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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

Kazuko Tajiri,*,† Kyoko Imanaka-Yoshida,‡,§ Akihiro Matsubara,*,¶ Yusuke Tsujimura,* Michiaki Hiroe,‖ Tetsuji Naka,# Nobutake Shimojo,† Satoshi Sakai,† Kazutaka Aonuma,† and Yasuhiro Yasutomi*,¶

**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

*Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki 305-0843, Japan; †Department of Cardiovascular Medicine, Majors of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; ‡Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan; §Mie University Matrix Biology Research Center, Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan; ¶Division of Immunoregulation, Department of Molecular and Experimental Medicine, Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan; ‖Department of Cardiology, National Center for Global Health and Medicine, Shinjuku, Tokyo 162-8655, Japan; and #Laboratory of Immune Signal, National Institute of Biomedical Innovation, Ibaragi, Osaka 565-0871, Japan

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Address correspondence and reprint requests to Dr. Yasuhiro Yasutomi, Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan. E-mail address: yasutomi@nibio.go.jp

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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 $2.5 \times 10^5$ 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 $1.5 \times 10^7$ $CD4^+CD62L^+$ 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 $0.25 \times 10^5$ 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 $5 \times 10^6$ $CD4^+$ 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

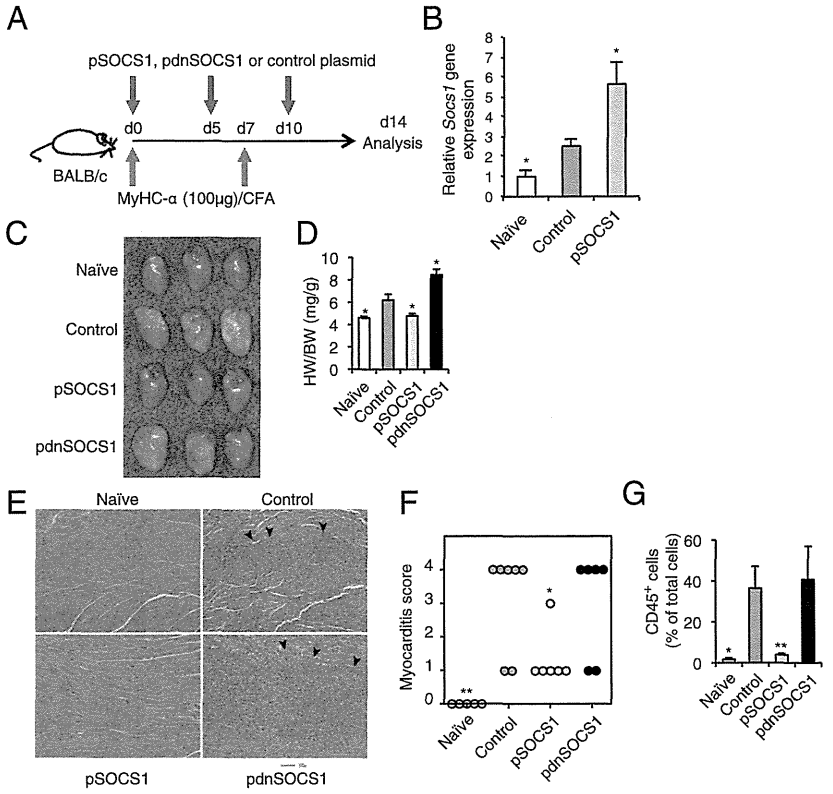


**FIGURE 1.** Amelioration of EAM and heart failure by SOCS1 DNA administration. (**A**) BALB/c mice were immunized twice, on days 0 and 7, with 100 μg of MyHC-α and treated with pSOCS1, pdnSOCS1, or control plasmid on days 0, 5, and 10. (**B**) QRT-PCR for the *Socs1* gene. RNA samples were obtained 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

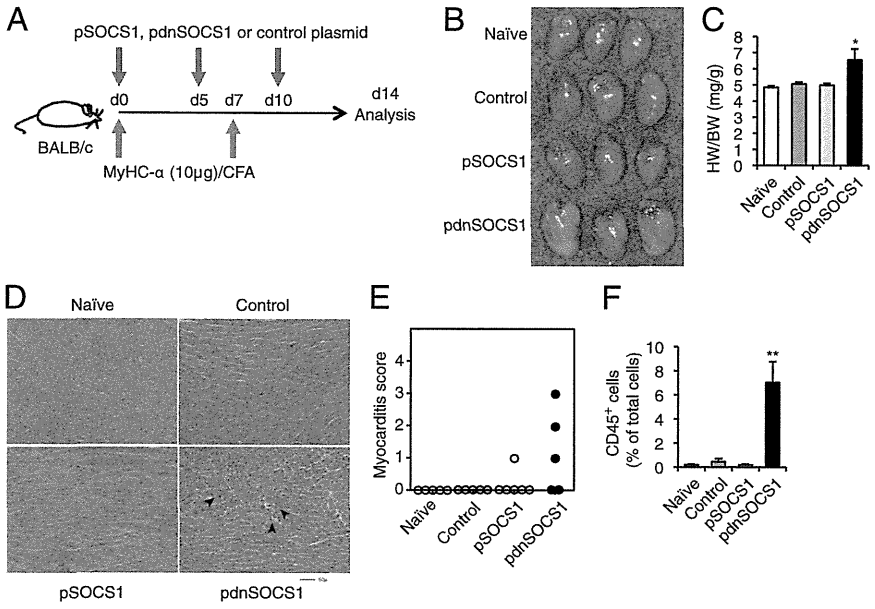


**FIGURE 2.** Increased susceptibility to EAM induced by inhibition of SOCS1. (**A**) Mice were immunized twice, on days 0 and 7, with 10 μg of MyHC-α emulsified 1:1 in PBS/CFA and treated with pSOCS1, pdnSOCS1, or control plasmid on days 0, 5, and 10. (**B**) Representative gross hearts (day 14) of 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

