We then estimated the amount of Ca^{2+} leak under the selective downregulation of SERCA. The time course of Ca^{2+} leak from the SR did not differ between the SLN-TG and NTG hearts (Fig. 4D) ($n=29$ for SLN-TG, $n=28$ for NTG).

4. Discussion

The primary result of the present study is that the selective modulation of the SERCA activity can produce large changes in the Ca^{2+} transient without changing the amount of Ca^{2+} leak from the SR.

In the present study, we used saponin-skinned preparations to estimate the SR function, in which a separate estimation of Ca^{2+} uptake and Ca^{2+} leak can be made using the same preparation [5]. A previous report suggests that a large change in the SERCA activity produces a relatively small change in the Ca^{2+} content in the SR [3,15]. In this study, in the SERCA-TG mouse heart, the expression level of SERCA proteins was increased 1.68 ± 0.10-fold compared with that observed in the NTG hearts (see Supplementary Figure II), and the amplitude and time course of the Ca^{2+} transient was largely affected (see Fig. 1). Although the maximal Ca^{2+} content was unaltered in the SERCA-TG hearts, the Ca^{2+} content of the SR in each beat changed. The Ca^{2+} content of the SR during diastole is determined by the balance between Ca^{2+} uptake and Ca^{2+} leak in the SR. If changes in the SERCA activity directly modulate Ca^{2+} leak, SERCA-dependent changes in the Ca^{2+} content can be off-set by the altered Ca^{2+} leak. However, the lack of significant changes in Ca^{2+} leak in the SERCA-TG heart does not support this possibility. We also showed that a selective decrease in the SERCA activity can produce a large decrease in the amplitude and significant slowing of the time course of the Ca^{2+} transient in SLN-TG heart preparations. The result showing that the significant attenuation of Ca^{2+} uptake did not change the amount of Ca^{2+} leak from the SR in the SLN-TG hearts also suggests that changes in the Ca^{2+} content upon the selective modulation of the SERCA activity do not have a significant effect on Ca^{2+} leak. Under our experimental conditions in which the SERCA activity was chronically modulated, the maximal Ca^{2+} content of the SR was not affected, although the Ca^{2+} content observed under the beat to beat conditions was altered according to the results of Ca^{2+} transient measurement (see Figs. 1 and 3). The unaltered maximal Ca^{2+} content of the SR may explain why the selective modulation of SERCA did not influence Ca^{2+} leak in our system.

Impairment of the SERCA activity is well documented under patho-physiological conditions. Under conditions of heart failure, reduced Ca^{2+} uptake due to inhibition of the SERCA activity has been reported in both animal models and human failing hearts [16–20]. Therefore, the upregulation of the SERCA activity is a promising therapeutic strategy for treating heart failure, and recent observations of gene therapy for heart failure have strongly focused on the SERCA activity as a target of gene transfer in animal models as well as human heart failure [21–23]. However, the increase in the SERCA activity also serves to increase the beat to beat Ca^{2+} content in the SR, which can cause Ca^{2+} leak through the RyR [24]. Abnormal Ca^{2+} leak through the RyR has also been extensively investigated in various heart failure models. Marks's group originally identified the novel mechanism by which the protein kinase A (PKA)-dependent phosphorylation of the RyR dissociates FKBP12.6, which in turn leads to Ca^{2+} leak through the RyR [4]. The phosphorylation-dependent modulation of RyR has therefore been considered an important mechanism regulating Ca^{2+} leak, especially under pathological conditions such as heart failure and ventricular arrhythmia [25,26]. Three independent Ser residues in cardiac RyR are known to be phosphorylated by protein kinases. PKA mainly phosphorylates Ser2808 whereas Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) mainly phosphorylates Ser2814 [27]. Ser2030 is also a target of PKA-dependent phosphorylation in RyR, however, the importance of this residue is questionable [28]. The first report stated that the PKA-dependent hyperphosphorylation of RyR2 accelerated the dissociation of FKBP12.6 from RyR, which destabilized the Ca^{2+} release channel to increase Ca^{2+} leak [4]. This idea is fits well with the increased activity of the sympathetic nervous system in subjects with heart failure. However, other experimental data suggested that the CaMKII-dependent phosphorylation of RyR is important for pathological Ca^{2+} leak [7,29,30]. In addition, the identification of an exchange protein that was directly activated by cAMP (Epac) further supported the importance of CaMKII-dependent phosphorylation through beta-adrenergic receptor stimulation in the heart [31–33]. Very recently, it has been reported that both CaMKII and PKA functionally regulate RyR but have differential roles in human cardiac pathology [34]. Therefore, abnormal Ca^{2+} leak through the RyR may play a significant role in heart failure and may be another promising target of treatment. Our results demonstrating the absence of changes in Ca^{2+} leak upon the selective modulation of the SERCA activity support the utility of gene therapy targeting the SERCA activity. Recent observations in animal models and the findings of a clinical trial support the favorable effects of SERCA-mediated gene therapy [35–37].

Recently, another target of gene therapy for Ca^{2+} handling has been proposed. S100A1 is an EF-hand Ca^{2+} binding protein that exhibits a conformational change upon binding to Ca^{2+} and interacts with various functional proteins [38]. In cardiac muscle, S100A1 has been found to modulate the intracellular Ca^{2+} handling process. For example, S100A1 interacts with SERCA to increase the SERCA activity and with RyR to inhibit Ca^{2+} leak from the SR [39]. S100A1 also modulates the myofilament sensitivity to Ca^{2+}, which can alter cardiac contractility. Therefore, S100A1 may be the ideal therapeutic target for treating heart failure due to both the activation of SERCA and the inhibition of Ca^{2+} leak. However, elucidating the role of S100A1 in gene therapy for heart failure must wait for the results of pre-clinical and clinical trials [40].

5. Limitations of this study

(1) Although we determined the protein expression levels of various functional modulators in cardiac excitation–contraction coupling, we did not assess the phosphorylation levels of proteins such as phospholamban and RyR. (2) Our measurements of the SR function were obtained under *in vitro* conditions, and the results must be extrapolated into the heart *in vivo*. (3) The SERCA-TG and SLN-TG models are not disease models (the mice did not exhibit heart failure). (4) Both transgenic mice demonstrated cardiac-specific overexpression, but not conditional overexpression. (5) Because we used a high concentration of EGTA (1 mmol/L) in the experimental solution, which might inhibit SERCA activity, the speed of the Ca^{2+} uptake by SERCA under our experimental conditions is much lower than that observed under physiological conditions *in situ*. (6) Because we could not evaluate the detailed effects of ATP (4 mmol/L) on RyR2 due to the nature of our experimental system used for the $Ca^{2\pm}$ leak assay, we might have underestimated the amount of $Ca^{2\pm}$ leak under our experimental conditions at (physiological) nmol/L levels of cytosolic $Ca^{2\pm}$ [41,42].

6. Conclusion

In the present study, we found the selective modulation of the SERCA activity to have a profound effect on cardiac excitation–contraction coupling due to alteration of Ca^{2+} uptake into the SR, without significant changes in Ca^{2+} leak from the SR.

These results support the utility of gene therapy targeting SERCA and related molecules.

Conflict of interest statement

The authors declare that there are no conflicts of interest associated with this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2013.10.005.

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Suppressor of Cytokine Signaling 1 DNA Administration Inhibits Inflammatory and Pathogenic Responses in Autoimmune Myocarditis

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Suppressor of Cytokine Signaling 1 DNA Administration Inhibits Inflammatory and Pathogenic Responses in Autoimmune Myocarditis

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Myocarditis and subsequent dilated cardiomyopathy are major causes of heart failure in young adults. Myocarditis in humans is highly heterogeneous in etiology. Recent studies have indicated that a subgroup of myocarditis patients may benefit from immune-targeted therapies, because autoimmunity plays an important role in myocarditis as well as contributing to the progression to cardiomyopathy and heart failure. Suppressor of cytokine signaling (SOCS) 1 plays a key role in the negative regulation of both TLR- and cytokine receptor-mediated signaling, which is involved in innate immunity and subsequent adaptive immunity. In this study, we investigated the therapeutic effect of SOCS1 DNA administration on experimental autoimmune myocarditis (EAM) in mice. EAM was induced by s.c. immunization with cardiac-specific peptides derived from α myosin H chain in BALB/c mice. In contrast to control myocarditis mice, SOCS1 DNA-injected mice were protected from development of EAM and heart failure. SOCS1 DNA administration was effective for reducing the activation of autoreactive CD4$^+$ T cells by inhibition of the function of Ag-presenting dendritic cells. Our findings suggest that SOCS1 DNA administration has considerable therapeutic potential in individuals with autoimmune myocarditis and dilated cardiomyopathy. ***The Journal of Immunology*****, 2012, 189: 2043–2053.**

Dilated cardiomyopathy (DCM) is a potentially lethal disorder of various etiologies for which no treatment is currently satisfactory (1); it often results from enteroviral myocarditis (2, 3). Many patients show heart-specific autoantibodies (3, 4), and immunosuppressive therapy can improve cardiac function in DCM patients who show no evidence of viral or bacterial genomes in heart biopsy samples (5). These observations suggest that autoimmunity plays an important role in myocarditis as well as contributing to the progression to cardiomyopathy and heart failure (6).

Experimental autoimmune myocarditis (EAM) is a model of postinfectious myocarditis and cardiomyopathy (7). A number of proinflammatory cytokines, including IL-1β, IL-6, IL-12, TNF-α, and GM-CSF, have been shown to contribute to the development of autoimmune myocarditis in animal models and human cases (8–13). EAM is a CD4$^+$ T cell-mediated disease (7, 14), and activation of self-Ag–loaded dendritic cells (DCs) is critical for expansion of autoreactive CD4$^+$ T cells. Activation of TLRs and IL-1 type 1 receptor and their common downstream signaling adaptor molecule, MyD88, in self-Ag–presenting DCs is also critical for the development of EAM (11, 15, 16). Compared with inhibition of a single cytokine, a more effective treatment might be inhibition of various signaling pathways to induce production of cytokines through both innate and adaptive immunity. One strategy that could accomplish this would be to target shared cytokine and TLR signal transduction pathways using suppressor of cytokine signaling (SOCS) molecules.

Recent lines of evidence indicate that SOCS proteins, originally identified as negative-feedback regulators in cytokine signaling, are involved in the regulation of TLR-mediated immune responses (17, 18). The SOCS family is composed of eight members: cytokine-inducible Src homology 2 domain-containing protein and SOCS1 to SOCS7 (19, 20). SOCS1 plays a key role in the negative regulation of both TLR-mediated signaling and cytokine receptor-mediated signaling, which are involved in innate immunity and subsequent adaptive immunity (21). The expression of SOCS1 is induced by various cytokines, including IFN-γ, IL-4, and IL-6, and also by TLR ligands, such as LPS and CpG-DNA (22). Several studies have demonstrated that SOCS1 is a negative regulator of LPS-induced macrophage activation and plays an essential role in suppression of systemic autoimmunity mediated by DCs (23–25). Thus, SOCS1 regulates not only adaptive immunity

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; dnSOCS1, dominant-negative suppressor of cytokine signaling 1; EAM, experimental autoimmune myocarditis; FS, fractional shortening; KO, knockout; LV, left ventricular; LVEDd, left ventricular end-diastolic dimension; LVESd, left ventricular end-systolic dimension; MyHC-α, cardiac myosin-specific peptide; pdnSOCS1, plasmid vector encoding dominant-negative suppressor of cytokine signaling 1; pSOCS1, plasmid vector encoding suppressor of cytokine signaling 1; QRT-PCR, quantitative real-time RT-PCR; SOCS, suppressor of cytokine signaling.

