Immunoglobulin Treatment Ameliorates Myocardial Injury in Experimental Autoimmune Myocarditis Associated With Suppression of Reactive Oxygen Species

Chiharu Kishimoto, MD, PhD ; Masaomi Nimata, BM ; Taka-aki Okabe, BM ; Keisuke Shioji, MD, PhD

Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University

Running head: Immunoglobulin and ROS in myocarditis

Total word count: 4056

Conflict of interest: We have no conflicts to disclose.

Potential Reviewers: Dr. McNamara DM (mcnamaradm@upmc.edu)

Dr. Ravetch JV (ravetch@rockefeller.edu)

Dr. Yuan Z (zuyiyuan@mail.xjtu.edu.cn)

Address correspondence to:

Chiharu Kishimoto, MD, PhD

Department of Cardiovascular Medicine,

Graduate School of Medicine, Kyoto University

54 Kawaracho, Shogoin, Sakyo-ku,

Kyoto 606-8507, JAPAN

Tel: (81) 75-751- 3197

Fax: (81) 75-751- 4281

E-mail: kkishi@kuhp.kyoto-u.ac.jp

Abstract

Aims: We tested the hypothesis that immunoglobulin ameliorated experimental autoimmune myocarditis (EAM) in mice attributing to the suppression of reactive oxygen species (ROS)-mediated myocardial injury. **Methods:** We intraperitoneally administered intact type of human immunoglobulin (Ig) or $F(ab')_2$ fragments of human immunoglobulin, 1g/kg/day daily for 3 weeks, to male BALB/c mice with heart failure due to EAM. **Results:** The results showed that intact type of Ig, but not $F(ab')_2$ type, reduced the severity of myocarditis by comparing the heart weight/body weight and lung weight/body weight ratios, pericardial effusion score, macroscopic and microscopic scores. Tissue superoxide production was marked in untreated mice with EAM, which was suppressed by the treatment of immunoglobulins. The cytotoxic activities of lymphocytes in mice with EAM treated with Ig were reduced compared with untreated controls. The shift from Th_1 toward Th_2 cytokine balance was demonstrated by the treatment of immunoglobulins both in vitro and in vivo. **Conclusion:** ROS may be involved in the development of myocarditis. Intact Ig ameliorates myocardial damage in mice with myocarditis associated with suppression of ROS and cytotoxic activity of lymphocytes. suppressive effects for cytotoxic myocardial injury.

Introduction

In humans, acute myocarditis is a potentially lethal disease, and frequently precedes the development of dilated cardiomyopathy (DCM). Two mechanisms to explain how myocarditis develops into DCM have been proposed; one is a persistent viral infection, and the other is a progressive autoimmune myocardial injury [1]. The autoimmune giant cell myocarditis in rats mimics human fulminant myocarditis in the acute phase [2].

Recent reports indicated that immunoglobulin treatment suppressed the severity of acute myocarditis in clinical studies and experimental animals [3-5]. Moreover, we found that immunoglobulin therapy exhibited an anti-inflammatory action via inhibitory Fc receptor (Fc receptor II B) associated with the suppression of dendritic cells in an experimental autoimmune myocarditis (EAM) model [4,5]. However, the effects of immunoglobulin upon autoimmune heart failure are still unknown.

Recent studies have reported the significant participation of oxidative myocardial injury in the development of heart failure [6-8]. The purpose of the present study was to examine the effects of immunoglobulins on an EAM model, focusing on the inhibitory effects on oxidative myocardial injury.

Materials and Methods

Immunization

Autoimmune heart failure with EAM was induced by immunization with porcine cardiac myosin in 6-week-old male BALB/c mice as previously described [4-6]. Porcine cardiac myosin (Sigma) was injected subcutaneously in the foot pads with 0.1

ml of myosin (10 mg/ml) mixed with an equal volume of Freund's complete adjuvant (FCA) supplemented with Mycobacterium tuberculosis H37Ra (Difco) on days 1 and 8. Control mice were immunized with FCA alone.

Medication

Mice with EAM were divided into three groups; they were intraperitoneally treated with phosphate buffered saline (PBS) (n=15), intact immunoglobulin (Ig) (n=12) (Venoglobulin-IH, Welfide Corporation, Japan, a polyethylene glycol treated human immunoglobulin,1g/kg/day), and $F(ab')_2$ fragments (n=12) (Gamma-Venin, Aventis Corp,1g/kg/day) daily for 3 weeks. The dose was chosen because of the previous studies [4,5]. In addition, immunoglobulin antigenicity between different species (human and mouse) did not appear to be a problem, as described before [4,5]. In parallel with this protocol, additional control groups were age-matched unimmunized mice treated for 3 weeks with PBS (n=4), Ig (n=4), and $F(ab')_2$ fragments (n=4). All the mice were sacrificed at the end of the experiment, and the sera were processed for cytokine assay. The organs were weighed, and the ratio of organ weight-to-body weight was calculated. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1985). The protocol was approved by the Institutional Animal Research Committee of Kyoto University, and the details of procedures were approved by the Ethics Committee.

Histopathology

At sacrifice, macroscopic findings were graded on a scale of 0 to +4.

Microscopic findings for, myocardial necrosis, and cellular infiltration were graded on a scale of 0 to +4, as previously described [4,5,9]. After macroscopic examination, a part of the ventricles was embedded in optical cutting temperature (OCT) compound for immunohistochemistry; remnant tissues were processed for other studies.

Immunohistochemical study

We used an immunoperoxidase technique to perform immunohistochemistry for interleukin-1β (IL-1β) staining and cell surface markers, as previously described [4,5,9]. In brief, anti-macrophage (anti-Mφ, M3/84, 1:400, PharMingen), anti-CD4 (GK1.5, 1:50, PharMingen), and anti-CD8 (53-6.7, 1:50, PharMingen) antibodies were applied to acetone-fixed cryosections. After being washed, the sections were then exposed to second antibodies (horseradish peroxidase-conjugated antibodies), and the antibody was visualized with diaminobenzidine. Sections were counterstained. The positive-staining cells of heart tissue were counted blindly by two observers in six fields at × 400 magnification (within a 1-mm^2 grid). The total positive-staining cells of the six fields were recorded as the number of infiltrating cells in the lesions, and the percentages of positive cells were calculated, as previously described [4,5,9].