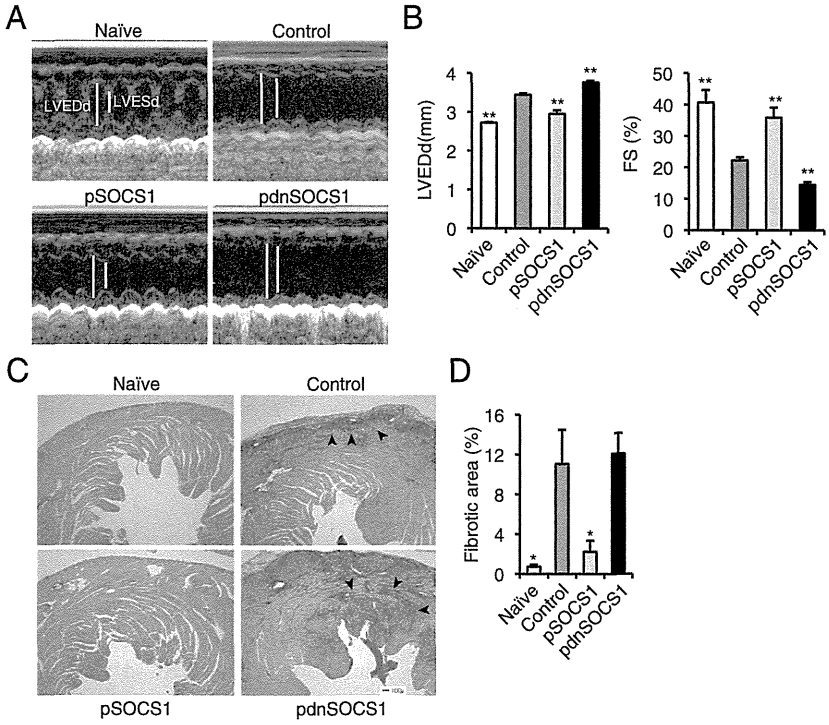


**FIGURE 3.** SOCS1 DNA administration prevents progression to heart failure. (**A** and **B**) Echocardiography was performed on naive and immunized mice on day 35. (A) Representative M-mode echocardiograms. Bars indicate LVESd and LVEDd. Bar graphs (B) represent LVEDd and percentage of FS from the 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.

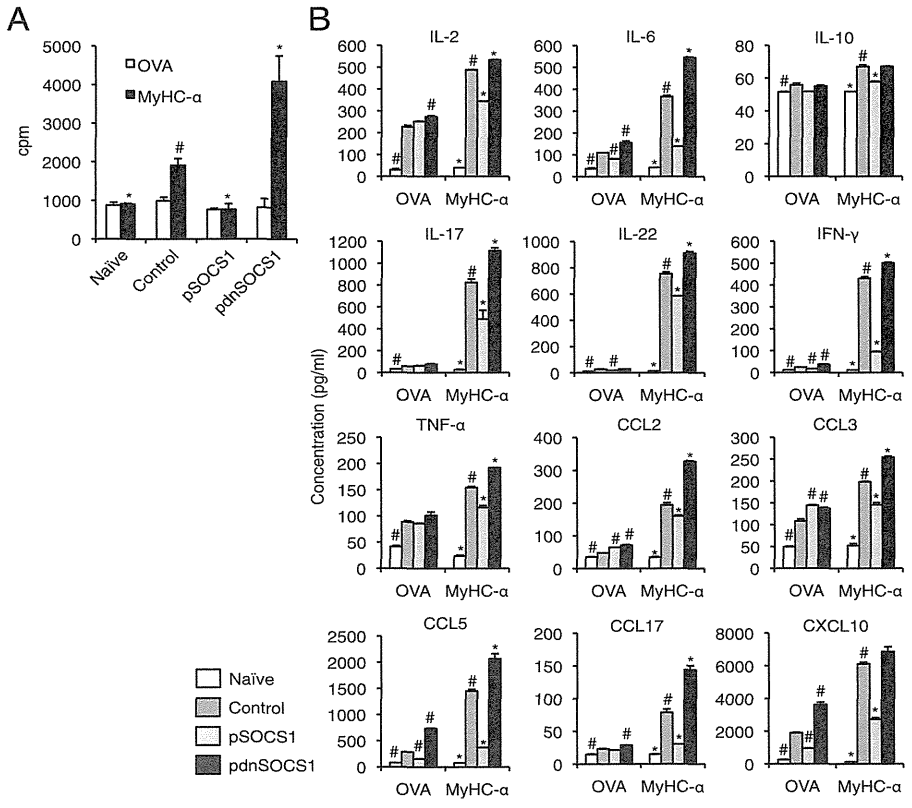


**FIGURE 4.** Impaired expansion of heart-specific $CD4^+$ T cells in pSOCS1-treated mice. (**A**) Splenocytes were isolated from naive and EAM mice treated with pSOCS1, pdnSOCS1, or control plasmid on day 14 and restimulated in vitro with MyHC-α or OVA peptide for 72 h. Proliferation was assessed by 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

