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# Single systemic administration of Ag85B of mycobacteria DNA inhibits allergic airway inflammation in a mouse model of asthma

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**Abstract:** The immune responses of T-helper (Th) and T-regulatory cells are thought to play a crucial role in the pathogenesis of allergic airway inflammation observed in asthma. The correction of immune response by these cells should be considered in the prevention and treatment of asthma. Native antigen 85B (Ag85B) of mycobacteria, which cross-reacts among mycobacteria species, may play an important biological role in host-pathogen interaction since it elicits various immune responses by activation of Th cells. The current study investigated the antiallergic inflammatory effects of DNA administration of Ag85B from *Mycobacterium kansasii* in a mouse model of asthma. Immunization of BALB/c mice with alum-adsorbed ovalbumin followed by aspiration with aerosolized ovalbumin resulted in the development of allergic airway inflammation. Administration of Ag85B DNA before the aerosolized ovalbumin challenge protected the mice from subsequent induction of allergic airway inflammation. Serum and bronchoalveolar lavage immunoglobulin E levels, extent of eosinophil infiltration, and levels of Th2-type cytokines in Ag85B DNA-administered mice were significantly lower than those in control plasmid-immunized mice, and levels of Th1- and T-regulatory-type cytokines were enhanced by Ag85B administration. The results of this study provide evidence for the potential utility of Ag85B DNA inoculation as a novel approach for the treatment of asthma.

**Keywords:** immunotherapy, asthma, Ag85B, mycobacteria, allergy

## Introduction

Asthma is characterized by airway hyperresponsiveness to a variety of specific and nonspecific stimuli, chronic pulmonary inflammation with eosinophilia, excessive mucus production, and high serum immunoglobulin E (IgE) levels. T-helper-2 (Th2) cells are thought to play a crucial role in the initiation, progression, and persistence of asthma in association with the production of interleukin-4 (IL-4), IL-5, and IL-13.<sup>1-3</sup> Bronchoalveolar lavage (BAL) T-cells from human asthmatics have been reported to express elevated levels of IL-4 and IL-5 messenger ribonucleic acid (mRNA).<sup>4,5</sup> Although the correction of this deviation to Th2-type immune responses is considered to be necessary to achieve therapeutic and preventive effects on asthma, it is not sufficient to obtain therapeutic effects in many cases. Another subset of T-cells, T-regulatory (Treg) cells, has been reported to be important in the development of allergic diseases such as asthma.<sup>6</sup> Many studies have suggested that effective immunotherapy for allergic diseases is associated with immune deviation from a disease-promoting Th2 response towards a Th1 response, with Treg cells having appropriate functions.<sup>7</sup> However, the induction of both subsets of cells – Th1 and Treg cells – for the treatment of asthma using immunological strategic tools is very difficult.

Administration of mycobacteria, including the bacillus Calmette–Guerin, has been thought to be effective for preventing the development of asthma by induction of Th1-type immune responses and inhibition of IgE by the production of IL-21 from natural killer T-cells.<sup>8–10</sup> However, the relationship between bacillus Calmette–Guerin infection or mycobacteria immunization and asthma in humans is controversial because of the many causative factors affecting the induction of immune responses by mycobacteria, eg, human genetic background, mycobacteria strains, and environmental factors (reviewed in Arnoldussen et al).<sup>11</sup> From these findings, bacterial products from mycobacteria for immunotherapy against allergic disease should eliminate the harmful effects of host genetic factors, environmental factors, and strain specificity of mycobacteria.

Antigen 85B (Ag85B) is one of the most dominant protein antigens secreted from all mycobacterial species and has been shown to induce substantial Th cell proliferation and vigorous Th1 cytokine production.<sup>12</sup> Moreover, the induction of Th1-type immune responses by immunization of Ag85B was enhanced by presensitization with bacillus Calmette–Guerin.<sup>13,14</sup> From these findings, the effectiveness of Ag85B DNA as immunotherapy for tumor disease and as a vaccine adjuvant for infectious disease, by its ability to induce Th1-type immune responses, was also reported.<sup>13,14</sup> The current study investigated whether Ag85B DNA from *Mycobacterium kansasii* can inhibit the development of allergic airway inflammation as a novel immunotherapy.

## Material and methods

### Induction of allergic inflammation in mice

BALB/c female mice used in this study were handled according to ethical guidelines approved by the Institutional Animal Care and Use Committee of National Institute of Biomedical Innovation, Japan. The mice were sensitized to ovalbumin (OVA; Sigma-Aldrich, St Louis, MO) and challenged with aerosolized OVA according to a modification of the method of Nishikubo et al.<sup>15</sup> Briefly, mice were subcutaneously immunized with 10 µg OVA complexed with alum on days zero and 14. On days 21–25 after the first immunization, mice were challenged with an aerosol of 5% OVA in phosphate-buffered saline in a chamber for 20 minutes.

### Administration of DNA

Mice were intraperitoneally administered 50 µg plasmid DNA encoding Ag85B DNA once on day –7, zero, 14, or 21. An empty plasmid vector (pcDNA™ 3.1; Life Technologies, Carlsbad, CA) was used as a control (Figure 1A).

### BAL fluid collection

BAL fluid was obtained by injecting and recovering two 0.5 mL aliquots of phosphate-buffered saline via a tracheal cannula. BAL fluid and sera were collected 25 days after the first OVA immunization. Cells in the BAL fluid were counted using a hemacytometer, and the differentials were determined by utilizing light microscopy to count 300 cells on Cytospin® preparations (Thermo Fisher Scientific, Waltham, MA). The concentration of inflammatory protein was measured by Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA).

### Quantitation of IgE

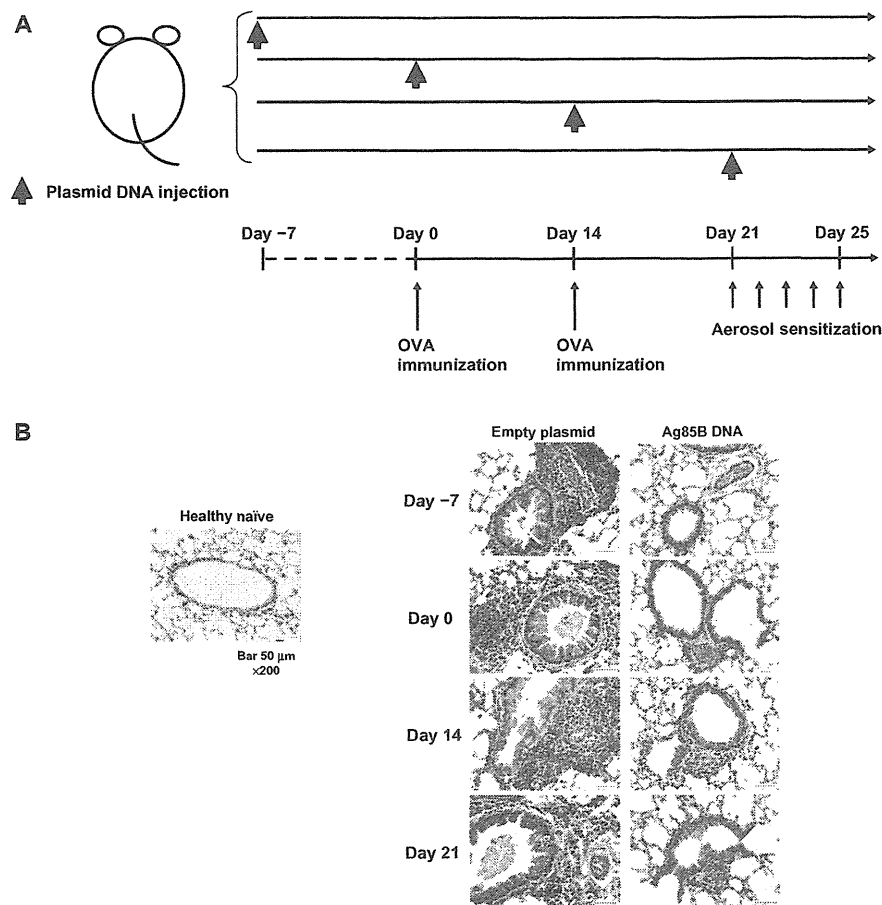
IgE levels in sera were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the procedure recommended by the manufacturer (Shibayagi Co, Ltd, Shibukawa, Japan).

