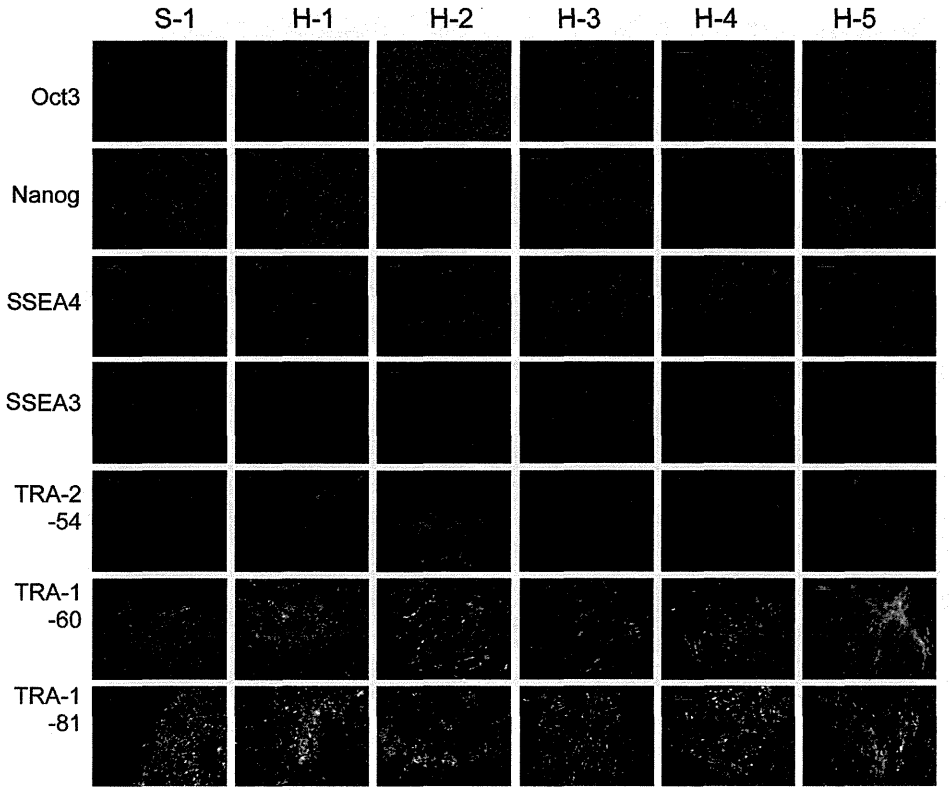

Fig. 2. Immunofluorescence analysis of undifferentiated markers in iPS cells. All cell lines expressed Oct-3, Nanog, SSEA4, TRA-2-54, TRA-1-60 and TRA-1-81, but not SSEA3, which was the same result as in cynomolgus monkey ES cell lines. Nuclei were counterstained with Hoechst 33342.

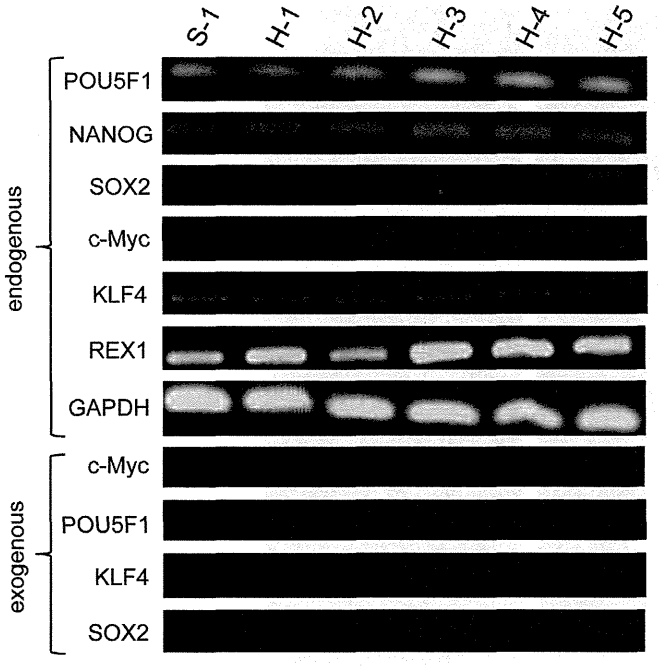

Fig. 3. Gene expression analysis by RT-PCR in iPS cell lines. All cell lines expressed endogenous POU5F1, SOX2, c-MYC, KLF4, Nanog and REX1, but not exogenous POU5F1, SOX2, c-MYC and KLF4, with the exception that exogenous c-MYC was expressed in the H-2 line.

spontaneously differentiated cells from EBs (Fig. 6E–H). In addition, in EBs at days 7, 14, 21 and 28 of culture, we examined the expression of the differentiated markers by RT-PCR. The expression of Brachyury, Pax6 and VASA was detected continuously, and the expression of AFP, GATA4 and CDX2 was detected partially (Fig. 7). In particular, expression of VASA was up-regulated as the *in vitro* culture progressed, and for AFP and CDX2, strong expression was identified in the later stages of the *in vitro* culture.

4. Discussion

We succeeded in establishing iPS cells from cynomolgus monkey somatic cells by using genes taken from the monkeys themselves. The examined iPS cell lines had characteristics similar to other primate ES cell lines. Our study will thus contribute to the development of research resources for a wide range of medical investigations using cynomolgus monkeys. To the best of our knowledge, this is the first report to describe the establishment of iPS cell lines by using cynomolgus monkey genes.

Although ES cells have the potential to make a major contribution to regenerative medicine, until recently, public concerns over the ethics of destroying early human embryos had limited their use. Takahashi and Yamanaka (2006) performed a landmark study to address this problem. They succeeded in directly inducing pluripotent stem cells from somatic cells in mice. By applying this technique, iPS cell lines have been established in humans (Takahashi et al., 2007; Yu et al., 2007), rats (Liao et al., 2009; Li et al., 2009), pigs (Esteban et al., 2009; Wu et al., 2009), rhesus monkeys (Liu et al., 2008), common marmosets (Tomioka et al., 2010), rabbits (Honda et al., 2010) and cynomolgus monkeys (Okahara-Narita et al., 2012). However, the establishment of iPS cell lines using a species' own genes has been achieved only in mice, humans and rhesus monkeys, while in the other species, genes taken from humans or mice were used (Liao et al., 2009; Li et al., 2009; Esteban et al., 2009; Wu et al., 2009; Tomioka et al., 2010; Honda et al., 2010; Okahara-Narita et al., 2012). We here reported on the establishment of iPS cells generated from somatic cells by using genes taken from cynomolgus monkeys.

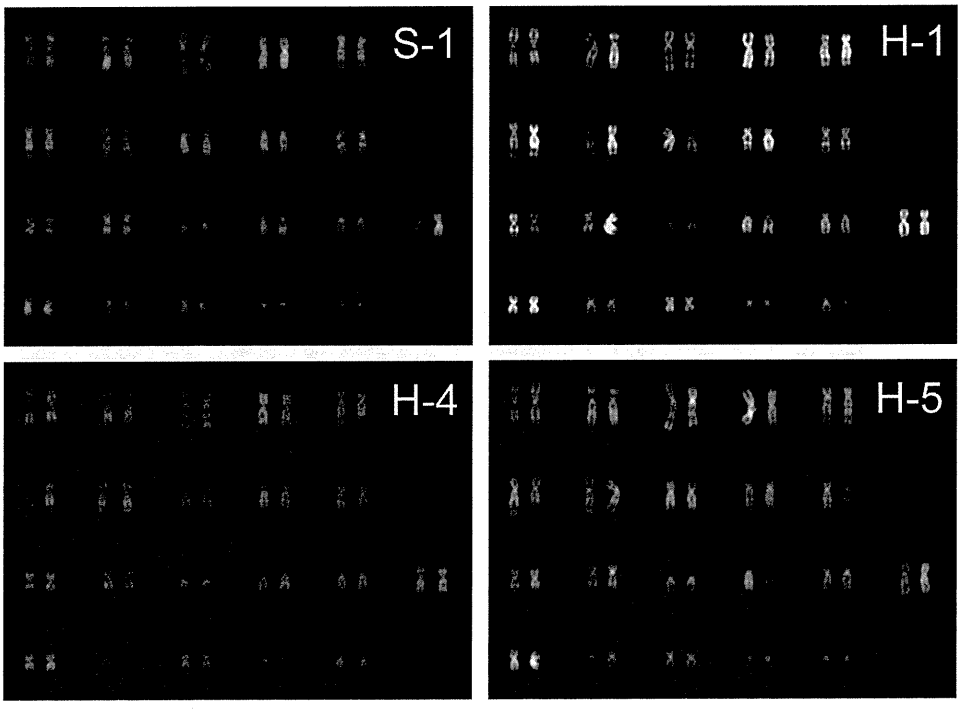

Fig. 4. Karyotyping analysis revealed that 82% (41/50), 86% (43/50), 78% (39/50) and 82% (41/50) of the S-1 (passage 23), H-1 (passage 28), H-4 (passage 23) and H-5 (passage 26) lines examined had a normal chromosome number of 40 and sex chromosomes XX.