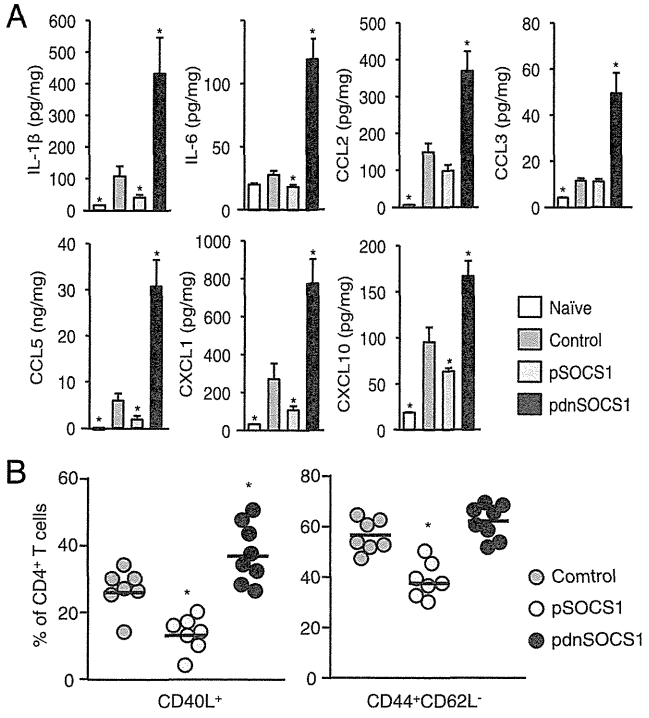


**FIGURE 5.** Cytokine and chemokine responses and $CD4^+$ T cell differentiation in the heart. (**A**) Myocardial tissues were homogenated and processed by ELISA to detect cytokines and chemokines on day 14. Bar graphs show group means ± SEM of 8–16 mice/group. Results of one of three representative 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-

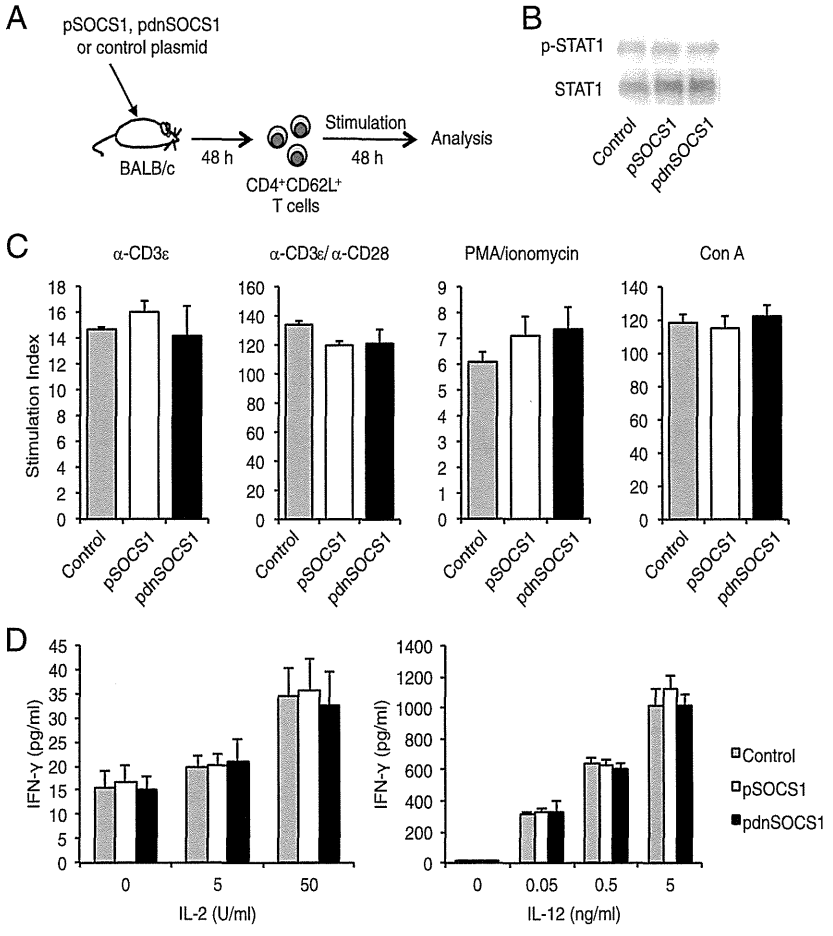


**FIGURE 6.** Primary responses of $CD4^+$ T cells from pSOCS1-, pdnSOCS1-, and control plasmid-treated mice. (**A**) $CD4^+CD62L^+$ T cells from mice injected with pSOCS1, pdnSOCS1, or control plasmid were stimulated with IFN-γ, anti-CD3ε, anti-CD3ε, anti-CD28, PMA/ionomycin, and Con A in the presence 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