### Determination of cytokine production

Lymphocytes obtained from thoracic lymph nodes of immunized mice ( $5 \times 10^6$ ) were cultured with 10 µg/mL OVA in 24-well culture plates at a volume of 2 mL. After incubation at 37°C in a humidified incubator (5% carbon dioxide) for 48 hours, culture supernatants were collected and analyzed for production of interferon-γ (IFN-γ; Life Technologies) or IL-4 (Quantikine®; R&D Systems, Minneapolis, MN) by an ELISA assay according to the manufacturer's protocol (Life Technologies). The amounts of IL-5 and IL-13 in BAL fluid were also measured by an ELISA kit (R&D Systems) 25 days after the first OVA immunization.

### Detection of cytokine mRNA from lymphocytes using real-time polymerase chain reaction

Total RNA was purified from OVA-stimulated or fetal calf serum (control)-stimulated spleen cells using Isogen (Nippon Gene Co, Ltd, Tokyo, Japan) following the manufacturer's instructions. For the real-time reaction, a reverse transcription system (Promega Corporation, Fitchburg, WI) was used. Polymerase chain reaction was performed in a total volume of 50 µL of 1 × polymerase chain reaction buffer (Takara Shuzo, Kyoto, Japan) containing 0.5–1.0 µg of complementary DNA, 0.25 mM of each deoxyribonucleotide triphosphate, 2 µM of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo). The specific primer pairs used were described previously.<sup>15</sup> The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 seconds at 56°C, extension for 1 minute at 73°C, and denaturation for 30 seconds at 93°C. The reaction products were



**Figure 1** Inhibition of the development of allergic inflammation in lungs by administration of Ag85B DNA vaccine. (A) Experimental design used to investigate the effects of Ag85B DNA vaccine on OVA-induced asthma. Mice were subjected to an OVA sensitization scheme,<sup>15</sup> and 50 μg of Ag85B DNA vaccine was intraperitoneally injected once on days -7, 0, 14, or 21. A control plasmid was also administered on the same day. (B) Results of histopathological examination of lungs of mice that had been administered Ag85B DNA or control DNA. All tissues were obtained 25 days after the first OVA immunization. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Abbreviations:** Ag85B, antigen 85B; OVA, ovalbumin.

analyzed on 2% agarose, Tris-buffered ethylenediaminetetraacetic acid gel. Photographs of the gels were scanned, and band intensities were measured using a densitometer (CS Analyzer 3.0; ATTO Corporation, Tokyo, Japan). The quantity of cytokine mRNA was determined by the ratio of cytokine and beta actin band intensities. The profiles shown are representative of three independent experiments.

### Histopathological examinations

Histopathological examinations of the lungs of the mice that had been administered Ag85B DNA or control DNA were performed. All tissues were obtained 25 days after the first OVA immunization. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Results for healthy naïve mice and control plasmid DNA-immunized mice are also shown.

### Statistical analysis

Statistical analyses were performed using the Mann–Whitney U test and the Kruskal–Wallis test. Values are expressed as mean ± standard deviation. A 95% confidence limit was considered to be significant ( $P < 0.05$ ).

### Results

#### Inhibition of the development of allergic inflammation in the lung by administration of Ag85B DNA

Mice were sensitized to OVA and challenged with aerosolized OVA as described previously.<sup>11</sup> These mice were intraperitoneally administered 50 μg plasmid DNA encoding Ag85B once on day -7, zero, 14, or 21. An empty plasmid vector (pcDNA 3.1) was used as a control (Figure 1A).

Histopathological examinations of the lungs of mice injected with Ag85B DNA or control DNA and the lungs of healthy naïve mice were performed 25 days after the first inoculation of the plasmid. The lungs of mice that were administered Ag85B DNA on days -7, zero, and 14 did not show any pathological abnormalities compared with those of healthy naïve mice, but the lungs of mice that were administered Ag85B DNA on day 21 showed mild inflammation due to infiltration of eosinophils (Figure 1B). Mice administered the control plasmid did not show any inhibitory effects on the development of allergic inflammations. These results indicated that Ag85B DNA administration was effective for inhibiting the development of allergic inflammation, especially in the early phase of antigen sensitization.

### Marked inhibition of allergic immune responses by administration of Ag85B DNA

The levels of protein, total cells, eosinophils, lymphocytes, and neutrophils in BAL fluid from mice immunized with Ag85B DNA vaccine were significantly lower than those in BAL fluid from mice vaccinated with control DNA (Figure 2A–F). Administration of Ag85B DNA also resulted in a significant reduction in the level of OVA-specific IgE (Figure 2G). The concentrations of Th2-type cytokines (IL-5 and IL-13) in BAL fluid from mice immunized with Ag85B DNA vaccine were significantly lower than those in BAL fluid from control mice (Figure 3A and B). These inhibitory effects on the development of allergic inflammation were correlated with day of Ag85B DNA injection. Injection on an early day was more effective for inhibiting the development of allergic inflammation. These results were also confirmed by histopathological observation.

### Effects of Ag85B DNA administration on the production of IL-4 and IFN- $\gamma$ in response to OVA

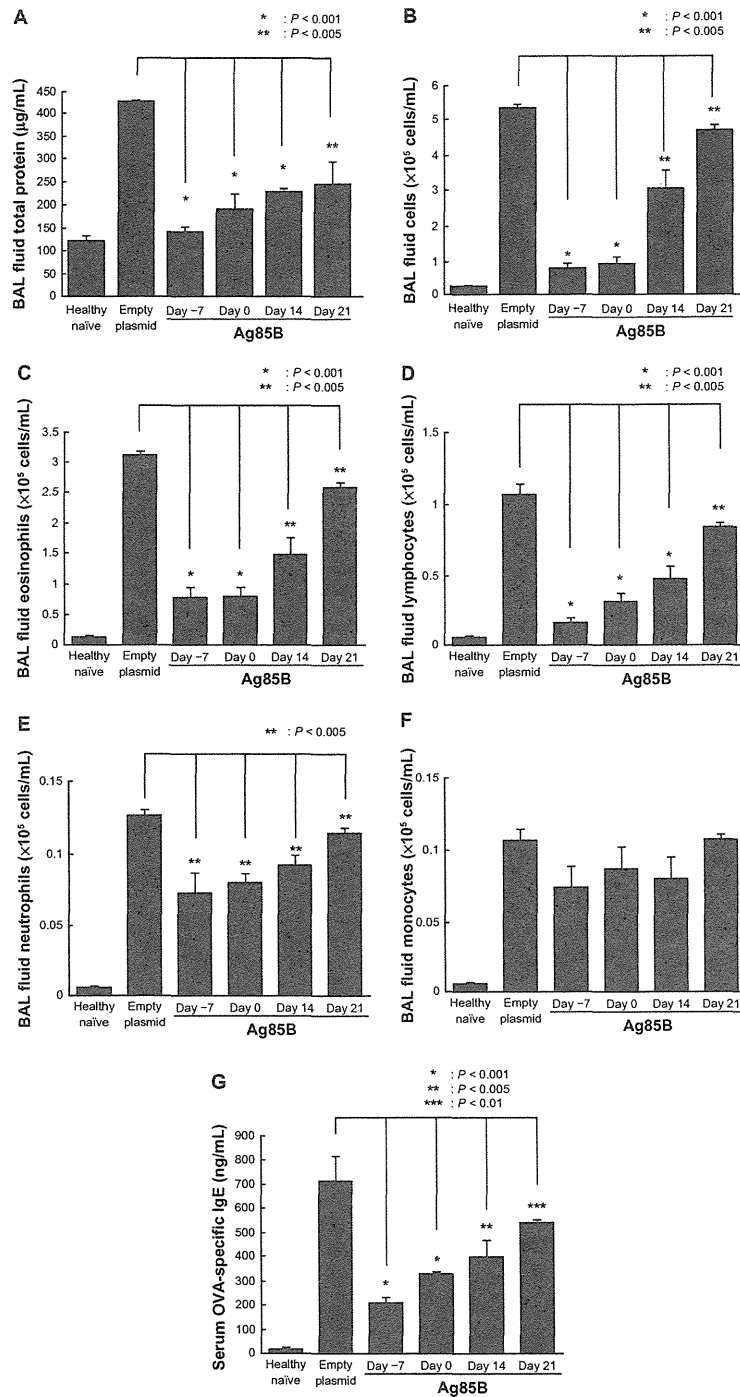
The production of OVA-specific cytokines in lymph node cells after *in vitro* stimulation with OVA were assessed. The lymphocytes obtained from thoracic lymph nodes were stimulated *in vitro* with OVA for 48 hours. IL-4 and IFN- $\gamma$  levels were measured in culture supernatants by ELISA. The level of IL-4 in culture supernatants from cells of Ag85B DNA-immunized mice was much lower than in culture supernatants from cells of control mice (Figure 4A). On the other hand, the production level of IFN- $\gamma$  in Ag85B DNA-immunized mice was significantly higher than in control DNA-immunized mice (Figure 4B).