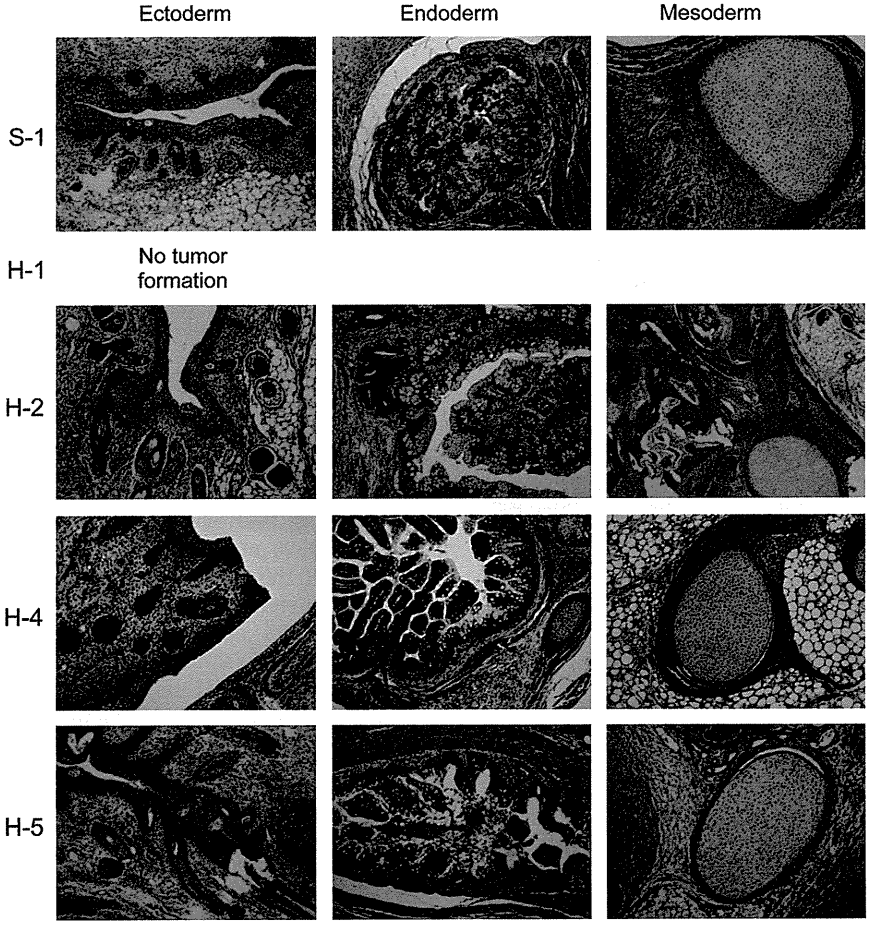

Fig. 5. Histological analysis of teratomas formed from iPS cells. Four of the five iPS cell lines transferred into immunodeficient mice formed tumors, but not the H-1 line. Histological analysis revealed that the formed tumors were teratomas consisting of ectoderm (skin), endoderm (gut) and mesoderm (cartilage and bone) tissues.

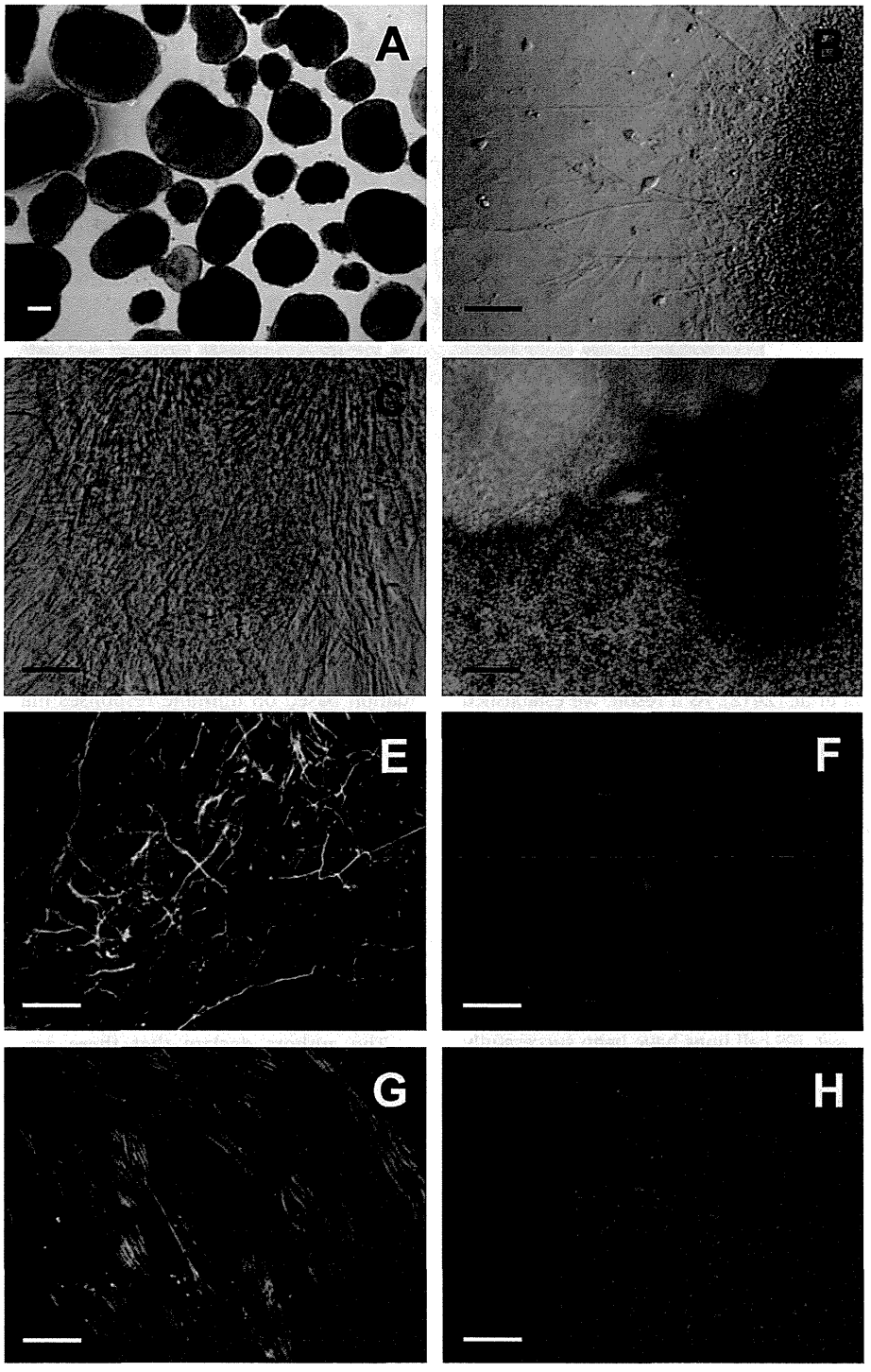

Fig. 6. Analysis of *in vitro* differentiation in iPS cells. Most EBs formed solid-type clusters (A). EBs outgrew on tissue culture dishes and spontaneously differentiated into various cells such as neuron-like cells (B), beating myocardial-like cells (C) and pigment cells (D). Immunofluorescence analysis confirmed the expression of β-tubulin III (E) (ectoderm), Foxa2 (F) (endoderm), and α-smooth muscle (G) and Brachyury (H) (mesoderm) in the cells spontaneously differentiated from EBs. Nuclei were counterstained with Hoechst 33342 (E–H). The bar represents 100 μm.