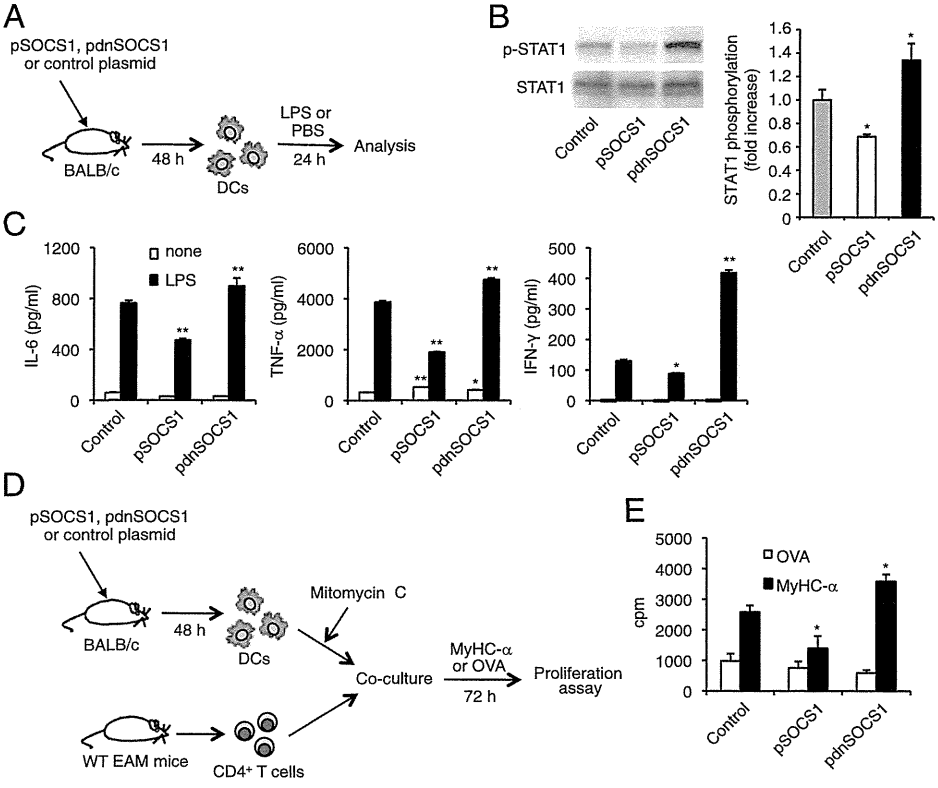


**FIGURE 7.** Functional capacities of DCs from pSOCS1-, pdnSOCS1-, and control plasmid-treated mice. (**A**) DCs from mice treated with pSOCS1, pdnSOCS1, or control plasmid were stimulated with LPS for 24 h. (**B**) STAT1 phosphorylation of DCs was assessed by Western blotting. Densitometry ratios of 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-