Western Blotting

The myocardial lysates underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were sequentially transferred to a membrane (Millipore) that was incubated with an anti-IL-1 β (Secrotec) antibody, then with a peroxidase-linked secondary antibody (Amersham). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Chemiluminescence was

detected and semiquantitatively analized using the NIH Image system[9].

In situ detection of superoxide production

To evaluate in situ superoxide production from hearts, unfixed frozen cross sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method [9-11]. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10-μm thick sections, and incubated with 10 μM DHE at 37°C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling.

For quantification of ethidium fluorescence from hearts, fluorescence (intensity × area) was measured using the high-power image monitor.

Electron spin resonance spectroscopy

The portions of excised heart were homogenized in cold PBS (0.1g heart/ml), and were then added to 0.05ml of 9.0ml of 5.5′-dimethyl-1-pyrroline-1-oxide (DMPO) (Labotec). The generation of hydroxyl radicals ($\cdot$ OH) was observed as DMPO-$\cdot$OH adduct on a JEL-FR30 spectrometer, as described previously [13,14]. Quantification of the DMPO signal intensity was performed by comparing the observed signal to a standard Mn^{2+} marker; the hydroxyl radical signal relative to the internal standard of manganese ion was calculated.

Cell culture

To culture popliteal lymph node (LN) cells, LN cells were harvested from mice at sacrifice, and single-cell suspensions were obtained by passing through a stainless steel mesh screen. Cells were suspended in RPMI-1640 supplemented with fetal calf serum (FCS), 1% sodium pyruvate, 1% nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol and penicillin-streptomycin mixture.

Cytotoxicity assays

LN cells from mice treated with or without immunoglobulin were used as effector cells. F-2 cells (murine endothelial cells) plated in 96-micro-well plates (2×10^4 / well) were labeled with sodium chromate at 1μci/well (^{51}Cr, Amersham International) for 1 hour. After labeled target cells had been washed with PBS three times, LN cells were incubated at an effector-target (E/T) ratio of 50: 1 and 100 : 1 for 4 hours. The supernatant was collected and the radioactivity of ^{51}Cr released into the supernatant was measured by a gamma counter. The percentage of cytotoxicity was calculated using the formula

$$\% \text{ cytotoxicity} = (E - S) / (M - S) \times 100$$

where E is the counts per minute (cpm) released in the presence of effector cells, S is the spontaneous cpm released from target cells incubated in the medium, and M is the maximal cpm released from target cells incubated with 2% Triton X-100 [15,16].

Cytokine assays

The serum levels of cytokines in mice with and without EAM were determined in vivo by enzyme-linked immunosorbent assay (ELISA).

In the in vitro study, U937 cells (macrophage-like cells bearing Fc receptors [4])

were stimulated with 10μg/ml lipopolysaccharide (LPS). Ig or F(ab′)$_2$ fragments

(0.6mg/ml) was added to the medium 30 min before LPS stimulation. Forty-eight

hours later, cytokines in the medium was assayed by ELISA using commercially

available kits (R&D System).

Statistics

All values were expressed as means±standard deviation (SD). One way analysis

of variance (ANOVA), following by Fisher protected least significant difference test,

was performed. A value of p<0.05 was considered statistically significant.

Results

Histopathology, organ weights in mice with EAM (Table 1)

There was no death during the study period in all the groups.

At sacrifice, the hearts showed severe and diffuse discolored myocarditis in mice

immunized with cardiac myosin. Extensive injuries to myocytes with inflammatory

changes and multinucleated giant cells associated with IL-1β e xpression were

observed (Figures 1, 2, 3). Treatment with Ig, but not with F(ab′)$_2$ fragments, reduced

the severity of the disease (Table 1), as assessed by measuring organ weight/body

weight, macroscopic and microscopic scores. The percentages of macrophages, CD4^{+},

and CD8^{+} T cells recruited into the lesions were significantly reduced by the treatment

of Ig and F(ab′)$_2$ fragments (Table 1). Myocardial IL-1 β expression (17kDa),

determined by immunohistochemistry and Western blotting, was upregulated in mice

with EAM, and was suppressed by the treatment with Ig, but not with F(ab′) fragments

(Figures 2, 3).

Reactive oxygen species (Table 1)

There was no significant difference in staining intensity with DHE among 3 unimmunized groups (data not shown). However, there was an increase in DHE staining in cardiac sections from untreated group. Namely, the brightness of DHE-stained hearts from untreated mice with EAM was stronger than that from immunoglobulins-treated mice with EAM (Table 1, Figure 2).

Electron spin resonance spectrometric analysis (Table 2)

The formation of hydroxyl radical was detected in the myocarditic heart homogenates and was decreased in the Ig- and $F(ab')_2$ fragments-treated myocarditic heart homogenates compared with the untreated ones. Thus, the hydroxyl radical signals relative to the internal standard of manganese ion in the Ig- and $F(ab')_2$ fragments-treated hearts were lower compared with those in the untreated ones.

Cytotoxic activity of lymphocytes (Table 2)

At E/T ratios of 50:1 and 100:1, the cytotoxic activities of lymphocytes from mice with EAM treated with Ig, but not with $F(ab')_2$ fragments, were significantly suppressed compared with those from mice with EAM treated with PBS.

Cytokine production (Tables 3 and 4)

Serum cytokines were elevated in mice with EAM. However, there was a decrease of cytokine level associated with a tendency of the shift from Th_1 toward Th_2 balance in mice with EAM treated with Ig and $F(ab')_2$ fragments (Table 3).

Cytokine production was markedly increased by LPS stimulation. Both the

immunoglobulin preparations suppressed LPS-induced increased cytokine production on U937 cells in a dose-dependent manner. However, Ig had more potent cytokine suppressive effects associated with the tendency favoring Th_2 response than $F(ab')_2$ fragments (Table 4).

Discussion

The present findings clearly demonstrated that Ig, but not $F(ab')_2$ fragments, sufficiently suppressed the development of EAM, and that the cardioprotection may be due to the suppression of both the production of ROS and cytotoxic activity of lymphocytes against the heart via Fc portion of the immunoglobulin. The analysis of behavior of cytokines showed that immunoglobulin preparations induced anti-inflammatory effects, and that although treatment with $F(ab')_2$ caused the in vitro anti-inflammatory effects to some extent, Ig induced a clear shift from Th_1 toward Th_2 cytokine balance.