### Expression of cytokine mRNA in pulmonary lymph node cells after stimulation with OVA

The production of OVA-specific cytokines was also confirmed by mRNA levels of Th1-type cytokines (IFN- $\gamma$ , IL-2, and IL-12) and Th2-type cytokines (IL-4, IL-5, and IL-13) (Figure 5A–C). Lymph node cells from Ag85B DNA vaccine-immunized mice showed strong IFN- $\gamma$ , IL-2, and IL-12 expression and weak IL-4, IL-5, and IL-13 expression of mRNA, whereas control DNA-immunized mice showed the completely opposite results. The cells from control mice showed strong mRNA expression of Th2-type cytokines and weak mRNA expression of Th1-type cytokines (Figure 5A–C). It has been reported that therapeutic effects against asthma by administration of the culture supernatant of *M. vaccae* were derived from Treg cells by the induction of IL-10 and transforming growth factor- $\beta$ .<sup>16</sup> In the current study, mRNA expression levels of IL-10 and transforming growth factor- $\beta$  in lymph node cells obtained from mice immunized with Ag85B DNA were much higher than those in lymph node cells obtained from control mice after *in vitro* stimulation with OVA (Figure 5A and D). Another Th17 cell lineage, which is associated with allergen-induced airway allergic inflammation, was also assessed by the mRNA expression of cytokines. In the current experiment, mRNA expression of IL-17 was seen in both control DNA-immunized and Ag85B-DNA immunized mice after stimulation with OVA, with no difference in the mRNA expression levels of IL-17 between these groups (Figure 5A and E). The mRNA expression of IL-23 was also assessed since IL-23 is associated with the maturation of Th17 cells.<sup>17</sup> Expression of IL-23 mRNA was observed at the same level in all samples (Figure 5A and E). Inhibitory effects on the development of allergic inflammation are readily obtained in a mouse model of asthma through the administration of Ag85B DNA. These effects of immunotherapy by Ag85B DNA are due to activation of the immune responses of Th1 and Treg cells and inhibition of the responses of Th2 cells as a result of the enhancement of responses of Th1 and Treg cells.

### Discussion

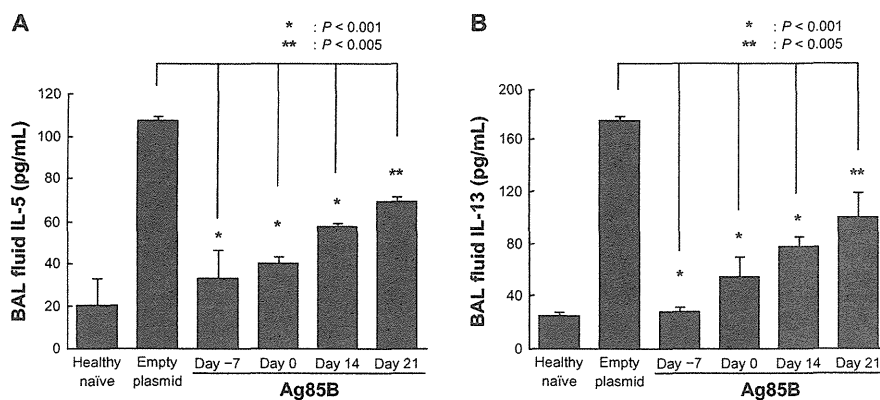
Current treatments of nonspecific immunosuppressive therapy for asthma, such as administration of glucocorticoids, are not satisfactory. Although these treatments are highly effective for controlling disease, most patients must continue to take these drugs throughout their lives. Moreover, these drugs have side effects, and asthma cannot be controlled by these drugs in up to 30% of patients. Given the high prevalence



**Figure 2** Marked inhibition of the development of allergic inflammation by administration of Ag85B DNA. BAL fluid was obtained by injecting and recovering two 0.5 mL aliquots of phosphate-buffered saline via a tracheal cannula. Cells in the lavage fluid were counted using a hemacytometer, and the differentials were determined by utilizing light microscopy to count 300 cells on Cytospin® preparations (Thermo Fisher Scientific, Waltham, MA). The concentration of inflammatory protein was measured by Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). Results for healthy naive mice and control plasmid DNA-immunized mice are also shown. (A) Total protein, (B) number of cells, (C) eosinophils, (D) lymphocytes, (E) neutrophils, and (F) monocytes in BAL fluid from experimental animals were investigated. (G) The degrees of ovalbumin-specific immunoglobulin E responses in sera collected from experimental mice were also analyzed. Immunoglobulin E levels in sera were measured using enzyme-linked immunosorbent assay kits according to the procedure recommended by the manufacturer (Shibayagi Co, Ltd, Shibukawa, Japan). BAL fluid and sera were collected 25 days after the first ovalbumin immunization.

**Notes:** Data are representative of at least three independent experiments; values shown are the means and standard deviations of five mice per group; statistical analysis was performed using the Mann–Whitney U test and the Kruskal–Wallis test.

**Abbreviations:** Ag85B, antigen 85B; BAL, bronchoalveolar lavage.



**Figure 3** IL-5 and IL-13 production in BAL fluid. Amounts of (A) IL-5 and (B) IL-13 in BAL fluid were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) 25 days after the first ovalbumin immunization.

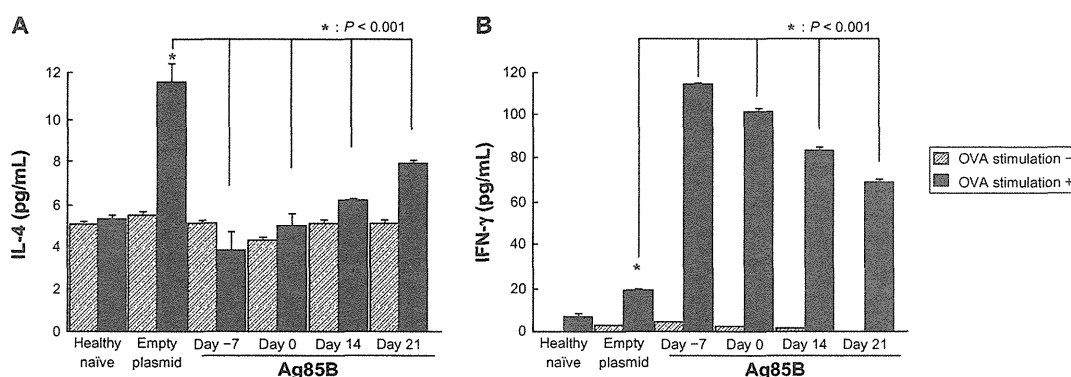
**Notes:** Data are representative of at least three independent experiments; values shown are means and standard deviations of five mice per group; statistical analysis was performed using the Mann-Whitney U test and the Kruskal-Wallis test.

**Abbreviations:** Ag85B, antigen 85B; BAL, bronchoalveolar lavage; IL, interleukin.

of this disease, improved and more effective therapeutic strategies are needed. The results of many studies have suggested that effective immunotherapy for allergic disease is associated with immune deviation from a disease-promoting Th2 response towards a Th1 response, with Treg cells having appropriate functions (reviewed in Takeda et al).<sup>18</sup> In the current study, the applicability of plasmid encoding complementary DNA of Ag85B from mycobacteria DNA to gene therapy of asthma was assessed. Although the introduced DNA is expressed predominantly by somatic cells, it is known that a relatively small but biologically significant number of dendritic cells are transfected with the inoculated DNA.<sup>19-21</sup> Moreover, it was recently reported that systemic inoculation of a plasmid DNA may cause dendritic cell

activation through direct transfection into dendritic cells.<sup>22</sup> It was demonstrated that inhibitory effects on the development of allergic inflammation are readily obtained in a mouse model of asthma through the administration of Ag85B DNA, even with only a single administration before or after antigen sensitization.