The cynomolgus monkey iPS cell lines that we established were found to be similar to other primate ES cell lines, including that of cynomolgus monkeys, with respect to characteristics such as morphology, the expression of undifferentiated markers, pluripotency and karyotypic stability. However, the expression of SSEA3, which is one of the ES cell-specific markers, could not be confirmed in the cynomolgus monkey iPS cell lines, although these cell lines could form teratomas consisting of three embryonic germ cells. This feature, the ambiguous expression of SSEA3, was also seen in the cynomolgus monkey ES cell lines (Suemori et al., 2001), while the ES cell lines of other primates, including humans, rhesus monkeys and common marmosets, express SSEA3 (Thomson et al., 1995, 1996, 1998; Sasaki et al., 2005). The results showed that, like primate ES cells, the iPS cell lines established in this study were pluripotent stem cell lines.

We succeeded in establishing iPS cells by using the amphotropic-type retroviral vectors produced from Plat-A cells (Kitamura et al., 2003) for introduction of transgenes into cynomolgus monkey

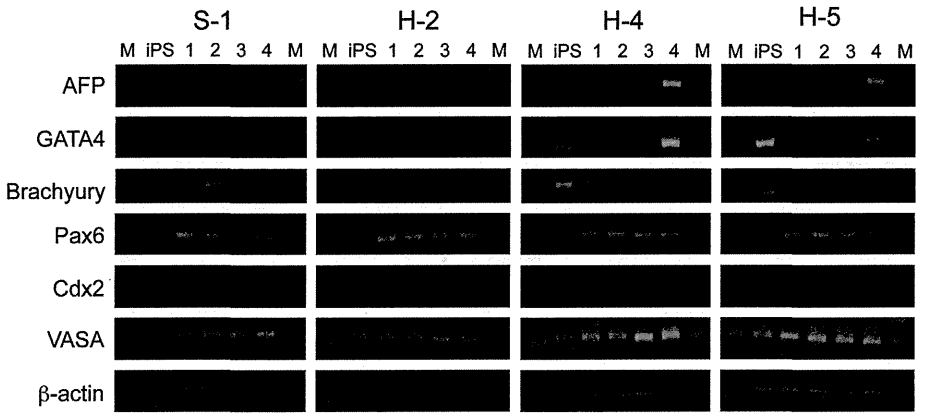

Fig. 7. Gene expression analysis of differentiation markers by RT-PCR in EBs from iPS cells. Expression of differentiation markers was detected by RT-PCR in EBs at days 7, 14, 21 and 28 of culture using four iPS cell lines. Endoderm (AFP, GATA4), mesoderm (Brachyury), ectoderm (Pax6), trophectoderm (CDX2) and germ cell (VASA) markers were analyzed.

somatic cells. Initially, when we examined the introduction of the GFP gene into cells derived from newborn skin and fetal liver, we confirmed the positivity for GFP expression in 77% and 68% of cells, respectively. Because of this highly efficient introduction, we applied the amphotropic retroviral vectors to direct introduction of four transgenes without the introduction of the ecotropic-type retrovirus receptor gene in accordance with the methods of Takahashi et al. (2007) and Okahara-Narita et al. (2012). As a result, we established the iPS cell lines from two kinds of somatic cells. These results showed that the amphotropic-type retroviral vectors could be used to derive pluripotent stem cells, such as mouse and human somatic cells, from cynomolgus monkey somatic cells by introducing the monkey POU5F1, SOX2, KLF4 and c-MYC genes.

The iPS cell lines established in this study expressed the endogenous undifferentiated marker genes POU5F1, NANOG, REX1, SOX2, KLF4 and c-MYC. In contrast, no expression of the transgenes POU5F1, SOX2 and KLF4 was observed in any of the cell lines, while c-MYC expression was observed in a single line. No expression of the four transgenes was observed in five of six cell lines.

In the common marmoset, iPS cell lines have been successfully established by the introduction of five or six genes, but not by the introduction of four genes (Tomioka et al., 2010). However, an important difference between this previously reported study and our present work is that the former used human genes. We and Liu et al. (2008) showed that iPS cell lines could be established using four transgenes from the same species without the introduction of the ecotropic-type retrovirus receptor gene in cynomolgus and rhesus monkeys. The results using two macaque monkeys demonstrate that, by using genes taken from the same species, it may be possible to achieve reprogramming of monkey somatic cells simply and to establish iPS cells suitable for medical research in primates. Furthermore, this may enable detailed analysis of the mechanisms underlying the reprogramming. Elucidating the nature of these mechanisms may in turn contribute to the establishment of an effective method for deriving iPS cells in a completely undifferentiated state without the need to integrate the transgenes into the genome.

We confirmed that, in the established iPS cell lines, expression of VASA, one of the germ cell marker genes (Castrillon et al., 2000; Toyooka et al., 2000), was up-regulated as the *in vitro* culture progressed. This suggested that the iPS cells have the potential to differentiate into germ cells. The differentiation from pluripotent stem cells into germ cells is being studied actively (Clark et al., 2004; Park et al., 2009; Aflatoonian et al., 2009), and the application of this technology to humans is anticipated. The cynomolgus monkeys may predominate in the study of human reproductive medicine because they are annual breeders, unlike rhesus monkeys, which are seasonal breeders.

We succeeded in the establishment of iPS cell lines using four genes taken from the cynomolgus monkeys themselves. When cells differentiated from the iPS cells established with genes from different species are transplanted into monkeys, reactivation of the genes may induce immune responses. Thus an appropriate evaluation based on immune responses may not be possible due to the reactivation of genes from different species. The iPS cell lines that we established will exclude this possibility. In addition, the genes from different species might have unexpected disadvantageous effects. Fortunately, unlike in mice, it is possible to conduct a long-term investigation in monkeys. Therefore, to evaluate the iPS cells established by genes from the same or different species, we plan a long-term investigation of post-transplantation into monkeys in the future.

Author disclosure statement

The authors declare that there are no conflicting financial interests.

Acknowledgments

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Pitavastatin Regulates Helper T-Cell Differentiation and Ameliorates Autoimmune Myocarditis in Mice