Several studies have described the participation of oxidative stress in the pathogenesis of heart failure [6-8,17,18]. Recent reports indicated that myocardial injury was produced by reactive oxygen species (ROS), and that lipid peroxidation was caused by ROS, resulting in cell membrane damage in heart failure [19-21]. Also, recent studies have definitely established the key role of the free radicals in the pathogenesis of heart failure by demonstrating that agents that inhibit the generation of free radical confer cardiovascular benefit.

In the present study, it was demonstrated that the numbers of macrophages, $CD4^+$, and $CD8^+$ T cells, and IL-1β-positive inflammatory cells were markedly

reduced by immunoglobulins treatment. Decreased myocardial IL-1 β expression by Ig treatment was confirmed by Western blotting. Accordingly, it may be that the beneficial effects of immunoglobulins in EAM may be partly due to the suppression of inflammatory events in the myocardium. The molecular mechanisms of the agents in the suppression of inflammatory cytokines are not fully understood. However, the inhibition of signal transduction pathways, such as immunoreceptor tyrosine-based inhibitory motif via Fc receptor, which is particularly important in the regulation of cytokines by Fc portion of immunoglobulin, has been postulated [22-25].

Next, Ig and $F(ab')_2$ fragments scavenged the generation of hydroxyl radicals in heart homogenates ex vivo. It was demonstrated in the electron spin resonance spectroscopy study that immunoglobulins treatment protected against cellular protein oxidative damage in vivo. It was already demonstrated that free radicals, especially hydroxyl radicals, play an important role in the development of heart failure in this animal model [13,14]. Also, in the current study in DHE staining, superoxide production was markedly suppressed in Ig-treated group and moderately suppressed in $F(ab')_2$ fragments-treated group. Although the reason why Fab portion has the free radical scavenging action to some extent is unclear, the Fc portion of immunoglobulin may act for radical scavengers, resulting in less severe myocardial damage.

Another important role of immunoglobulin is an immunomodulation. Although whether immunoglobulin directly affects the host immune status or not is unknown, it was demonstrated in this study that the overall effects of the drug could induce the

suppression of EAM associated with the reduction of cytotoxic activity; i.e., Ig suppressed the cytotoxic myocardial injury in mice with EAM. Indeed, the cytotoxic activities of lymphocytes against F-2 cells in mice with EAM treated with immunoglobulins were reduced compared with untreated controls.

Immune dysfunction in autoimmune diseases is known to be caused by an imbalance between Th_1 and Th_2. Th_1 cytokines play critical roles in the differentiation of cardiac myosin-specific Th_1 cells and the pathogenesis of EAM [2,4,5]. Although the precise mechanisms of cytokine shift from Th_1 to Th_2 by the drug is unknown, immunoglobulin preparations, especially Ig, induced the potent Th_1 cytokine-suppressive effect, resulting in the favoring Th_2 cytokine response both in vitro and in vivo. Indeed, recent reports have indicated that treatment with Ig induced cytokine shift from Th_1 to Th_2 in patients with recurrent spontaneous abortion [26], and that pioglitazone, a peroxisome proliferator-activated receptor γ activator and clarithromycin, a major macrobide antibiotic, ameliorate EAM associated with the modulation of Th_1 / Th_2 cytokine balance [27-29]. Accordingly, the cytokine shift from Th_1 to Th_2 may be in part due to the results associated with the amelioration of the condition[30].

Oxidative stress and free radicals are known to impair normal cardiac function. Up to now there is increasing evidence for the generation of ROS reactive to the systemic and local inflammatory reactions in various diseases and for the significant role of free radicals upon the antigen presenting system [31,32]; inflammation itself and ROS may affect the function of antigen-presenting system. Recently, the

activating Fc receptor-coupled factor, Mincle, has been identified [33]. In addition, various tissues may generate ROS upon the exposure to Fc receptors-antigen complexes [31-33]. Accumulating evidence suggests the enhancement or modulation of antigen-presentating activity by ROS with and without Ig treatment [34-36]. The present study might have important implications for oxidative myocardial injury in relation to immune system.

In conclusion, Ig ameliorates EAM in mice. The cardioprotection of Ig is associated with the suppression of myocardial ROS production as well as the suppressive effects for cytotoxic myocardial injury concomitant with cytokine shift from Th_1 toward Th_2 balance.

References

[1]   Kawai C. From myocarditis to cardiomyopathy: mechanisms of inflammation and cell death. Circulation 1999; 99: 1091-1100.

[2]   Kodama M, Matsumoto Y, Fujiwara M, Masani F, Izumi T, Shibata A. A novel experimental model of giant cell myocarditis induced in rats by immunization with cardiac myosin fraction. Clin Immunol Immunopathol 1990; 57: 250-262.

[3]   McNamara DM, Holubkov R, Starling RC, Dec GW, Loh E, Torne-Amione G, et al. Controlled trial of intravenous immune globulin in recent-onset dilated cardiomyopathy. Circulation 2001; 103: 2254-2259.

[4]   Shioji K, Kishimoto C, Sasayama S. Fc receptor-mediated inhibitory effect of immunoglobulin therapy on autoimmune giant cell myocarditis: concomitant suppression of the expression of dendritic cells Circ Res. 2001; 89: 540-546.

[5]   Shioji K, Yuan Z, Kita T, Kishimoto C. Immunoglobulin treatment suppressed adoptively transferred autoimmune myocarditis in severe combined immunodeficient mice. Am J Physiol 2004; 287: H2619-2625.

[6]   Bergamini C, Cicoira M, Rossi A, Vassanelli C. Oxidative stress and hyperuricaemia: pathophysiology, clinical relevance, and therapeutic implications in chronic heart failure. Eur J Heart 2009; 5: 444-452.

[7]   Amir O, Paz H, Rogowski O, Barshai M, Sagiv M, Shnizer S, et al. Serum oxidative stress level correlates with clinical parameters in chronic systolic heart failure patients. Clin Cardiol 2009; 4: 199-203

[8]   Rogowski O, Shnizer S, Wolff R, Lewis BS, Amir O. Increased serum levels of oxidative stress are associated with hospital readmissions due to acute heart failure. Cardiology 2011; 1; 33-37.

[9] Yuan Z, Kishimoto C, Shioji K. Beneficial effects of low dose benidipine in acute autoimmune myocarditis: suppressive effects on inflammatory cytokines and inducible nitric oxide synthase. Circulation J 2003; 67: 545-550.

[10]   Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 1998; 82: 1298-1305.

