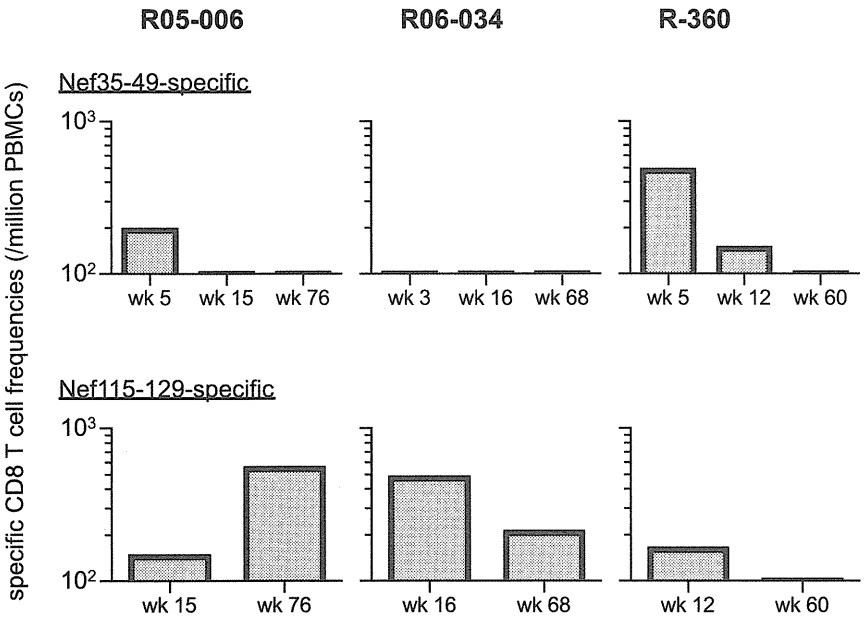

Figure 4. SIV Nef-specific CD8$^+$ T-cell responses in macaques R05-006, R06-034, and R-360. Nef_{35-49}-specific (upper panels) and $Nef_{115-129}$-specific (lower panels) CD8$^+$ T-cell responses were examined at indicated time points after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g004

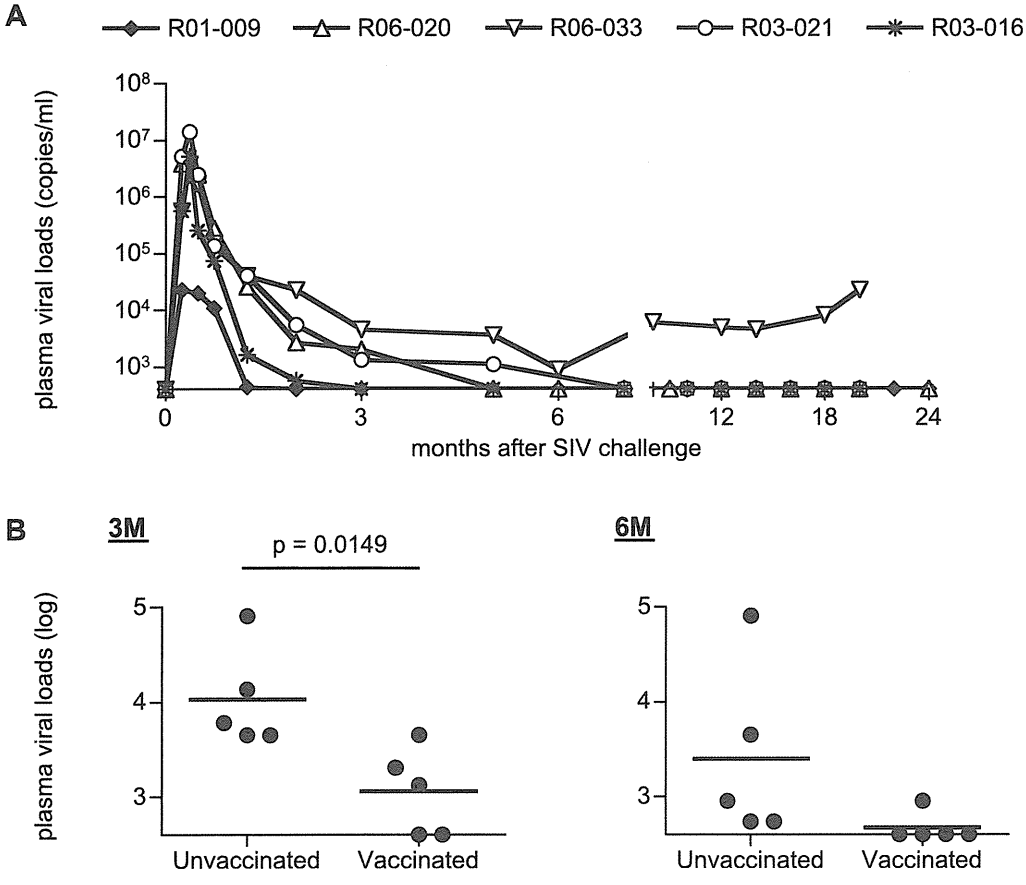

Figure 5. Plasma viral loads after SIVmac239 challenge in vaccinated D$^+$ macaques. (A) Changes in plasma viral loads after challenge vaccinated macaques possessing MHC-I haplotype D. (B) Comparison of plasma viral loads at 3 months (left panel) and 6 months (right panel) between five unvaccinated D$^+$ and five vaccinated D$^+$ animals. Viral loads at 3 months in vaccinated animals were significantly lower than those in the unvaccinated ($p = 0.0149$ by t-test).
doi:10.1371/journal.pone.0054300.g005

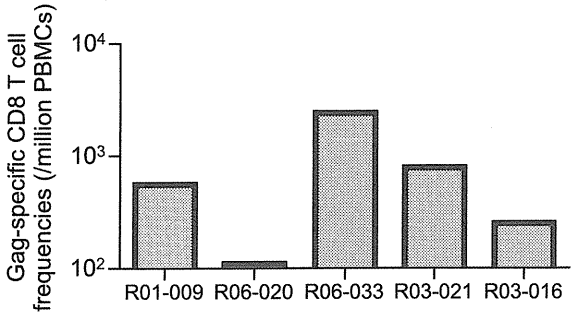

Figure 6. SIV Gag-specific CD8$^+$ T-cell responses in vaccinated D$^+$ macaques at week 2 after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g006

significant difference at 3 months after SIV challenge ($p = 0.2436$ by t-test), but viral loads in the former D$^+$ animals became significantly lower than the latter after 6 months ($p = 0.0360$ at 6 months and $p = 0.0135$ at 9 months by t-test; Fig. 1). Four of these five macaques sharing MHC-I haplotype D showed low viral loads, less than 5×10^3 copies/ml, after 6 months, whereas macaque R01-012 maintained relatively higher viral loads.

Predominant Nef-specific CD8$^+$ T-cell Responses

We examined SIV antigen-specific CD8$^+$ T-cell responses by detection of antigen-specific IFN-γ induction. In the very acute phase, we did not have enough PBMC samples for measurement of individual SIV antigen-specific CD8$^+$ T-cell responses and focused on examining Gag-specific CD8$^+$ T-cell responses in most animals. At week 2 after challenge, Gag-specific CD8$^+$ T-cell responses were undetectable in four of five animals (Fig. 2).

