weight ratio (0.41 ± 0.02% vs. 0.55 ± 0.03%, $P = 0.002$) in Group S_{14} were significantly lower than that in Group C_{14} (*Table 1*). The area ratio of myocarditis in microscopic grading was significantly lower in Group S_{14} than that in Group C_{14} (15.4 ± 4.2% vs. 37.2 ± 7.3%, $P = 0.036$) (*Figure 4A–G*).

Group S_{21} vs. Group C_{21}

Echocardiography revealed a significant difference in LVEF between Groups S_{21} and C_{21} (78 ± 2% vs. 67 ± 4%, $P = 0.049$) (*Figure 3B*). The heart weight/body weight ratio in Group S_{21} was significantly lower than that in Group C_{21} (0.39 ± 0.02% vs. 0.48 ± 0.02%, $P = 0.009$) (*Table 1*). The area of myocarditis was significantly smaller in Group S_{21} than that in Group C_{21} (16.0 ± 3.8% vs. 44.3 ± 4.4%, $P < 0.001$) (*Figure 4G*).

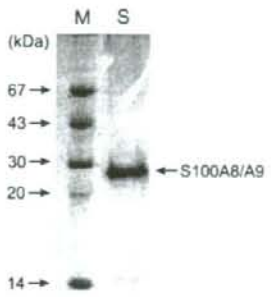

Figure 2 Purification of recombinant S100A8/A9. SDS–PAGE was performed as described in the Methods section. Lane S: purified recombinant S100A8/A9. As determined by densitometry, the purity of recombinant S100A8/A9 as a protein concentration was ~93%. Lane M: this lane contains molecular mass markers.

Effect of treatment with recombinant S100A8/A9 on mRNA expression of proinflammatory cytokines

Real-time RT–PCR analysis was performed to assess the expression of IL-1β, IL-6, and TNF-α. The mRNA expression of these proinflammatory cytokines in the myocardium was significantly depressed in Group S_{14} compared with Group C_{14}. However, between Groups S_{21} and C_{21}, there were no significant differences in the mRNA expression of IL-1β, IL-6, and TNF-α (*Figure 5A*).

Effect of treatment with recombinant S100A8/A9 on serum proinflammatory cytokine concentrations

The serum IL-1β and IL-6 concentrations in Group S_{14} markedly decreased compared with those in group C_{14} ($P = 0.008$ and $P = 0.019$, respectively). The serum TNF-α concentration in Group S_{14} tended to decrease compared with that in Group C_{14}. On Day 21, there was a significant difference only in the serum concentration of IL-1β ($P = 0.002$) between Groups S_{21} and C_{21} (*Figure 5B*).

Suppression of NF-κB expression in the heart of S100A8/A9-treated rats

To evaluate NF-κB activity, we immunohistochemically examined the expression of the nuclear p65 protein in the myocardium. This revealed that the expression of p65 was enhanced in Group