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but also innate immunity by suppressing hyperactivation of macrophages and DCs.

In this study, we describe the therapeutic effect of SOCS1 DNA administration using plasmid DNA encoding SOCS1 for EAM. SOCS1 DNA therapy reduces myocarditis by regulating DC populations during EAM.

Materials and Methods

Animals

BALB/c mice and CB17.SCID mice were purchased from CLEA Japan. We used 5–7-wk-old male mice. All animals were cared for according to ethical guidelines approved by the Institutional Animal Care and Use Committee of the National Institute of Biomedical Innovation.

Immunization protocols

Mice were immunized with 100 μg cardiac myosin-specific peptide (MyHC-$\alpha_{614-629}$) Ac-RSLKLMATLFSTYASADR-OH (Toray Research Center) emulsified 1:1 in PBS/CFA (1 mg/ml; H37Ra; Sigma-Aldrich) on days 0 and 7 as described previously (12). For DC immunization, bone marrow-derived DCs (BMDCs) were generated as described (26). BMDCs were pulsed overnight with 10 μg/ml MyHC-α peptide and stimulated for another 4 h with 0.1 μg/ml LPS (Sigma-Aldrich) and 5 μg/ml anti-CD40 (BD Pharmingen) (15). Recipient mice received 2.5×10^5 pulsed and activated BMDCs i.p. on days 0, 2, and 4 and were killed 10 d after the first injection.

Plasmid construction and DNA transfection

Mouse SOCS1 cDNA and dominant-negative SOCS1 (dnSOCS1) cDNA were subcloned into the mammalian vector pcDNA3.1-myc/His(-) using oligonucleotide primers containing restriction sites for XhoI and EchoRI at the 5′ and 3′ ends, respectively. MyHC-α/CFA-immunized mice were injected i.p. with 100 μg of plasmid DNA in 200 μl PBS on days 0, 5, and 10. BMDC-transferred mice and $CD4^+$ T cell adoptive-transferred SCID mice were treated with plasmid DNA on days 0 and 5.

Histopathologic examination

Myocarditis severity was scored on H&E-stained sections using grades from 0–4: 0, no inflammation; 1, <25% of the heart section involved; 2, 25–50%; 3, 50–75%; and 4, >75%. To quantify the fibrotic area, ventricular sections were stained with Sirius Red. The fibrotic area was calculated as the sum of all areas stained positive for Sirius Red divided by the sum of all myocardial areas in each mouse. Two independent researchers scored the slides separately in a blinded manner.

Flow cytometry

Heart inflammatory cells were isolated and processed as described (15, 27). Cells were stained using fluorochrome-conjugated mouse-specific Abs against CD45, CD4, CD3e, CD44, CD62L, and CD40L (BD Biosciences). Samples were analyzed on an FACSCalibur cell sorter (BD Biosciences).

Measurements of cytokines and chemokines

Hearts were homogenized in media containing 2.5% FBS. Supernatants were collected after centrifugation and stored at −80°C. For in vitro stimulation assay of primary $CD4^+$ T cells, naive $CD4^+CD62L^+$ T cells were isolated from the spleens by MACS ($CD4^+CD62L^+$ T Cell Isolation Kit II; Miltenyi Biotec). A total of 1.5×10^7 $CD4^+CD62L^+$ cells were then stimulated with recombinant mouse IL-2 (R&D Systems) or recombinant mouse IL-12 (R&D Systems). Concentrations of cytokines and chemokines in the heart homogenates or culture supernatants were measured with Quantikine ELISA kits (R&D Systems).

Proliferative responses of T cells

Proliferation of T cells was assessed as previously described (28). Briefly, mice were immunized as described above, and the spleens collected on day 14. Cells were cultured with 5 μg/ml MyHC-α for 72 h and pulsed with 0.5 μCi [^{3}H]thymidine 8 h before being measured with a β counter. For in vitro stimulation assay of primary $CD4^+$ T cells, naive $CD4^+CD62L^+$ T cells were isolated from the spleens by MACS ($CD4^+CD62L^+$ T Cell Isolation Kit II; Miltenyi Biotec). A total of 10^5 $CD4^+CD62L^+$ cells were then stimulated with 5 μg/ml anti-CD3ε, 5 μg/ml anti-CD3ε, 1 μg/ml anti-CD28, 50 ng/ml PMA, and 500 ng/ml ionomycin or with 1 μg/ml Con A together with 0.25×10^5 DCs. Proliferative responses were assessed after 48 h in 2.5% RPMI 1640 medium by measurement of the [^{3}H]thymidine incorporation.

Western blot analysis

Total lysates from $CD4^+$ T cells or DCs were immunoblotted and probed with Abs directed against STAT1 (Santa Cruz Biotechnology) and p-STAT1 protein (Cell Signaling Technology). HRP-conjugated goat anti-rabbit IgG (Bio-Rad) was used to identify the binding sites of the primary Ab.

Adoptive transfer of T cells

Splenocytes were collected from diseased mice and cultured with 5 μg/ml MyHC-α for 48 h. A total of 5×10^6 $CD4^+$ T cells were purified by using anti-CD4 magnetic beads (Miltenyi Biotec) and injected i.p. into the SCID mice. The mice were killed 10 d after the injection.

Quantitative real-time RT-PCR

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA by reverse transcriptase (Takara). Quantitative real-time RT-PCR (QRT-PCR) analysis was performed with LightCycler (Roche Diagnostics). Primers for mouse *Socs1* were 5′-GTGGTTGTGGAGGGTGAGAT-3′ (sense) and 5′-CCTGAGAGGTGGGATGAGG-3′ (antisense). Primers for mouse *Hprt* were 5′-TCCTCCTCAGACCGCTTTT-3′ (sense) and 5′-CCTGGTTCATCATCGCTAATC-3′ (antisense). Data were normalized by the level of *Hprt* expression in each sample.

Echocardiography

Transthoracic echocardiography was performed on animals on day 35 by using a Prosound α6 with a 10-MHz transducer (Aloka). The left ventricular (LV) chamber dimensions were measured from the M-mode. Two independent investigators who conducted the echocardiography were unaware of the treatment status.

Statistical analysis

All data were expressed as means ± SEM. Statistical analyses were performed using the two-tailed *t* test or Mann–Whitney *U* test for experiments comparing two groups. The p values <0.05 were considered statistically significant.

Results

SOCS1 DNA administration inhibits the development of EAM

To examine the effect of in vivo gene delivery of *Socs1* on the pathogenesis of EAM, BALB/c mice were injected with a mammalian expression plasmid vector encoding SOCS1 (pSOCS1) during the course of EAM induction (Fig. 1A). QRT-PCR analysis revealed elevated expression of *Socs1* in the control EAM heart (Fig. 1B). Importantly, in the SOCS1 DNA-administered mice, *Socs1* was strongly expressed in the heart. By day 28, *Socs1* gene expression was significantly elevated in the pSOCS1-treated heart as compared with the controls (Supplemental Fig. 1). Gross cardiac enlargement and edema were reduced in mice with EAM that received pSOCS1 as compared with those in control empty plasmid DNA-administered EAM mice (Fig. 1C). The heart-to-body weight ratio in the pSOCS1-injected mice was significantly decreased as compared with that in the control plasmid-administered mice (Fig. 1D). The pSOCS1-injected EAM mice had a significantly lower myocarditis severity score and fewer infiltrating inflammatory cells than did the control plasmid-injected mice (Fig. 1E–G). The empty vector [pcDNA3.1-myc/His(-)] was used as the control and did not have any effects on EAM in our experiments (data not shown).

Recently, Hanada et al. (29) demonstrated that dnSOCS1, which has a point mutation (F59D) in a functionally critical kinase inhibitory region of SOCS1, strongly augmented cytokine-dependent JAK-STAT activation both in vivo and in vitro as an antagonist of SOCS1. We examined the effect of dnSOCS1 on the clinical course of EAM. Mice administered a plasmid vector

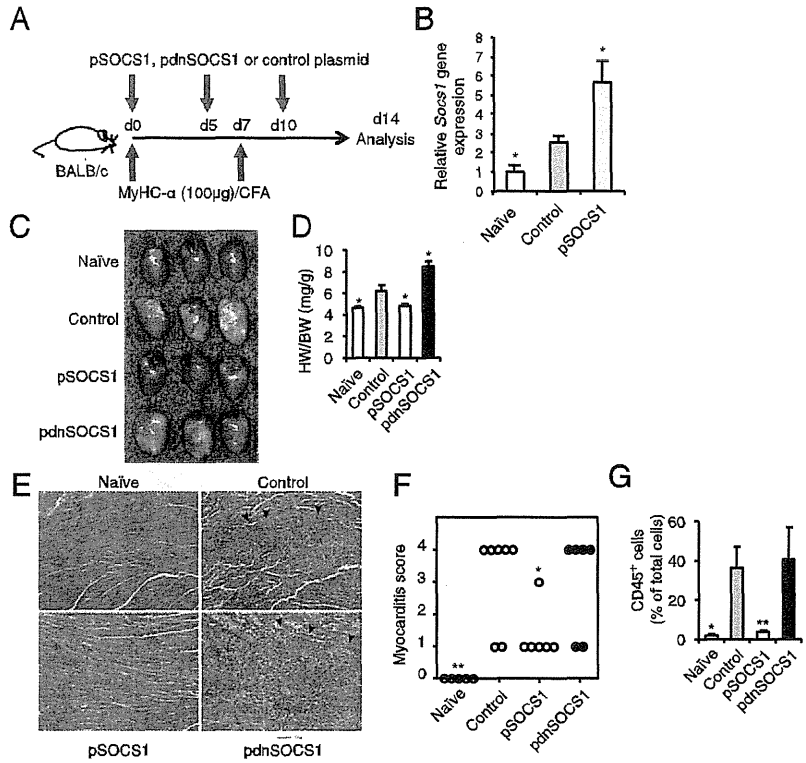

FIGURE 1. Amelioration of EAM and heart failure by SOCS1 DNA administration. (**A**) BALB/c mice were immunized twice, on days 0 and 7, with 100 μg of MyHC-α and treated with pSOCS1, pdnSOCS1, or control plasmid on days 0, 5, and 10. (**B**) QRT-PCR for the *Socs1* gene. RNA samples were obtained from hearts of immunized mice on day 14 and used as a template for QRT-PCR. Results represent the average gene induction in five independent heart samples. (**C**) Representative gross hearts (day 14) of naive and EAM mice treated with the indicated plasmid. (**D**) Heart-to-body weight ratios of naive and EAM mice with indicated treatment (n = 5 mice/group). (**E**) Representative H&E-stained sections of hearts from naive and immunized mice. Arrowheads indicate infiltrating cells. Scale bar, 50 μm. (**F**) Myocarditis severity in heart sections stained with H&E (n = 5–7 mice/group). (**G**) Flow cytometry analysis of $CD45^+$ heart infiltrates of naive and immunized mice (n = 5–7 mice/group). Data are representative of at least two independent experiments. Error bars represent means ± SEM. $*p < 0.05$, $**p < 0.01$ compared with control.

encoding dnSOCS1 (pdnSOCS1) showed augmentation of gross heart enlargement, edema, and heart-to-body weight ratio (Fig. 1C, 1D). However, the myocardial leukocyte infiltration and myocarditis scores were not significantly different between the pdnSOCS1- and control plasmid-administered mice (Fig. 1E–G).