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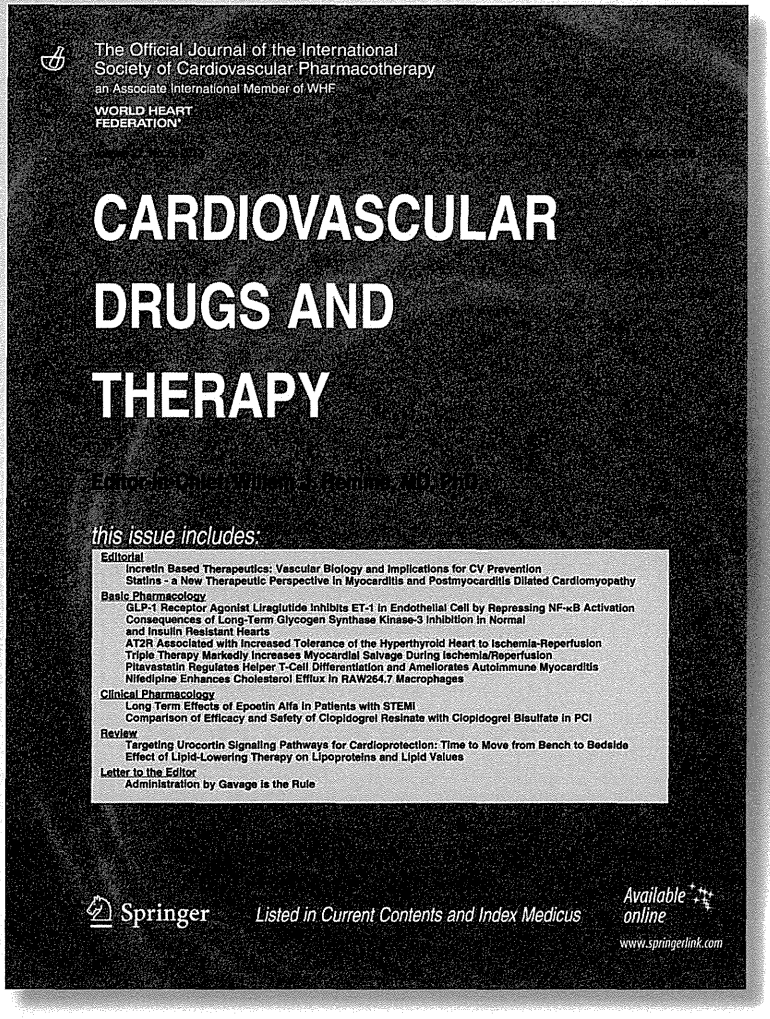

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ORIGINAL ARTICLE

Pitavastatin Regulates Helper T-Cell Differentiation and Ameliorates Autoimmune Myocarditis in Mice

Kazuko Tajiri · Nobutake Shimojo · Satoshi Sakai · Tomoko Machino-Ohtsuka · Kyoko Imanaka-Yoshida · Michiaki Hiroe · Yusuke Tsujimura · Taizo Kimura · Akira Sato · Yasuhiro Yasutomi · Kazutaka Aonuma

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Abstract
Purpose Experimental autoimmune myocarditis (EAM) is a mouse model of inflammatory cardiomyopathy, and the involvement of T helper (Th) 1 and Th17 cytokines has been demonstrated. Accumulated evidence has shown that statins have anti-inflammatory and immunomodulatory effects; however, the mechanism has not been fully elucidated. This study was designed to test the hypothesis that pitavastatin affects T cell-mediated autoimmunity through inhibiting Th1 and Th17 responses and reduces the severity of EAM in mice.
Methods The EAM model was established in BALB/c mice by immunization with murine α-myosin heavy chain. Mice were fed pitavastatin (5 mg/kg) or vehicle once daily for 3 weeks from day 0 to day 21 after immunization.
Results Pitavastatin reduced the pathophysiological severity of the myocarditis. Pitavastatin treatment inhibited the phosphorylation of signal transducer and activator of transcription (STAT)3 and STAT4, which have key roles in the Th1 and Th17 lineage commitment, respectively, in the heart, and suppressed production of Th1 cytokine interferon-γ and Th17 cytokine interleukin-17 from autoreactive $CD4^+$ T cells. In in vitro T-cell differentiation experiments, pitavastatin-treated T cells failed to differentiate into Th1 and Th17 cells through inhibiting the transcription of T-box expressed in T-cells (T-bet) and RAR-related orphan receptor γt (RORγT) which have critical roles in the development of Th1 and Th17 cells, respectively, and this failure was rescued by adding mevalonate.
Conclusions Pitavastatin inhibits Th1 and Th17 responses and ameliorates EAM. These results suggest that statins may be a promising novel therapeutic strategy for the clinical treatment of myocarditis and inflammatory cardiomyopathy.

Keywords Autoimmune myocarditis · Statin · Helper T cells · Inflammation

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Introduction

Dilated cardiomyopathy (DCM) is a potentially lethal disorder of various etiologies for which no treatment is currently satisfactory [1]. Many patients show heart-specific autoantibodies [2, 3], and immunosuppressive therapy can improve their cardiac function [4]. These observations suggest that

autoimmunity plays an important role in myocarditis as well as contributes to the progression to DCM and heart failure [5]. Animal models have greatly advanced our knowledge of the pathogenesis of myocarditis and inflammatory cardiomyopathy. Experimental autoimmune myocarditis (EAM) induced by cardiac myosin immunization is a model of postinfectious myocarditis and DCM [6–8]. EAM represents a $CD4^+$ T cell-mediated disease [6, 9, 10] and has been considered to be associated with both interferon (IFN)-γ producing T helper (Th)1 cells and interleukin (IL)-17 producing Th17 cells [10].

Statins are orally administered competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate. As effective cholesterol-lowering agents, statins have been extensively used for prevention of cardiovascular disease [11]. In the past few years, accumulated evidence from animal experiments and clinical studies has shown that statins have anti-inflammatory and immunomodulatory effects. The effects of statins on the immune system are pleiotropic and include inhibition of T-cell activation, proliferation, and migration [12–14]. Reportedly, atorvastatin was able to promote shifting of the T-cell response from a pro-inflammatory Th1 to an anti-inflammatory Th2 profile in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis mediated by Th1 cells in the central nervous system [15, 16]. Furthermore, simvastatin was able to inhibit IL-17 secretion which plays a critical role in the development of autoimmune diseases, in $CD4^+$ T cells derived from relapsing remitting multiple sclerosis patients [17]. Although this evidence suggests that statins can inhibit Th1 and Th17 inflammatory responses in autoimmune diseases, the mechanism has not been fully elucidated.

Based on these effects of statins, this study was designed to test the hypothesis that pitavastatin affects T cell-mediated autoimmunity through inhibiting Th1 and Th17 responses and reduces the severity of EAM in mice.

Materials and Methods

Study Approval

All animal experiments were approved by the Institutional Animal Experiment Committee of the University of Tsukuba.

Mice

BALB/c mice and CB17.SCID mice were purchased from CLEA Japan. We used 5- to 7-week-old male mice.

Immunization Protocols

The mice were immunized with 100 μg of murine cardiac α-myosin heavy chain (MyHC-α) peptide (MyHC-$\alpha_{614-629}$ [Ac-RSLKLMATLFSTYASADR-OH]; Toray Research Center) emulsified 1:1 in phosphate buffered saline (PBS)/complete Freund's adjuvant (CFA) (1 mg/ml; H37Ra; Sigma-Aldrich) on days 0 and 7 as described previously [10, 18].

In Vivo Pitavastatin Treatment

Pitavastatin was obtained from the Kowa Company and diluted with distilled water before use. Mice were fed pitavastatin (5 mg/kg) or vehicle once daily by gavage feeding for 3 weeks from day 0 to day 21 after immunization. In some experiments, EAM mice were treated with 0.05, 0.5 or 5 mg/kg of pitavastatin.

Histopathologic Examination

Myocarditis severity was scored on hematoxylin and eosin (H&E)–stained sections using grades from 0 to 4: 0, no inflammation; 1, less than 25 % of the heart section involved; 2, 25 to 50 %; 3, 50 to 75 %; and 4, more than 75 % as described previously [10, 18]. Two independent researchers scored the slides separately in a blinded manner.

Flow Cytometric Analyses and Intracellular Cytokine Staining

Heart inflammatory cells were isolated and processed as previously described [19, 20]. For the flow cytometric analysis of the surface markers and cytoplasmic cytokines, the cells were stained with directly conjugated fluorescence antibodies and analyzed with a FACSCalibur instrument (BD Biosciences). For the analysis of the intracellular cytokine production, the cells were stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA), 750 ng/ml of ionomycin (Sigma-Aldrich) and 10 μg/ml of brefeldin A (eBioscience) for 5 h. Fluorochrome-conjugated, mouse-specific monoclonal antibodies purchased from eBioscience, included CD4, forkhead box P3 (Foxp3), IFN-γ, IL-17A, T-box expressed in T-cells (T-bet) and RAR-related orphan receptor γt (RORγT).