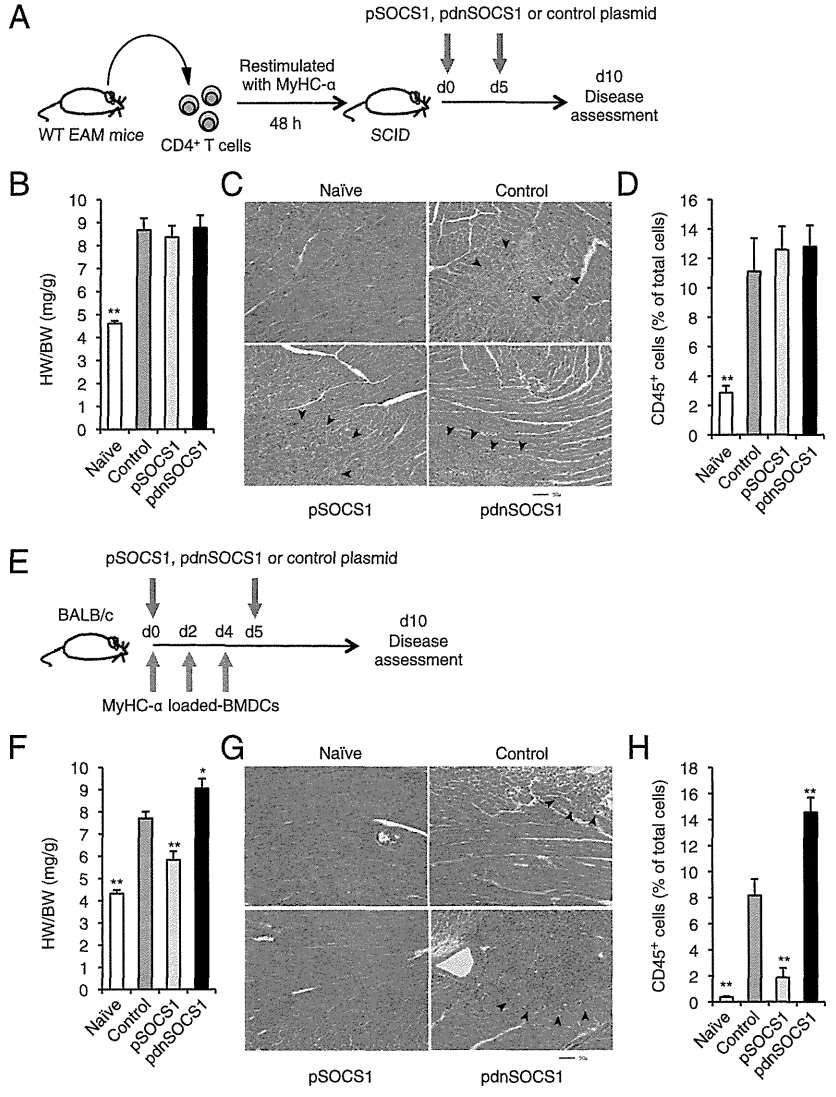


**FIGURE 8.** pSOCS1 administration inhibited the development of myocarditis induced by cardiac Ag-loaded BMDC injection but not by heart-specific $CD4^+$ T cells. (**A–D**) $CD4^+$ T cells were purified from diseased mice and restimulated in vitro with MyHC-α for 48 h before transfer into SCID recipients. 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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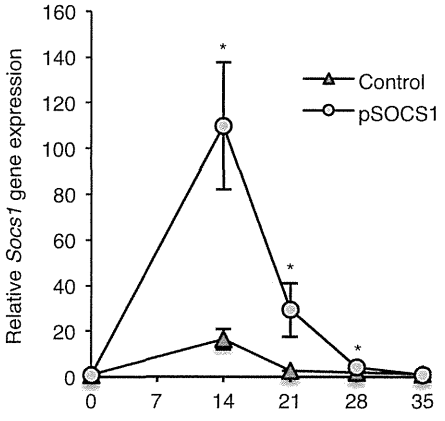
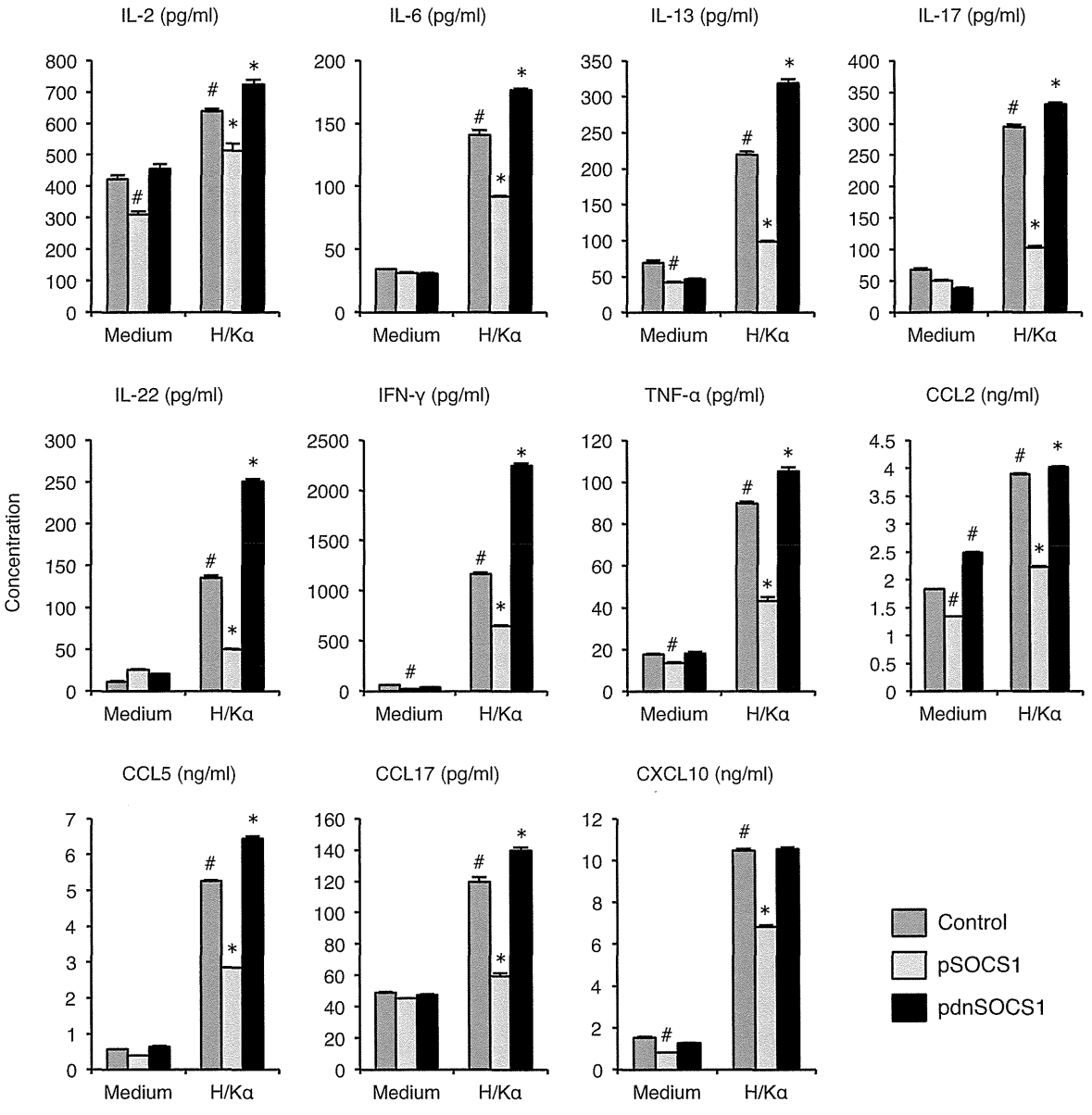
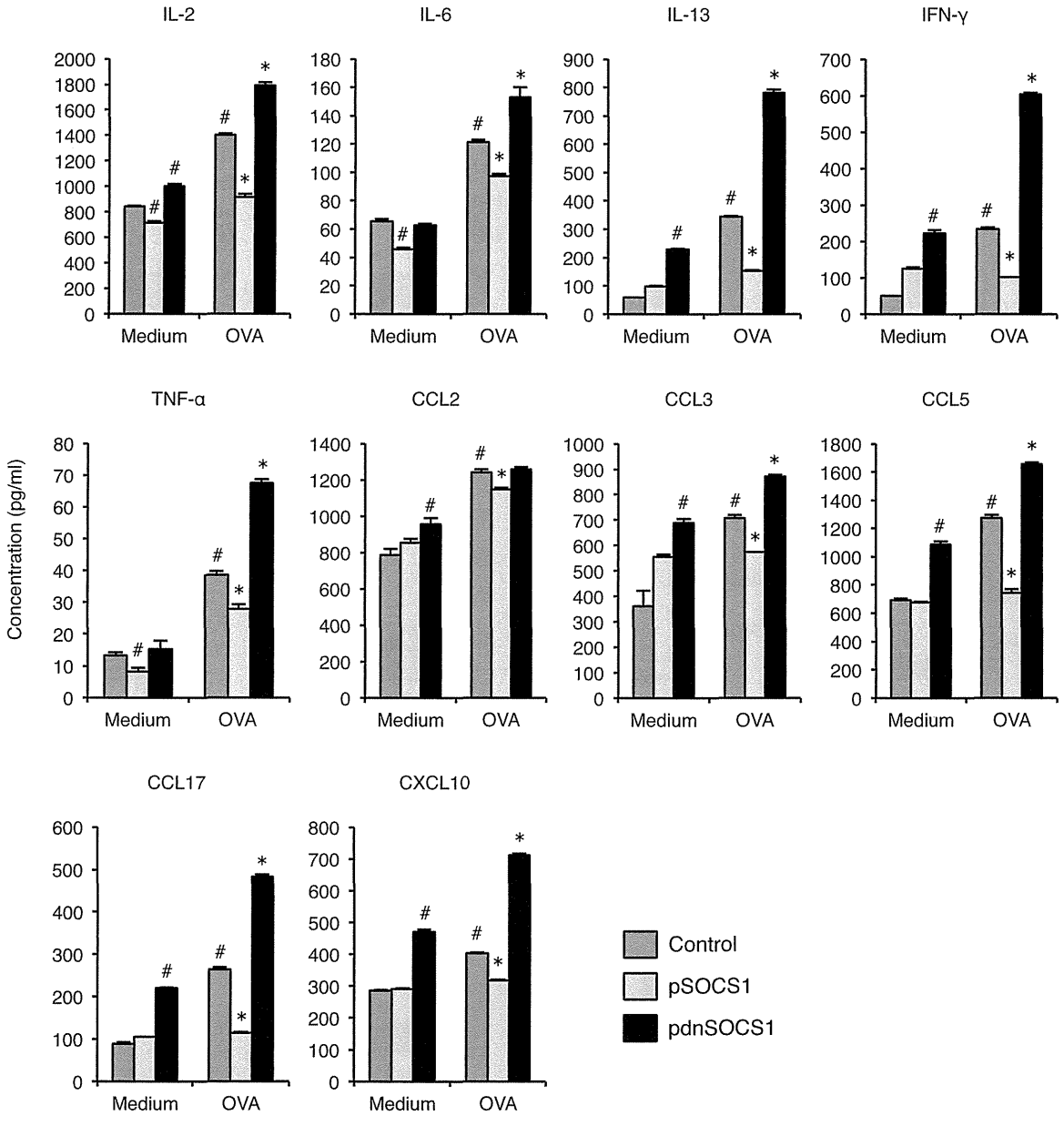