To clarify the adverse effect of dnSOCS1 DNA administration on the development of EAM, we used mice immunized with a tithe amount (10 μg) of MyHC-α instead of the usual amount of peptide for EAM development (Fig. 2A). Those MyHC-α–immunized mice injected with the control plasmid or pSOCS1 did not develop myocarditis (Fig. 2B–F). However, immunized mice injected with pdnSOCS1 developed myocarditis with inflammatory infiltrates (Fig. 2B–F). Thus, administration of pSOCS1 is effective against the development of EAM, and the inhibition of SOCS1 by use of a SOCS1 antagonist adversely affects myocarditis.

SOCS1 DNA administration prevents progression of heart failure and fibrosis after myocarditis

Some patients diagnosed with myocarditis after viral, bacterial, or protozoal infection develop heart failure (2). On day 35 of the present experiment, mice immunized with MyHC-α showed increased LV end-diastolic dimensions (LVEDd) and LV end-systolic dimensions (LVESd) and decreased fractional shortening (FS); however, pSOCS1-injected mice showed almost normal chamber size and LV function (Fig. 3A, 3B). In contrast, LV dysfunction and chamber dilatation in pdnSOCS1-administered mice were manifested as significant increases in LVEDd and LVESd and decrease in FS (Fig. 3A, 3B). In these EAM models, on day 35, hearts from myocarditis mice showed interstitial fibrosis without active leukocyte infiltration. The fibrotic area in mice administered pSOCS1 was significantly smaller than that in control plasmid-injected mice (Fig. 3C, 3D). Although pdnSOCS1-injected mice developed severe cardiac fibrosis, the difference between the fibrotic areas in pdnSOCS1- and control plasmid-injected mice was not statistically significant (Fig. 3C, 3D). These inhibitory effects of pSOCS1 on the development of fibrosis and heart failure were considered to be the result of inhibition of myocardial inflammation because myocarditis developed mice injected with pSOCS1 on day 14, 21, and 28 did not show inhibitory effects on fibrosis and heart failure (data not shown).

Cardiac myosin-specific $CD4^+$ T cell response and cytokine production

Autoimmune myocarditis is a $CD4^+$ T cell-mediated disease (7, 15). Proliferative responses of $CD4^+$ T cells after in vitro restimulation with MyHC-α were not clearly seen in pSOCS1-injected mice; however, the proliferation of $CD4^+$ T cells from pdnSOCS1-injected mice was enhanced (Fig. 4A). Production of IL-2, IL-6, IL-10, IL-17, IL-22, IFN-γ, TNF-α, CCL2, CCL3, CCL5, CCL17, and CXCL10 by $CD4^+$ T cells from EAM mice was enhanced by in vitro restimulation with the MyHC-α epitope peptide. This cardiac-Ag–specific cytokine production by $CD4^+$ T cells was decreased in the supernatants of in vitro MyHC-α–restimulated $CD4^+$ T cells from pSOCS1-administered mice but

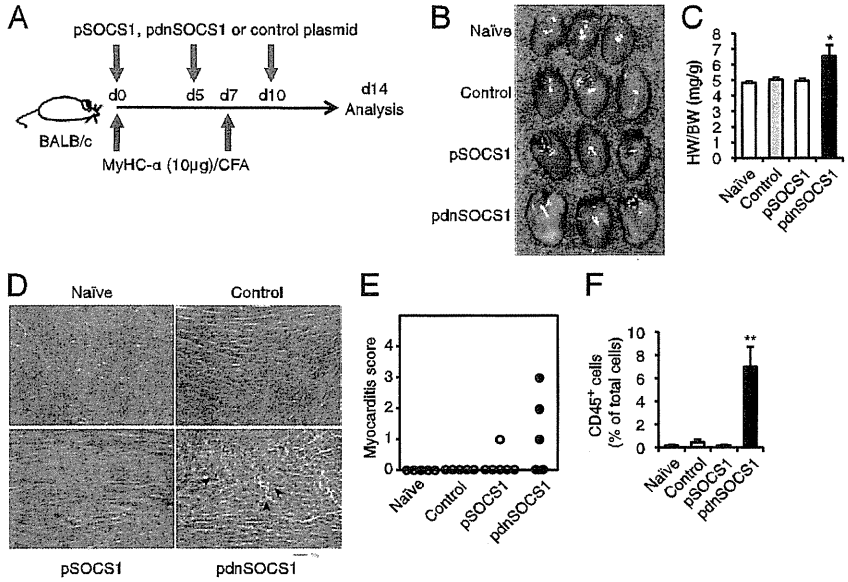

FIGURE 2. Increased susceptibility to EAM induced by inhibition of SOCS1. (**A**) Mice were immunized twice, on days 0 and 7, with 10 μg of MyHC-α emulsified 1:1 in PBS/CFA and treated with pSOCS1, pdnSOCS1, or control plasmid on days 0, 5, and 10. (**B**) Representative gross hearts (day 14) of naive and 10 μg of MyHC-α–immunized mice treated with the indicated plasmid. (**C**) Heart-to-body weight ratios of naive and immunized mice ($n = 5$ to 6 mice/group). (**D**) Representative H&E-stained sections of hearts from naive and immunized mice. Arrowheads indicate infiltrating cells. Scale bar, 50 μm. (**E**) Myocarditis severity in heart sections stained with H&E ($n = 5$ to 6 mice/group). (**F**) Flow cytometry analysis of $CD45^+$ heart infiltrates of naive and immunized mice ($n = 5$ mice/group). Data are representative of at least two independent experiments. Error bars represent means ± SEM. $*p < 0.05$, $**p < 0.01$ compared with control.

was increased in the supernatants of these cells from pdnSOCS1-administered mice (Fig. 4B). In contrast, cardiac-Ag–specific production of IL-1β, IL-10, and CXCL1 was not detected in the culture supernatants of in vitro-restimulated $CD4^+$ T cells from control plasmid-, pSOCS1-, or pdnSOCS1-injected mice (data not shown). Taken together, these results indicate that SOCS1 DNA

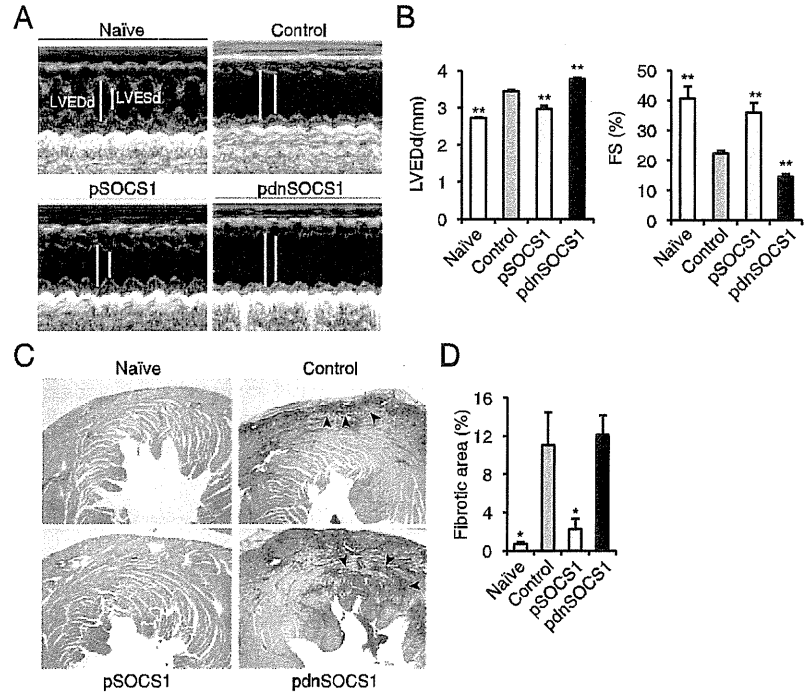

FIGURE 3. SOCS1 DNA administration prevents progression to heart failure. (**A** and **B**) Echocardiography was performed on naive and immunized mice on day 35. (A) Representative M-mode echocardiograms. Bars indicate LVESd and LVEDd. Bar graphs (B) represent LVEDd and percentage of FS from the indicated animals ($n = 9$ mice/group). The percentage FS was calculated according to the following formula: FS (%) = (LVEDd − LVESd)/LVEDd. (**C** and **D**) Heart tissue sections were stained with Sirius Red and analyzed for fibrosis at day 35. Representative Sirius Red-stained sections of hearts. Scale bar, 50 μm. (C) Arrowheads indicate fibrotic area. (D) The degree of fibrosis was calculated as the percentage of the fibrotic area in relation to the total heart area ($n = 5$ mice/group). Data are representative of at least two independent experiments. Error bars represent means ± SEM. $*p < 0.05$, $**p < 0.01$ compared with control.

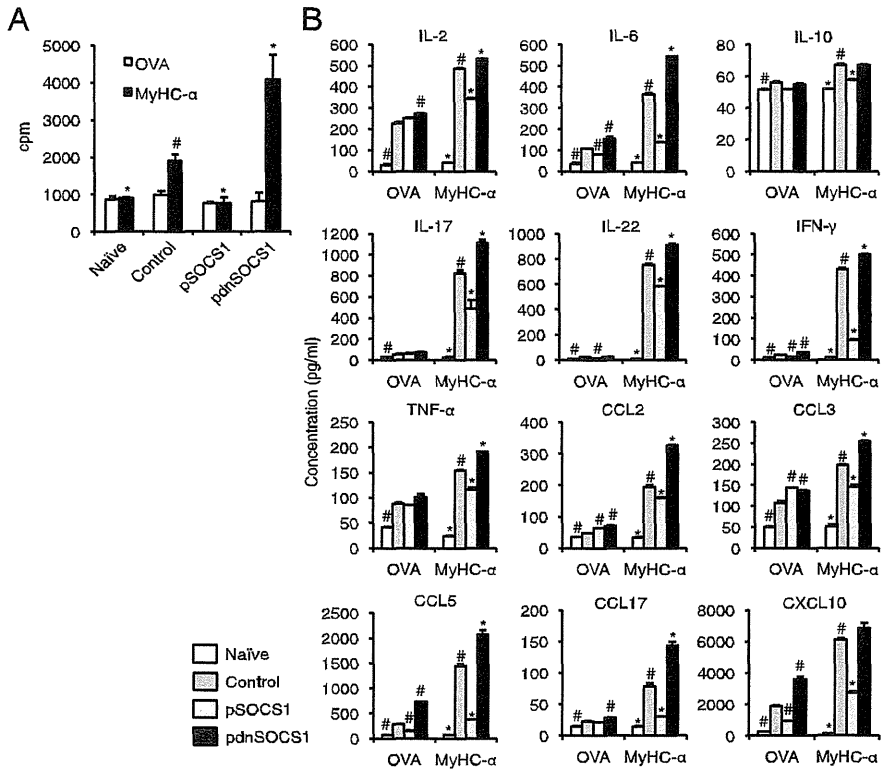

FIGURE 4. Impaired expansion of heart-specific $CD4^+$ T cells in pSOCS1-treated mice. (**A**) Splenocytes were isolated from naive and EAM mice treated with pSOCS1, pdnSOCS1, or control plasmid on day 14 and restimulated in vitro with MyHC-α or OVA peptide for 72 h. Proliferation was assessed by measurement of [^{3}H]thymidine incorporation. Data represent means ± SEM of triplicates from one of three independent experiments. (**B**) Cytokines and chemokines in the culture supernatants of splenocytes were measured by ELISA after 48 h of restimulation with MyHC-α or OVA peptide. Data are expressed as mean ± SEM from triplicate culture wells. Results of one of two representative experiments are shown. $^*p < 0.05$ compared with MyHC-α–stimulated control, $^\#p < 0.05$ compared with OVA-stimulated control.

delivery inhibits the activation of myosin-specific $CD4^+$ T cells and strongly suggest that impaired $CD4^+$ Th cell function prevents EAM development in pSOCS1-injected mice after immunization with cardiac self-Ag.