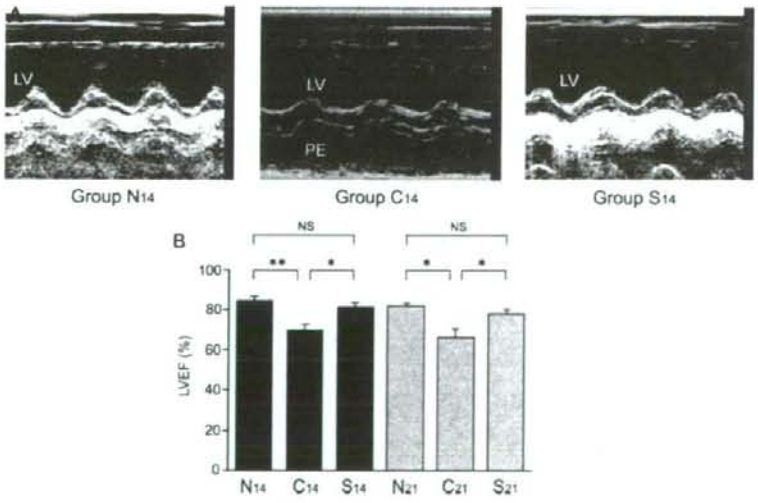

Figure 3 Echocardiographic findings. (*A*) Representative M-mode echocardiographic findings. Non-treated EAM rats (Group C_{14}) showed reduced left ventricular ejection fraction (LVEF) and pericardial effusion on Day 14. Recombinant S100A8/A9 administration from Days 8 to 13 (Group S_{14}) improved LVEF and suppressed pericardial effusion. (*B*) EF (%) in Groups N_{14}, C_{14}, S_{14}, N_{21}, C_{21}, and S_{21}. *$P < 0.05$; **$P < 0.01$.

Table 1 Effect of S100A8/A9 on heart weight, body weight, and heart/body weight ratio

	Day 14			Day 21		
	Normal control (Group N_{14}, $n = 5$)	EAM (Group C_{14}, $n = 10$)	EAM with S100A8/A9 (Group S_{14}, $n = 10$)	Normal control (Group N_{21}, $n = 5$)	EAM (Group C_{21}, $n = 10$)	EAM with S100A8/A9 (Group S_{21}, $n = 10$)
Heart weight (g)	0.89 ± 0.02	$1.21 \pm 0.06^{\#}$	$1.02 \pm 0.03^{*}$	0.97 ± 0.01	$1.16 \pm 0.04^{\#}$	$0.99 \pm 0.05^{*}$
Body weight (g)	279 ± 6	$221 \pm 5^{\#\#}$	$248 \pm 6^{\#,**}$	297 ± 4	$242 \pm 4^{\#\#}$	$256 \pm 6^{\#\#}$
Heart/body weight ratio (%)	0.32 ± 0.004	$0.55 \pm 0.03^{\#\#}$	$0.41 \pm 0.02^{**}$	0.33 ± 0.004	$0.48 \pm 0.02^{\#\#}$	$0.39 \pm 0.02^{**}$

$^{\#}P < 0.05$ (vs. Group N).
$^{\#\#}P < 0.01$ (vs. Group N).
$^{*}P < 0.05$ (vs. Group C).
$^{**}P < 0.01$ (vs. Group C).