[11] Takaya T, Kawashima S, Shinohara M, Yamashita T, Toh R, Sasaki N, et al. Angiotensin II type 1 receptor blocker telmisartan suppresses superoxide production and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice. Atherosclerosis 2006; 186: 402-410.

[12] Neilan TG, Blake SL, Ichinose F, Raher MJ, Buys ES, Jassel DS et al. Disruption of nitric oxide synthase 3 protects against the cardiac injury, dysfunction, and mortality induced by doxorubicin. Circulation 2007; 116: 506-514.

[13] Shioji K, Kishimoto C, Nakamura H, Masutani H, Yuan Z, Oka S, et al. Overexpression of thioredoxin-1 in transgenic mice attenuates adriamycin-induced cardiotoxicity. Circulation 2002; 106: 1403-1409.

[14] Nimata M, Okabe T, Hattori M, Yuan Z, Shioji K, Kishimoto C. MCI-186 (edaravone), a novel free radical scavenger, protects against acute autoimmune

myocarditis in rats. Am J Physiol 2005; 289: H 2514-2518.

[15] Kishimoto C, Takada H, Hiraoka Y, Shinohara H, Kitazawa M. Protection against murine coxsackievirus B3 myocarditis by T cell vaccination. J Moll Cell Cardiol 2000; 32:2269-2277.

[16] Kishimoto C, Hiraoka Y, Takamatsu N, Kamiya H, Ochiai H. An in vivo model of autoimmune post-coxsackievirus B3 myocarditis in severe combined immunodeficiency mouse. Cardiovasc Res 2003; 60: 397-403.

[17] Tsutsui H, Ide T, Hayashidani S, Suematsu N, Shiomi T, Wen J, et al. Enhanced generation of reactive oxygen species in the limb skeletal muscles from a murine infarct model of heart failure. Circul 2001; 104:134-136.

[18] Miyamoto M, Kishimoto C, Shioji K, Lee JD, Shimizu H, Ueda T, et al. Cutaneous arteriolar thioredoxin expression in patients with heart failure. Circul J 2003; 67: 116-118.

[19] Belch JJ, Bridges AB, Scott N, Chopra M. Oxygen free radicals and congestive heart failure. Br Heart J 1991; 65: 245-248.

[20] Dhalla AK, Hill MF, Singal PK. Role of oxidative stress in transition of hypertrophy to heart failure. J Am Coll Cardiol 1996; 28: 506-514.

[21] Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, et al. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium Circ Res. 2000; 86:152-157.

[22] Ravetch JV, Bolland S. IgG Fc receptor. Annu Rev Immunol 2001; 19:275-290.

[23] Park SY, Ueda S, Ohno H, Hamano Y, Tanaka M, Shiratori T, et al. Resistance of Fc receptor-deficient mice to fatal glomerulonephrits. J Clin Invest 1998; 102:1229-1238.

[24] Suzuki Y, Gomez-Guerrero C, Shirato I, López-Franco O, Gallego-Delgado J, Sanjuán G, Lazaro A, et al. Pre-existing glomerular immune complexes induce polymorphonuclear cell recruitment through an Fc receptor-dependent respiratory burst: potential role in the perpetuation of immune nephritis. J Immunol 2003; 170:3243-3253.

[25] Radeke HH, Janssen-Graalfs I, Sowa EN, Chouchakova N, Skokowa J, Löscher F, et al. Opposite regulation of type II and III receptors for immunoglobulin G in mouse glomerular mesangial cells and in the induction of anti-glomerular basement membrane (GBM) nephritis. J Biol Chem 2002; 277: 27535-27544.

[26] Yamada H, Morikawa M, Furuta I, Kat EH, Shimada K, Iwabuchi K et al. Intravenous immunoglobulin treatment in women with recurrent abortions; increased cytokine levels and reduced Th1/Th2 lymphocyte ratio in peripheral blood. Am J Reprod Immunol 2003, 2: 84-89.

[27] Yuan Z, Liu Y, Liu Y, Zhang J, Kishimoto C, Wang Y, et al. Peroxisome proliferation-activated receptor-gamma ligands ameliorate experimental autoimmune myocarditis. Cardiovasc Res 2003; 59: 685-694.

[28] Hasegawa H, Takano H, Zou Y, Qin Y, Hizukuri K, Odaka K, Toyozaki T, Komuro I. Pioglitazone, a peroxisome proliferator-activated receptor γ activator,

ameliorates experimental autoimmune myocarditis by modulating Th_1/Th_2 balance. J Moll Cell Cardiol 2005; 38: 257-265.

[29] Ogawa M, Suzauki J, Hishikari K, Takayama K, Tanaka H, Isobe M. Clarithromycin attenuates acute and chronic reuection via matrix metalloproteinase suppression in murine cardiac transplantation. J Am Coll Cardiol 2008; 51: 1977-1985.

[30] Anthony RM, Kobayashi T, Wermeling F, Ravetch J. Intravenouos gammaglobulin suppresses inflammation through a novel Th2 pathway. Nature 2011; 475:110-114.

[31] Liu J, Yoshida Y, Yamashita U. Suppressive effect of reactive oxygen species on CD 40-induced B cell activation. FEBS Lett 2007; 581: 5043-5049.

[32] Swindle EJ, Coleman JW, DeLeo FR, Metcalfe DD. Fc epsilon RI-and Fc gamma receptor-mediated production of reactive oxygen species by mast cells is lipoxygenase-and cyclooxygenase-dependent and NADPH oxidase-independent. J Immunol 2007; 179: 7059-7071.

[33] Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol 2008; 9: 1179-1188.

[34] Aukrust P, Müller F, Nordøy I, Haug CJ, Frøland SS. Modulation of lymphocyte and monocyte activity after intravenous immunoglobulin administration in vivo. Clin Exp Immunol 1997; 107: 50-56.

[35] Hehner SP, Breitkreutz R, Shubinsky G, Unsoeld H, Schulze-Osthoff K, Schmitz ML, Dröge W. Enhancement of T cell receptor signaling by a mild oxidative shift in the intracellular thiol pool. J Immunol 2000; 165: 4319-4328.

[36] Griffiths HR. Is the generation of neo-antigenic determinants by free radicals central to the development of autoimmune rheumatoid disease? Autoimmun Rev. 2008; 7: 544-549.

Figure legends

Figure 1. <u>Histopathology</u>

Diffuse myocardial necrosis and cellular infiltration with giant cells (arrowheads) were shown in the inflammatory lesions. Ig, but not $F(ab')_2$ type, markedly suppressed the severity of EAM (Insets are transverse sections of myocardium).

