Whitney U test was used for the evaluation of severity scores. One-way ANOVA with the Tukey–Kramer or Bonferroni test was used for multiple comparisons. Data are presented as mean ± SEM. P-values of <0.05 were considered to be statistically significant.

3. Results

3.1 The infiltration of Th17 cells into inflamed myocardium is associated with disease severity of EAM

To address the dynamics of Th17 cells during EAM, we examined the expression of RORγt, a critical transcriptional factor for Th17 differentiation, in EAM hearts (Figure 1A and B). After induction of EAM, infiltrating inflammatory cells increased in number with the peak at Day 21 and started to decrease. Consistent with the EAM severity, the expression of RORγt and IL-17 mRNA was enhanced in a similar manner, suggesting that the infiltration of Th17 cells is correlated with EAM severity. Since previous studies have proposed that Th1 response is activated in EAM, we examined the expression of T-bet, Th1-related transcriptional factor, and found that T-bet was up-regulated in EAM hearts (Figure 1C). We also confirmed that IL-6 was induced in inflamed EAM hearts.

We characterized IL-17-producing cells that infiltrated into myocardium. The sections were prepared from EAM hearts and stained with anti-IL-17 antibody (Figure 2A). IL-17+ cells were detected in inflamed lesion of the hearts. To confirm that IL-17 was produced by T cells, CD3+ cells were prepared from EAM hearts and FACS analysis was performed (Figure 2B). CD3+IL-17+ cells were detected in heart-infiltrating cells and CD3+IL-17+ cell population was clearly distinct from CD3+IFN-γ+ cell population. Furthermore, we prepared T cell culture from control or EAM hearts and measured IL-17 production in vitro. In response to stimuli, CD3+ cells from EAM hearts produced IL-17, but not those from control mice. To address the involvement of Th17 cells in EAM, we generated EAM using heterozygous RORγt-eGFP knock-in (Rorc(γt)Lox+) mice, and revealed that GFP+ cells were primarily detected in lesion site in heart, indicating RORγt-expressing cells infiltrated into myocardium (Figure 2C). Finally, FACS analyses revealed that ~10% of CD4+ cells produce IL-17 (Figure 2D). These findings suggest that Th17 cells infiltrate into EAM hearts, and at least partially, contribute to IL-17 production.

3.2 STAT3 was activated in CD4+ cells in EAM hearts

RORγt is induced by STAT3 activation in Th17 cells. Therefore, we examined whether STAT3 was activated in CD4+ cells in EAM hearts (Figure 3). First, we performed immunohistological analyses to detect activated STAT3 using anti-p-STAT3 antibody in EAM hearts. Nuclear staining of p-STAT3 was detected not only in cardiomyocytes but also in infiltrating cells at Day 21 (Figure 3A). Next, to clarify whether STAT3 activation occurs in CD4+ cells of infiltrating cell population, CD4+ cells were prepared from lymph node by cell sorting and cell lysates were immunoblotted with anti-p-STAT3 antibody (Figure 3B and C). STAT3 activation was observed in CD4+ cells at Day 14, before the peak of the myocardial inflammation, while STAT3 phosphorylation was reduced to an undetectable level at Day 28 after the peak of inflammation.

3.3 The treatment with anti-IL-6R Ab prior to the immunization suppressed EAM

The previous study demonstrated that IL-6 gene ablation confers resistance to EAM.10 IL-6 was induced in EAM model, and STAT3—a downstream signal transducer of IL-6—was activated in CD4+ cells (Figures 1C and 3). Therefore, we investigated the effects of IL-6 signal blockade on EAM induction by administering IL-6R-blocking antibody MR16–1 at Day 0 prior to immunization (Figure 4). MR16-1 treatment dramatically reduced the infiltration of the inflammatory cells, while not control IgG (Figure 4A). Echocardiographical analysis has shown that the treatment of MR1-1 prevented EAM-induced cardiac dysfunction (see Supplementary material online, Table S1), accompanied by the reduced expression of brain natriuretic peptide (BNP) (see Supplementary material online, Figure S1). Consistently, the expression of RORγt and IL-17 was markedly suppressed in EAM hearts by MR16-1 (Figure 4B and C), suggesting that cardiac infiltration of Th17 cells was abrogated by MR16-1. Interestingly, the expression of T-bet and IFN-γ was not affected by MR16-1. Importantly, FACS analysis revealed that MR16-1 inhibit Th17 differentiation in lymph nodes (Figure 4D).