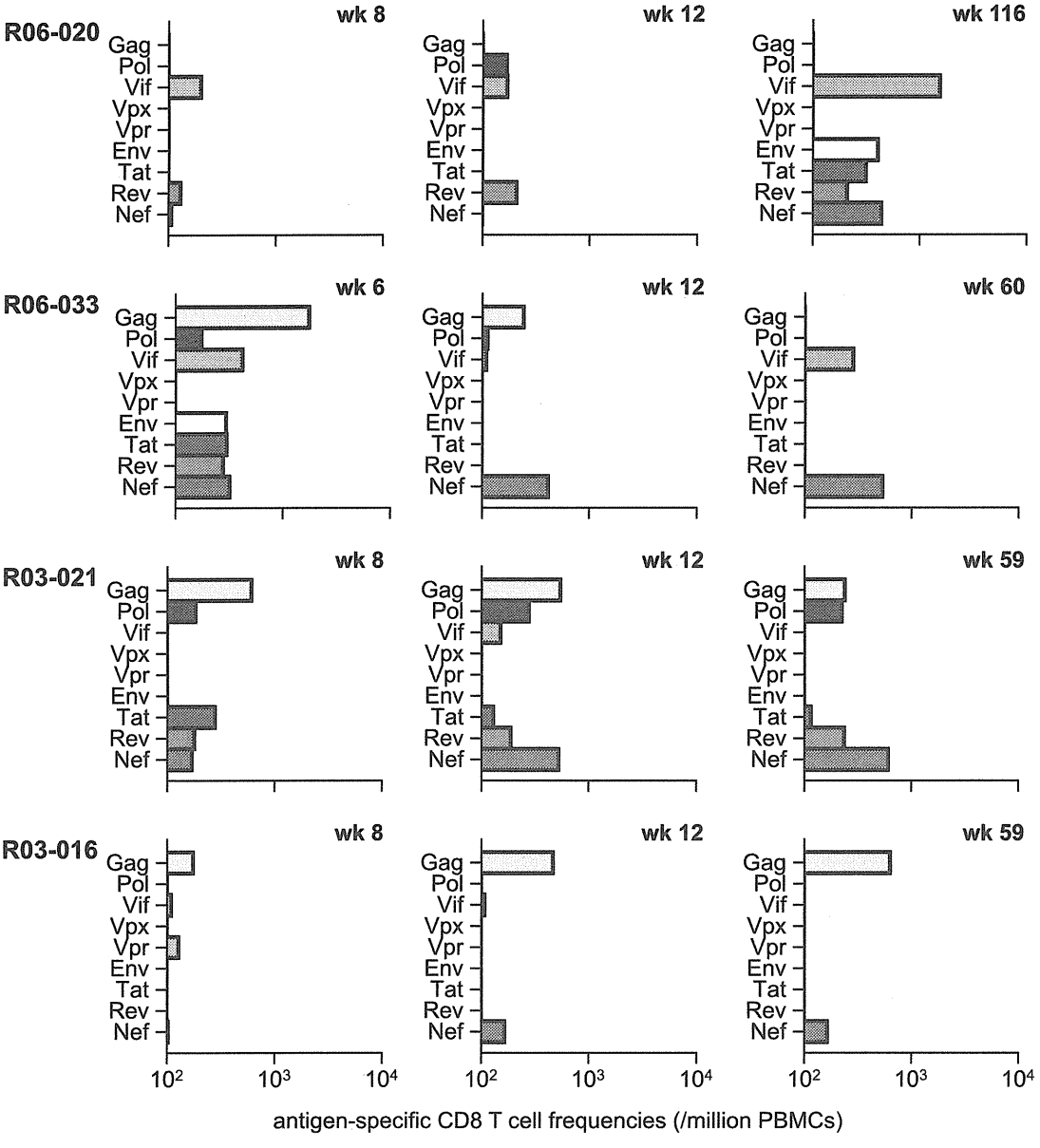

Figure 7. SIV antigen-specific CD8$^+$ T-cell responses in vaccinated D$^+$ animals after SIVmac239 challenge. Samples for this analysis were unavailable in macaque R01-009.
doi:10.1371/journal.pone.0054300.g007

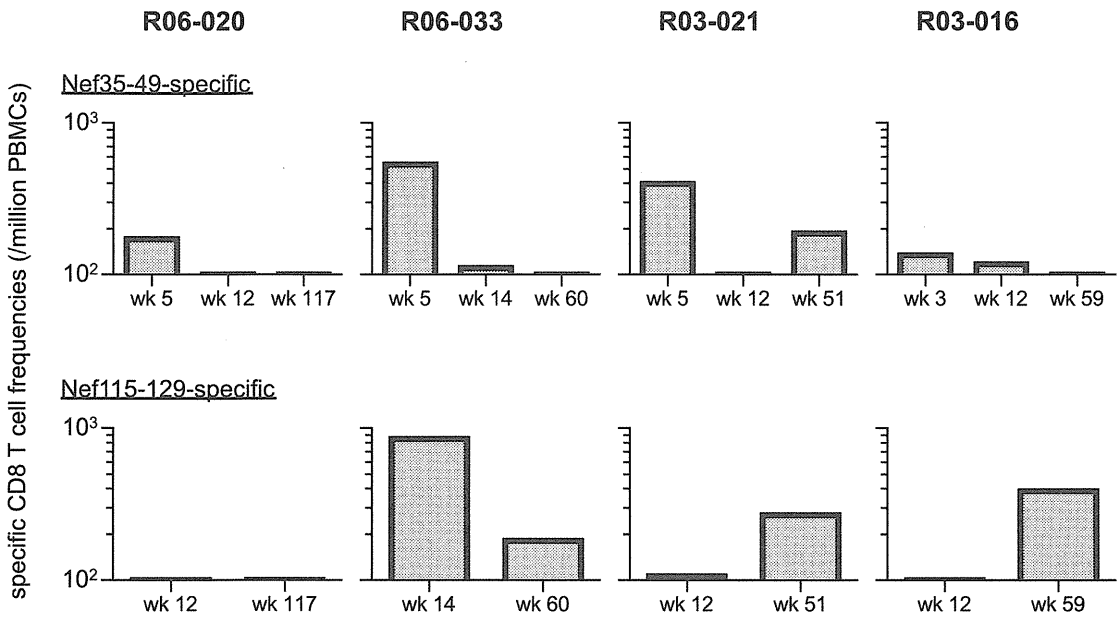

Figure 8. SIV Nef-specific CD8$^+$ T-cell responses in macaques R06-020, R06-033, R03-021, and R03-016. Nef_{35-49}-specific (upper panels) and $Nef_{115-129}$-specific (lower panels) CD8$^+$ T-cell responses were examined at indicated time points after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g008

We then examined CD8$^+$ T-cell responses specific for individual SIV antigens in the early and the late phases (Fig. 3). Nef-specific but not Gag-specific CD8$^+$ T-cell responses were predominant in most D$^+$ animals. Gag-specific CD8$^+$ T-cell responses were dominantly induced in macaque R08-005 showing very low set-point viral loads. Macaque R01-012 having higher viral loads showed poor CD8$^+$ T-cell responses in the early phase.