To evaluate whether pSOCS1 administration affects Ag-specific $CD4^+$ T cell function in other models, we injected plasmid DNA into an autoimmune gastritis model and an OVA-immunized model. In the autoimmune gastritis model, gastric-Ag–specific production of IL-2, IL-6, IL-13, IL-17, IL-22, IFN-γ, TNF-α, CCL2, CCL5, CCL17, and CXCL10 by $CD4^+$ T cells was decreased in pSOCS1-administered mice but increased in pdnSOCS1-administered mice (Supplemental Fig. 2). Lower amounts of cytokines (including IL-2, IL-6, IL-13, IFN-γ, TNF-α, CCL2, CCL3, CCL5, CCL17, and CXCL10) were also produced in $CD4^+$ T cells from pSOCS1-injected OVA-immunized mice (Supplemental Fig. 3). These results suggest that pSOCS1 administration may suppress Ag-specific $CD4^+$ T cell activation in various autoimmune diseases and foreign body infections.

SOCS1 DNA administration inhibits the production of proinflammatory cytokines and $CD4^+$ T cell differentiation in the heart

We also examined whether SOCS1 DNA administration has an effect on cytokine and chemokine milieu in the heart. On day 14 after MyHC-α immunization, heart homogenates from pSOCS1-injected mice had significantly decreased amounts of proinflammatory cytokines, including IL-1β and IL-6, and of myelotropic chemokines, including CCL5, CXCL1, and CXCL10 (Fig. 5A). In contrast, hearts from mice injected with pdnSOCS1 showed greatly increased amounts of proinflammatory cytokines and chemokines (Fig. 5A). SOCS1 protein has been shown to regulate T cell differentiation (17, 18). To determine the differentiation of $CD4^+$ T cells during EAM, we examined the heart-infiltrating $CD4^+$ T cell populations by FACS analysis. Activated $CD4^+$ T cells ($CD4^+CD40L^+$) and effector memory $CD4^+$ T cells ($CD44^+CD62L^-$) were reduced in the pSOCS1-injected mice (Fig. 5B). Thus, protection from EAM in pSOCS1-administered mice is associated with abrogation of proinflammatory cytokines, chemokines, and $CD4^+$ T cell differentiation in the heart.

SOCS1 DNA injection does not have a direct suppressive effect on $CD4^+$ T cell activation

To gain new insights into the mechanism of protection from myocarditis, we investigated whether pSOCS1 therapy directly affects $CD4^+$ T cell activation. Naive T cells ($CD4^+CD62L^+$ cells) were isolated from non-EAM mice injected with pSOCS1, pdnSOCS1, or control plasmid, and their primary responses to various stimuli were compared (Fig. 6A). As shown in Fig. 6B, there were no differences in IFN-γ–induced STAT1 activation among these $CD4^+$ T cells. There were also no differences in primary responses to stimulation with anti-CD3ε, anti-CD3ε/anti-CD28, PMA/ionomycin, or Con A presented by mitomycin C-treated wild-type DCs among pSOCS1-, pdnSOCS1-, and control plasmid-treated $CD4^+$ T cells (Fig. 6C). Chong et al. (30) demonstrated that SOCS1-deficient T cells produced substantially greater levels of IFN-γ in response to IL-2 or IL-12. From these findings, we assessed the production of IFN-γ from $CD4^+$ T cells by using the same experiments. In the culture supernatants of

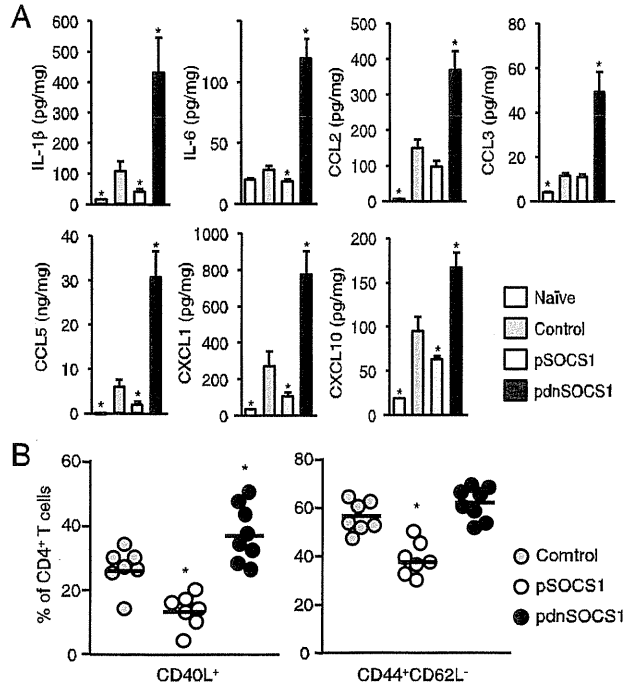

FIGURE 5. Cytokine and chemokine responses and $CD4^+$ T cell differentiation in the heart. (**A**) Myocardial tissues were homogenated and processed by ELISA to detect cytokines and chemokines on day 14. Bar graphs show group means ± SEM of 8–16 mice/group. Results of one of three representative experiments are shown. (**B**) Heart-infiltrating cells were isolated from EAM mice treated with indicated plasmid DNA. Cells were stained for CD4, CD40L, CD44, and CD62L. CD44 and CD62L expression are based on gates set from total $CD4^+$ T cells. Bar graphs show group means ± SEM of 5–9 mice/group. Data are representative of two independent experiments. *$p < 0.05$ compared with control.

$CD4^+$ T cells stimulated with IL-2 or IL-12, there were also no differences in IFN-γ production (Fig. 6D). These results indicate that in vivo administration of pSOCS1 does not directly affect $CD4^+$ T cell activation.

In vivo SOCS1 DNA administration inhibits DC function

Although $CD4^+$ T cell differentiation was inhibited in pSOCS1-treated mice (Fig. 5B), our results suggested that in vivo *Socs1* gene administration has no direct effect on $CD4^+$ T cell activation (Fig. 6). We therefore investigated whether in vivo pSOCS1 administration inhibits the function of Ag-presenting DCs by stimulation through the TLR pathway. DCs from mice administered pSOCS1, pdnSOCS1, or control plasmid were stimulated with LPS for 24 h (Fig. 7A). STAT1 phosphorylation was attenuated in DCs from pSOCS1-injected mice and enhanced in DCs from pdnSOCS1-injected mice (Fig. 7B). The production of proinflammatory cytokines, including IL-6, TNF-α, and IFN-γ, was inhibited in DCs from pSOCS1-injected mice and enhanced in DCs from pdnSOCS1-injected mice (Fig. 7C). These results indicate that in vivo administration of *Socs1* affects DC function. In the current study, the cardiac-Ag–specific proliferative response and cytokine production of $CD4^+$ T cells were inhibited in pSOCS1-injected EAM mice (Fig. 4). We next assessed the functional capability of DCs to prime and expand autoreactive $CD4^+$ T cells from mice injected with each plasmid as a measure of Ag-specific proliferative responses of $CD4^+$ T cells from MyHC-α–immunized mice. Myosin-specific $CD4^+$ T cells were cocultured with MyHC-α–pulsed DCs from pSOCS1-, pdnSOCS1-, and control plasmid-treated mice (Fig. 7D). Interestingly, the proliferative responses of $CD4^+$ T cells cocultured with DCs from pSOCS1-treated mice were much weaker than those of cells cultured with DCs from control plasmid-treated mice, and these proliferative responses of $CD4^+$ T cells were enhanced by coculturing with DCs from pdnSOCS1-administered mice (Fig. 7E). These results suggest that in vivo gene delivery of *Socs1* suppresses the functional capability of DCs to prime and expand autoreactive $CD4^+$ T cells.

SOCS1 DNA administration inhibits the development of myocarditis induced by cardiac myosin peptide-loaded BMDC transfer but not by $CD4^+$ T cell transfer

Functionally interposed SOCS1 is induced in various cell populations, including leukocytes, vascular cells, and cardiomyocytes (18, 31, 32). A mouse model of EAM was established by cell transfer using peptide-pulsed DCs or cardiac epitope-specific $CD4^+$ T cells (7, 14). The effects of pSOCS1 administration in mice transferred with $CD4^+$ T cells from mice with EAM were assessed. pSOCS1, pdnSOCS1, or control plasmid was injected into mice transferred with cardiac myosin-specific $CD4^+$ T cells (Fig. 8A). All mice transferred with $CD4^+$ T cells developed myocarditis, and no therapeutic effects were seen in pSOCS1-injected mice (Fig. 8B–D). Furthermore, pdnSOCS1 administration showed no adverse effect on the status of myocarditis induced by $CD4^+$ T cell transfer (Fig. 8B–D). These findings suggest that systemic injection of pSOCS1 is not effective for inhibition of autoreactive $CD4^+$ T cell activation and recruitment to the heart during myocarditis development. Next, we administered pSOCS1, pdnSOCS1, or control plasmid into mice transferred with MyHC-α–loaded BMDCs (Fig. 8E). Interestingly, pSOCS1 injection inhibited the development of myocarditis after MyHC-α–loaded BMDC transfer, and myocarditis deteriorated after administration of pdnSOCS1 (Fig. 8F–H). These results indicate that the therapeutic effects of SOCS1 DNA administration on EAM contribute to professional APCs such as DCs and also provide evidence for the potential utility of SOCS1 DNA inoculation as an approach to gene therapy for myocarditis.

Discussion

There have been no effective fundamental therapies for acute myocarditis; therefore, supportive care for LV dysfunction is the first line of treatment. Because patients generally present days to weeks after the initial viral infection, antiviral therapy has limited applicability in patients with acute viral myocarditis. The long-term sequelae of viral myocarditis appear to be related to abnormal cellular and humoral immunity; therefore, many clinicians believe that immunosuppression is beneficial for myocarditis treatment (2). In this study, we showed that administration of SOCS1 DNA is effective for inhibiting the development of EAM in BALB/c mice, suggesting a novel immunotherapy for myocarditis. To our knowledge, this is the first report showing that gene delivery of *Socs1* prevents autoimmune disease.