Cytokine ELISA

For the analysis of the cytokines and chemokines in the heart, the hearts were homogenized in media containing 2.5 % fetal bovine serum. The supernatants were collected after centrifugation and stored at −80 °C. The concentrations of cytokines and chemokines in the heart homogenates and

culture supernatants were measured with Quantikine ELISA kits (R&D Systems).

$CD4^+$ T-Cell Isolation

We used magnetic-activated cell sorting kits for the cell isolation ($CD4^+CD62L^+$ T Cell Isolation Kit II for naïve $CD4^+$ T-cell isolation and $CD4^+$ T Cell Isolation Kit II for $CD4^+$ T cell isolation, Miltenyi Biotec).

Peripheral Blood Mononuclear Cell (PBMC) Isolation

PBMCs were isolated as previously described [21]. Briefly, blood samples were diluted with PBS and the plasma was removed by centrifugation. To remove the red blood cells, the samples were incubated with ACK lysing buffer (Lonza).

Proliferative Responses of T Cells

The MyHC-α-specific T-cell proliferation was assessed as previously described [10, 22]. Briefly, the mice were immunized as described above, and the $CD4^+$ T cells were collected on day 14. The cells were cultured with 5 μg/ml of MyHC-α in the presence of antigen-presenting cells (APCs), and irradiated splenocytes, for 72 h and pulsed with 0.5 μCi of [^{3}H]-thymidine for 8 h before being measured with a beta counter.

Western Blot Analysis

Total lysates from $CD4^+$ T cells were immunoblotted and probed with primary Abs. The phosphorylated (p)-signal transducer and activator of transcription (STAT)3 was purchased from Cell Signaling. STAT3, STAT4, STAT6, p-STAT4 and p-STAT6 were purchased from Santa Cruz Biotechnologies. Horseradish peroxidase-conjugated secondary antibodies (Abs) (Cell Signaling) were used to identify the binding sites of the primary antibody.

Serum Troponin Determinations

Blood was collected from the mice at the time of sacrifice, and the serum levels of cardiac troponin I (TnI) were measured with an ELISA kit (mouse cardiac Tn-I, ultra sensitive; Life Diagnostics).

Reagents and Inhibitors

For the in vitro assay, reagents and inhibitors were used in the following concentrations: pitavastatin 1 μM, mevalonate 1 mM (Sigma), farnesyltransferase inhibitor (FTI-277) 20 μM (Sigma-Aldrich), geranylgeranyltransferase inhibitor (GGTI-298) 20 μM (Sigma-Aldrich), anti-CD3 1 μg/ml (R&D Systems), anti-CD28 1 μg/ml (Acris Antimodies), farnesylpyrophosphate (farnesyl-PP) 20 μM (Sigma-Aldrich) and geranylgeranylpyrophosphate (geranylgeranyl-PP) 20 μM (Sigma-Aldrich).

In Vitro Th Differentiation

Purified naïve $CD4^+CD62L^+$ T cells were treated for 24 h with pitavastatin, mevalonate, FTI-277, GGTI-298, or vehicle; then they were stimulated with anti-CD3 and anti-CD28 under Th1- or Th17- polarizing conditions for 48 h. Th1 condition: IL-12 (10 ng/ml) and anti-IL-4 antibody (10 μg/ml). Th17 condition: transforming growth factor (TGF)-β (10 ng/ml), IL-6 (20 ng/ml), IL-23 (20 ng/ml), anti–IL-4 (10 μg/ml), anti–IL-12 (10 μg/ml) and anti–IFN-γ (10 μg/ml). The cytokines and antibodies were obtained from R&D systems except for the TGF-β (BioLegend).

Adoptive T-Cell Transfer

$CD4^+$ T cells were collected from EAM mice and cultured with 5 μg/ml MyHC-α in the presence of irradiated splenocytes for 48 h. In some experiments, cells were cultured in the presence or absence of 1 μM of pitavastatin. 5×10^6 $CD4^+$ T cells were injected intraperitoneally into severe combined immunodeficiency (SCID) mice. The mice were killed 10 days after the transfer.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (QRT-PCR)

The total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of the total RNA by reverse transcriptase (Takara). QRT-PCR analysis was performed with LightCycler (Roche Diagnostics). The oligonucleotides used for the PCR amplification of the cytokines and receptors were the following: *Tbx21* forward, TCAACCAGCACCAGACAGAG; *Tbx21* reverse, AAACATCCTGTAATGGCTTGTG; *Rorc* forward, CCCTGGTTCTCATCAATGC; *Rorc* reverse, TCCAAATTGTATTGCAGATGTTC; *Socs3* forward, ATTTCGCTTCGGGACTAGC; *Socs3* reverse, AACTTGCTGTGGGTGACCAT; *Hprt* forward, TCCTCCTCAGACCGCTTTT; and *Hprt* reverse CCTGGTTCATCATCGCTAATC. The data were normalized by the level of the *Hprt* expression in each sample.

Statistical Analysis

Statistical analyses were performed using the two-tailed *t* test or Mann–Whitney *U* test, for experiments comparing two groups. For multiple comparisons, one-way analysis of

variance with Dunnett post-hoc test was used. P values< 0.05 were considered statistically significant.

Results

Pitavastatin Inhibits the Development of EAM

First, we sought to determine the in vivo effects of pitavastatin on EAM. BALB/c mice were fed pitavastatin (5 mg/kg) or vehicle (control) once daily by gavage feeding, starting from the beginning of the MyHC-α immunization and lasting to the end of the experiment. As shown in Fig. 1a, vehicle-treated mice (control) developed severe myocarditis with inflammatory infiltrates. In contrast, pitavastatin treatment of mice resulted in a significant reduction in the inflammatory infiltrates in the heart. Pitavastatin-treated mice had a significantly lower myocarditis severity score than did the control mice (Fig. 1b). The heart-to-body weight ratio in the pitavastatin-treated mice was significantly decreased compared to that in the control mice (Fig. 1c), as were the levels of the circulating cardiac TnI, a clinical marker of myocyte damage (Fig. 1d). We also examined whether pitavastatin treatment had an effect on the cytokine and chemokine milieu in the heart. On day 21 after the MyHC-α immunization, the heart homogenates from pitavastatin-treated mice had significantly decreased amounts of proinflammatory cytokines, including IL-1β and IL-6, and chemokines, including chemokine (C-C motif) ligand (CCL)2, CCL3, CCL5, CCL17, CCL20 and chemokine (C-X-C motif) ligand (CXCL)10 (Fig. 1e). Thus, pitavastatin ameliorated EAM development, which corresponded to the abrogation of the proinflammatory cytokines and chemokines in the heart.

Pitavastatin Induced SOCS3 Expression and Suppressed STAT3 and STAT4 Phosphorylation

EAM is a $CD4^+$ T cell-mediated disease [6, 20] and both Th1 and Th17 cells are linked to the promotion of EAM. Activated STAT4 has a key role in Th1 lineage commitment and STAT3 does so for Th17 [23–25]. Conversely, STAT6 is required for Th2-dependent lineage commitment [23]. Therefore, we examined whether pitavastatin treatment suppressed the formation of activated STAT3 and STAT4 or induced activation of STAT6. In vivo pitavastatin treatment inhibited STAT3 and STAT4 phosphorylation and promoted STAT6 phosphorylation in a dose-dependent manner (Fig. 2a). Accordingly, heart-infiltrating and circulating IFN-γ- and IL-17-producing $CD4^+$ T cells were reduced in pitavastatin-treated mice