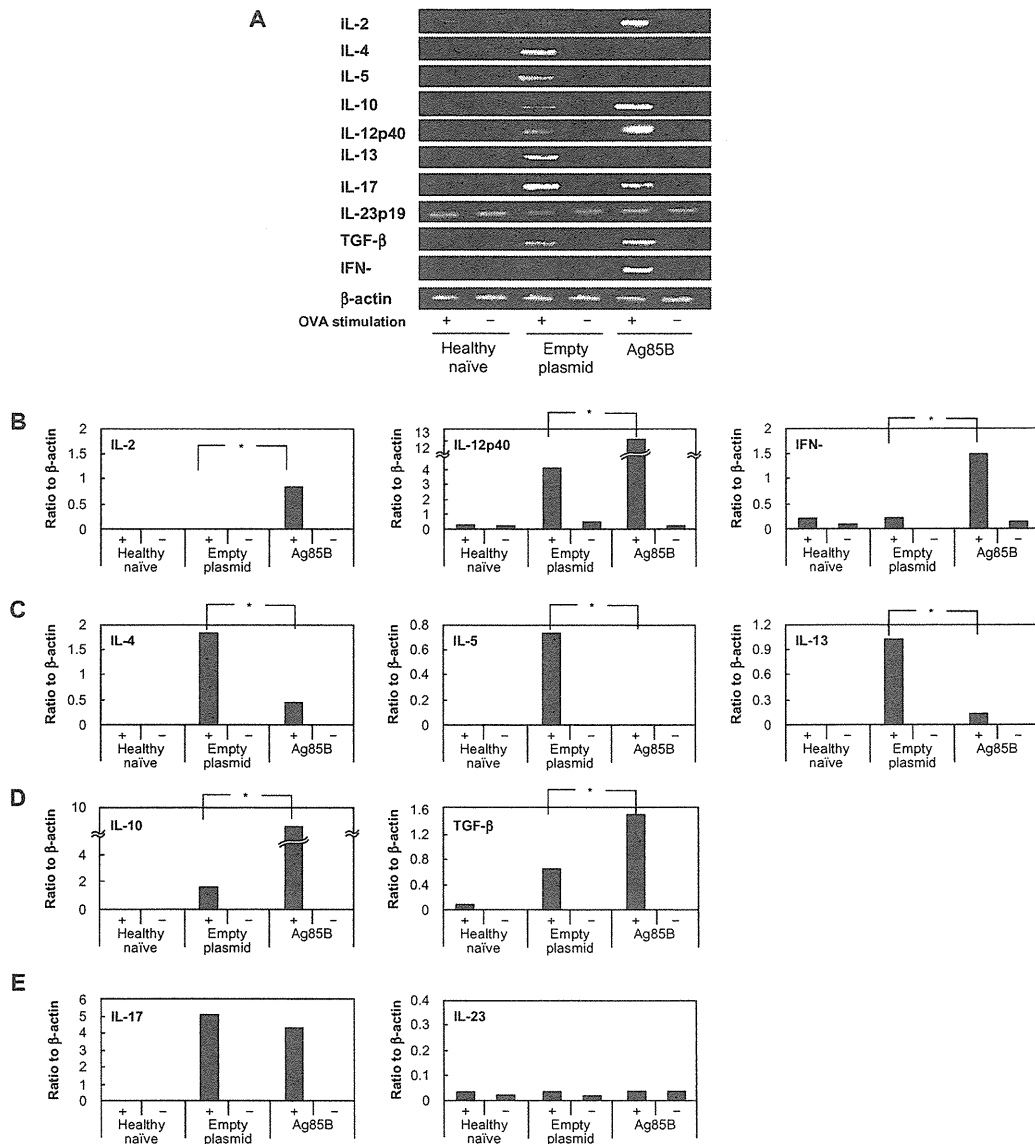
The mechanism of immune responses induced by Ag85B remains unclear. Various products having adjuvant activities, eg, lipopolysaccharide, cytosine-phosphodiester-guanine motif, and polyinosinic:polycytidylic acid, involve toll-like receptors (TLRs) and show augmentation of Th1-type immune responses.<sup>18</sup> It was previously reported that plasmid DNA encoding Ag85B stimulated the expression of TLR2, TLR3, and TLR4 mRNA. One possibility is that the induction



**Figure 4** IFN- $\gamma$  and IL-4 production in culture supernatant. Amounts of (A) IFN- $\gamma$  and (B) IL-4 in culture supernatant were measured by enzyme-linked immunosorbent assay 25 days after the first OVA immunization. Spleen cells from immunized mice ( $5 \times 10^6$ ) were cultured with  $10 \mu\text{g/mL}$  OVA in 24-well culture plates at a volume of 2 mL. After incubation at  $37^\circ\text{C}$  in a humidified incubator (5% carbon dioxide) for 96 hours, culture supernatants were quantified by using a standard enzyme-linked immunosorbent assay kit (Life Technologies, Carlsbad, CA).

**Notes:** Data are representative of at least three independent experiments; values represent mean and standard deviation of ten mice per group; statistical analysis was performed using the Mann-Whitney U test and the Kruskal-Wallis test.

**Abbreviations:** Ag85B, antigen 85B; IFN- $\gamma$ , interferon- $\gamma$ ; IL-4, interleukin-4; OVA, ovalbumin.



**Figure 5** Detection of cytokine messenger ribonucleic acid from lymphocytes using real-time polymerase chain reaction. Spleen cells were stimulated in vitro with OVA for 1 day in culture. Spleen cells stimulated with fetal calf serum were used as controls. Total ribonucleic acid was purified from the OVA-stimulated or fetal calf serum (control)-stimulated spleen cells using Isogen (Nippon Gene Co, Ltd, Tokyo, Japan) following the manufacturer's instructions. For the real-time reaction, a reverse transcription system (Promega Corporation, Fitchburg, WI) was used. Polymerase chain reaction was performed in a total volume of 50  $\mu$ L of 1  $\times$  polymerase chain reaction buffer (Takara Shuzo, Kyoto, Japan) containing 0.5–1.0  $\mu$ g of complementary DNA, 0.25 mM of each deoxyribonucleotide triphosphate, 2  $\mu$ M of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo). The specific primer pairs used were previously described.<sup>15</sup> The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 seconds at 56°C, extension for 1 minute at 73°C, and denaturation for 30 seconds at 93°C. (A) The reaction products were analyzed on 2% agarose, Tris-buffered ethylenediaminetetraacetic acid gels. (B–E) Photographs of the gels were scanned, and band intensities were measured using a densitometer (CS Analyzer 3.0; ATTO Corporation, Tokyo, Japan). The quantity of cytokine messenger ribonucleic acid was determined by the ratio of cytokine and beta actin band intensities.

**Notes:** \* $P < 0.005$ ; the profiles are representative of three independent experiments; statistical analysis was performed using the Mann-Whitney U test and the Kruskal–Wallis test.

**Abbreviations:** Ag85B, antigen 85B; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; OVA, ovalbumin; TGF- $\beta$ , transforming growth factor- $\beta$ .

of Th1-type immune responses by Ag85B is involved in innate immune responses. From this result, the activation of Th1 and Treg cells by Ag85B administration was thought to be involved in responses through stimulation of TLR2, TLR3, and TLR4, but not TLR9.<sup>14</sup> Various proteins derived

from pathogens promote Th1 responses through stimulation of TLRs and subsequently through secretion of cytokines.<sup>18</sup> It has also been reported that TLR signaling induces not only Th1-type immune responses but also secretion of various cytokines from Treg cells.<sup>23–27</sup> Moreover, recent studies have



indicated that Th1 cells produce IL-10 as well as Th1-type cytokines by Notch regulation-dependent signal transducer and activator of transcription-4 signaling.<sup>28</sup> From these findings, effective immunotherapy by induction of both Th1 cell and Treg cell responses is thought to be possible by using appropriate materials. In fact, an asthma model of mice immunized with culture supernatant of mycobacteria, *M. vaccae*, showed Th1 and Treg responses.<sup>16</sup> The results of the current study suggest that the administration of Ag85B DNA has several potential advantages due to the activation of Th1 and Treg cells for the prevention and treatment of asthma.