Animal models have greatly advanced our knowledge of the pathogenesis of myocarditis and inflammatory cardiomyopathy. Infection of BALB/c mice with either Coxsackievirus or murine CMV results in the development of acute myocarditis from days 7–14 postinfection that is characterized by myocyte damage due to viral cytotoxicity, and the infectious virus cannot be detected past day 14 of the infection (7). After elimination of viruses, mice showed autoimmune myocarditis, which is associated with mononuclear infiltration of the myocardium and production of autoantibodies to cardiac myosin (7), similar to the pathogenesis of autoimmune myocarditis in humans (3, 4, 33). These autoim-

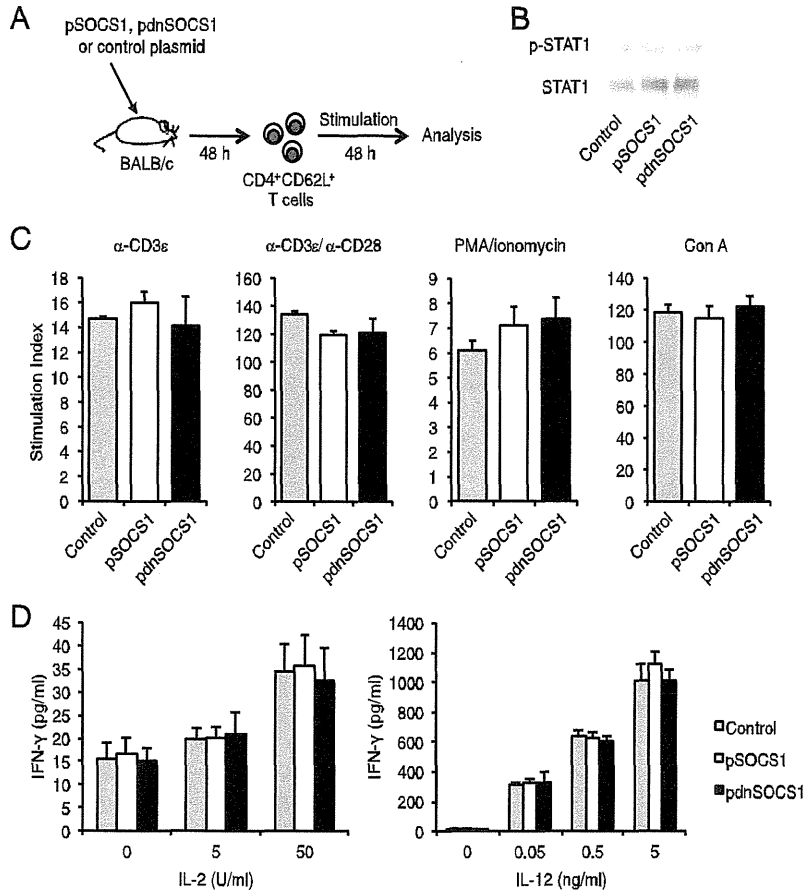

FIGURE 6. Primary responses of $CD4^+$ T cells from pSOCS1-, pdnSOCS1-, and control plasmid-treated mice. (**A**) $CD4^+CD62L^+$ T cells from mice injected with pSOCS1, pdnSOCS1, or control plasmid were stimulated with IFN-γ, anti-CD3ε, anti-CD3ε, anti-CD28, PMA/ionomycin, and Con A in the presence of wild-type DCs, IL-2, or IL-12. (**B**) STAT1 phosphorylation of $CD4^+$ T cells after IFN-γ treatment (10 ng/ml) was assessed by Western blotting. (**C**) T cell proliferation was measured after 48 h of culture. (**D**) IFN-γ in the culture supernatants was measured by ELISA. Values are expressed as means ± SEM of triplicate culture wells. Results of one of at least two representative experiments are shown.

mune responses are thought to be elicited by two mechanisms. One is molecular mimicry: responses to microbial Ags could result in the activation of T cells that are cross-reactive with self-Ags. Another possibility is bystander activation of autoreactive cells. APCs that have become activated in the inflammatory milieu of a pathogenic infection can stimulate the activation and proliferation of autoreactive T or B cells in a process known as bystander activation (reviewed in Ref. 34). Thus, immune responses to myocytes involving various innate and adaptive immune pathways were recognized during myocarditis development. The cardiac myosin peptide-immunized mouse EAM model reflects human autoimmune myocarditis and heart failure after elimination of infectious pathogens.

Recent studies have indicated that various microbes use the host's SOCS proteins for manipulating cytokine receptor signaling as one of the strategies to evade immune responses (35, 36). Coxsackievirus usually infects cardiomyocytes and induces the expression of SOCS1 and SOCS3 in cardiomyocytes, which can result in evasion of immune responses and facilitation of virus replication by inhibition of JAK–STAT signaling (32, 37). These findings indicate that it may be harmful to administer SOCS1 DNA in the acute phase of infectious myocarditis because it may augment viral replication by inhibition of IFN signaling. The effect of SOCS1 transduction on viral myocarditis has been examined by Yasukawa et al. (32). The SOCS1-transgenic mice infected with CVB3 showed increased myocardial injury, virus replication, and mortality. In contrast, they also showed that SOCS1 inhibition in the heart through adeno-associated virus-mediated expression of dnSOCS1 increased resistance to the acute cardiac injury caused by CVB3 infection. These results were acceptable because SOCS proteins have emerged as frequent targets of viral exploitation. Furthermore, when administrating JAK inhibitors, such as SOCS, active serious infections should have been resolved before the start of treatment. It is considered to be inappropriate to use JAK inhibitors for a person with infectious disease or their possibility with consideration for complication of infection (38–40). In contrast, the overactive autoimmune responses triggered by microbial pathogens can persist after elimination of infectious pathogens (7). Therefore, we examined the efficacy of SOCS1 transfection by using EAM induced by cardiac autoantigen immunization in the absence of viral infection. In the current study, we clearly showed the efficacy of *Socs1* gene transfer as an immunosuppressive therapy for myocarditis under infectious pathogen-free conditions in an EAM mice model. The results of a recent randomized, double-blind, placebo-controlled study showed that immunosuppressive therapy, including prednisone and azathioprine, was effective in patients with myocarditis and inflammatory cardiomyopathy and without evidence of the myocardial viral genome (41). These findings indicate that *Socs1* gene transfer can be effective to treat some clinical

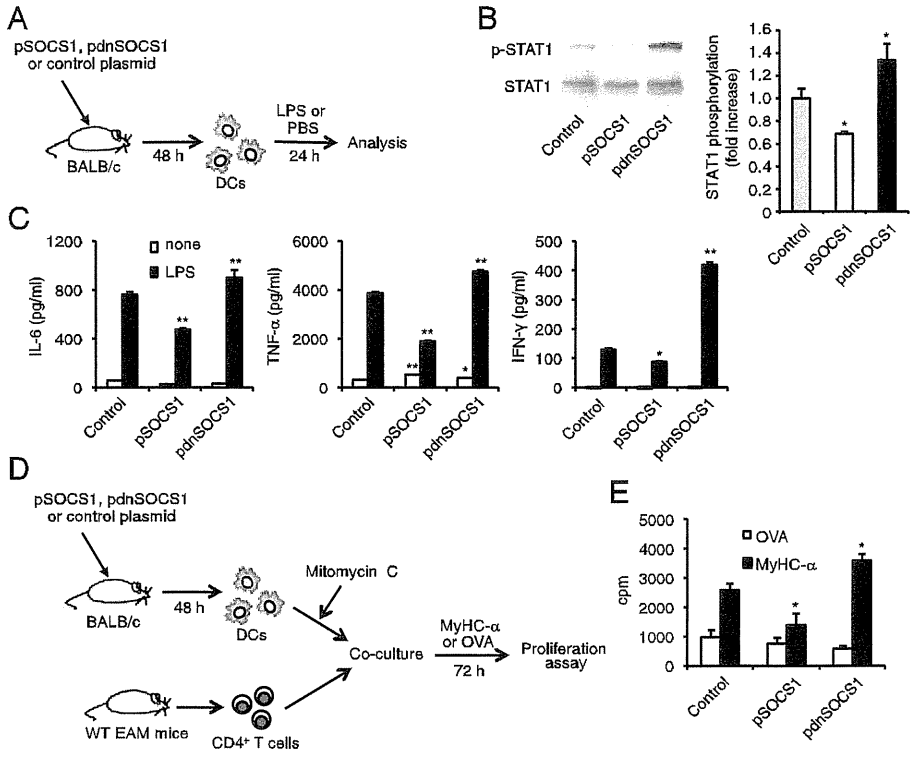

FIGURE 7. Functional capacities of DCs from pSOCS1-, pdnSOCS1-, and control plasmid-treated mice. (**A**) DCs from mice treated with pSOCS1, pdnSOCS1, or control plasmid were stimulated with LPS for 24 h. (**B**) STAT1 phosphorylation of DCs was assessed by Western blotting. Densitometry ratios of pSTAT1/STAT1 are shown as fold induction, the ratio for DCs from control plasmid-injected mice being set at 1. Results are means of five independent experiments ± SEM. Blots are representative of experiments performed a minimum of three times. (**C**) IL-6, TNF-α, and IFN-γ in the culture supernatants were measured by ELISA. Values indicate means ± SEM of triplicate culture wells from one of three independent experiments. (**D** and **E**) Heart-specific $CD4^+$ T cells from EAM mice were restimulated with MyHC-α or OVA peptide on DCs from mice treated with control plasmid, pSOCS1, or pdnSOCS1 for 72 h before measurement of [^{3}H]thymidine incorporation. Each value represents mean ± SEM cpm values of six different culture wells. Results of one of three representative experiments are shown. $*p < 0.05$, $**p < 0.01$ compared with control.

cases of myocarditis and inflammatory cardiomyopathy associated with autoimmunity and without the virus genome in the myocardium, as well as EAM in mice.

In the current study, we demonstrated that the administration of plasmid DNA encoding SOCS1 did not affect autoreactive $CD4^+$ T cell function (Fig. 6) and adoptive transfer of autoreactive $CD4^+$ T cells was able to induce myocarditis in SOCS1 DNA-administered SCID mice (Fig. 8A–D), suggesting that SOCS1 DNA does not suppress either $CD4^+$ T cell recruitment or accumulation of other inflammatory cells in the heart. In contrast, the introduced SOCS1 DNA inhibited the activation of DCs producing proinflammatory cytokines (Fig. 7C). In fact, inhibition of the phosphorylation of STAT1 molecules was observed in DCs from mice injected with SOCS1 DNA (Fig. 7B). In addition, the proliferative responses of $CD4^+$ T cells cocultured with DCs from pSOCS1-treated mice were much weaker than those of cells cultured with DCs from control plasmid-injected mice (Fig. 7E). These results suggest that the inoculated SOCS1 DNA may have been transfected into DCs and impaired DC function in vivo. Contrary to expectations, we could not find evidence of direct transfection of inoculated DNA into DCs in the heart, spleen, peritoneal cavity, or lymph nodes. Although the introduced DNA is expressed predominantly by somatic cells (e.g., cardiomyocytes, keratinocytes, and fibroblasts), it is known that relatively small but biologically significant numbers of DCs are transfected with the inoculated DNA (42–44). Based on this fact, the inoculated SOCS1 DNA may have inhibited DC activation through the direct transfection into DCs; however, our data do not exclude the possibility of another indirect mechanisms.