The data presented here indicate that MR16-1 suppressed EAM induction by inhibiting Th17 cell differentiation; however, it is not clear whether MR16-1 inhibits Th17 migration into myocardium. To address this issue, MR16-1 was administered at Day 7, when the inflammatory cell infiltration was initiated (Figure 1), and the disease severity was estimated (Figure 5). After EAM inflammation started, MR16-1 treatment failed to exhibit the suppressive effects on
disease severity. Importantly, FTY720, which is known to inhibit T cell migration, inhibited EAM induction, when FTY720 treatment started at Day 7 (Figure 5A and B). Consistently, the cardiac expression of RORγt was suppressed by FTY720, while not by MR16-1 treatment at Day 7 (Figure 5B). Thus, it is unlikely that IL-6 blockade prevents the infiltration of the inflammatory cells.

3.4 RORγt-deficient mice were resistant to EAM

RORγt is responsible for Th17 differentiation. Finally, to elucidate the causality between IL-6-mediated induction of Th17 differentiation and EAM, we generated RORγt-deficient mice using RORγt-eGFP knock-in

![Figure 1](image-url) The expression of RORγt mRNA was correlated with disease severity. (A) Invasive heart-infiltrating cells in cardiomyocytes were observed by HE staining on Day 21 after first immunization (bar: 20 μm), and severity score was estimated as described in Methods. (B) Severity score was estimated on indicated days. The expression of RORγt and IL-17 mRNA was measured on indicated days and normalized with that of GAPDH. The expression level was shown as a ratio to the average value of Day 0. (C) The expression level of T-bet, IFN-γ, or IL-6 mRNA in inflamed heart tissue was measured by quantitative RT–PCR on Day 21. The gene expression level was normalized with that of GAPDH and shown as a ratio to the average of the control group. Data are shown ± SEM. The number of mice was as follows: control, n = 4; EAM, n = 8. *P < 0.05 vs. control (Mann–Whitney U test or one-way ANOVA followed by Tukey–Kramer test).
Figure 2 IL-17-producing cell was significantly increased and RORγt+ cells were infiltrated to inflamed myocardium. (A) Immunofluorescence analysis of IL-17 (green) expression in EAM heart on Day 21. Hoechst 33258 (blue) staining was performed to identify nuclei (bar: 20 μm). (B) CD3+ cells were prepared from EAM heart using FACS (left panel). Cells were re-stimulated with PMA and ionomycin for 3 h. IL-17 production was measured in culture medium by ELISA (right panel). The number of mice was as follows: control, n = 7; EAM, n = 7. *P < 0.05 vs. control (Student’s t-test). Data are shown ± SEM. (C) Heart section were prepared from RORγt-eGFP (Rorc(gftgfp/+), gfp/+ or wild-type (+/-)) mice and stained with Hoechst 33258 (blue). (D) FACS analysis of IL-17- and IFN-γ-producing CD4+ cells from inflamed hearts on Day 21. Values represent the percentages of Th17 and Th1 cell. The experiments were performed three times with similar results.

Figure 3 STAT3 was activated in CD4+ cells in lymph node in the early phase of EAM induction. (A) The sections were prepared from EAM heart on Day 21 and co-stained with anti-p-STAT3 (green) and anti-sarcomeric α-actinin (red) antibodies. Hoechst 33258 (blue) staining was performed to show nuclei (bar: 20 μm). P-STAT3 positive cardiomyocytes (arrow) and heart-infiltrating cells (arrowhead) are shown. (B) CD4+ T cells from axillary lymph node were isolated using cell-sorting system on Days 14, 21, and 28 after the first immunization. Values represent the percentages of CD4+ cell in lymphocytes. (C) The cell lysates were prepared from CD4+ T cells and immunoblotted with anti-phospho-STAT3-specific antibody (p-STAT3). Blots were reprobed with anti-STAT3 antibody (total STAT3).
In RORγt-deficient mice (gfp/gfp), EAM severity was markedly reduced, compared with wild-type (+/+) and heterozygous (gfp/+ ) mice. There was no significant difference in the disease severity between wild-type (+/+) and heterozygous (gfp/+ ) mice. The expression of IL-17 was completely abolished in the hearts of RORγt-deficient mice, indicating that the major population of IL-17-producing cells in EAM is Th17 cells. These data indicated that the induction of Th17 cells through RORγt is essential for EAM.