A=Normal mouse, B=Untreated mouse with EAM, C=Ig-treated mouse with EAM, D= $F(ab')_2$ fragments-treated mouse with EAM.

($\times$ 180)

Figure 2. <u>IL-1β staining, macrophage infiltration and DHE staining</u>

Intense IL-1β staining (brown areas) and positive macrophage infiltrations (arrows) were shown in the myocardium in untreated (B, inset $\times$ 380) and $F(ab')_2$ fragments-treated (D) mice with EAM, but not in Ig treated (C) mouse with EAM. In DHE staining, superoxide production was demonstrated by red fluorescence labeling. White arrows indicate the increased DHE stainings. Ig and $F(ab')_2$ fragments suppressed the expressions of DHE compared with the untreated mouse with EAM.

Each left panel=IL-1β staining, middle panel=macrophage staining, and right panel=DHE staining.

A=Normal mouse, B=Untreated mouse with EAM, C=Ig-treated mouse with EAM, D= $F(ab')_2$ fragments-treated mouse with EAM.

($\times$ 180)

Figure 3. <u>Myocardial IL-1 β expression</u>

(A) Western blot analysis of IL1 β (17kDa).

Representative Western blot analysis showed decreased expression of IL1 β in Ig-treated mouse (lane 2) compared with control mouse (lane 1). GAPDH=glyceraldehyde-3-phosphate dehydrogenase.

(B) Densitometric analysis of relative protein levels.

The   expression of IL1 β in Ig-treated mice (lane 2), but not in F(ab′)$_2$ fragments-treated mice (lane 3), was decreased compared with control mice(lane1).
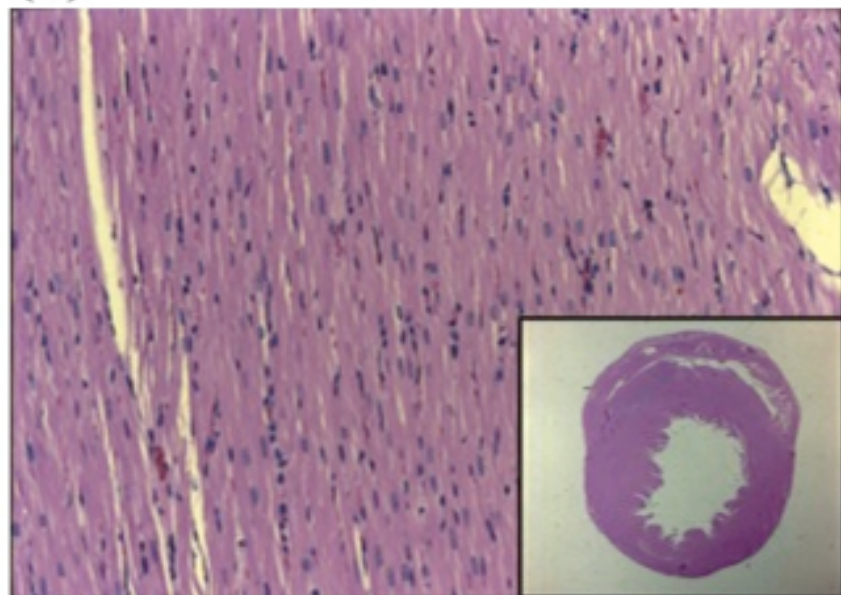
Values are derived from 5 animals and represent a ratio to GAPDH.

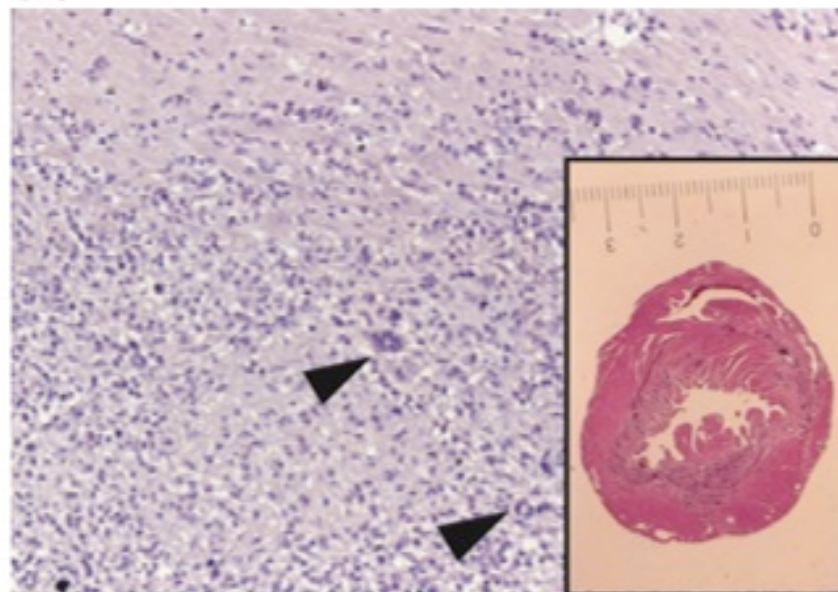lane1=untreated mouse with EAM, lane2= Ig-treated mouse with EAM, lane3= F(ab′)$_2$ fragments treated mouse with EAM. *P<0.01 vs lane 1.
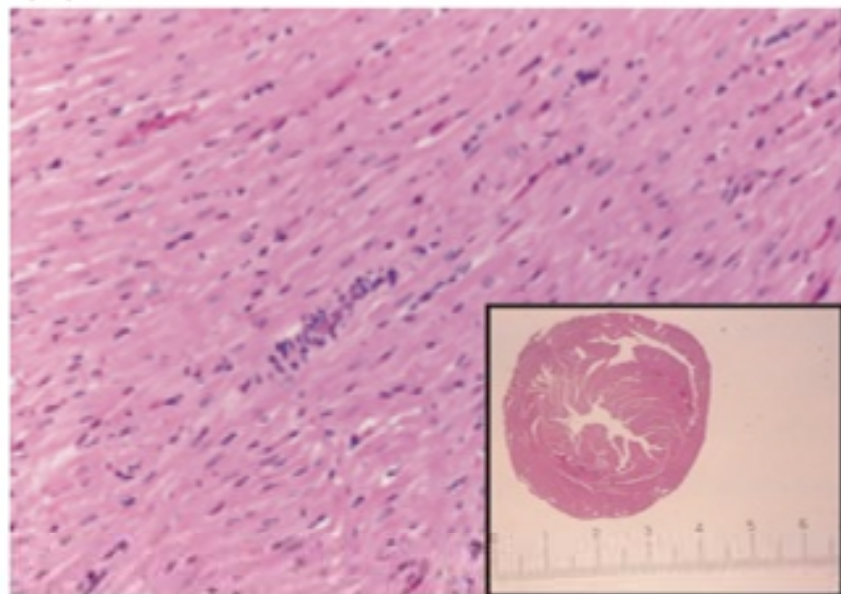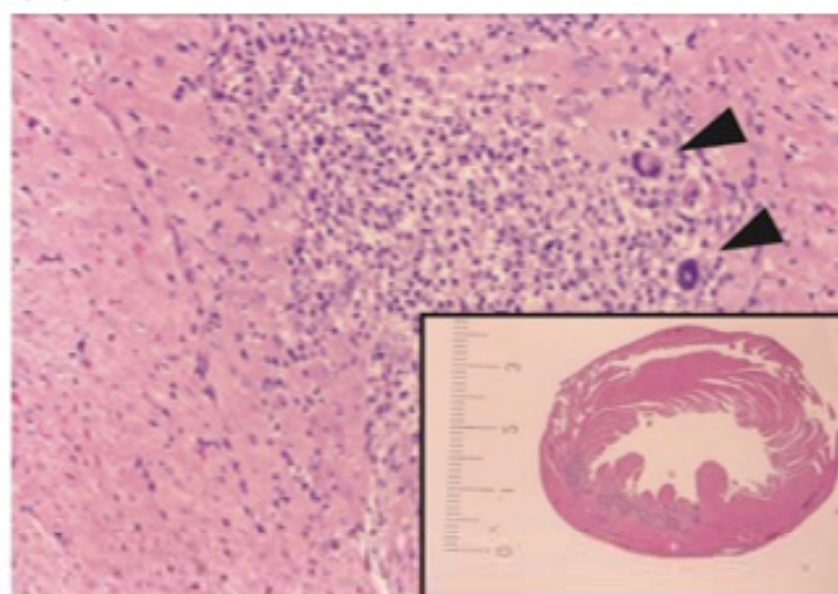
Figure 1

