Immunoglobulin treatment ameliorates myocardial injury in experimental autoimmune myocarditis associated with suppression of reactive oxygen species.

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

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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 Th₁ toward Th₂ 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.

**Keywords:** myocarditis; reactive oxygen species; immune system; immunoglobulins
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-mm² 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 analyzed 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 (·OH) was observed as DMPO-·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 Mn²⁺ 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, $5 \times 10^{-5}$ 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 ($2 \times 10^4$ / 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

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

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 expression 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.
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 Th1 toward Th2 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 Th2 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 Th1 toward Th2 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-1β 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 Th1 and Th2. Th1 cytokines play critical roles in the differentiation of cardiac myosin-specific Th1 cells and the pathogenesis of EAM [2,4,5]. Although the precise mechanisms of cytokine shift from Th1 to Th2 by the drug is unknown, immunoglobulin preparations, especially Ig, induced the potent Th1 cytokine-suppressive effect, resulting in the favoring Th2 cytokine response both in vitro and in vivo. Indeed, recent reports have indicated that treatment with Ig induced cytokine shift from Th1 to Th2 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 Th1 / Th2 cytokine balance [27-29]. Accordingly, the cytokine shift from Th1 to Th2 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-presenting 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 Th1 toward Th2 balance.
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Figure legends

Figure 1. Histopathology

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.

(× 180)

Figure 2. IL-1β staining, macrophage infiltration and DHE staining

Intense IL-1β staining (brown areas) and positive macrophage infiltrations (arrows) were shown in the myocardium in untreated (B, inset × 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.

(× 180)
Figure 3. Myocardial IL-1β expression

(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

(A) IL-1β  MΦ  DHE

(B) IL-1β  MΦ  DHE

(C) IL-1β  MΦ  DHE

(D) IL-1β  MΦ  DHE
Figure 3

A

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<th>Immunization</th>
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<th>n</th>
<th>HW/BW (mg/g)</th>
<th>LuW BW (mg/g)</th>
<th>LiW BW (mg/g)</th>
<th>Macroscopic Score</th>
<th>Pericardial Effusion Score</th>
<th>Microscopic Score (0~+4)</th>
<th>Positive Cells (%)</th>
<th>Ethidium Fluorescence (units)</th>
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<td>+FCA</td>
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<td>±1.4</td>
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<td>±1.0</td>
<td>±5.3 * ±4.9 *</td>
<td>±2.6 *</td>
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*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 tissue 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 recorded 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. Cytotoxic activity and ESR spectrometric analysis

<table>
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<tr>
<th>Immunization</th>
<th>Peritoneal Injection</th>
<th>Against F-2 cells (%)</th>
<th>Hydroxyl Radical Signal Relative to Internal Standard</th>
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<td>50:1</td>
<td>100:1 (E/T)</td>
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<td>PBS</td>
<td>11.2±3.3</td>
<td>14.2±3.6</td>
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<td></td>
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<td>(n=5)</td>
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<tr>
<td>Myosin + FCA</td>
<td>Ig</td>
<td>8.4±4.0*</td>
<td>9.2±3.3*</td>
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<td>(n=5)</td>
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<tr>
<td>Myosin + FCA</td>
<td>F(ab’)2</td>
<td>10.5±5.7</td>
<td>10.7±4.1</td>
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<tr>
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<td></td>
<td>(n=5)</td>
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</table>

*P<0.05 vs PBS.

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

<table>
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<tr>
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<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
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<td>114±26**</td>
<td>2.7±1.5**</td>
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<td>Myosin + FCA</td>
<td>F(ab’)_2</td>
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<td>176±45**</td>
<td>5.7±3.1*</td>
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<td>FCA alone</td>
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<tr>
<td>FCA alone</td>
<td>F(ab’)_2</td>
<td>4</td>
<td>&lt;10.6</td>
<td>&lt;0.1</td>
<td>&lt;5.0</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>Conditions</th>
<th>LPS (10μg/ml)</th>
<th>PBS</th>
<th>Ig</th>
<th>F(ab’)_2</th>
<th>IL-1β (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>&lt;10.6</td>
<td>&lt;0.1</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>175±22</td>
<td>52.2±7.5</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>&lt;10.6</td>
<td>&lt;0.1</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>35±17**</td>
<td>10.7±5.5**</td>
<td>23.5±5.0</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>&lt;10.6</td>
<td>&lt;0.1</td>
<td>5.0</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>56±15**</td>
<td>40.0±6.2*</td>
<td>12.2±3.8</td>
</tr>
</tbody>
</table>

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.