**Supplementary Figure 1. *Socs1* gene expression in the heart.** RNA samples were obtained from EAM hearts on days 0, 14, 21, 28 and 35, and used as a template for QRT-PCR. Results represent the average gene induction in five to six independent heart samples. Results of one of two representative experiments are shown. *$P < 0.05$ compared to control.




**Supplementary Figure 2. Impaired cytokine production by $H^+/K^+$ ATPase α (H/Kα)-specific $CD4^+$ T cells in pSOCS1-treated mice.** BALB/c mice were immunized twice, on days 0 and 7, with 100 μg of H/Kα p253-277 in an emulsion with CFA and treated with pSOCS1, pdnSOCS1 or control plasmid on days 0, 5 and 10. Splenocytes were isolated from mice on day 14 and cultured in the absence or presence of H/Kα peptide (1 μg/ml) for 72 h. Cytokines and chemokines in the culture supernatants were measured by ELISA. Data are expressed as mean ± SEM from triplicate culture wells. Results of one of two representative experiments are shown. $*P < 0.05$ compared to H/Kα stimulated control and $\#P < 0.05$ compared to unstimulated control.




**Supplementary Figure 3. Impaired cytokine production by OVA-specific CD4$^+$ T cells in pSOCS1-treated mice.** BALB/c mice were immunized twice, on days 0 and 7, with 100 μg of OVA p323-339 in an emulsion with alum and treated with pSOCS1, pdnSOCS1 or control plasmid on days 0, 5 and 10. Splenocytes were isolated from mice on day 14 and cultured in the absence or presence of OVA peptide (5 μg/ml) for 72 h. Cytokines and chemokines in the culture supernatants were measured by ELISA. Data are expressed as mean ± SEM from triplicate culture wells. Results of one of two representative experiments are shown. *$P < 0.05$ compared to OVA stimulated control and #$P < 0.05$ compared to unstimulated control.

—Mini Review—

# Cryopreservation of the Ovary

Tadashi Sankai[1]*, Norio Owada[2] and Koichi Kyono[3]

[1]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Ibaraki 305-0843, Japan
[2]ABI Co. Ltd., Chiba 270-0133, Japan
[3]Kyono ART Clinic, Miyagi 980-0014, Japan

**Abstract:** The removal, cryopreservation, and subsequent reimplantation of ovaries would make it possible to treat a young cancer patient and improve her quality of life by preserving her fertility. The current technology requires cutting the ovary into pieces before freezing and does not support preservation of the whole ovary. The ovary has a complex endocrinologic function. It is composed of cells of different form and character and contains oocytes at various stages of development. Successful cryopreservation, transplantation, and functional rehabilitation of the whole ovary would have broad significance, not only for ovaries but also for other organs such as the liver, kidney, and heart. Ovarian cryopreservation technology would lead the way to the establishment of a biological bank for frozen internal organs.