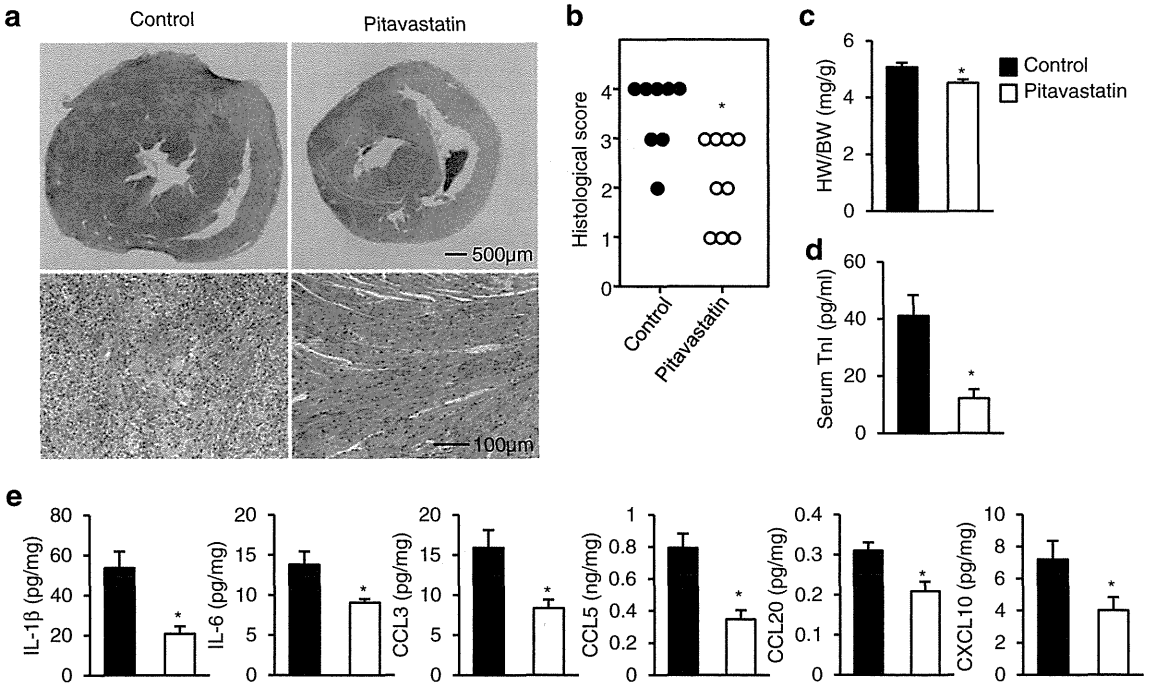

Fig. 1 Pitavastatin ameliorates the EAM development. BALB/c mice were immunized twice, on days 0 and 7, with 100 μg of cardiac myosin epitope peptide (MyHC-α) and treated with pitavastatin (5 mg/kg) or vehicle (control) for 3 weeks from day 0 to day 21 after immunization. **a** Representative H&E-stained sections of the hearts. Scale bars, 500 or 100 μm. **b** Myocarditis severity in heart sections (n=8–9 per group). **c** Heart-to-body weight ratios of control and pitavastatin-treated mice (n=5 per group). **d** Circulating troponin I (TnI) concentration (n=5 per group). **e** Production of cytokines and chemokines in the hearts. Myocardial tissues of vehicle- and pitavastatin-treated EAM mice were homogenated and processed by ELISA to detect the cytokines and chemokines on day 21. The *bar graphs* show the group means ± SEM of 8 mice per group. The results of one of two representative experiments are shown. Data are expressed as the mean ± SEM. *P<0.05 vs. control

(Fig. 2b and c). Foxp3$^+$ regulatory T cells (Tregs) are known to play a crucial role in preventing autoimmune disorders and actively controlling autoimmune responses [26, 27]. Therefore, we examined the effect of pitavastatin on Tregs in EAM. There were no differences in the frequencies of Foxp3$^+$ Tregs in the heart and PBMCs (Fig. 2d). The MyHC-α-specific proliferative responses of CD4$^+$ T cells were reduced by pitavastatin treatment in a dose-dependent manner (Fig. 2e). We also found that the MyHC-α-specific production of IFN-γ and IL-17 was reduced in supernatants of CD4$^+$ T cells from pitavastatin-treated mice (Fig. 2f). We could not detect any Th2 cytokine IL-4 production from the heart and splenic CD4$^+$ T cells in either the control or pitavastatin-treated mice.

STAT activation is partially regulated by the expression of inhibitory SOCS proteins. We thus assessed the expression of SOCS3 and STAT phosphorylation in pitavastatin-treated T cells. Vehicle-treated T cells downregulated the *Socs3* mRNA expression after activation, whereas pitavastatin-treated T cells retained a higher expression (Fig. 2g). Mevalonate reversed the effects of pitavastatin on the *Socs3* expression (Fig. 2g). In contrast, pitavastatin treatment inhibited STAT3 and STAT4 phosphorylation, and mevalonate reversed the inhibitory effects of pitavastatin on the STAT activation (Fig. 2h). Such observations demonstrate that pitavastatin upregulates the