(A)

(B)

(C)

(D)

Figure 2

Figure 3

A

lane   1   2   3

⟵ IL-1β

⟵ GAPDH

B

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

*
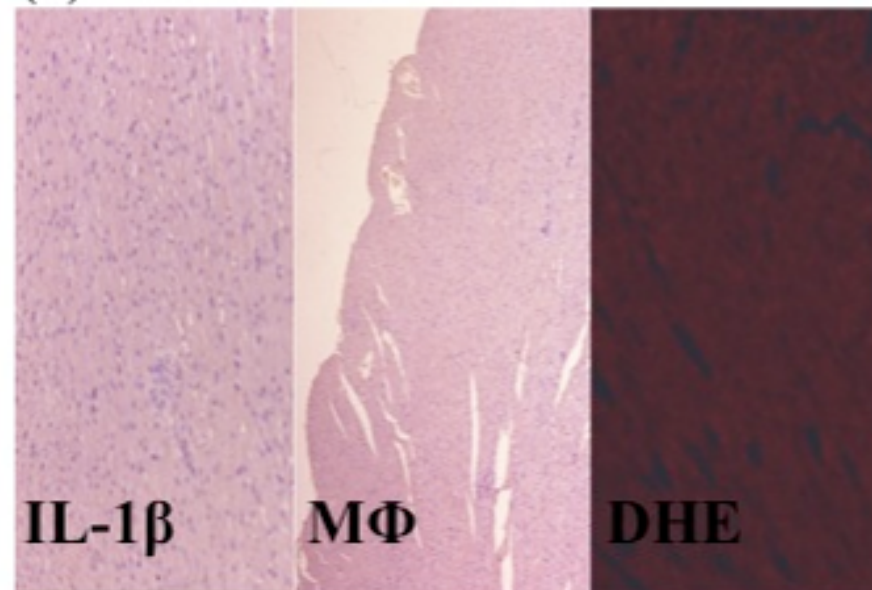