Among four D$^+$ animals controlling SIV replication with less than 5×10^3 copies/ml of plasma viral loads after 6 months, Gag-specific CD8$^+$ T-cell responses were dominant only in macaque R08-005, while efficient Nef-specific CD8$^+$ T-cell responses were induced in the remaining three, suggesting possible contribution of Nef-specific CD8$^+$ T-cell responses to SIV control in these three controllers (R05-006, R06-034, and R-360). We then attempted to localize Nef CD8$^+$ T-cell epitopes shared in these animals and found Nef_{35-49}-specific and $Nef_{115-129}$-specific CD8$^+$ T-cell responses (Fig. 4), although we did not have enough samples for mapping the exact epitopes.

Reduction of Viral Loads in the Early Phase of SIV Infection by Prophylactic Vaccination

We also investigated SIVmac239 infection of additional five, vaccinated Burmese rhesus macaques sharing the MHC-I haplotype D. These animals received a prophylactic DNA/SeV-Gag vaccination. In four of these five vaccinated macaques, plasma viremia became undetectable after 6 months, while macaque R06-033 showed persistent viremia (Fig. 5A). Difference in viral loads between unvaccinated and vaccinated D$^+$ animals was unclear in the acute phase, but the latter vaccinees showed significant reduction in viral loads compared to those in the former unvaccinated at 3 months ($p = 0.0360$; Fig. 5B). After 6 months, unvaccinated animals also showed reduced viral loads, and the difference in viral loads between unvaccinated and vaccinated became unclear.

In contrast to unvaccinated D$^+$ animals, all five vaccinated animals elicited Gag-specific CD8$^+$ T-cell responses at week 2 after challenge (Fig. 6), reflecting the effect of prophylactic vaccination. We then examined CD8$^+$ T-cell responses specific for individual SIV antigens in these vaccinated animals (Fig. 7). Samples for this analysis were unavailable in vaccinated macaque R01-009. Vaccinated animals except for macaque R06-020 showed dominant Gag-specific CD8$^+$ T-cell responses even at 1–2 months. However, Gag-specific CD8$^+$ T-cell responses became not dominant after 1 year, while Nef-specific or Vif-specific CD8$^+$ T-cell responses became predominant, instead, in most vaccinees except for macaque R03-016.

Like three unvaccinated macaques (R05-006, R06-034, and R-360), vaccinated D$^+$ animals induced Nef_{35-49}-specific and $Nef_{115-129}$-specific CD8$^+$ T-cell responses after SIV challenge (Fig. 8). In analyses of three unvaccinated (Fig. 4) and four vaccinated animals (Fig. 8), Nef_{35-49}-specific CD8$^+$ T-cell responses were induced in the early phase in six animals but mostly became undetectable in the chronic phase. $Nef_{115-129}$-specific CD8$^+$ T-cell responses were also induced in most animals except for macaque R06-020 which showed $Nef_{112-126}$-specific ones in the chronic phase (data not shown). Macaques R05-006, R03-021, and R03-016 showed efficient $Nef_{115-129}$-specific CD8$^+$ T-cell responses not in the early phase but in the chronic phase. In contrast, vaccinated animal R06-033 that failed to control viremia showed higher $Nef_{115-129}$-specific CD8$^+$ T-cell responses in the early phase than those in the chronic phase.

Selection of Mutations in Nef CD8$^+$ T-cell Epitope-coding Regions

To see the effect of selective pressure by Nef-specific CD8$^+$ T-cell responses on viral genome mutations, we next analyzed nucleotide sequences in viral *nef* cDNAs amplified from plasma RNAs obtained at several time points after SIV challenge. Nonsynonymous mutations detected predominantly in Nef_{35-49}-coding and $Nef_{115-129}$-coding regions were as shown in Fig. 9. Remarkably, all the unvaccinated and vaccinated D$^+$ animals showed rapid selection of mutations in the Nef_{35-49}-coding region in 3 months. On the other hand, mutations in the $Nef_{115-129}$-coding region were observed in the late phase in all the three

		Nef35-49						Nef115-129				
Nef		36	37	41	42	44		119	122	124	125	126
		E	D	Q	S	G		M	F	K	E	K
R01-012	1M		*G									
	3M		*G									
	14M	*G	*G									
	24M	*G	*G			*E						
R05-006	1M					*E						
	3M			R								
	16M			R								
	24M			R								R
R06-034	1M											
	3M	*G										
	10M	*G	*G			*E						*R
	18M	G				E						R
R08-005	1M											
	3M				*F							
	6M	G										
	14M				F							
	24M	*G			F							
R-360	1M											
	3M		*G									
	6M		G									
	12M		G					T	L			
	20M		G					T	L			
R06-020	1M											
	3M	*K										
	11M		G	R								
R06-033	1M											
	3M	*G										
	6M		*G									
	14M		G			*E					K	E
R03-021	1M											
	3M				*F							
	14M	G								R		
R03-016	1M	*K		*R								
	4M	K										
	12M	K										

Figure 9. Predominant non-synonymous mutations in Nef_{35-49}-coding and $Nef_{115-129}$-coding regions of viral cDNAs in D^+ animals after SIVmac239 challenge. Amino acid substitutions are shown. Detection of similar levels of wild-type and mutant sequences at the residue is indicated by asterisks. Samples for this analysis were unavailable in macaque R01-009.
doi:10.1371/journal.pone.0054300.g009

unvaccinated animals eliciting $Nef_{115-129}$-specific $CD8^+$ T-cell responses. These mutations were also detected in two of three vaccinated animals eliciting $Nef_{115-129}$-specific $CD8^+$ T-cell responses.

We also analyzed viral *gag* sequences to see the effect of Gag-specific $CD8^+$ T-cell pressure on viral genome mutations in vaccinated animals (data not shown). Our previous study [35] showed rapid selection of a mutation leading to a glutamine (Q)-to-lysine (K) change at the 58th residue in Gag (Q58K) at week 5 in vaccinated macaque R01-009, although no more samples were available for this sequencing analysis. This Q58K mutation results in escape from Gag_{50-65}-specific $CD8^+$ T-cell recognition. In the present study, macaque R03-016 showed rapid selection of a mutation leading to a K-to-asparagine (N) change at the 478th residue in Gag in 1 month. These results may reflect rapid disappearance of detectable plasma viremia in 1 or 2 months in these two vaccinees. Macaque R06-020 showed selection of a *gag* mutation in 3 months, while other two vaccinees (R06-033 and R03-021) selected no *gag* mutation in the early phase.