Immunization with mycobacteria or mycobacteria products has been reported to inhibit the development of allergic disease.<sup>29–32</sup> However, various causative factors affect immune responses by mycobacteria. It was reported as a notable point that the efficacy of mycobacteria in preventing allergic inflammation of asthma was strongly affected by *Nramp1* alleles.<sup>33</sup> Several host genetic factors, including natural resistance-associated macrophage protein 1 (NRAMP1),<sup>34</sup> vitamin D receptor (VDR),<sup>35,36</sup> and Mendelian susceptibility to mycobacterial disease,<sup>37</sup> have been reported to be involved in responses to mycobacteria (reviewed in Casanova and Abel).<sup>38</sup> Differences in immune responses induced by different mycobacteria strains have also been reported. The differential immune responses were mediated by lipid-extracted molecules of mycobacteria.<sup>39</sup> Moreover, environmental factors are important for immune responses induced by mycobacteria in therapy for atopic diseases.<sup>40,41</sup> Presensitization of mycobacteria in the natural environment affects the induction of Th1-type immune responses by mycobacteria vaccination.<sup>9,42,43</sup> However, the specific components of mycobacteria that inhibit the development of allergic responses have not been reported. Ag85B is a single component of mycobacteria, and this product might not be affected by various other mycobacteria factors involved in immune responses. In fact, Th1-type immune responses induced by Ag85B are not affected by *Nramp* in mice.<sup>44,45</sup>

Wu et al demonstrated the effects of intranasal administration of Ag85B in a mouse model of asthma.<sup>46</sup> It was previously reported that Ag85B has strong adjuvant activities involving Th1 immune responses.<sup>14</sup> Intranasal administration of a plasmid DNA (DNA vaccine) with adjuvant activities has been considered to be inappropriate for human use. Intranasal inactivated influenza vaccine, with adjuvant, induced Bell's palsy in humans. Therefore, intranasal inactivated influenza vaccine with adjuvant is no longer in clinical use.<sup>47</sup> Systemic administration of a plasmid DNA (DNA vaccine) is better than intranasal administration if the same effects of the plasmid

DNA can be induced. The current study demonstrated the usefulness of Ag85B DNA vaccine and provided evidence of the potential utility of Ag85B DNA vaccine for the prevention and treatment of asthma, even with only a single systemic administration before or after antigen sensitization.

## Conclusion

The correction of immune response should be considered in the prevention and treatment of asthma. Ag85B has potential utility for the prevention and treatment of asthma even with only a single administration.

## Acknowledgments

This work was supported by Health Science Research Grants from the Ministry of Health, Labor, and Welfare of Japan and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

## Disclosure

The authors report no conflicts of interest in this work.

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## CD16<sup>+</sup> natural killer cells play a limited role against primary dengue virus infection in tamarins

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Received: 29 July 2011 / Accepted: 12 November 2011  
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**Abstract** CD16 is a major molecule expressed on NK cells. To directly assess the role of natural killer (NK) cells in dengue virus (DENV) infection *in vivo*, CD16 antibody-treated tamarins were inoculated with a DENV-2 strain. This resulted in the transient depletion of CD16<sup>+</sup> NK cells, whereas no significant effects on the overall levels or kinetics of plasma viral loads and antiviral antibodies were observed in the treated monkeys when compared to control monkeys. It remains elusive whether the CD16<sup>-</sup> NK subpopulation could play an important role in the control of primary DENV infection.

**Keywords** Dengue virus · Tamarin · NK cells · CD16

DENV is one of the most serious mosquito-borne virus affecting humans, with 2.5 billion people at risk in tropical and subtropical regions around the world each year [12]. A wide variety of clinical manifestations have been noted, which range from asymptomatic, mild febrile illness (dengue fever [DF]) to dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), a life-threatening illness. It has been shown that humans with a secondary heterologous DENV infection are at a higher risk of contracting severe dengue disease [10, 26]. DHF/DSS occurs in infants during primary DENV infection, predominantly in the second half of the first year of life, when maternal antibodies have low residual neutralizing activity [11, 17].

NK cells are a component of the innate immune system that plays a central role in host defense against viral infection and tumor cells. It has been shown that infection by some viruses, such as herpes simplex virus-1, influenza virus or ectromelia poxvirus, can be controlled by NK cells in mice [15]. Yet the most compelling evidence for a role

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T. Yoshida and T. Omatsu contributed equally to this study.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00705-011-1178-6) contains supplementary material, which is available to authorized users.

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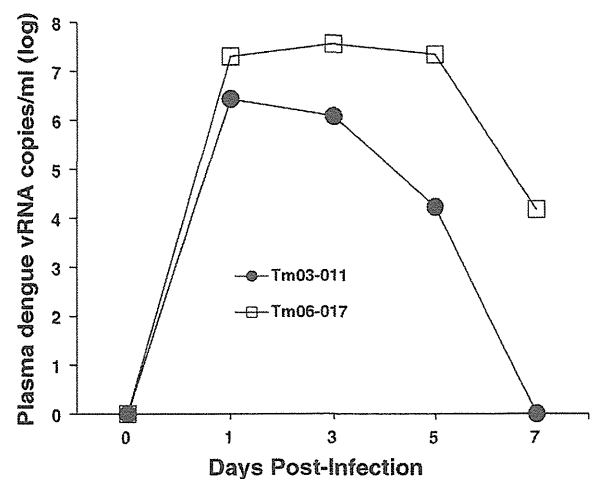
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of NK cells in early defense against viruses was obtained in a study showing increased susceptibility to murine cytomegalovirus (MCMV) after NK cell depletion and increased resistance after adoptive transfer of NK cells [23]. Defects in NK cell activity, such as decreased production of interferon (IFN)- $\gamma$  or cytotoxicity, render mice more susceptible to MCMV infection [23]. NK cells can kill virus-infected cells by using cytotoxic granules or by recognizing and inducing lysis of antibody-coated target cells (antibody-dependent cell cytotoxicity) via an Fc-binding receptor such as CD16 [21].

Early activity of NK cells may be important for clearing primary DENV infection [24]. In a DENV mouse model, mice experimentally infected with DENV showed increased NK cell levels [24]. A significant increase in the frequency of NK cell circulation was also shown in patients who developed an acute dengue disease [2]. In addition, patients with a mild dengue disease have elevated NK cell rates when compared to those with severe dengue diseases [9, 27]. Moreover, Kurane *et al.* [14] reported that human blood NK cells are cytotoxic against DENV-infected cells in target organs via direct cytolysis and antibody-dependent cell-mediated cytotoxicity. It was also shown that the intracellular cytotoxic granule, TIA-1, was up-regulated early in NK cells in the acute phase of DENV infection and that NK-activating receptor NKp44 was involved in virus-mediated NK activation through direct interaction with DENV envelope protein [2, 13]. These results suggest that the early activation of NK cells contributes to the prevention of the severe dengue disease. However, based on quantitative and functional analyses in animal models *in vivo*, defining the contribution of NK cells to suppression of DENV replication *in vivo* has been necessary.

We have recently reported that common marmosets (*Callithrix jacchus*) are highly permissive to DENV infection [22]. These New World monkeys, being nonhuman primates, are considered to have an immune system similar to that of humans [28, 29]. The present study was initiated to investigate the role of NK cells in controlling DENV during primary infection in our nonhuman primate model.

The animals were cared for in accordance with National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare, and all protocols were approved by our Institutional Animal Study Committee. Eight tamarins (*Saguinus midas* and *Saguinus labiatus*) were used in this study. As marmosets and tamarins are closely related monkey species and are classified as members of the Callitrichinae, we expected that tamarins would also be permissive to DENV infection, like marmosets. To check the permissiveness of tamarins to DENV, 2 tamarins were infected with DENV-2 (DHF0663 strain:  $6.7 \times 10^7$  PFU/ml) subcutaneously or intravenously (Fig. 1).