In the EAM model, activation of TLRs on self-Ag–presenting DCs is essential for the expansion of autoreactive $CD4^+$ T cells to induce myocarditis and heart failure (15). We previously reported that *Tlr4* mutant C3H/HeJ mice are resistant to development of EAM (45). Furthermore, IL-1 type 1 receptor signaling on DCs is critical for autoimmune myocarditis development (11). MyD88 is a crucial common adaptor molecule that mediates both TLRs and IL-1 type 1 receptor activation (46, 47), and MyD88 signaling in DCs is critical for the induction of EAM (16). SOCS1 negatively regulates the MyD88-dependent pathway by interacting with both IL-1R–associated kinase and NF-κB (17), which results in a decrease in the induction of inflammatory cytokines such as TNF-α and IL-6. In fact, production of these inflammatory cytokines was inhibited by the administration of SOCS1 DNA in the current study (Fig. 7C). Although nearly all TLRs recruit MyD88, other specific adaptor proteins function downstream of particular TLRs. One such adaptor molecule is Toll/IL-1R domain-containing adaptor protein/Mal. SOCS1 also binds to tyrosine-phosphorylated Mal through its interaction with Bruton's tyrosine kinase, leading to the suppression of Mal-dependent p65 phosphorylation and transactivation of NF-κB (48). Another important mechanism of the suppression of APC activation by SOCS1 is inhibition of the secondary activated JAK–STAT pathway (49, 50). The Toll/IL-1R domain-containing adaptor protein-inducing IFN-β–IFN-regulatory factor 3 pathway rapidly induces IFN-β, which in turn activates JAK–STAT1 and contributes to the expression of IFN-

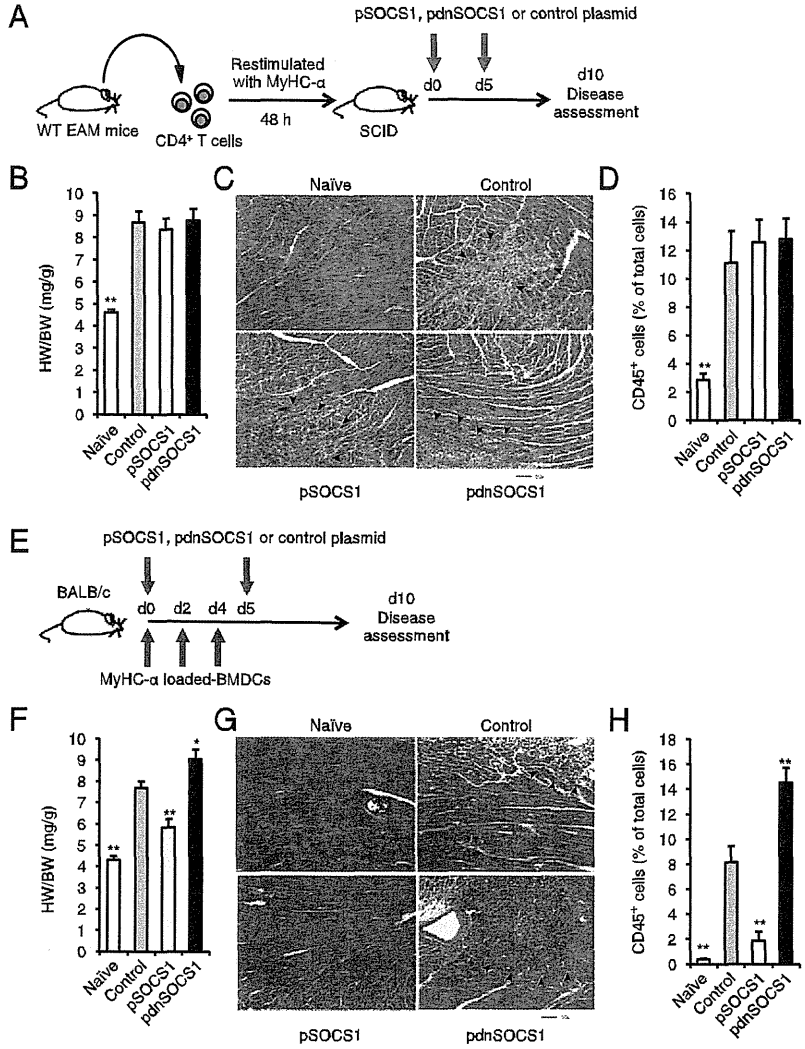

FIGURE 8. pSOCS1 administration inhibited the development of myocarditis induced by cardiac Ag-loaded BMDC injection but not by heart-specific $CD4^+$ T cells. (**A–D**) $CD4^+$ T cells were purified from diseased mice and restimulated in vitro with MyHC-α for 48 h before transfer into SCID recipients. pSOCS1, pdnSOCS1, or control plasmid was injected on days 0 and 5 after the transfer. Heart-to-body weight ratios (B; $n = 5$ mice/group), representative H&E-stained sections of hearts (C), and results of flow cytometry analysis of $CD45^+$ heart infiltrates (D; $n = 5$ mice/group) of naive and adoptive transferred mice at day 10. Arrowheads indicate infiltrating cells. Scale bar, 50 μm. (**E–H**) Mice were immunized with activated MyHC-α– or control OVA peptide-pulsed DCs on days 0, 2, and 4. Mice immunized with MyHC-α–pulsed DCs were treated with pSOCS1, pdnSOCS1, or control plasmid on days 0 and 5. Heart-to-body weight ratios (F; $n = 6$–12 mice/group), representative H&E-stained sections of hearts (G), and results of flow cytometry analysis of $CD45^+$ heart infiltrates (H; $n = 5$ mice/group) of naive and transferred mice at day 10. Arrowheads indicate infiltrating cells. Scale bar, 50 μm. Data are expressed as means ± SEM. Data are representative of at least two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared with control.

inducible genes (51). Moreover, Kimura et al. (52) showed that LPS can activate JAK2 and STAT5, which are involved in IL-6 induction, and that SOCS1 selectively inhibits this process. Thus, SOCS1 negatively regulates several activation pathways in DCs. The present study indicates that pSOCS1 administration is a possible therapy against various diseases caused by overshooting of DCs.

IFN-γ has been shown to be a downregulatory cytokine, as evidenced by exacerbated myocarditis in IFN-γR knockout (KO), IFN-γ KO, and T-bet KO mice (9, 53, 54). In contrast, Th17 cells have recently been implicated in the pathogenesis of various types of autoimmune diseases (reviewed in Ref. 55); however, IL-17 deficiency did not significantly impact the severity of EAM (56). Though these gene-ablated mice provided us with much important information, these studies do not necessarily lead to an effective therapy. In this study, we showed that SOCS1 DNA administration inhibited a broad array of cytokine production from $CD4^+$ T cells (Fig. 4B) and effectively reduced myocardial inflammation (Fig. 1). Compared with inhibition of a single cytokine, SOCS1 DNA therapy could be a more useful therapy that inhibits various signaling pathways to induce production of cytokines.

In the current study, SOCS1 DNA administration was efficacious against EAM development, and inhibition of SOCS1 molecules by SOCS1 antagonist DNA administration enhanced the severity of myocarditis. We demonstrated that SOCS1 DNA administration inhibits the stimulation of self-Ag–presenting DCs inducing cardiac myosin-specific $CD4^+$ T cell responses in peripheral compartments in vivo. Given the availability of clinically effective drugs targeting SOCS1, our findings show new therapeutic perspectives for the treatment of autoimmune myocarditis and cardiomyopathy.

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Disclosures

The authors have no financial conflicts of interest.

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Original contribution

Sarcoidosis does not belong to or overlap with immunoglobulin G4–related diseases based on an assessment of serum immunoglobulin G4 levels in cardiac and noncardiac sarcoidosis☆

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Summary Although sarcoidosis may exhibit histopathologic features similar to those of a newly emerging clinical entity, immunoglobulin G4–related sclerosing disease, sarcoidosis is currently not considered to be associated with immunoglobulin G4–related immunoinflammation. Not many studies on this association have been reported. We investigated serum immunoglobulin G4 levels among patients with sarcoidosis with or without cardiac involvement (cardiac sarcoidosis and non–cardiac sarcoidosis patients). The mean serum immunoglobulin G4 level among the 65 patients with sarcoidosis was 56.8 ± 43.0 mg/dL, which did not significantly differ between patients with cardiac sarcoidosis (54 ± 48 mg/dL, n = 12) and patients without cardiac sarcoidosis (58 ± 42 mg/dL; n = 53). Serum level of soluble interleukin 2 receptor, a potent marker that may reflect sarcoidosis activity, was elevated in cardiac sarcoidosis (910 ± 683 U/L) and noncardiac sarcoidosis (689 ± 399 U/L) but did not significantly differ between the groups. Immunohistochemistry of cardiac or lymph node specimens from patients with cardiac sarcoidosis showed only sparse or no infiltration of immunoglobulin G4–positive lymphocytes, in contrast to the moderate to severe infiltration of CD68-positive macrophages and CD45-positive lymphocytes. Although the number of study subjects was small, these findings collectively suggest that regardless of the presence or absence of cardiac involvement, sarcoidosis does not belong to or overlap with immunoglobulin G4–related sclerosing disease.

1. Introduction

Since elevation of serum immunoglobulin G4 (IgG4) levels and tissue infiltration of IgG4-positive plasma cells were first characterized in autoimmune pancreatitis [1], similar findings have been observed in a wide variety of disorders, such as retroperitoneal fibrosis, inflammatory

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Table 1 Patient characteristics

Characteristics	Cardiac involvement (+) (n = 12)	Cardiac involvement (−) (n = 53)	*P*
Age (y)	52.3 ± 13.4	55.6 ± 16.0	NS
Men	6 (50%)	23 (43%)	NS
Body mass index	23.8 ± 3.8	22.6 ± 2.8	NS
History of smoking	5 (42%)	18 (34%)	NS
Accompanying diseases			
Hypertension	2 (17%)	15 (28%)	NS
Diabetes mellitus	2 (17%)	7 (13%)	NS
Dyslipidemia	6 (50%)	19 (36%)	NS
Coronary artery diseases	0 (0%)	4 (8%)	NS
Heart failure	11 (92%)	2 (4%)	<.001
Systolic blood pressure (mm Hg)	110 ± 24	127 ± 16	.006
Diastolic blood pressure (mm Hg)	72 ± 16	74 ± 9	NS
Heart rate (beats per minute)	74 ± 17	72 ± 8	NS
Electrocardiogram			
Complete or advanced AV block	5 (42%)	4 (8%)	.002
Ventricular tachycardia	3 (25%)	0 (0%)	<.001
Echocardiogram			
LV end-diastolic diameter (mm)	59.7 ± 14.4	47.1 ± 4.8	<.001
LV ejection fraction	41.3 ± 22.1	66.8 ± 7.4	<.001
Medication			
ACEI/ARB	4 (33%)	9 (20%)	NS
Calcium channel antagonists	1 (8%)	10 (19%)	NS
β-Blockers	4 (33%)	2 (4%)	.002
Diuretics	6 (50%)	1 (2%)	<.0001
Digitalis	3 (25%)	1 (2%)	.003
Spironolactone	2 (17%)	0 (0%)	.003
Statin	6 (50%)	14 (26%)	NS
Amiodalon	3 (25%)	0 (0%)	.001
Laboratory findings			
Brain natriuretic peptide (pg/mL)	350.9 ± 378.4	116.1 ± 242.1	.048
CRP (mg/dL)	2.43 ± 5.35	0.18 ± 0.33	.003
White blood cell count (/μL)	6860 ± 2810	5740 ± 1640	NS
Hemoglobin level (g/L)	14.5 ± 1.3	13.9 ± 1.3	NS
Platelet count (103/μL)	191 ± 57	242 ± 51	.003
Erythrocyte sedimentation rate (mm/h)	17.5 ± 9.9	13.8 ± 12.1	NS
Aspartate aminotransferase (IU/L)	38.5 ± 34.8	24.5 ± 11.7	.019
Alanine aminotransferase (IU/L)	27.3 ± 14.6	24.6 ± 15.2	NS
Blood urea nitrogen (mg/dL)	19.4 ± 6.4	15.8 ± 4.5	.022
Creatinine level (mg/dL)	0.96 ± 0.29	0.76 ± 0.23	.020

NOTE. Data are presented as mean ± SD for continuous variables.