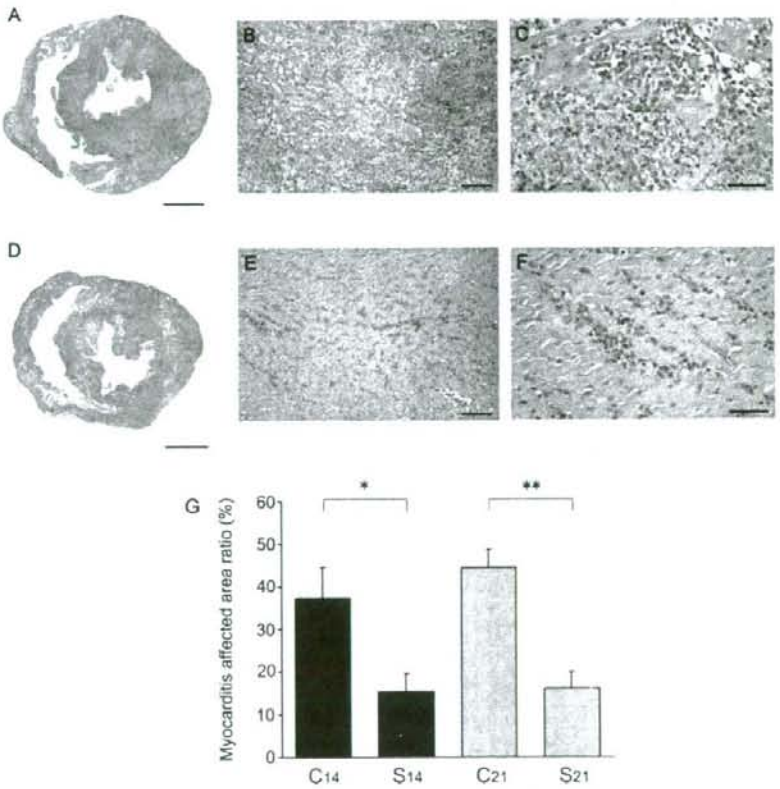

Figure 4 Representative cross-sections of heart. (*A*) Histopathological findings of specimen obtained from the midportion of the left ventricle of a vehicle rat (Group C_{14}). (*B* and *C*) Severe infiltration of relatively large mononuclear cells was observed in myocardium (Group C_{14}). (*D*) Cross-section of heart from a rat treated with recombinant S100A8/A9 (Group S_{14}). (*E* and *F*) Less severe infiltration of inflammatory cells is revealed (Group S_{14}). (*G*) Myocarditis-affected area ratio in the respective groups. Bar indicates 5 mm in (A and D), 100 μm in (B and E), and 50 μm in (C and F). $^{*}P < 0.05$; $^{**}P < 0.001$.

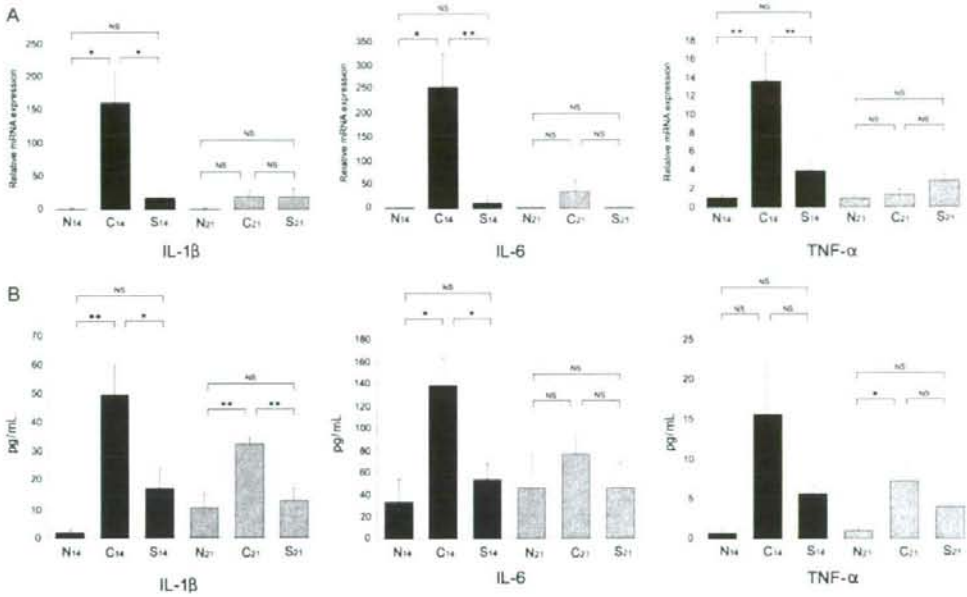

Figure 5 Effect of recombinant S100A8/A9 on proinflammatory cytokines: mRNA expression in EAM heart and serum concentrations. (*A*) Total RNA was extracted from the heart and real-time RT–PCR analysis was performed. Bar graphs show relative mRNA expression of IL-1β, IL-6, and TNF-α. $^{*}P < 0.05$, $^{**}P < 0.01$. (*B*) Serum IL-1β, IL-6, and TNF-α concentrations were determined using an ELISA method. The *Y*-axis represents the concentrations of each proinflammatory cytokine. $^{*}P < 0.05$; $^{**}P < 0.01$.