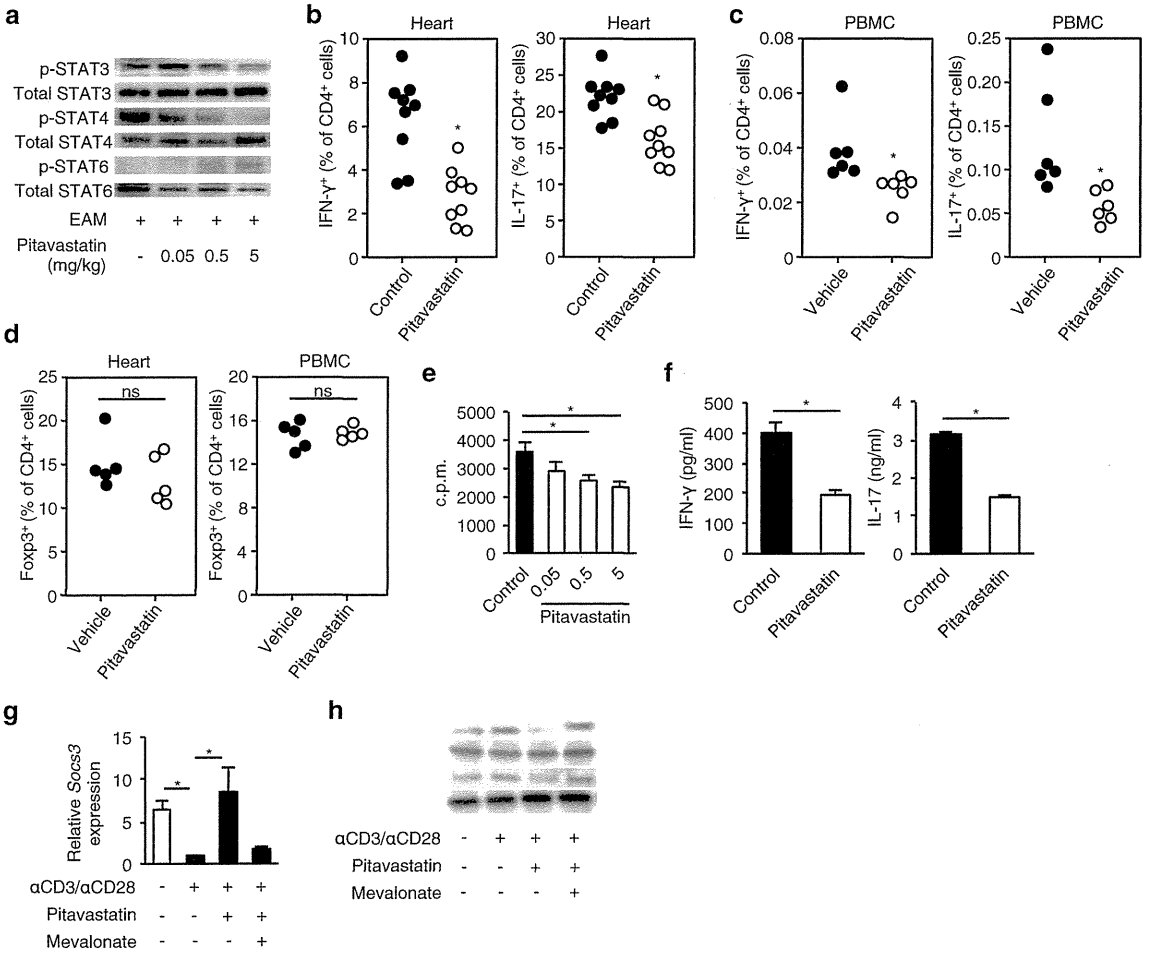

Fig. 2 Pitavastatin increases the SOCS3 expression and suppresses phosphorylation of STAT3 and STAT4. **a** STATs activation in CD4$^+$ T cells. CD4$^+$ T cells were isolated from the heart of vehicle-treated EAM mice, and EAM mice treated with 0.05, 0.5 or 5 mg/kg of pitavastatin. The phosphorylated and total STAT3, STAT4 and STAT6 proteins were detected by Western blot analysis. **b–d** Flow cytometry analysis of IFN-γ- or IL-17-producing CD4$^+$ T cells or Fop3+ Tregs in the hearts and PBMC from 5 mg/kg of pitavastatin- or vehicle-treated mice (n=5–9 per group). **e** CD4$^+$ T cells were isolated from EAM mice treated with pitavastatin or vehicle on day 14 and restimulated with MyHC-α in the presence of APCs (irradiated splenocytes) for 72 h. Proliferation was assessed by a measurement of the [^{3}H]-thymidine incorporation. **f** The MyHC-α-specific IFN-γ and IL-17 production in the culture supernatant of CD4$^+$ T cells. CD4$^+$ T cells were isolated from 5 mg/kg of pitavastatin- or vehicle-treated EAM mice and cultured with 5 μg/ml of MyHC-α in the presence of APCs for 2d. **g** The *Socs3* mRNA expression in CD4$^+$ T cells treated for 18 h with the indicated agents and then stimulated with anti-CD3 and anti-CD28 for 6 h. The data were normalized for the basal gene expression in anti CD3- and anti CD28-treated cells. **h** STATs activation in CD4$^+$ T cells. CD4$^+$ T cells were treated as described in (**g**), and the phosphorylated and total STAT3 and STAT4 proteins were detected by Western blot analysis. Data are expressed as the mean ± SEM from triplicate culture wells. The results of one of two representative experiments are shown. *P<0.05

SOCS3 expression, which inhibits the Th1 and Th17 differentiation through the inhibition of the STAT3 and STAT4 phosphorylation.

Pitavastatin Regulates Th1 and Th17 Differentiation in an Isoprenylation-Dependent Manner

Next, we assessed the effect of pitavastatin on the Th differentiation. Pitavastatin-treated T cells failed to differentiate into IFN-γ-producing Th1 or IL-17-producing Th17 cells under the appropriate skewing conditions in vitro (Fig. 3a). Pitavastatin blunted the production of IFN-γ and IL-17 in the culture supernatant, which was reversed by mevalonate supplement (Fig. 3b). Thus pitavastatin interferes with the Th1 and Th17 differentiation.

As a result of the HMG-CoA reductase inhibition, statins regulate the biosynthesis of mevalonate pathway-derived isoprenoids, leading to reduced isoprenylation of protein targets (Fig. 4a). To confirm the involvement of isoprenylated proteins in the T-cell differentiation, we investigated the effects of a farnesyltransferase inhibitor (FTI-277) and a geranylgeranyltransferase inhibitor (GGTI-298) on $CD4^+$ T cells. Under Th1 skewing conditions, both FTI-277 and GGTI-298 inhibited the Th1 differentiation (Fig. 4b and c). In contrast, FTI-277 inhibited the Th17 differentiation, whereas GGTI-298 did not (Fig. 4b and c). We also examined whether farnesyl-PP and geranylgeranyl-PP could reverse the inhibitory effects of pitavastatin on the Th1/Th17 differentiation. As shown in Fig. 3b, farnesyl-PP reversed the inhibitory effects of pitavastatin on both the Th1 and Th17 differentiation, however, geranylgeranyl-PP only did so on the Th1 differentiation. Collectively, these results suggest that pitavastatin prevents Th1 and Th17 differentiation by inhibiting the biosynthesis of isoprenoids.

Pitavastatin Influenced the Expression of Transcription Factors in T Cells

The transcription factors, T-bet and RORγT, have critical roles in the development of Th1 and Th17 cells, respectively [28–30]. Next we sought to determine if these transcription factors were affected by pitavastatin. Pitavastatin-treated T cells

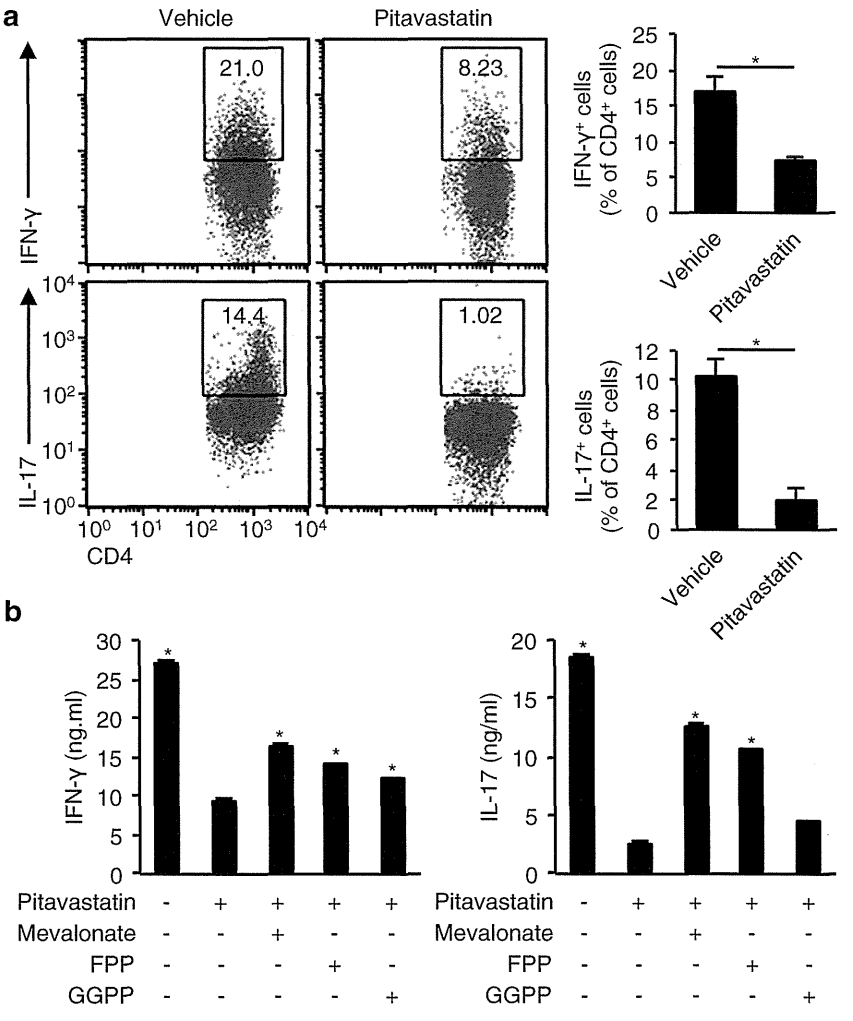

Fig. 3 Pitavastatin inhibits the Th1 and Th17 differentiation. **a** The IFN-γ and IL-17 production in $CD4^+$ T cells treated with pitavastatin overnight, followed by 2 d of stimulation with anti-CD3 and anti-CD28 in Th1- or Th17-skewing conditions. The numbers in the outlined areas indicate the percent of cytokine-producing $CD4^+$ T cells. **b** The IFN-γ and IL-17 concentration in the culture supernatant of $CD4^+$ T cells treated with pitavastatin with or without mevalonate, farnesyl-PP (FPP) or geranylgeranyl-PP (GGPP) overnight, followed by 2 d of stimulation as in **a**. Data are expressed as the mean ± SEM from triplicate culture wells. The data are from 1 of 2 experiments performed, with similar results. *$P<0.05$