Abbreviations: AV, atrioventricular; LV, left ventricle; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; CRP, C-reactive protein; and NS, not significant.

abdominal aortic aneurysm [2], Mikulicz disease, and Sjoegren syndrome [3], leading to the proposal of a new clinicopathologic entity, IgG4-related sclerosing disease [4]. Although the clinical spectrum of IgG4-related sclerosing disease or its identity as a novel clinical entity has not been established, it has been proposed that diagnosis of IgG4-related sclerosing disease can be defined by elevated serum IgG4 and histopathologic features such as greater than 50% infiltration of IgG4/IgG-positive plasma cells [5]. Currently, sarcoidosis is not considered to be IgG4 related. However, a small fraction of IgG4-related sclerosing disease may be misdiagnosed as other lymphoproliferative diseases because of the resemblance of clinicopathologic pictures [6] as well as the fact that a substantial fraction of autoimmune pancreatitis, the first disease to be diagnosed as IgG4 related, may occur concomitantly with other autoimmune diseases, including sarcoidosis [7].

Recent studies have suggested that cardiac involvement, although less common, may be one of the features of IgG4-related sclerosing disease [8,9]. Sarcoidosis with cardiac involvement, termed *cardiac sarcoidosis*, is reported to be more prevalent in Japan than in the United States and is responsible for approximately 58% to 85% of deaths from sarcoidosis [10-12]. To date, there has been little information

available regarding whether serum IgG4 levels are increased in patients with sarcoidosis, especially those who have been diagnosed with cardiac involvement. Therefore, we analyzed serum IgG4 levels in 65 patients with sarcoidosis, including 12 patients with cardiac sarcoidosis.

2. Materials and methods

2.1. Study patients and diagnosis of cardiac sarcoidosis

The study was approved by the Ethical Committee of the Osaka Medical College, Osaka, Japan, and Hayama Heart Center, Kanagawa, Japan. Sixty-five patients with active sarcoidosis who attended our hospital and/or Hayama Heart Center between 2002 and 2010 were enrolled in the current study. Among these patients, 12 (6 men and 6 women) were diagnosed as having cardiac sarcoidosis according to the diagnostic guidelines proposed by the Japan Society of Sarcoidosis and Other Granulomatous Disorders [13].

2.2. Laboratory measurements

Serum IgG4 levels, soluble interleukin 2 receptor (sIL-2R), and C-reactive protein (CRP) were measured by turbidimetry (SRL, Tokyo, Japan), enzyme-linked immunosorbent assay, and a latex agglutination immunophotometric assay. Serum levels of angiotensin-converting enzyme (ACE) were measured by the Kasahara method [14]. The upper reference ranges of IgG4, sIL-2R, and ACE were 105 mg/dL, 519 U/mL, and 21.4 U/L, respectively.

2.3. Histologic and immunohistochemical examinations

Biopsy specimens of 5 of the 12 patients with cardiac sarcoidosis enrolled in the study were available. In addition, a biopsy specimen was available for 7 other patients with cardiac sarcoidosis. Specimens of left ventricular (LV) myocardium or lymph nodes were obtained from surgically excised LV muscles, biopsy, or autopsy. Sections of paraffin-embedded specimens with a thickness of 4 to 6 μm were incubated with antibodies against cell surface markers (CD45 [Leica, Newcastle, UK], CD38 [Leica], and CD68 [DAKO, Glostrup, Denmark]) or IgG4 (Cappel, Cochranville, PA), and antigens were visualized by using the 3,3-diaminobenzidine tetrahydrochloride method (ScyTek Laboratories, Logan, UT).

Table 2 Biomarkers

Characteristics	Cardiac involvement (+) (n = 12)	Cardiac involvement (−) (n = 53)	*P*
Immunoglobulin G4 (mg/dL)	53.5 ± 48.5	57.5 ± 42.1	NS
sIL-2R (U/mL)	910 ± 683	689 ± 399	NS
ACE (U/mL)	19.1 ± 10.8	19.4 ± 7.5	NS

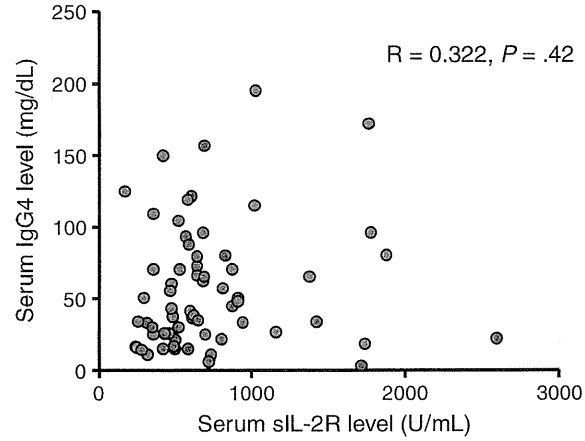

Fig. 1 Scatter plot of serum IgG4 and sIL-2R levels in patients with sarcoidosis. Patients with cardiac sarcoidosis and non–cardiac sarcoidosis patients are represented by red and blue circles, respectively.

2.4. Statistical analysis

Data are expressed as mean ± SD for continuous variables and as number (percentage) for categorical variables. Spearman correlation analysis was performed to estimate correlations between variables. Comparison between 2 groups was performed by Wilcoxon rank sum test or unpaired Student *t* test. $P < .05$ was considered to be statistically significant.

3. Results

3.1. Patient characteristics

The mean age and prevalence of male sex did not significantly differ between the groups (Table 1). Compared with non–cardiac sarcoidosis patients, heart failure, complete atrioventricular block, and ventricular tachycardia were found to be more prevalent in patients with cardiac sarcoidosis. Use of cardiac medications, such as β-blockers, diuretics, digitalis, spironolactone, and amiodarone, was more frequent in patients with cardiac sarcoidosis.

3.2. Laboratory data

Serum levels of CRP, aspartate aminotransferase, blood urea nitrogen, and creatinine were significantly higher in patients with cardiac sarcoidosis. No statistical difference was found between serum levels of IgG4, sIL-2R, and ACE of patients with cardiac sarcoidosis and non–cardiac sarcoidosis patients (Table 2). Among the 12 patients with cardiac sarcoidosis, 2 (17%), 9 (75%), and 3 (25%) had IgG4, sIL-2R, and ACE levels higher than the upper normal

Table 3 Histologic and immunohistochemical findings in tissues from patients with cardiac sarcoidosis

Age			Tissue examined	Sampling	Preoperative clinical diagnosis	Echocardiogram		Serum data		Immunohistochemistry			
Case	(y)	Sex				LV diastolic diameter	LVEF (%)	IgG4 (mg/dL)	sIL-2R (U/mL)	CD38	CD68	CD45	IgG4
1	60	M	Myocardium	Excised LV	DCM	88	24	NA	NA	1+	3+	3+	-
2	63	M	Myocardium	Excised LV	DCM	85	21	NA	NA	2+	3+	3+	1+
3	51	F	Myocardium	Excised LV	Unclassified CM	62	29	NA	NA	2+	3+	3+	1+
4	51	F	Myocardium	Excised LV	DCM	68	35	NA	NA	1+	3+	3+	-
5	36	M	Myocardium	Excised LV	DCM	73	8	23.3	2600	2+	3+	3+	1+
6	45	M	Myocardium	Excised LV	DCM	81	11	121	601	2+	2+	3+	-
7	49	F	Myocardium	Biopsy	Unclassified CM	66	24	21.4	491	2+	3+	3+	-
8	55	M	Myocardium	Biopsy	DCM	69	26	NA	NA	NA	2+	3+	-
9	48	F	Myocardium	Excised LV	DCM	83	28	NA	NA	1+	3+	3+	-
10	43	F	Myocardium	Autopsy	DCM	71	22	NA	NA	1+	2+	2+	-
11	72	M	Lymph nodes (mediastinal)	Biopsy	DCM, MDL	56	41	14.6	577	2+	3+	3+	-
12	54	F	Lymph nodes (cervical)	Biopsy	Unclassified CM	44	40	93.5	567	2+	3+	3+	1+

Abbreviations: LV, left ventricle; DCM, dilated cardiomyopathy; CM, cardiomyopathy; MDL, mediastinal lymphadenopathy; LVEF, LV ejection fraction; IgG, immunoglobulin G; and NA, not assessed.

limit, respectively. Among the non–cardiac sarcoidosis patients, 7 (13%), 32 (60%), and 22 (42%) had IgG4, sIL-2R, and ACE levels higher than the upper normal limit, respectively. The correlation between IgG4 and sIL-2R was found to be nonsignificant (Fig. 1).

3.3. Histologic and immunohistochemical analysis

Of the 12 patients whose serum IgG4 levels were available, the histologic specimens of 5 patients were also available (Table 3). The gross photograph from one of the patients (case 10 in Table 3) demonstrates dilated and partially thinned LV wall with scar lesion formation, and sarcoid granulomas and interstitial fibrosis are shown microscopically in these lesions (Fig. 2). In the patients listed in Table 3, the cardiac tissue showed sarcoid granulomas with multinucleated giant cells in cases 1 to 10 (Supplementary Figure). On the other hand, in cases 11 and 12, the cardiac tissues had been obtained by endomyocardial biopsy, which showed granulomatous degeneration with inflammatory cell infiltrates, and multinucleated giant cells that occasionally contained asteroid bodies can be observed. In the latter 2 cases, although multinucleated giant cells were not apparent (Supplementary Figure), cardiac sarcoidosis was diagnosed with the histopathologic findings in lymph node tissues showing sarcoid granulomas and the presence of cardiomyopathy. In the tissue sample of a patient (case 6), who had slightly elevated levels of IgG4 and sIL-2R, increased infiltration of CD45-positive T lymphocytes and CD68-positive macrophages was observed. In contrast, CD38-positive B lymphocytes and IgG4-positive cells were sparse (Fig. 3). In lymph node specimens from another patient with cardiac sarcoidosis (case 11) who had normal serum IgG4 levels, increased infiltration of CD45-positive T lymphocytes and CD68-positive macrophages was found; however, IgG4-positive cells as well as CD38-positive B lymphocytes were sparse (Fig. 4). IgG4 staining of heart specimens that were judged to be 1+ (Table 3) is shown in Fig. 5.