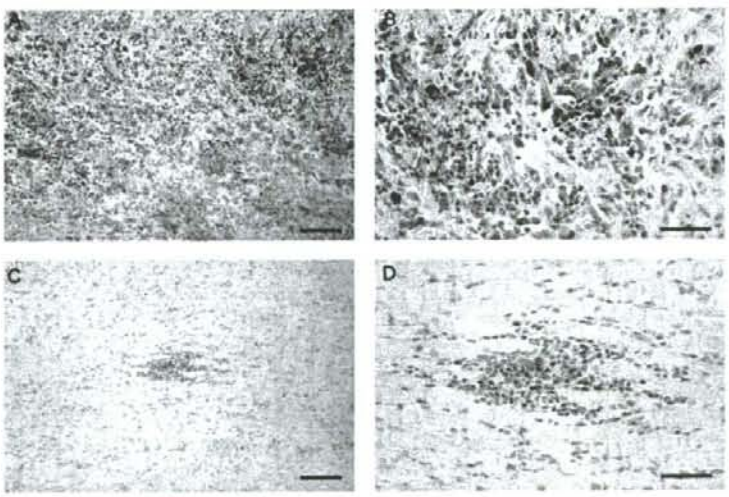

Figure 6 Representative results of immunohistochemical detection of NF-κB. Enhanced expression of NF-κB (p65) was observed in non-treated EAM heart (Group C_{14}) (*A* and *B*), while recombinant S100A8/A9 (Group S_{14}) suppressed expression (*C* and *D*). Bar indicates 100 μm in (A and C) and 50 μm in (B and D).

C_{14}, whereas in Group S_{14}, it was significantly suppressed (*Figure 6*). There was no detectable expression of p65 in naive rat hearts in Group N_{14}.

In vivo binding between S100A8/A9 and proinflammatory cytokines

To confirm the *in vivo* binding of S100A8/A9 with IL-1β, IL-6, and TNF-α, we used the ELISA test for S100A8/A9 to the supernatant of the homogenized heart tissue from EAM rats treated with recombinant S100A8/A9 (Group S_{14}). We demonstrated three kinds of S100A8/A9–proinflammatory cytokine complexes in heart tissue. As determined by ELISA, these complexes were quantitatively measured (*Figure 7*).

Discussion

We first examined the localization of endogenous S100A8/A9 in the inflamed myocardium of EAM rats. Fluorescent immunohistochemistry revealed that mononuclear cells both positive for CD68 and S100A8/A9 infiltrated into the myocardium to a great extent. This suggested that S100A8/A9 might play an important role in the pathogenesis of acute inflammation in the EAM model. However, it remains unclear how endogenous S100A8/A9 acts in this myocarditis model.

The pathogenic mechanism of the EAM model involves three sequential processes: (i) autoreactive macrophages and T lymphocytes are activated and expanded by a fragment of cardiac myosin; (ii) activated macrophages and T lymphocytes are recruited to the target organ; and (iii) effector–target interaction occurs.[14] Thus, inflammation of the EAM model consists mainly of macrophages and T lymphocytes. During the inflammatory phase, proinflammatory and Th1-type cytokines (e.g. IL-1β, IL-6, TNF-α, IFN-γ, and IL-2) are produced, which induce inflammation.[15,16] Pilot studies of the EAM model have shown that the expression of IL-1β, IL-6, and TNF-α in heart tissue had already increased on Day 14 when histological myocarditis did not reach the severest phase. Therefore, to verify our hypothesis that S100A8/A9 suppresses the inflammation of EAM by neutralizing the activity of proinflammatory cytokines, we intraperitoneally injected the recombinant S100A8/A9 into immunized EAM rats from Days 8 to 13 and sacrificed them on Day 14 or 21. On Day 14, data such as echocardiographic parameters, heart weight/body weight ratio, and histological assessment revealed that acute inflammation in recombinant S100A8/A9-treated EAM rats (Group S_{14}) was significantly suppressed compared with the vehicle group that had not received S100A8/A9 (Group C_{14}). Additionally, on Day 21, when histologically severe myocarditis was reported to be observed in EAM rats,[15] the area affected with myocarditis in the S100A8/A9-treated EAM rats (Group S_{21}) was significantly smaller than that in the vehicle group (Group C_{21}).