4. Discussion

In this study, we have demonstrated that IL-6 plays important roles in Th17 induction, which is essential for the initiation of EAM. First, we have demonstrated that the expression of RORγt, Th17-specific transcriptional factor, is correlated with the disease severity in EAM hearts. Consistently, immunofluorescent analysis and FACS analysis showed that Th17 cells infiltrated into EAM hearts. Interestingly, STAT3—a downstream signalling molecule of IL-6—was phosphorylated in CD4^+ cells from EAM hearts, suggesting that STAT3 activation in CD4^+ cells contributes to the pathogenesis of EAM. Importantly, blockade of IL-6R, prior to immunization, prevented the development of EAM, accompanied by the reduced expression of RORγt in hearts, while IL-6R blockade—after the initiation of the inflammation—failed to exhibit the inhibitory effects on EAM and RORγt expression. Finally, RORγt-gene ablation conferred the resistance to EAM, indicating that IL-6-mediated induction of RORγt is essential for the initiation of EAM.
Previous studies have reported that Th17 cells are involved in the pathogenesis of EAM. Importantly, the transplantation of EAM heart-derived Th17 cells after in vitro expansion led to the onset of EAM, indicating that the enhancement of Th17 cell function is sufficient to promote the development of EAM; however, the requirement of Th17 cells in this disease substantially remained to be elucidated. Here, by generating RORγt-null mice, we have uncovered that Th17 cells are requisite for the development of EAM. Interestingly, Th17 cells were a small population of CD4+ cells; only ~10% of total heart-infiltrating CD4+ cells were positively stained with anti-IL-17 antibody. A recent study has shown that IL-17-blocking antibody attenuated the severity of EAM. In comparison with the treatment with IL-17A-blocking antibody (see Supplementary material online, Figure S1), RORγt-gene ablation exhibited more remarkable suppression of EAM, probably because Th17 cells produce not only IL-17A but also various kinds of pro-inflammatory cytokines, such as IL-17F, IL-23, and IL-6, and these cytokines might co-operatively promote the inflammation. Therefore, Th17 cells may hierarchically orchestrate inflammatory reactions in EAM. In this context, Th17 cells could be a therapeutic target of EAM.

The previous study reported that IL-6-gene ablation results in the resistance to EAM; however, how IL-6 is involved in EAM has not been revealed. In this study, we have demonstrated that IL-6 contributes mainly to the initiation of Th17 differentiation through the induction of RORγt. Blockade of IL-6R prior to immunization prevented EAM, accompanied by reduced expression of RORγt in the hearts, while IL-6 blockade failed to suppress EAM once the inflammation started. Moreover, Th17 differentiation in lymph nodes was abrogated in the mice treated with anti-IL-6R antibody before immunization. Thus, IL-6 is indispensable for the initiation of EAM, but dispensable for the progression of the inflammation in EAM model. Consistently, since cardiac expression of RORγt is not affected by IL-6R-blocking antibody once the inflammation was initiated, Th17 responses are maintained at least partially in an IL-6-independent pathway. In this context, Th17 cells could be a therapeutic target of EAM.
become manifest, while blockade of IL-6R after immunization even after the disease has failed to exhibit beneficial effects on the chronic inflammation of our study, we did not address the IL-6-independent pathway responsible for the maintenance of Th17 response; however, IL-23 could be a candidate cytokine because IL-23 is involved in the survival of Th17 cells.

Blockade of IL-6R is effective in other animal autoimmune disease models, such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE), which are induced by the injection of tissue-specific antigen. Interestingly, in EAE model, IL-6R blockade suppressed Th1 response as well as Th17 response, while neither IL-6R blockade nor RORγt-gene ablation affected Th1 in EAM. Therefore, the importance of Th1 in the pathogenesis of autoimmune diseases is likely to depend on the diseased tissues. Consistently, in T-bet-null mice, the development of EAE is suppressed, while that of EAM is exacerbated.

EAM is thought to be a rodent model of viral infection-induced myocarditis. In this study, we have shown that blockade of IL-6R suppressed the initiation of EAM, providing the clinical proposal that IL-6R blockade with tocilizumab (a clinically available IL-6R-blocking antibody) just after viral infection, would prevent the transition from acute to chronic myocarditis. Of note, although IL-6R blockade failed to exhibit beneficial effects on the chronic inflammation of our EAM model once Th17 response was initiated, there might be a difference in immune response between the murine model and human disease. Indeed, tocilizumab remarkably exhibits beneficial effects in the treatment of rheumatoid arthritis, even after the disease has become manifest, while blockade of IL-6R after immunization shows the limited effects in experimental autoimmune arthritis. Therefore, the chronic Th17-mediated inflammation might be more dependent on IL-6 in human disease than in murine model. Clinical trial for the application of tocilizumab to the patients with chronic myocarditis after viral infection would make clear this point.

In conclusion, we have demonstrated that IL-6 plays crucial roles in Th17 differentiation through the induction of RORγt, as a critical event for the initiation of EAM. IL-6R blockade is a promising therapeutic and/or preventive strategy against autoimmune myocarditis.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgement

We thank Yasuko Murao and Wakako Okamoto for her excellent secretary work.

Conflict of interest: T.K. has a patent for MR16-1.

Funding

This study was supported by Grant-in-Aid for Scientific Research (B) from Japan Society for the Promotion of Science, by the Osaka Foundation for Promotion of Clinical Immunology, and by Grant-in-Aid from Knowledge Cluster Initiative (2nd Stage) of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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