4. Discussion

In the current study, we measured serum IgG4 levels in 65 patients who were diagnosed with sarcoidosis. The mean serum IgG4 level was 56.8 ± 43.0 mg/dL, and 9 (14%) patients had an IgG4 level above the upper reference range (105 mg/dL). In addition, among the 12 patients with cardiac sarcoidosis, 2 (17%) had an IgG4 level above the upper reference range. The mean IgG4 level did not differ significantly between patients with cardiac sarcoidosis and non–cardiac sarcoidosis patients. On the other hand, 41 (63%) of the 65 patients with sarcoidosis had increased serum levels of sIL-2R, a marker that may reflect sarcoidosis

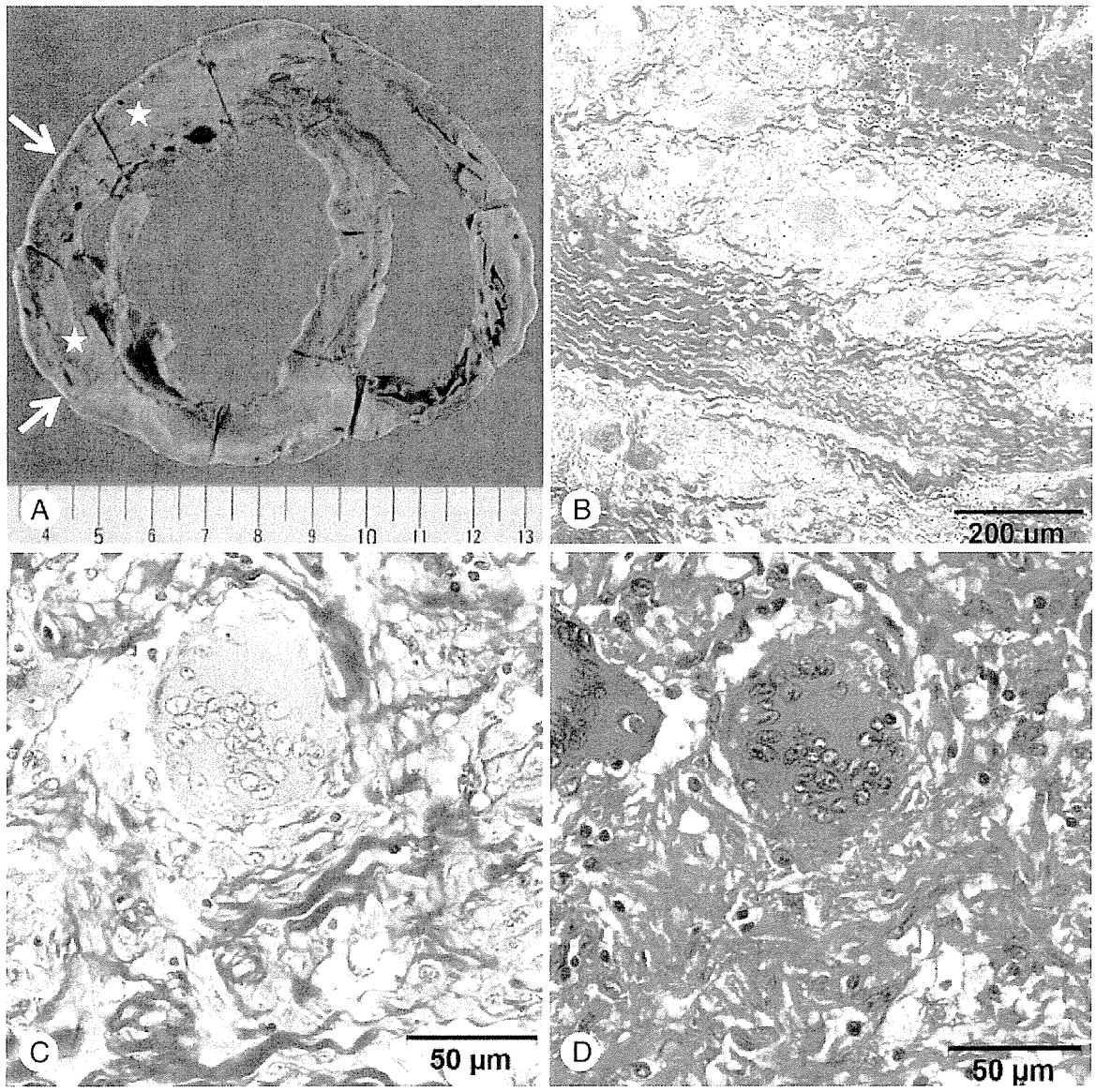

Fig. 2 Gross anatomy and histologic findings of cardiac sarcoidosis (case 10 in Table 3). A, Macroscopic analysis. The dilated LV wall showed thinning (arrows), and the scar-like white lesion was observed (asterisks). B, C, and D, Microscopically, many sarcoid granulomas and interstitial fibrosis were evident. B and C, Masson-trichrome staining. D, Hematoxylin-eosin staining. (Original magnification: ×100 in B and ×400 in C and D.)

activity [15], although the mean sIL-2R level did not differ corresponding to the presence or absence of cardiac involvement. Together with the finding that the relationship between serum IgG4 and sIL-2R was insignificant, these results suggest that serum IgG4 levels may not represent a biomarker for or reflect the disease activity of sarcoidosis, regardless of cardiac involvement.

IgG4-related sclerosing disease is a newly emerging disease entity, and a certain fraction of various lymphoproliferative disorders, such as Mikulicz disease, Sjoegren syndrome, and Castleman disease, may be IgG4 related [3,16]. However, whether IgG4-related sclerosing disease is truly a separate clinical entity needs to be elucidated, and if yes, the extent covered by this disease requires further evaluation to avoid diagnostic confusion [16]. We propose the possibility that other disorders that occasionally present similar clinical features, such as sarcoidosis, Wegener granulomatosis, and malignant lymphoma, should be ruled out before the diagnosis of IgG4-related sclerosing disease or alternatively, IgG4+ multiorgan lymphoproliferative disease is made [5].

As previously discussed, sarcoidosis is one of the diseases that should be distinguished from IgG4-related sclerosing disease. Whether a certain fraction of IgG4-related sclerosing disease has been misdiagnosed as sarcoidosis or whether a certain fraction of sarcoidosis overlaps with IgG4-related

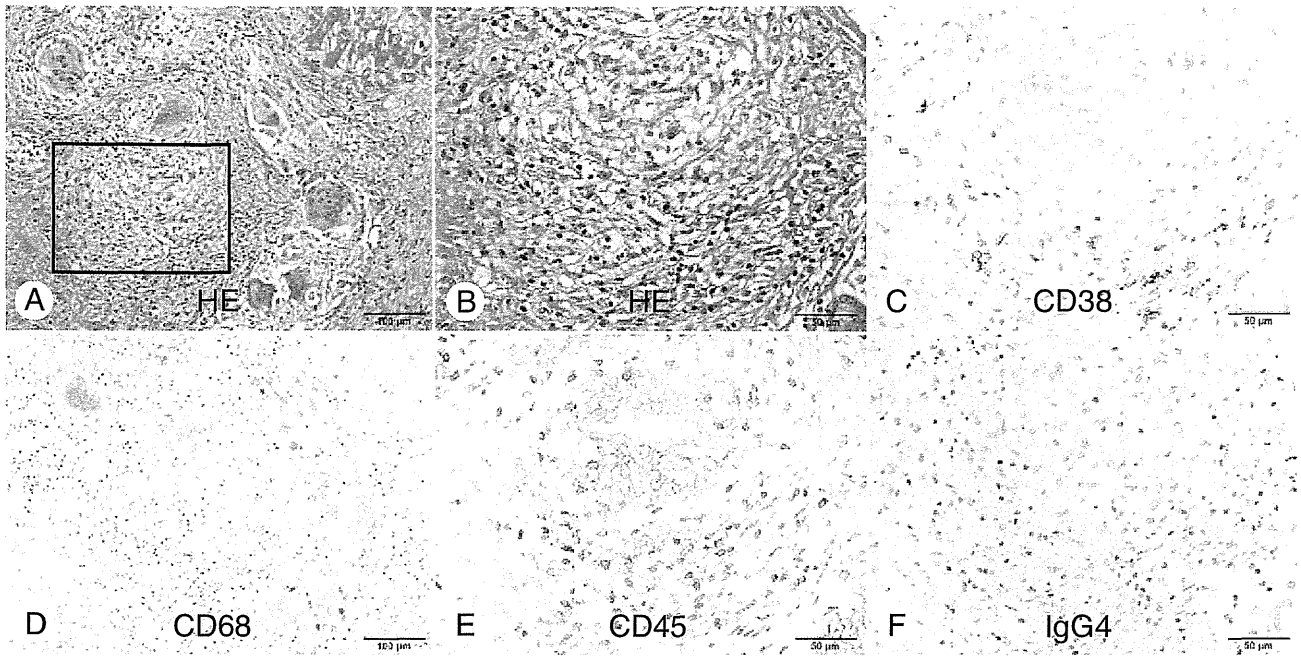

Fig. 3 Histologic and immunohistochemical analyses of the cardiac specimen from a patient with cardiac sarcoidosis (case 6 in Table 3). All panels were from serially cut sections. A, Hematoxylin and eosin staining. Large noncaseating sarcoid granulomas are observed. They are mainly composed of lymphocytes, macrophages, multinucleated giant cells, and interstitial components. B, Higher-magnification image of the boxed area in A. C, CD38 staining showing CD38-positive B cells. D, CD68 staining. CD68-positive macrophages and multinucleated giant cells are observed. E, CD45 staining showing CD45-positive T cells. F, IgG4 staining. (Original magnification: ×40 in A and D and ×100 in B, C, E, and F.)

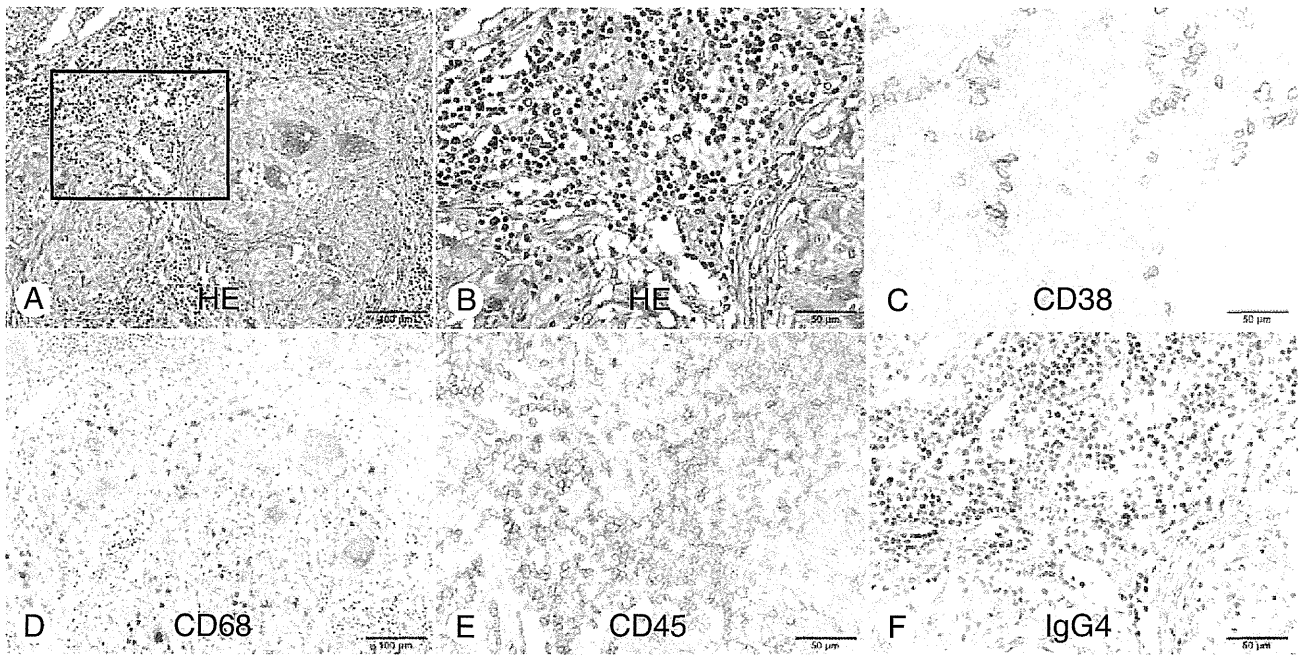

Fig. 4 Histologic and immunohistologic analyses of the mediastinal lymph node from a patient with cardiac sarcoidosis (case 11 in Table 3). All panels were from serially cut sections. A, Hematoxylin and eosin staining. B, Higher-magnification image of the boxed area in A. C, CD38 staining. D, CD68 staining. CD68-positive macrophages and multinucleated giant cells are observed. E, CD45 staining. F, IgG4 staining. (Original magnification: ×40 in A and D and ×100 in B, C, E, and F.)