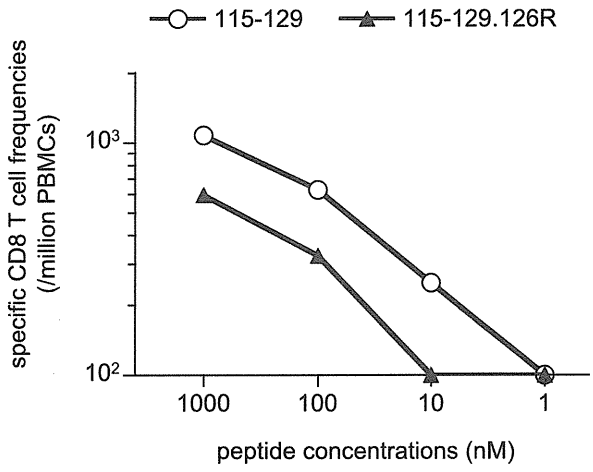

Figure 10. IFN-γ induction in $CD8^+$ T cells after stimulation with the wild-type or the mutant peptide. PBMCs obtained at week 31 from macaque R06-033 were stimulated by coculture with B-LCL pulsed with indicated concentrations of the wild-type $Nef_{115-129}$ peptide (open circles, 115–129, LAIDMSHFIKEKGGL) or the mutant $Nef_{115-129}$ peptide with a K126R alteration (closed triangles, 115–129.126R, LAIDMSHFIKERGGL).
doi:10.1371/journal.pone.0054300.g010

Discussion

HIV infection in humans with polymorphic MHC-I genotypes induces various patterns of viral antigen-specific $CD8^+$ T-cell responses. Previous studies have found several protective MHC-I alleles associated with lower viral loads and slower disease progression in HIV/SIV infection [7,13,14,16,17]. Elucidation of the mechanisms of viral control associated with individual protective MHC-I alleles would contribute to HIV cure and vaccine-based prevention. Because $CD8^+$ T-cell responses specific for some MHC-I-restricted epitopes can be affected by those specific for other MHC-I-restricted epitopes due to immunodominance [29,46,47], macaque groups sharing MHC-I genotypes at the haplotype level are useful for the analysis of cooperation of multiple epitope-specific $CD8^+$ T-cell responses. Previously, we reported a group of Burmese rhesus macaques sharing MHC-I haplotype *90-120-Ia* (A), which dominantly induce Gag-specific $CD8^+$ T-cell responses and tend to show slower disease progression after SIVmac239 challenge [21]. In the present study, we presented another type of protective MHC-I haplotype, which is not associated with dominant Gag-specific $CD8^+$ T-cell responses. Significant reduction of viral loads in unvaccinated macaques possessing this D haplotype compared to those in D^- macaques was observed after 6 months. Analysis of SIV infection in macaques sharing this protective MHC-I haplotype would lead to understanding of $CD8^+$ T-cell cooperation for viral control.

Analyses of antigen-specific $CD8^+$ T-cell responses after SIVmac239 challenge indicate that this MHC-I haplotype D is associated with predominant Nef-specific $CD8^+$ T-cell responses. Nef-specific $CD8^+$ T-cell responses were efficiently induced in all SIV controllers, whereas Gag-specific $CD8^+$ T-cell responses were dominant in only one of them. We found Nef_{35-49}-specific and $Nef_{115-129}$-specific $CD8^+$ T-cell responses shared in D^+ animals. We were unable to determine the MHC-I alleles restricting these epitopes, but these responses are not usually induced in our

previous D^- cohorts and considered to be associated with this MHC-I haplotype D.

Sequencing analysis of viral genomes showed rapid selection of mutations in the Nef_{36-44}-coding region within 3 months in all the D^+ animals. This is consistent with our results that Nef_{35-49}-specific $CD8^+$ T-cell responses were mostly induced in the early phase but undetectable in the chronic phase. These mutations were not consistently selected in our previous D^- cohorts and thus considered as MHC-I haplotype D-associated mutations. This suggests strong selective pressure by Nef_{35-49}-specific $CD8^+$ T-cell responses in the acute phase of SIVmac239 infection in D^+ macaques, although it remains undetermined whether these mutations result in viral escape from Nef_{35-49}-specific $CD8^+$ T-cell recognition.

$Nef_{115-129}$-specific $CD8^+$ T-cell responses were detected in six D^+ animals. In five of them, nonsynonymous mutations in the $Nef_{119-126}$-coding region were observed in the chronic phase. At least, we confirmed viral escape from $Nef_{115-129}$-specific $CD8^+$ T-cell recognition by a mutation leading to a K-to-arginine (R) (K126R) substitution at Nef residue 126 (Fig. 10). The number of nonsynonymous substitutions per the number of sites estimated to be nonsynonymous (dN) exceeded that estimated to be synonymous (dS) during the evolution process of $Nef_{115-129}$-coding region, but the value did not show statistically significant difference from that of neutral selection. Among three unvaccinated animals that controlled SIV replication without dominant Gag-specific $CD8^+$ T-cell responses, amino acid substitutions in the $Nef_{119-126}$-coding region were observed in a year in macaques R06-034 and R-360 but after 2 years in macaque R05-006. The former two animals tended to show earlier increases in plasma viral loads in the chronic phase, while the latter R05-006 maintained higher frequencies of $Nef_{115-129}$-specific $CD8^+$ T-cell responses. $Nef_{115-129}$-specific $CD8^+$ T-cell responses were efficient in the chronic phase in vaccinated controllers R03-021 and R03-016 but decreased in R06-033 that failed to contain SIV replication. Although a possible effect of this haplotype-associated factors other than $CD8^+$ T-cell responses such as NK activity on SIV infection [48,49,50] remains undetermined, these results imply involvement of Nef-specific $CD8^+$ T-cell responses in the SIV control associated with MHC-I haplotype D.

Unvaccinated macaque R08-005 dominantly elicited Gag antigen-specific $CD8^+$ T-cell responses and showed rapid selection of a mutation encoding Gag 257 residue, which was not observed in any other D^+ animals. Nef-specific $CD8^+$ T-cell responses were detectable only at week 2 in the acute phase (data not shown) and a mutation in the Nef_{42}-coding region was rapidly selected. It is speculated that those dominant Gag-specific $CD8^+$ T-cell responses associated with the second, non-D MHC-I haplotype were effective in this animal. Nef_{35-49}-specific $CD8^+$ T-cell responses may not be efficient due to immunodominance but exert some suppressive pressure on viral replication.

DNA/SeV-Gag vaccination resulted in earlier reduction of viral loads after SIV challenge. Vaccinees showed significantly lower viral loads at 3 months than those in unvaccinated animals. Gag-specific $CD8^+$ T-cell responses were elicited at week 2 in all the vaccinees but not in the unvaccinated except for one animal R08-005. No *gag* mutations were shared in the vaccinees in the acute phase, but three of them showed rapid selection of individual non-synonymous mutations in *gag*. Rapid selection of mutations in the Nef_{36-44}-coding region was consistently detected even in these vaccinees. These results suggest broader $CD8^+$ T-cell responses consisting of dominant vaccine antigen Gag-specific and inefficient naive-derived Nef-specific ones in the acute phase. In three vaccinated animals, Gag-specific $CD8^+$ T-cell responses became lower or undetectable, and instead, Nef-specific $CD8^+$ T-cell responses became predominant in the chronic phase.

In summary, we found a protective MHC-I haplotype not associated with dominant Gag-specific $CD8^+$ T-cell responses in SIVmac239 infection. Our results in D^+ macaques suggest suppressive pressure by Nef_{35-49}-specific and $Nef_{115-129}$-specific $CD8^+$ T-cell responses on SIV replication, contributing to reduction in set-point viral loads. DNA/SeV-Gag-vaccinated D^+ animals induced Gag-specific $CD8^+$ T-cell responses in addition to Nef-specific ones after SIV challenge, resulting in earlier containment of SIV replication. This study presents a pattern of SIV control with involvement of non-Gag antigen-specific $CD8^+$ T-cell responses, contributing to accumulation of our knowledge on HIV/SIV control mechanisms.