**Key words:** **********, **********, **********

## Introduction

Cryopreservation of living cells is an established technology, and the cell banking system provides a source of materials for all aspects of medical research. Clinically, the freezing of mature unfertilized eggs and fertilized eggs has been widely applied to human fertility treatments. What is the difference between freezing ovaries and freezing cells, oocytes, and preimplantation embryos? The difference is the size of the sample. Internal organs are composed of various tissues, and tissues are formed by cells. The egg is the largest cell in the body, but the internal organs are much larger. The size of the sample for freezing affects such factors as temperature change, infiltration of the cryoprotectant, and generation of ice crystals. Recent advances in technology have increased the opportunity to freeze whole ovaries. However, there are still problems to be solved. Here we discuss the current state of the technology for the cryopreservation of whole ovaries.

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*To whom correspondence should be addressed.
e-mail: sankai@nibio.go.jp

## Utility of Ovary Freezing

Cryopreservation of the ovary is useful for preserving resources in research using laboratory animals as disease models and for transgenic studies. Clinically, this technology is shifting from the research area to practical uses for maintenance of fertility and improving the quality of life of cancer patients [1–11]. It has even been applied to patients with Turner syndrome, whose ovarian follicles are lost with age. In younger patients, ovary freezing could be used when the vaginal collection of eggs is difficult. At present, human fertility is supported by the use of assisted reproductive techniques such as *in vitro* fertilization and intracytoplasmic sperm injection into mature or immature oocytes. The probability of achieving conception depends on the method of egg collection before the ovary is frozen. Therefore, ovary freezing will expand the range of current applications in the medical technology.

## Current State of Ovary Cryopreservation

Ovary cryopreservation was developed using animals [12–16] such as mice and monkeys. Ovary freezing is believed to be a useful means for preserving reproductive cells, but the technology to achieve conception is not yet at the practical stage. The first child to be born from the transplantation of frozen-thawed sliced ovaries was reported in 2004 [3, 4]. Since then, human births resulting from the use of this technology have been widely reported [5–7]. For

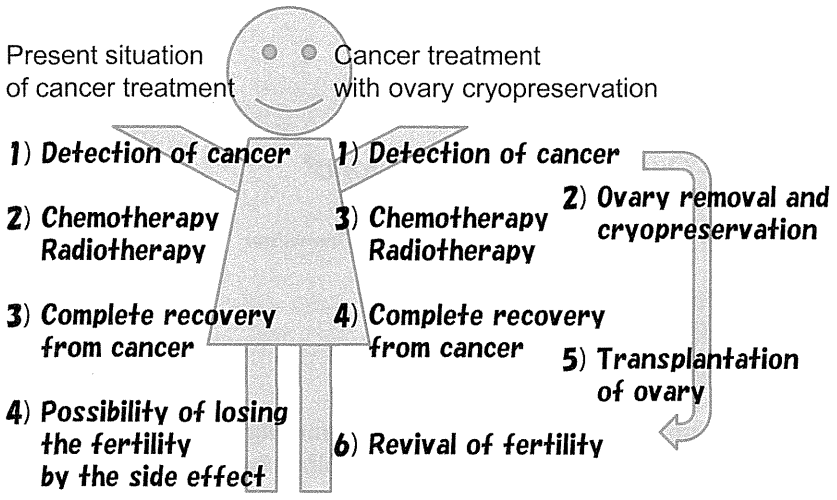


**Fig. 1.** Example of the application of ovary cryopreservation. The fertility of a cancer patient may be lost due to the side-effects of the treatment of the cancer cells with chemotherapy and radiotherapy (Left side). Before the treatment of the cancer, the ovary can be removed and preserved (Right side).

cancer patients, the ovaries are removed before the start of cancer treatment, and the frozen-thawed ovaries are retransplanted to the patient when the cancer has been successfully treated, because there is a possibility that the ovarian germ cells may undergo damage along with the cancer cells during treatment. One of the new cancer treatments preserves the fertility of the patient, but this technique is complicated (Fig. 1).

The current method for ovary freezing involves slowly freezing slices of the ovary. This method was devised to improve the success rate of freezing because the whole ovary is too large to be frozen using current methods. It is common to use a cryoprotectant for cell freezing. By slicing off a large piece of ovary tissue or cutting it into smaller pieces, the effect of the cryoprotectant is known. However, there can be physiological and biological problems with slicing or cutting the ovary. The organ slice begins to lose cells at the contact site of the slice with the screen insert, and thereby reduces organ slice architectural stability and viability. We believe that many problems can be avoided if it were possible to freeze the whole ovary, and into whole ovary preservation has already begun.

## Theme for the Future

Thinly slicing the ovary or cutting it into small pieces is an excellent way for the cryoprotectant to infiltrate the specimen and for an even temperature to be maintained. However, the number of ovarian follicles that exist in a cut ovary (10 mm × 10 mm × 1 mm) is thought to be limited, and many of them can be lost during the process of freezing, thawing and transplantation. It seems that small ovarian follicles (such as a primordial ovarian follicle) can survive. Therefore, it is currently necessary to wait for several months to confirm the functionality of a transplanted ovary. Furthermore, the long-term maintenance of ovarian function cannot be guaranteed. It is necessary to analyze in detail the living cells in the thawed ovary. Cells (oocytes) at various stages and cells with endocrinologic function exist in the ovary. The state of each cell cannot always be determined. Moreover, it will be necessary to verify the effect of cryoprotectant and its side-effects after transplantation.

It is not known why a transplanted ovary sometimes does not function. Therefore, the problems of cryopreservation cannot be solved by focusing on the freezing technology alone. It is also necessary to perfect the thawing method. In the thawing method, it is extremely difficult to thaw both the inside and the outside of large internal organs under the same conditions. It is difficult to achieve success according to the fundamental principles of physics in freezing theory, even though the theory has been clarified [17–23]. When one does not achieve an excellent result by analyzing a frozen-thawed organ transplantation sample, it should be considered that there should be

problems in both the freezing and the thawing technology. Thus, the further development of thawing technology is an important area of research.