**Fig. 1** Levels of vRNA in DENV-infected tamarins. Tamarins were infected subcutaneously or intravenously with DENV at a dose of  $6.7 \times 10^7$  PFU/ml. The vRNAs were detected in plasma by real-time PCR. Tm03-011, subcutaneous infection; Tm06-017, intravenous infection

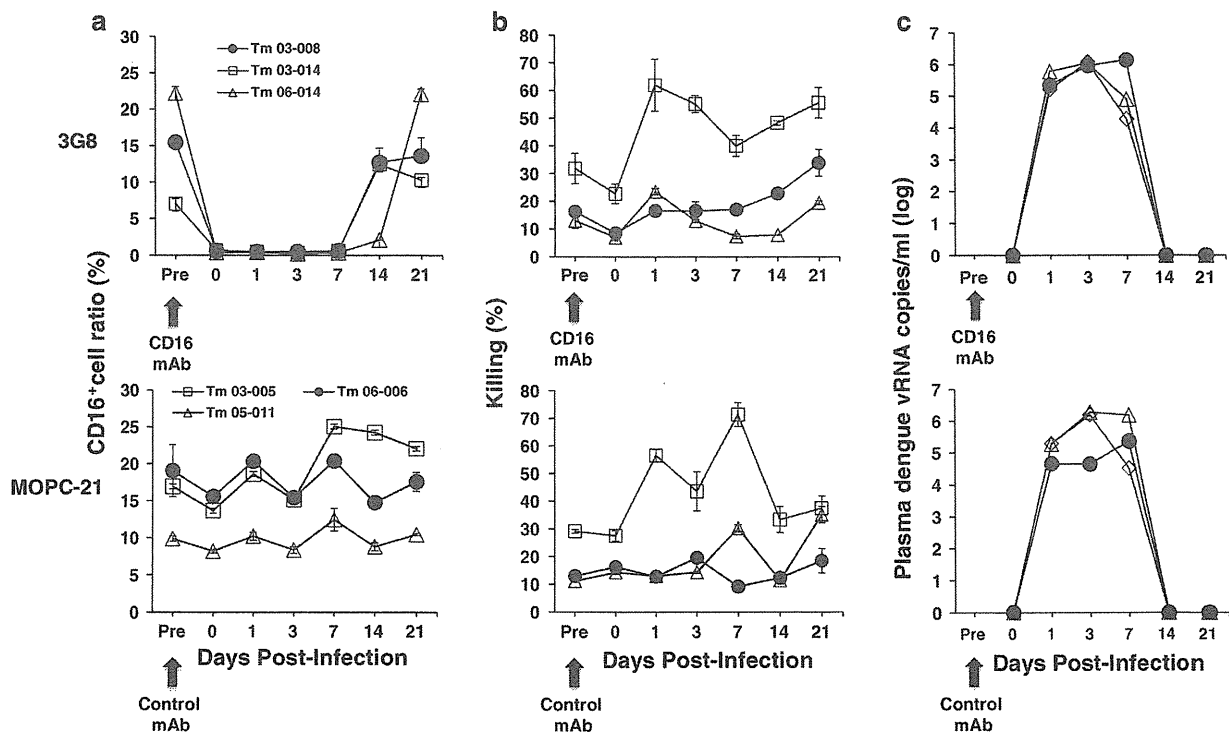
Dengue viral RNA (vRNA), which was quantified using real-time PCR as previously described [22], was detected in plasma samples from the tamarins on day 1 post-infection. For each of the two tamarins (Tm03-011, Tm06-017), the plasma vRNA levels reached  $2.7 \times 10^6$  copies/ml and  $2.0 \times 10^7$  copies/ml on day 1 post-infection, respectively, and were detectable on days 3 and 5. These results indicate that tamarins are also permissive to DENV infection, which is consistent with the results obtained by using marmosets [22].

Next, we sought to assess the role of NK cells in DENV infection *in vivo*. In this regard, *in vivo* depletion of NK cells by the administration of NK-specific monoclonal antibody (mAb) was considered to be straightforward to directly address the question. We employed a new method by which an anti-CD16 mAb 3G8 [7] but not a control mAb MOPC-21 efficiently depleted a major NK population expressing CD16 in tamarins, as we recently reported [29]. The mouse anti-human CD16 mAb 3G8 was produced in serum-free medium and purified using protein A affinity chromatography. Endotoxin levels were confirmed to be lower than 1 EU/mg. Four red-handed tamarins and two white-lipped tamarins (*Saguinus labiatus*) were used in this experiment. Three tamarins were intravenously administered 3G8 at a dose of 50 mg/kg, while others were given a control mAb MOPC-21. One day later, both mAb-treated tamarins were subcutaneously inoculated with  $3 \times 10^5$  PFU/ml of DENV-2 DHF0663 strain on the basis of a previous report that a single mosquito might inject between  $10^4$  and  $10^5$  PFU of DENV into a human [20]. It was confirmed that at 1–3 days after the 3G8 mAb treatment, CD16<sup>+</sup> cells were almost completely depleted in the

tamarins followed by recovery to the initial levels at around 2 weeks after administration, while the cells were maintained at the initial levels in the monkeys with MOPC-21 (Fig. 2a). In addition, it is noteworthy that the ratios of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and CD20<sup>+</sup> B cells were not affected by the administration of the 3G8 mAb (Supplementary Figure 1). In the case of administration of mAb MOPC-21, we confirmed no significant effect on CD16<sup>+</sup> cells (Supplementary Figure 2). The killing activities of the peripheral blood mononuclear cells (PBMCs) taken from the 3G8-treated monkeys were reduced at day 1 post-antibody-treatment, followed by an increase irrespective of depletion of CD16<sup>+</sup> NK cells at day 2 post-antibody-treatment (1 day after DENV inoculation), suggesting that the CD16<sup>-</sup> NK population may be activated by DENV infection (Fig. 2b). Plasma viral loads in both mAb-treated monkeys rose to 10<sup>5</sup> copies/ml by day 1 after infection and then reached a peak at 10<sup>6</sup> copies/ml on day 3 or day 7, followed by a rapid decline, with values dipping below the detectable level by day 14 after infection (Fig. 2c). These results suggested that CD16<sup>+</sup> NK cells apparently did not contribute to DENV replication in the acute phase in our tamarin model.

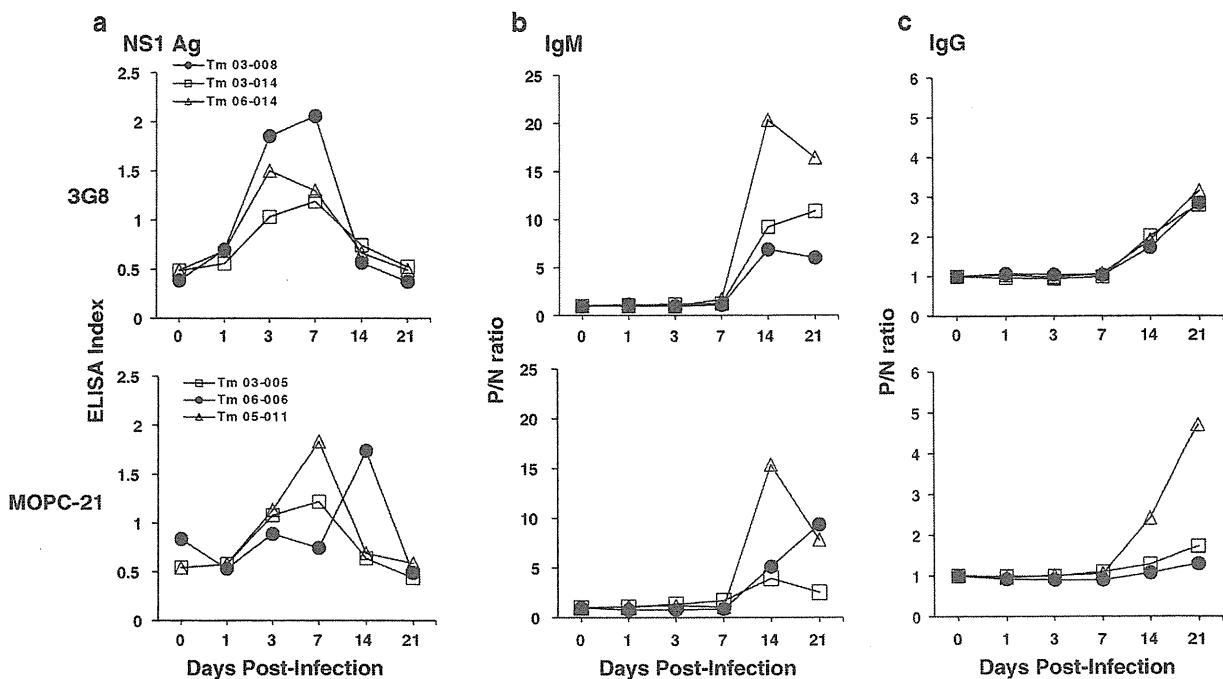
It was reported previously that non-structural glycoprotein NS1 is essential for flavivirus viability and that the NS1 protein circulates during the acute phase of disease in the plasma of patients infected with DENV [1]. Epidemiological studies have demonstrated that secreted NS1 levels are correlated with viremia levels and are higher in cases of DHF than in dengue fever (DF) early in illness [16]. Thus, it has been suggested that NS1 might be a useful marker as an indicator of the severity of dengue disease. We have used the level of the NS1 antigen as an alternative diagnostic marker to examine the effects of CD16 antibody treatment on DENV replication. The NS1 was measured by Platelia Dengue NS1 Ag assay (BioRad). Antigenemia was observed in these infected monkeys between 3-14 days post-infection. Serum IgM and IgG specific for DENV antigens were measured by ELISA. DENV-specific IgM or IgG antibody was equally detected in both mAb-treated monkeys (Fig. 3).