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Author Contributions

Performed animal experiments: HS TM TI YK. Performed MHC-I typing: TKN AK. Conceived and designed the experiments: NT TM. Performed the experiments: NT TN YT HY AT. Analyzed the data: NT HY T. Shiino TM. Contributed reagents/materials/analysis tools: MI AI HH T. Shu MH. Wrote the paper: NT TM.

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ORIGINAL RESEARCH

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.[1–3] 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).[4,5] 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.[6] 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.[7] However, the induction of both subsets of cells – Th1 and Treg cells – for the treatment of asthma using immunological strategic tools is very difficult.

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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.[8–10] 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).[11] 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.[12] Moreover, the induction of Th1-type immune responses by immunization of Ag85B was enhanced by presensitization with bacillus Calmette–Guerin.[13,14] 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.[13,14] 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.[15] 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 hematocytometer, 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 (5×10^6) 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.[15] 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

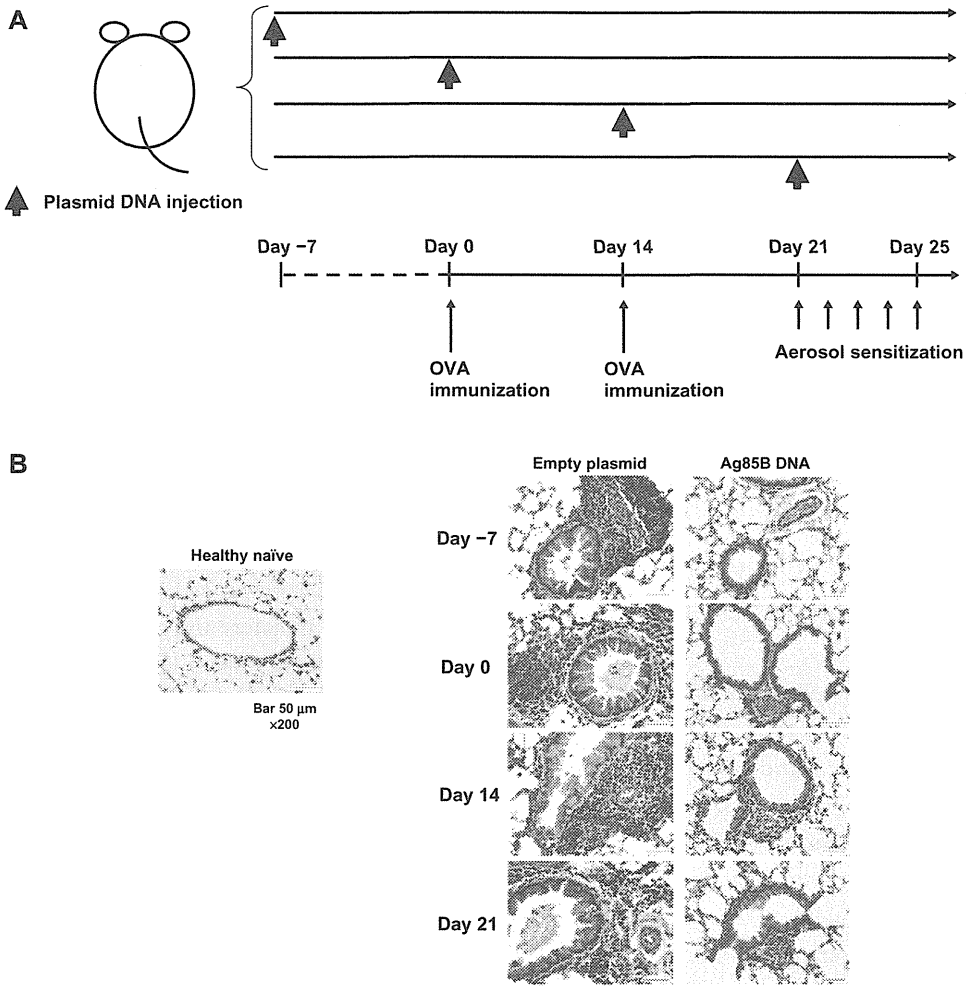

Figure 1 Inhibition of the development of allergic inflammation in lungs by administration of Ag85B DNA vaccine. (**A**) Experimental design used to investigate the effects of Ag85B DNA vaccine on OVA-induced asthma. Mice were subjected to an OVA sensitization scheme,[15] and 50 μg of Ag85B DNA vaccine 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.[11] 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-γ 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-γ 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-γ 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-γ, 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-γ, 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-β.[16] In the current study, mRNA expression levels of IL-10 and transforming growth factor-β 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.[17] 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