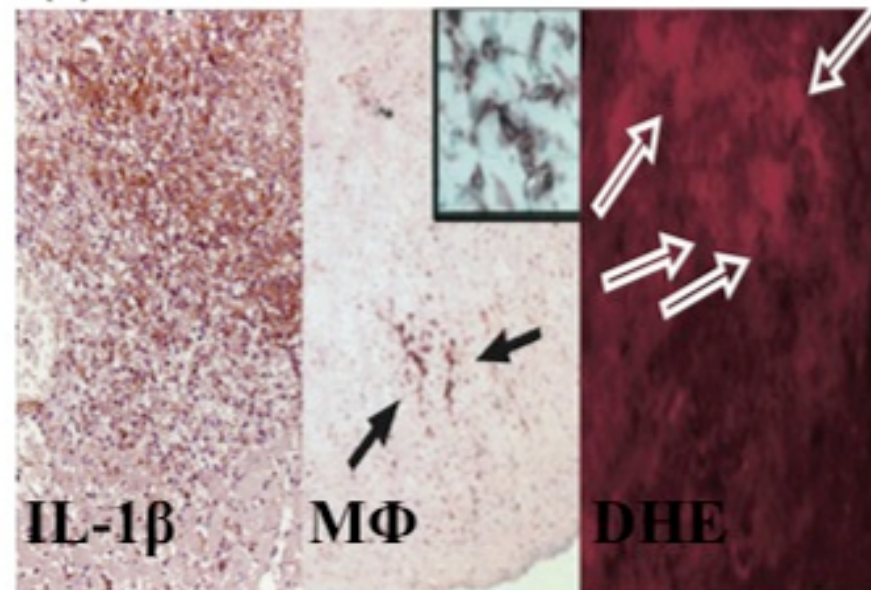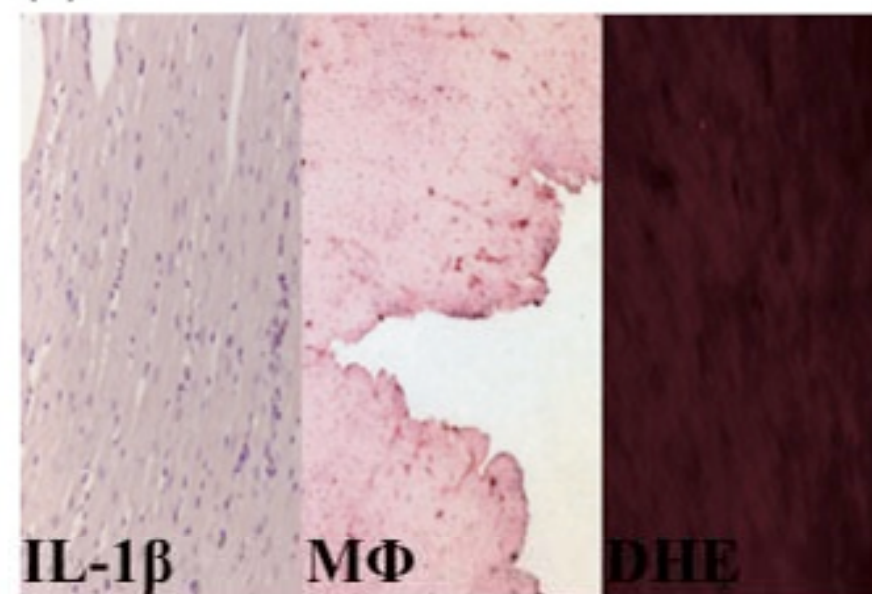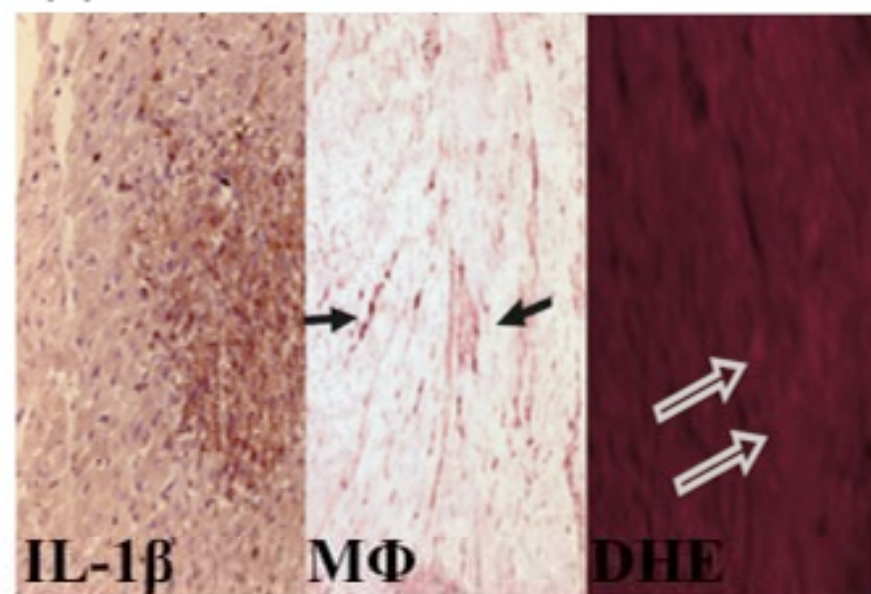
lane   1   2   3
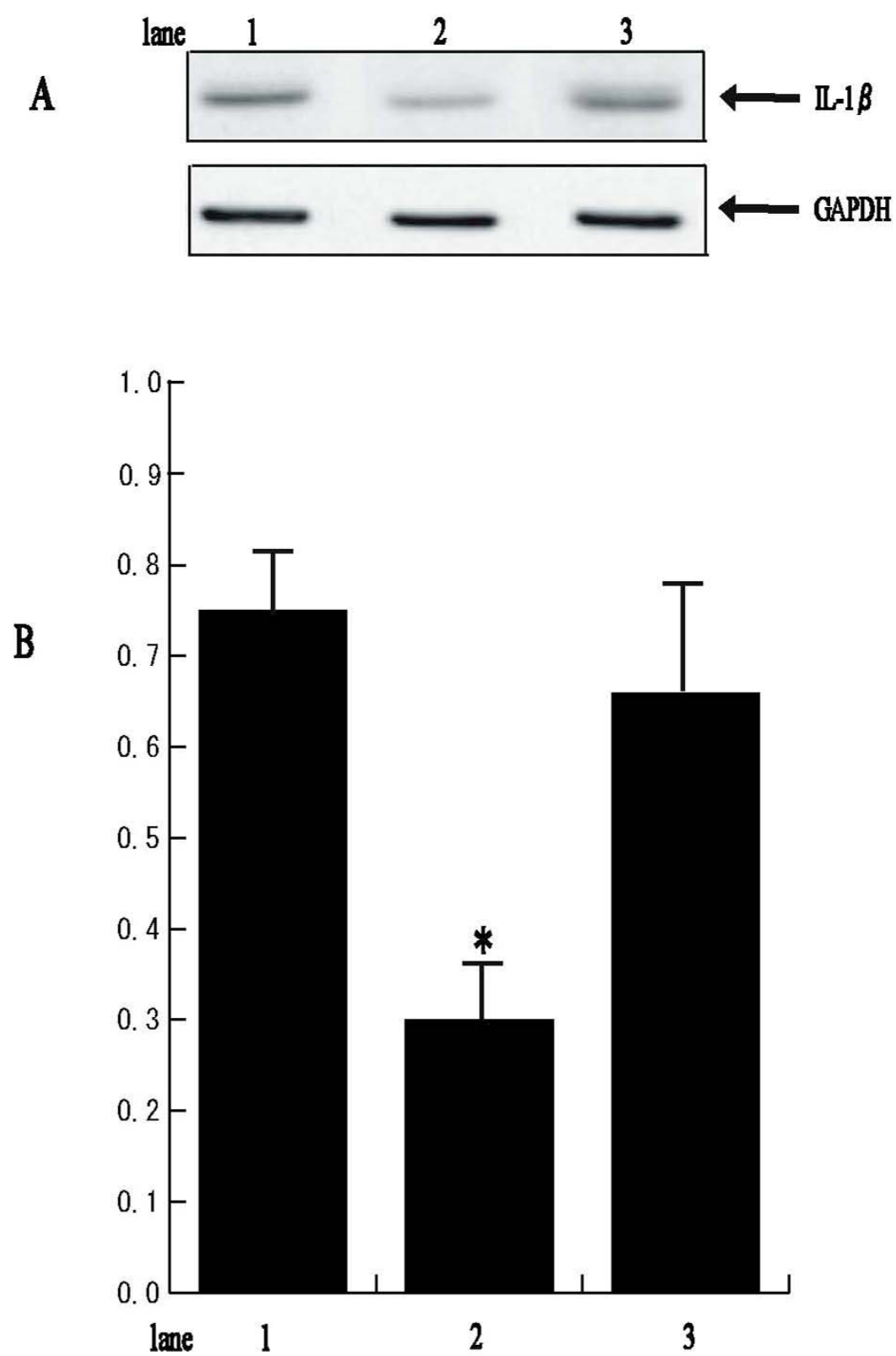
Table 1. <u>Histological analysis</u>