We recently demonstrated that marmosets are permissive to DENV infection [22]. In this study, we found that tamarins are also permissive to DENV infection (Fig. 1). Moreover, we also investigated the role of NK cells against early DENV infection using *in vivo* depletion of CD16<sup>+</sup>



**Fig. 2** Ratios of CD16<sup>+</sup> NK cells, killing activity of PBMCs, and vRNA in DENV-infected tamarins after treatment with 3G8 or MOPC-21 mAb. Tamarins were infected subcutaneously with DENV at a dose 3x10<sup>5</sup> PFU/ml after treatment with 50 mg/kg of 3G8 or

MOPC-21 mAb. **a** Ratios of CD16<sup>+</sup> NK cells were determined in whole-blood specimens. **b** The activities of NK cells were determined in PBMCs of tamarins by NK cytotoxic assay. **c** The vRNAs were detected in plasma by real-time PCR



**Fig. 3** Levels of NS1 antigen and DENV-specific IgM and IgG in plasma samples from DENV-infected tamarins after treatment with 3G8 or MOPC-21 mAb. The levels of NS1 antigen and DENV-specific IgM and IgG in plasma were measured by ELISA. **a** ELISA index of NS1 antigen, **b** positive/negative (P/N) ratio of DENV-specific IgM, **c** P/N ratio of DENV-specific IgG in plasma samples

from DENV-infected tamarins after administration of the 3G8 or MOPC-21 mAb. The P/N ratio was calculated as the optical density of the test sample divided by that of a negative sample. P/N ratios  $< 2$  and  $\geq 2$  were considered to be negative and positive, respectively. Top, 3G8; bottom, MOPC-21 mAb

NK cells in tamarins and found that the depletion of CD16<sup>+</sup> NK cells had almost no effect on DENV replication (Fig. 2), indicating that this NK subpopulation is unlikely to contribute to controlling DENV replication. Interestingly, these results imply that the CD16<sup>-</sup> NK subpopulation may have a critical role of controlling DENV infection *in vivo*.

Using our model, we investigated the role of NK cells *in vivo* against DENV infection, which remains to be elucidated in several aspects. We previously reported that almost complete *in vivo* depletion of the CD16<sup>+</sup> NK subpopulation was not able to completely remove the NK-mediated cytotoxic activity in tamarins [29]. In this study, despite a transient but substantial reduction in the CD16<sup>+</sup> NK cell number following 3G8 treatment in tamarins, DENV replication was comparable to that in monkeys that received the control mAb. The NK-mediated cytotoxic activity was augmented in both study groups, indicating that CD16<sup>-</sup> NK cells were responsible for the cytotoxic activity and suggesting that they might play a role in controlling DENV replication.

The next question is how CD16<sup>-</sup> NK cells may regulate DENV infection. One possibility regarding CD16<sup>-</sup> NK cells is that CD56<sup>+</sup> or CD57<sup>+</sup> NK cells are involved in

controlling DENV infection. Human NK cells are classically divided into two functional subsets based on their cell-surface density of CD56 and CD16, i.e., CD56<sup>bright</sup>CD16<sup>-</sup> immunoregulatory cells and CD56<sup>dim</sup>CD16<sup>+</sup> cytotoxic cells. Both subsets have been characterized extensively regarding their different functions, phenotypes, and tissue localization [8]. The NK cell number is maintained by a continuous differentiation process associated with the expression of CD57 that results in NK cells with poor responsiveness to cytokine stimulation but high cytolytic capacity [3, 18]. The second possibility is that CD16<sup>-</sup> NK cells have a non-cytolytic helper function. Generally, it is well known that NK cells possess both a cytolytic and a non-cytolytic helper function. It has been suggested that cytokine production is carried out by CD56<sup>bright</sup>CD16<sup>-</sup> NK cells [4–6]. Interferon (IFN)- $\gamma$  secreted by NK cells has shown potent antiviral effects against DENV infection in early phases [25]. One aspect of the NK helper function arises from recent evidence indicating that NK cells can be induced to function as non-cytotoxic helper cells following stimulation with interleukin-18 [19]. This cytokine induces IFN- $\gamma$  secretion from NK cells and thus enables dendritic cells (DCs) to secrete IL-12, leading to Th1 polarization [19]. It is possible that CD16<sup>-</sup> NK cells, which have poor

cytotoxic activity but an enhanced ability to secrete cytokines and then lead to a Th1 response, are preserved during 3G8 administration. The persistence of this minor CD16<sup>-</sup> NK cell subpopulation could exert an antiviral effect through INF- $\gamma$ -mediated pathways despite the depletion of CD16<sup>+</sup> NK cells. The third possibility is that CD16<sup>+</sup> NK cells of tamarins play pivotal roles against bacterial infections and cancer progression but not DENV-infected cells. We will address these possibilities for the roles of the NK subpopulation in the future studies.

In conclusion, this study provides a DENV *in vivo* replication model in tamarins and new information on the possible role of CD16<sup>+</sup> NK cells in DENV replication *in vivo*. It remains elusive whether the CD16<sup>+</sup> and CD16<sup>-</sup> NK subpopulations could play an important role in the control of primary DENV infection.

**Acknowledgments** We would like to give special thanks to members of the Corporation for Production and Research of Laboratory Primates for technical assistance. We also would like to give special thanks to Ms. Tomoko Ikoma and Ms. Mizuho Fujita for technical assistance. Moreover, we appreciate Dr. Keith A. Reimann (the NIH Nonhuman Primate Reagent Resource R24 RR016001, NIAID contact HHSN272200900037C) for providing CD16 antibody. This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan (to Hirofumi Akari and Ichiro Kurane). This research was also supported by the Environment Research and Technology Development Fund (D-1007) from the Ministry of the Environment of Japan (to Tomoyuki Yoshida and Hirofumi Akari).

**Conflict of interest** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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  $\alpha$  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<sup>+</sup> 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.

**D**ilated 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-1 $\beta$ , IL-6, IL-12, TNF- $\alpha$ , 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<sup>+</sup> T cell-mediated disease (7, 14), and activation of self-Ag-loaded dendritic cells (DCs) is critical for expansion of autoreactive CD4<sup>+</sup> 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- $\gamma$ , 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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Received for publication December 13, 2011. Accepted for publication June 5, 2012.

This work was supported by Health Science Research grants from the Ministry of Health, Labor and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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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, knock-out; LV, left ventricular; LVEDd, left ventricular end-diastolic dimension; LVESd, left ventricular end-systolic dimension; MyHC- $\alpha$ , 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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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1103610

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  $\mu$ g 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  $\mu$ g/ml MyHC- $\alpha$  peptide and stimulated for another 4 h with 0.1  $\mu$ g/ml LPS (Sigma-Aldrich) and 5  $\mu$ g/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 EcoRI at the 5' and 3' ends, respectively. MyHC- $\alpha$ /CFA-immunized mice were injected i.p. with 100  $\mu$ g of plasmid DNA in 200  $\mu$ l PBS on days 0, 5, and 10. BMDC-transferred mice and CD4<sup>+</sup> 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  $-80^{\circ}\text{C}$ . For in vitro stimulation assay of primary CD4<sup>+</sup> T cells, naive CD4<sup>+</sup>CD62L<sup>+</sup> T cells were isolated from the spleens by MACS (CD4<sup>+</sup>CD62L<sup>+</sup> T Cell Isolation Kit II; Miltenyi Biotec). A total of  $1.5 \times 10^7$  CD4<sup>+</sup>CD62L<sup>+</sup> 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  $\mu$ g/ml MyHC- $\alpha$  for 72 h and pulsed with 0.5  $\mu$ Ci [<sup>3</sup>H]thymidine 8 h before being measured with a  $\beta$  counter. For in vitro stimulation assay of primary CD4<sup>+</sup> T cells, naive CD4<sup>+</sup>CD62L<sup>+</sup> T cells were isolated from the spleens by MACS (CD4<sup>+</sup>CD62L<sup>+</sup> T Cell Isolation Kit II; Miltenyi Biotec). A total of  $10^5$  CD4<sup>+</sup>CD62L<sup>+</sup> cells were then stimulated with 5  $\mu$ g/ml anti-CD3e, 5  $\mu$ g/ml anti-CD3e, 1  $\mu$ g/ml anti-CD28, 50 ng/ml PMA, and 500 ng/ml ionomycin or with 1  $\mu$ g/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 [<sup>3</sup>H]thymidine incorporation.