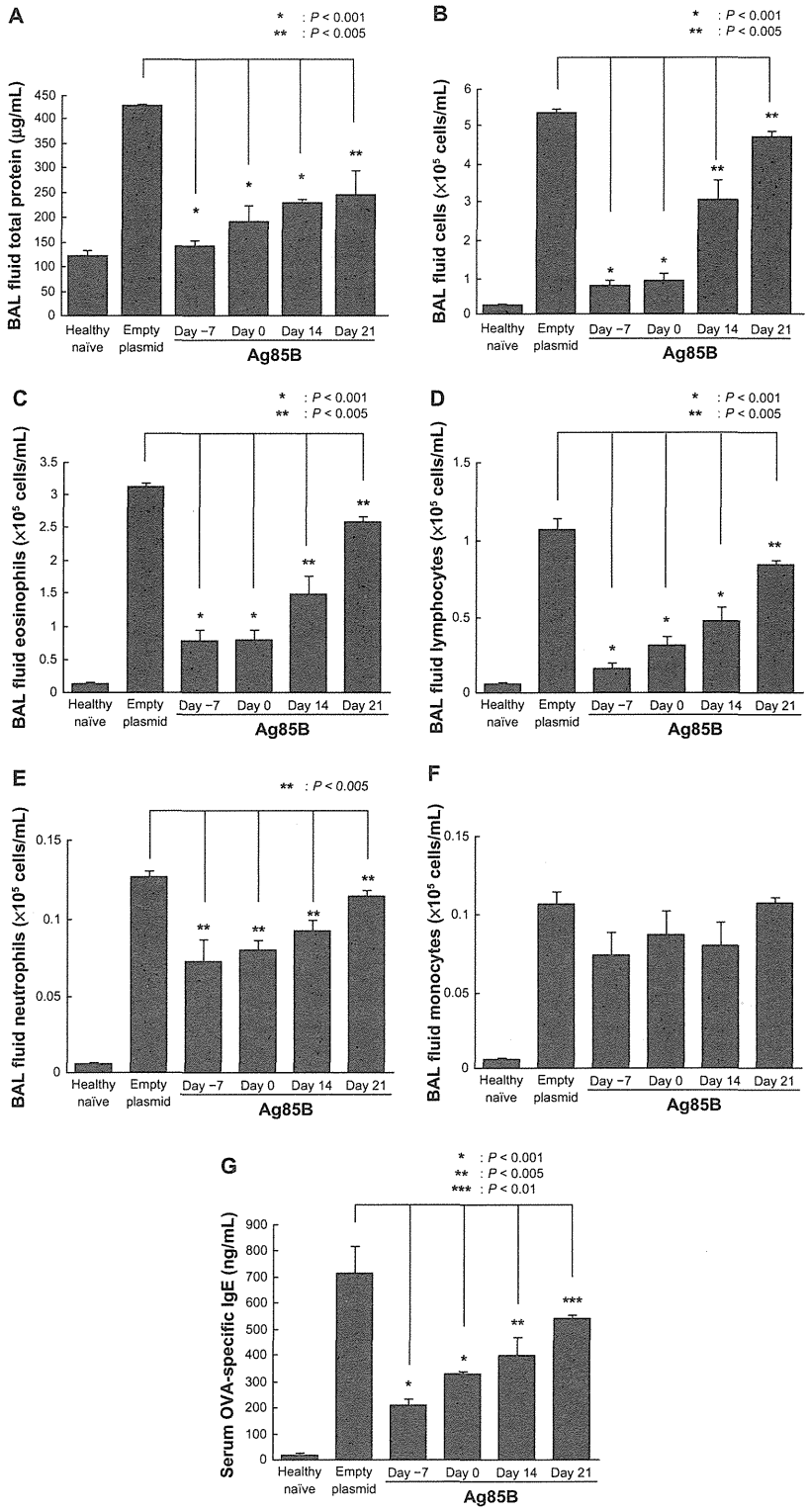

Figure 2 Marked inhibition of the development of allergic inflammation by administration of Ag85B DNA. BAL fluid was obtained by injecting and recovering two 0.5 mL aliquots of phosphate-buffered saline via a tracheal cannula. Cells in the lavage fluid were counted using a hematocytometer, and the differentials 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 naïve 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.

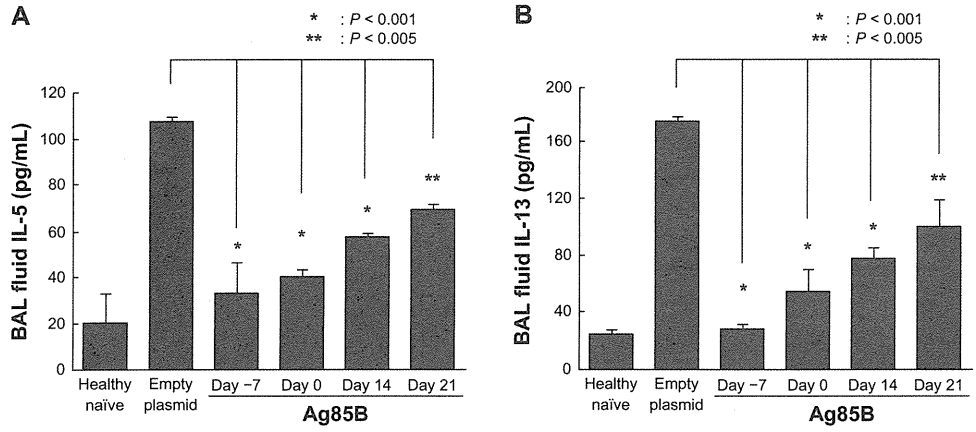

Figure 3 IL-5 and IL-13 production in BAL fluid. Amounts of (**A**) IL-5 and (**B**) IL-13 in BAL fluid were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) 25 days after the first ovalbumin immunization.
Notes: Data are representative of at least three independent 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).[18] 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.[19–21] Moreover, it was recently reported that systemic inoculation of a plasmid DNA may cause dendritic cell activation through direct transfection into dendritic cells.[22] 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.[18] It was previously reported that plasmid DNA encoding Ag85B stimulated the expression of TLR2, TLR3, and TLR4 mRNA. One possibility is that the induction

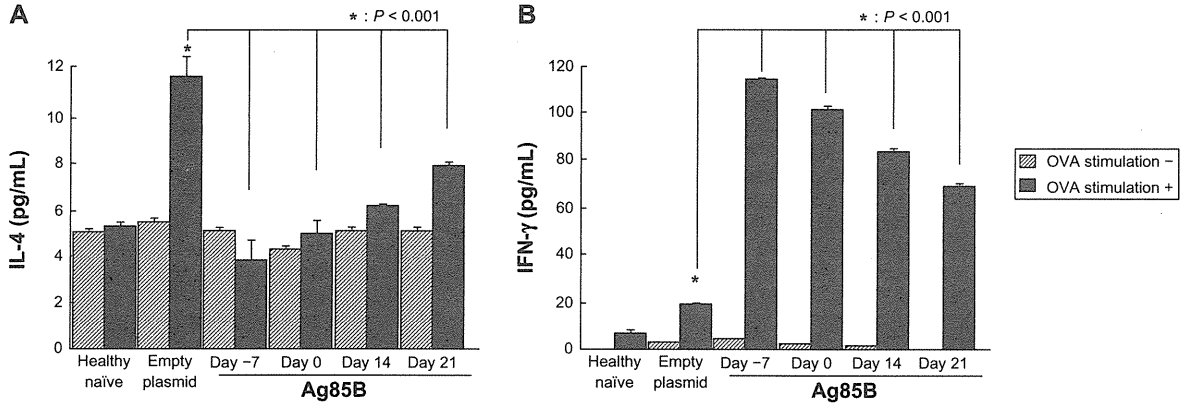

Figure 4 IFN-γ and IL-4 production in culture supernatant. Amounts of (**A**) IFN-γ and (**B**) IL-4 in culture supernatant were measured by enzyme-linked immunosorbent assay 25 days after the first OVA immunization. Spleen cells from immunized mice (5×10^6) were cultured with 10 μg/mL OVA in 24-well culture plates at a volume of 2 mL. After incubation at 37°C in a humidified incubator (5% carbon dioxide) for 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-γ, interferon-γ; IL-4, interleukin-4; OVA, ovalbumin.