These data indicate that treatment with S100A8/A9 inhibits the development of acute inflammation in EAM, and are in agreement with the results of our previous study that intraperitoneal injection of S100A8/A9 suppresses liver injury induced by lipopolysaccharides in rats.[8] In the present study, the mRNA expression of IL-1β, IL-6, and TNF-α in the myocardium was dramatically

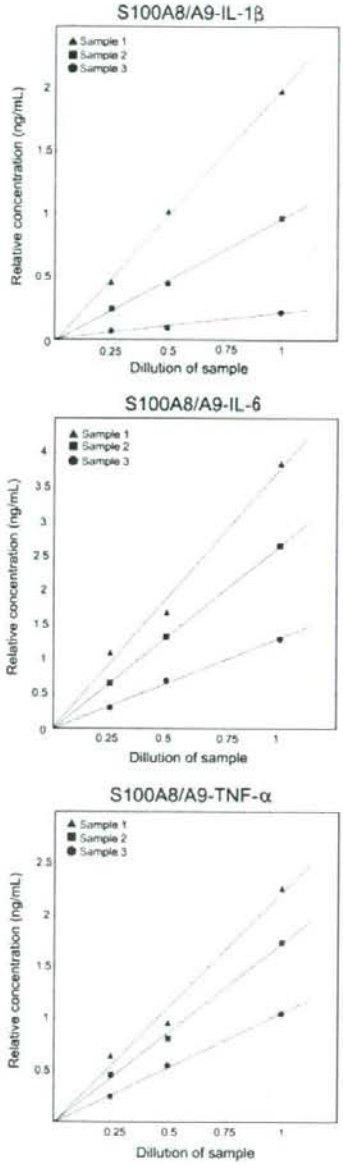

Figure 7 Evidence of binding of S100A8/A9 with proinflammatory cytokines. For detection of S100A8/A9–proinflammatory cytokine complexes, we used the ELISA plate coated with anti-S100A8/A9 monoclonal IgG (Mo2B9). Biotinylated anti-rat IL-1β, IL-6, and TNF-α IgGs were used as the second antibody. Finally, colour development was achieved by measuring horseradish peroxidase activity after incubation of streptavidin–horseradish peroxidase conjugate for 30 min. The reaction was significantly positive and quantitative, indicating the existence of S100A8/A9–cytokine complexes. This is a representative result of three samples.

suppressed in Group S_{14} compared with Group C_{14}, whereas there was no significant difference in the mRNA expression of these cytokines between Groups S_{21} and C_{21}. It has been reported that the mRNA expression of proinflammatory cytokines in the EAM model has only one peak during the inflammatory phase.[15] Thus, it may be said that mRNA levels of proinflammatory cytokines in our EAM model peaked around Day 14, and that recombinant S100A8/A9 ameliorated the peak expression of proinflammatory cytokines. On Day 21, there was no significant difference in mRNA levels of proinflammatory cytokines between Groups S_{21} and C_{21}, which may be due to the natural decrease in the mRNA expression of these cytokines in Group C_{21}.[15]

NF-κB is a rapid-response transcription factor that regulates the expression of the genes encoding cytokines, chemokines, and adhesion molecules. NF-κB exists in the cytoplasm as a heterodimer of 50-kDa (p50) and 65-kDa (p65) subunits associated with an inhibitory protein of the IκB family. When cells are stimulated, the IκB inhibitory protein is phosphorylated and dissociates from the NF-κB heterodimer, followed by translocation of free NF-κB into the nucleus.[17] In the cytokine network, in which the actions of certain cytokines are regulated by the activity of others, IL-1β and TNF-α both activate and are activated by NF-κB.[17–20] In addition, NF-κB-binding sequences have been found in the promoter regions of cytokine genes associated with inflammatory responses, including IL-6, TNF-α, IL-2, and the IL-2 receptor.[20,21] In our EAM model, immunohistochemical findings demonstrated that S100A8/A9 suppressed the activation of NF-κB as reflected by p65. Treatment with S100A8/A9 ameliorated the expression of proinflammatory cytokines and the activity of NF-κB; this suggests that S100A8/A9 has a suppressive function in the cytokine network.