### Western blot analysis

Total lysates from CD4<sup>+</sup> 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  $\mu$ g/ml MyHC- $\alpha$  for 48 h. A total of  $5 \times 10^6$  CD4<sup>+</sup> 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  $\mu$ g 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'-CC-TGGTTCATCATCGCTAATC-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  $\alpha 6$  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

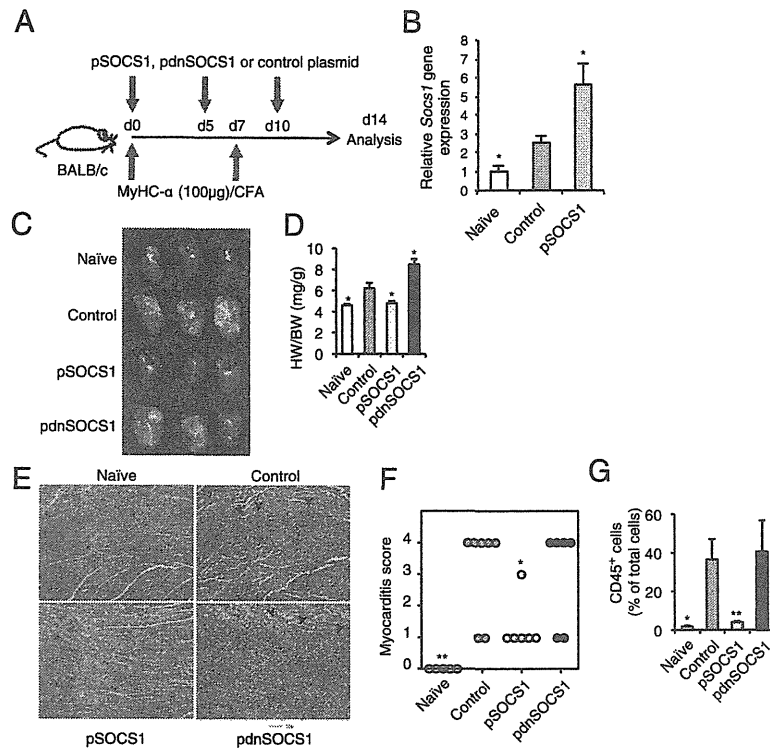
All data were expressed as means  $\pm$  SEM. Statistical analyses were performed using the two-tailed *t* test or Mann-Whitney *U* test for experiments comparing two groups. The *p* values <0.05 were considered statistically significant.

## 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  $\mu$ g of MyHC- $\alpha$  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  $\mu$ m. (F) Myocarditis severity in heart sections stained with H&E ( $n = 5$ –7 mice/group). (G) Flow cytometry analysis of CD45<sup>+</sup> 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  $\pm$  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–1G).

To clarify the adverse effect of dnSOCS1 DNA administration on the development of EAM, we used mice immunized with a tithe amount (10  $\mu$ g) of MyHC- $\alpha$  instead of the usual amount of peptide for EAM development (Fig. 2A). Those MyHC- $\alpha$ -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.

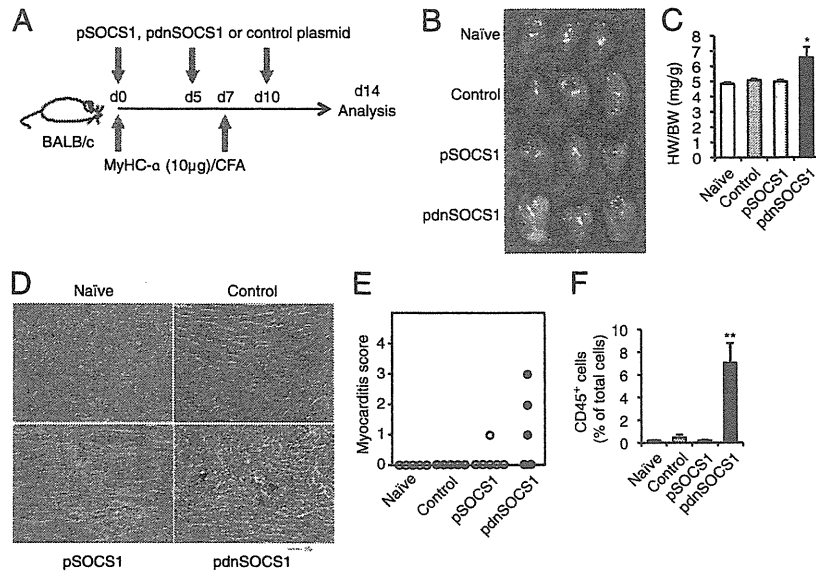
#### SOCS1 DNA administration prevents progression of heart failure and fibrosis after myocarditis

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- $\alpha$  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<sup>+</sup> T cell response and cytokine production

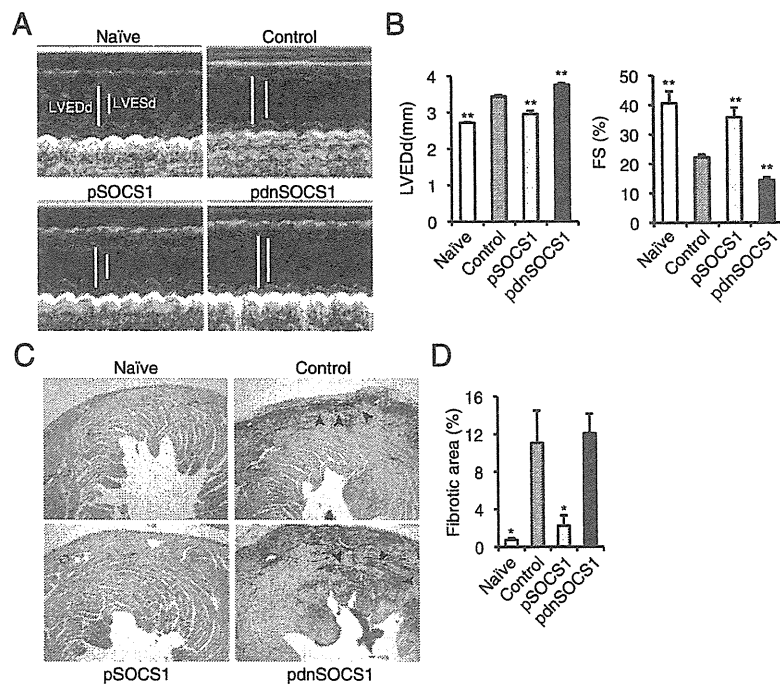
Autoimmune myocarditis is a CD4<sup>+</sup> T cell-mediated disease (7, 15). Proliferative responses of CD4<sup>+</sup> T cells after *in vitro* restimulation with MyHC- $\alpha$  were not clearly seen in pSOCS1-injected mice; however, the proliferation of CD4<sup>+</sup> T cells from pdnSOCS1-injected mice was enhanced (Fig. 4A). Production of IL-2, IL-6, IL-10, IL-17, IL-22, IFN- $\gamma$ , TNF- $\alpha$ , CCL2, CCL3, CCL5, CCL17, and CXCL10 by CD4<sup>+</sup> T cells from EAM mice was enhanced by *in vitro* restimulation with the MyHC- $\alpha$  epitope peptide. This cardiac-Ag-specific cytokine production by CD4<sup>+</sup> T cells was decreased in the supernatants of *in vitro* MyHC- $\alpha$ -restimulated CD4<sup>+</sup> 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  $\mu$ g of MyHC- $\alpha$  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  $\mu$ g of MyHC- $\alpha$ -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  $\mu$ m. (E) Myocarditis severity in heart sections stained with H&E ( $n = 5$  to 6 mice/group). (F) Flow cytometry analysis of CD45<sup>+</sup> heart infiltrates of naive and immunized mice ( $n = 5$  mice/group). Data are representative of at least two independent experiments. Error bars represent means  $\pm$  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-1 $\beta$ , IL-10, and CXCL1 was not detected in the

culture supernatants of in vitro-restimulated CD4<sup>+</sup> 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  $\mu$ m. (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  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  compared with control.