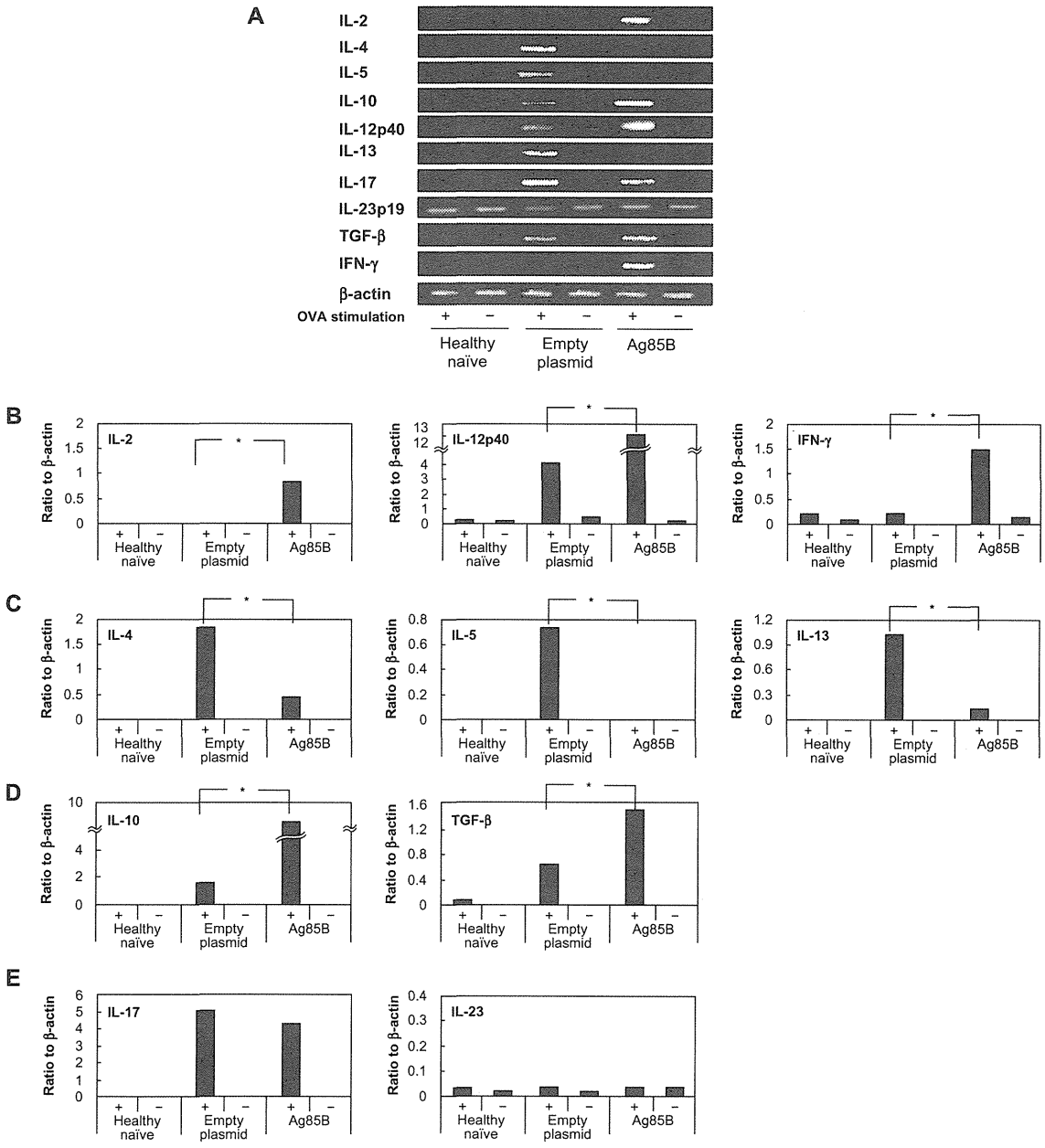

Figure 5 Detection of cytokine messenger ribonucleic acid from lymphocytes using real-time polymerase chain reaction. Spleen cells were stimulated in vitro with OVA for 1 day in culture. Spleen cells stimulated with fetal calf serum were used as controls. Total ribonucleic acid was purified from the 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 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 previously described.[15] 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-γ, interferon-γ; IL, interleukin; OVA, ovalbumin; TGF-β, transforming growth factor-β.

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.[14] Various proteins derived from pathogens promote Th1 responses through stimulation of TLRs and subsequently through secretion of cytokines.[18] It has also been reported that TLR signaling induces not only Th1-type immune responses but also secretion of various cytokines from Treg cells.[23–27] 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.[28] 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.[16] 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.[29–32] 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.[33] Several host genetic factors, including natural resistance-associated macrophage protein 1 (NRAMP1),[34] vitamin D receptor (VDR),[35,36] and Mendelian susceptibility to mycobacterial disease,[37] have been reported to be involved in responses to mycobacteria (reviewed in Casanova and Abel).[38] 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.[39] Moreover, environmental factors are important for immune responses induced by mycobacteria in therapy for atopic diseases.[40,41] Presensitization of mycobacteria in the natural environment affects the induction of Th1-type immune responses by mycobacteria vaccination.[9,42,43] 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.[44,45]

Wu et al demonstrated the effects of intranasal administration of Ag85B in a mouse model of asthma.[46] It was previously reported that Ag85B has strong adjuvant activities involving Th1 immune responses.[14] 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.[47] 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

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Disclosure

The authors report no conflicts of interest in this work.

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

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

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

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

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

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

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

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

Materials and Methods

Animals

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

Immunization protocols

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

Plasmid construction and DNA transfection

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

Histopathologic examination

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

Flow cytometry

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

Measurements of cytokines and chemokines

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

Proliferative responses of T cells

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

Western blot analysis

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

Adoptive transfer of T cells

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

Quantitative real-time RT-PCR

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

Echocardiography

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

Statistical analysis

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

Results

SOCS1 DNA administration inhibits the development of EAM

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

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

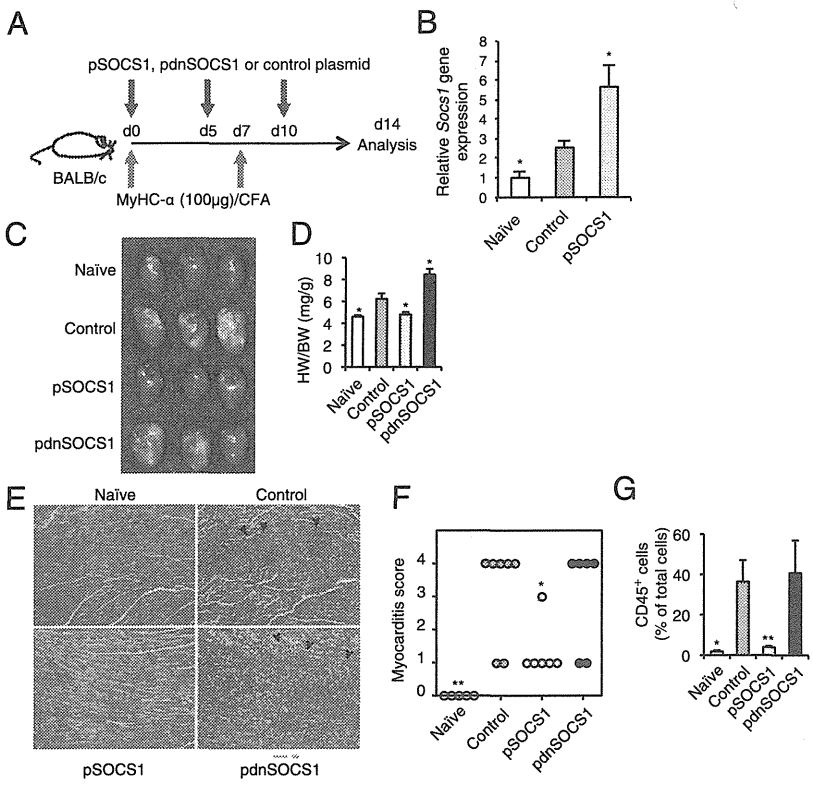

FIGURE 1. Amelioration of EAM and heart failure by SOCS1 DNA administration. (**A**) BALB/c mice were immunized twice, on days 0 and 7, with 100 μg of MyHC-α and treated with pSOCS1, pdnSOCS1, or control plasmid on days 0, 5, and 10. (**B**) QRT-PCR for the *Socs1* gene. RNA samples were obtained from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