Immunization	Peritoneal Injection	n	HW/BW (mg/g)	LuW/BW (mg/g)	LiW/BW (mg/g)	Macroscopic Score	Pericardial Effusion Score	Microscopic Score (0~+4) Necrosis	Infiltration	Positive Cells (%) Mφ	CD4	CD8	Ethidium Fluorescence (units)
Myosin +FCA	PBS	15	10.9 ±1.8	9.1 ±1.5	59.5 ±7.3	1.6 ±0.6	1.4 ±0.9	2.5 ±0.7	2.8 ±0.4	25.3 ±3.6	16.5 ±5.6	8.4 ±4.3	1.00 ±0.08
Myosin +FCA	Ig	12	8.3 ±2.0*	7.8 ±1.3*	48.9 ±9.3	0.2 ±0.6*	0.2 ±0.6*	0.9 ±1.0*	0.9 ±1.0*	4.5 ±2.5**	3.5 ±3.0**	3.0 ±2.5**	0.52 ±0.07**
Myosin +FCA	F(ab′)₂	12	10.6 ±1.5	8.5 ±1.7	53.3 ±8.8	1.4 ±1.8	1.0 ±0.9	2.4 ±1.1	2.4 ±1.0	15.7 ±5.3*	10.7 ±4.9*	5.6 ±2.6*	0.78 ±0.18*
FCA alone	PBS	4	7.6 ±2.2	6.9 ±1.8	48.7 ±8.6	0	0	0	0	- - -	- - -	- - -	- - - - -
FCA alone	Ig	4	7.3 ±1.0	6.6 ±1.7	47.0 ±5.7	0	0	0	0	- - -	- - -	- - -	- - - - -
FCA alone	F(ab′)₂	4	7.0 ±1.5	6.7 ±1.6	47.7 ±6.0	0	0	0	0	- - -	- - -	- - -	- - - - -

*P<0.05, **P<0.01 vs PBS.   (mean±SD)

BW=body weight, F(ab′)₂ = F(ab′)₂ fragments of immunoglobulin, FCA=Freund's complete adjuvant, HW=heart weight, Ig=intact immunoglobulin, LiW=Liver weight, LuW=Lung weight, Mφ=macrophage, PBS=phosphate-buffered saline.

The positive-staining cells of heart itissue were counted blindly by two observers in six fields at x 400 magnification (within a 1-mm2 grid),and the total positive standing cells of the six fields were redorded as the number of infiltrating cells in the lesions, as previously described [4,5,9]

There was no significant difference in staining with DHE among 3 control groups (FCA alone).

Table 2. <u>Cytotoxic activity and ESR spectrometric analysis</u>

Immunization	Peritoneal Injection	Against F-2 cells (%)		Hydroxyl Radical Signal Relative to Internal Standard
		50:1	100:1(E/T)	
Myosin + FCA	PBS	11.2±3.3	14.2±3.6	0.96±0.07
		(n=5)		(n=6)
Myosin + FCA	Ig	8.4±4.0*	9.2±3.3*	0.72±0.09**
		(n=5)		(n=6)
Myosin + FCA	F(ab′)$_2$	10.5±5.7	10.7±4.1	0.82±0.05*
		(n=5)		(n=6)

*P<0.05 vs PBS.   (mean±SD)

E=effector, F(ab')$_2$=F(ab')$_2$ fragments of immunoglobulin, FCA=Freund's complete adjuvant, Ig=intact immunoglobulin, PBS=phosphate-buffered saline, T=target.

The cytotoxic activity in non-myocarditic groups (FCA alone) was very low （≤ 3.0%) at each E/T ratio. The formation of hydroxyl radical was not detected in non-myocarditic heart homogenates.

Table 3. Serum cytokine concentrations

Immunization	Peritoneal Injection	n	IL-1β (pg/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)
Myosin + FCA	PBS	7	252±52	9.4±2.6	<5.0
Myosin + FCA	Ig	7	114±26**	2.7±1.5**	18.3±6.7
Myosin + FCA	F(ab′)₂	7	176±45**	5.7±3.1*	10.4±5.3
FCA alone	PBS	4	<10.6	<0.1	<5.0
FCA alone	Ig	4	<10.6	<0.1	<5.0
FCA alone	F(ab′)₂	4	<10.6	<0.1	<5.0

*P<0.05, **P<0.01 vs Control (Myosin + FCA). (mean±SD)

F(ab′)₂ = F(ab′)₂ fragments of immunoglobulin, FCA=Freund's complete adjuvant, Ig=intact immunoglobulin, IFN-γ=interferon-γ, IL-4=interleukin 4, IL-1β=interleukin-1β, PBS=phosphate-buffered saline.

Table 4. Effects of immunoglobulin preparations on cytokine production in vitro

LPS (10μg/ml)	Conditions			Il-1β (pg/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)
	PBS	Ig	F(ab′)₂			
−	+	−	−	<10.6	<0.1	<5.0
+	+	−	−	175±22	52.2±7.5	<5.0
−	−	+	−	<10.6	<0.1	<5.0
+	−	+	−	35±17**	10.7±5.5**	23.5±5.0
−	−	−	+	<10.6	<0.1	5.0
+	−	−	+	56±15**	40.0±6.2*	12.2±3.8

Each value was derived from 4 to 5 independent trials. (mean±SD)

*P<0.05, ** P<0.01 vs the 2nd panel value [LPS (+), PBS (+), Ig (-), F(ab′)₂ (-)].

F(ab′)₂ = F(ab′)₂ fragments of immunoglobulin, FCA=Freund's complete adjuvant, Ig=intact immunoglobulin, IFN-γ=interferon-γ, IL-4=interleukin 4, IL-1β=interleukin-1β, LPS=lipopoilysaccharide, PBS=phosphate-buffered saline.