Moreover, the implantation technique cannot be disregarded. It is necessary to make blood circulate within the ovary several hours or a few days after transplantation of the ovary. If the nutritional content of the cells is not supplied with blood, an individual cell cannot survive at body temperature. Ideally, each ovary is frozen with a blood vessel, and the blood vessel is sutured during transplantation. We are attempting to improve the method of cryopreservation of blood vessels to solve this problem.

In one clinical application of cryopreservation, the ovaries of female cancer patients are removed and frozen before cancer treatment begins, and then the ovary is transplanted after the cancer has been successfully treated. There will be no cancer cells in the ovaries, but because of the possibility of minimal residual disease (MRD), it is necessary to assess the transplant carefully. A new technology for MRD detection needs to be developed [24, 25]. Furthermore, an ovum, embryo or ovary should be selected for cryopreservation according to the patient's age and the degree of damage to the ovary, because of the close relationship between the age of a woman and the degree of ovarian function.

It is necessary to select the appropriate treatment for each patient who desires a natural pregnancy, such as assisted reproductive techniques involving ovary removal on only one side, whole ovary removal, or partial excision. Moreover, data should be accumulated about each technique to improve the survival rate of the ovary after transplantation and the timing of retransplantation to maintain ovarian function at the site of the graft.

## Upgrade of Technology and the Importance of Education

Theoretically, it is effective to freeze the whole ovary and to keep the ovary function long time when transplanting an ovary. If the loss of ovarian follicles can be reduced to the minimum, ovarian function should be maintained for a long time. To achieve this, it is necessary to develop freezing and thawing technologies for the whole ovary and to solve the problem of thrombosis during vascular anastomosis. The rate of pregnancy after such procedures is extremely low, although pregnancies have been reported in animals. However, live births after transplantation of the whole ovary have not been reported in humans [26].

Cryoprotectants cannot work properly on the whole ovary. However, the damage from ice crystals in cells can be reduced by slightly magnetizing the whole ovary during freezing, without a cryoprotectant. The following conclusions are possible: (i) a cryoprotectant is not needed, (ii) thrombosis can be prevented, and (iii) large growing follicles can survive. If this magnetization technique could be established, it would be possible to apply it not only to the ovary but also to other internal organs. This technology may even be useful in the field of organ transplantation.

Although research on the cryopreservation of internal organs started only recently, this technology should improve the ability to treat cancer in young patients and their quality of life after recovery [27, 28]. Medical researchers and physicians in various fields should cooperate in the development of this technology. We strongly encourage supplying accurate information about the technology not only to medical personnel but also to the general public.

## Possible Applications of Cryopreservation Technology in Other Fields, and Future Perspectives

The most important goal of cryopreservation technology is to maintain the function of the cell after freeze-thawing. The ovary is one of the sources of female reproductive cells and has an endocrinologic function. In other words, it is a complex internal organ that contains cells of various kinds and at various stages of development. Once the freezing of the ovary becomes feasible and the return to ovarian function after transplantation is certain, it may also be possible to freeze other internal organs such as the heart, liver, and kidneys. As we know that the cell-mediated immune response molecule, the major histocompatibility complex (MHC), varies greatly between individuals and mismatch of MHC antigen is an important factor in the acute rejection of the transplanted tissues. If an information bank of MHC antigen for frozen internal organs used in organ transplantation can be successfully developed, medical treatment with organ transplants could be greatly improved, since it is thought that a frozen internal organ banking system could serve the entire world (Fig. 2). In addition, the utility of the umbilical funiculus, including stem cells, is parallel to that of bone marrow stem cells. If freezing the umbilical funiculus were to become possible, the number of stem cells preserved would increase. Technological development might also

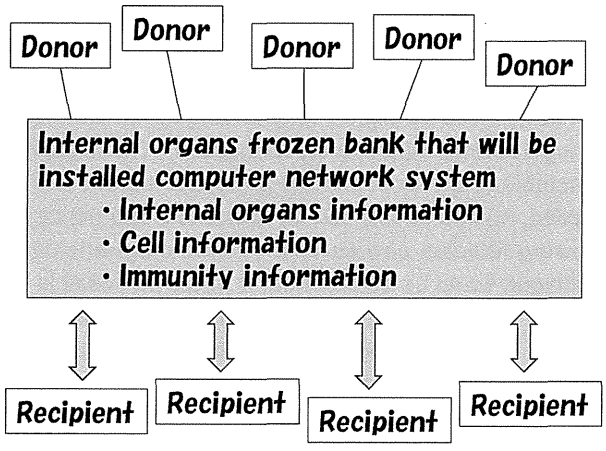


**Fig. 2.** Schematic diagram of an internal organ cryobank. Time can be saved in the transportation of internal organs and the matching of the donor and the recipient. The establishment of an organ cryobank would help to meet many of the challenges faced in transplantation of an organ between a donor and a recipient living elsewhere in the world.

enable the cryopreservation of blood.

Ovary cryopreservation technology contributes greatly to both laboratory animal research and clinical applications. Further advancement of organ cryopreservation technology is expected to occur through research into the freezing of different organs in the near future.

## Conclusion

The technology of ovary cryopreservation has progressed and has opened many possible doors. This technology will give hope to young women with cancer. Moreover, existing research suggests the possibility of constructing a bank for frozen internal organs.

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