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

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

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

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

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

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

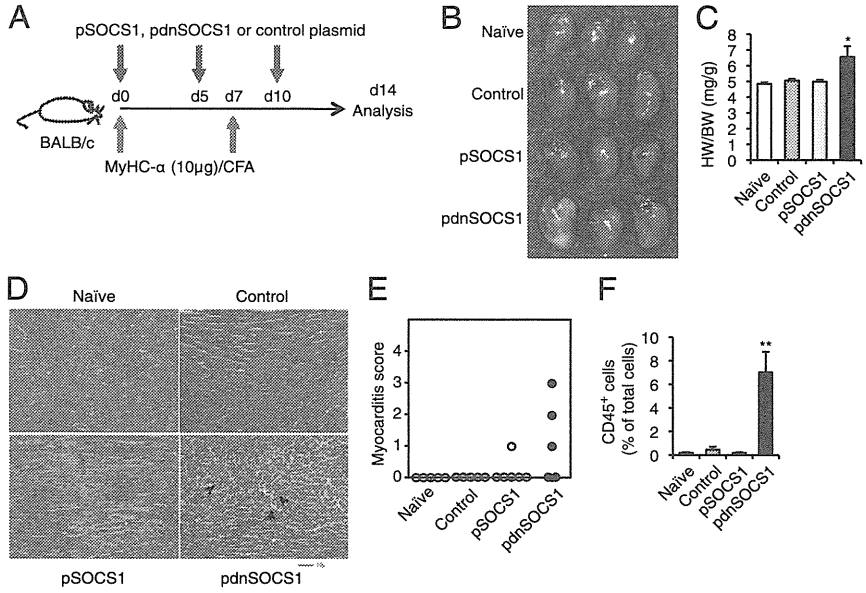

FIGURE 2. Increased susceptibility to EAM induced by inhibition of SOCS1. (**A**) Mice were immunized twice, on days 0 and 7, with 10 μg of MyHC-α emulsified 1:1 in PBS/CFA and treated with pSOCS1, pdnSOCS1, or control plasmid on days 0, 5, and 10. (**B**) Representative gross hearts (day 14) of naive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

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

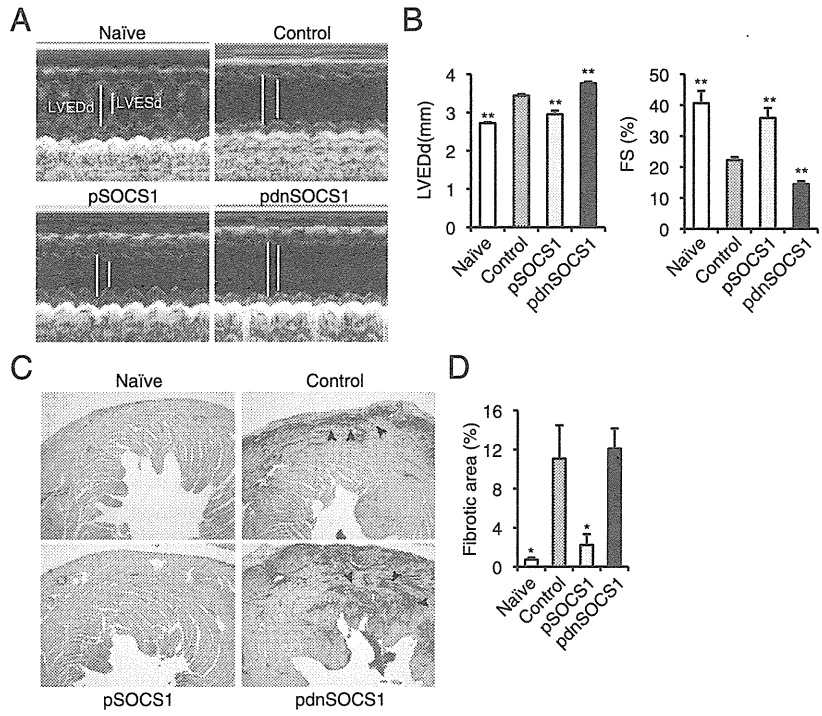

FIGURE 3. SOCS1 DNA administration prevents progression to heart failure. (**A** and **B**) Echocardiography was performed on naive and immunized mice on day 35. (A) Representative M-mode echocardiograms. Bars indicate LVESd and LVEDd. Bar graphs (B) represent LVEDd and percentage of FS from the indicated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

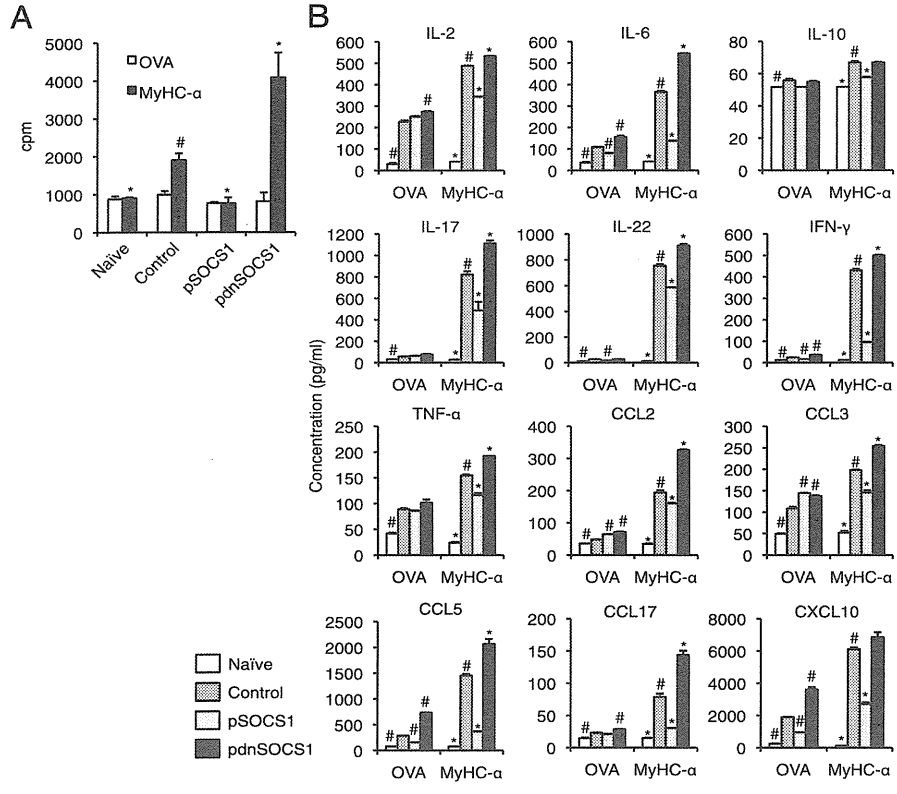

FIGURE 4. Impaired expansion of heart-specific $CD4^+$ T cells in pSOCS1-treated mice. (**A**) Splenocytes were isolated from naive and EAM mice treated with pSOCS1, pdnSOCS1, or control plasmid on day 14 and restimulated in vitro with MyHC-α or OVA peptide for 72 h. Proliferation was assessed by measurement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

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

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

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

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

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

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