In our previous study, affinity chromatography was performed using the purified S100A8/A9–Sepharose 4B column to confirm the binding of S100A8/A9 with proinflammatory cytokines.[8] A significant amount of IL-1β, IL-6, and TNF-α was eluted from the column, but anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β were not, indicating that S100A8/A9 binds with these proinflammatory cytokines *in vitro*.[8] However, the presence of the S100A8/A9–proinflammatory cytokine complexes was not documented *in vivo* in that study. Therefore, in the present study, effort was made to clarify the presence of these complexes *in vivo* using an antibody specific for S100A8/A9 in the ELISA system. It was established that in the acute phase of EAM, S100A8/A9 binds to at least three kinds of proinflammatory cytokines, IL-1β, IL-6, and TNF-α, *in vivo*. On the other hand, using anti-inflammatory cytokines IgGs as the second antibody, biotinylated anti-rat IL-4 and TGF-β IgGs, the reaction was not detected in the ELISA system (data not shown). It has been reported that extracellular S100A8/A9 interacts with binding sites on specific surface molecules, such as heparan sulfate, carboxylated glycans, and arachidonic acid.[22–24] Thus, S100A8/A9 may be a protein with multiple binding sites for many substances and may often bind to proinflammatory cytokines. The present study revealed that mRNA expression in the myocardium and also serum concentrations of proinflammatory cytokines were significantly decreased by the S100A8/A9 treatment. Possible causes of this effect may be the binding of the proinflammatory cytokines with S100A8/A9, as well as a decrease in cytokine production via the suppression of mRNA. Treatment with S100A8/A9 presents a new mechanistic approach for mitigating inflammation in the EAM model by trapping proinflammatory cytokines and altering the cytokine network.

Although the biological function of S100A8/A9 is yet to be described in detail, it has been proposed that S100A8/A9 has several functions, including antimicrobial activity, enhancement of transendothelial leucocyte migration, and induction of apoptosis.[25–27] While several studies to date have asserted that S100A8/A9 has a proinflammatory function,[28,29] few have revealed its anti-inflammatory function. The present study may shed some light on the novel anti-inflammatory function of S100A8/A9, which occurs by its binding to proinflammatory cytokines and modulating the cytokine network. Thus, treatment with S100A8/A9 is capable of neutralizing several kinds of proinflammatory cytokines, which may be unique because so-called anti-cytokine therapy generally targets a certain cytokine.

To summarize, we found that treatment with recombinant S100A8/A9 attenuated acute myocarditis in rats with EAM. At least three kinds of S100A8/A9 complexes with IL-1β, IL-6, and TNF-α were found in the inflamed organ tissues, which might have contributed to the reduction in acute inflammatory responses.

Study limitations

There are some limitations to the present study. First, the dosage of S100A8/A9 (1 mg/day) was chosen based on our previous study.[8] Myocarditis was not suppressed significantly when one-tenth of the dosage (0.1 mg/day) was given to EAM rats in the preliminary experiment. We did not attempt to use any other doses of S100A8/A9, which might have led to different results. Since only a single timing framework for S100A8/A9 administration was used in this study, the time dependency of the observed effects could not be confirmed. Further investigations on the efficacy of S100A8/A9 at different dosages and timings are therefore needed. Second, Th1/Th2 balance has been reported to play an important role in the pathogenesis of the inflammatory process in the EAM model.[13] As a counter-regulator of inflammatory cytokines, the suppressor of cytokine signalling (SOCS) family has also attracted attention.[30] However, we could not evaluate the involvement of Th1/Th2 balance and SOCS in the present study. Further studies on these factors are necessary.

Conflict of interest: none declared.

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