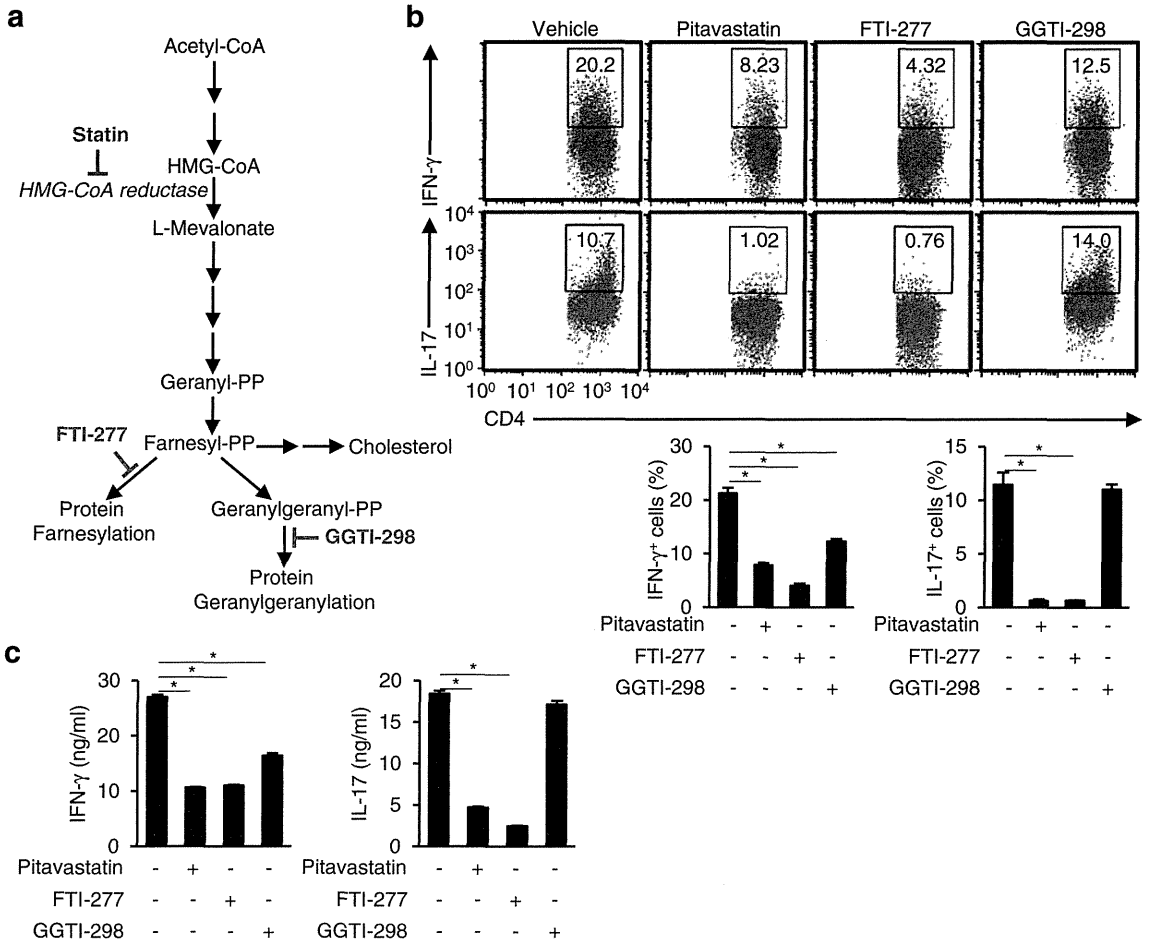

Fig. 4 *Inhibitors of isoprenylation prevent the Th1 and Th17 differentiation.* **a** The mevalonate pathway. Drug inhibitors are shown in red. Not all pathway intermediates are shown. **b** The IFN-γ and IL-17 production in $CD4^+$ T cells treated with pitavastatin, farnesyltransferase inhibitors (FTI-277) or geranylgeranyltransferase inhibitors (GGTI-298) overnight, followed by 2 d of stimulation as in Fig. 3a. The numbers in the outlined areas indicate the percent of cytokine-producing $CD4^+$ cells. **c** The IFN-γ and IL-17 accumulation in the culture supernatant of $CD4^+$ T cells treated as in **b**. Data are expressed as the mean ± SEM from triplicate culture wells. The results of one of two representative experiments are shown. $^*P<0.05$

failed to fully upregulate the T-bet mRNA (*Tbx21*) and protein under Th1 conditions and the RORγT mRNA (*Rorc*) and protein under Th17 conditions (Fig. 5a and b). Mevalonate reversed the effects of pitavastatin on the expression of these transcription factors (Fig. 5a and b). To confirm the involvement of isoprenylated proteins in the expression of transcription factors, we investigated the effects of FTI-277 and GGTI-298 on $CD4^+$ T cells. As shown in the Supplementary Figure, FTI-277 suppressed both the T-bet and RORγT, whereas GGTI-298 only suppressed the T-bet expression. These results suggested that pitavastatin affected the Th1 and Th17 differentiation through inhibiting the T-bet and RORγT transcription.

Adoptive Transfer of Pitavastatin-Treated $CD4^+$ T Cells Prevents Induction of EAM in Recipient Mice

If the myocarditis resistance of pitavastatin-treated mice indeed results from the inhibition of the Th differentiation, an adoptive transfer of pitavastatin-treated autoreactive $CD4^+$ T cells would fail to induce myocarditis in the recipient mice. To test this hypothesis, we isolated $CD4^+$ T cells from EAM mice either given or not given pitavastatin treatment and transferred them into SCID mice. All of the recipient mice that received $CD4^+$ T cells from vehicle-treated mice developed severe myocarditis (Fig. 6a and b). In contrast, mice that received donor $CD4^+$ T cells from mice treated with pitavastatin were protected from severe myocarditis.

In order to test whether in vitro pitavastatin treatment affects the effector T-cell function, we isolated $CD4^+$ T cells from EAM mice and treated them with pitavastatin or FTI-277 in vitro. Pitavastatin and FTI-277 inhibited the MyHC-α-specific IFN-γ and IL-17 production (Fig. 6c). We also found that in vitro pitavastatin-treated $CD4^+$ T cells failed to induce myocarditis in the recipients (Fig. 6d and e). These data suggest that pitavastatin was able to impair not only the priming but also the effector phases of T-cell responses, and

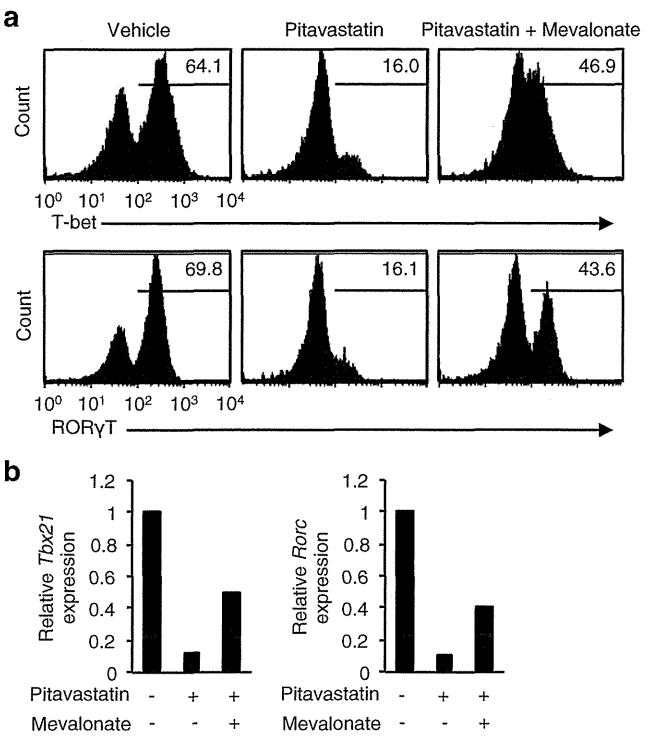

Fig. 5 Control of the induction of transcription factors by pitavastatin. **a** T-bet and RORγT expression in $CD4^+$ T cells treated with pitavastatin with or without mevalonate overnight, followed by 2 d of stimulation with anti-CD3 and anti-CD28 in Th1- or Th17-skewing conditions. The numbers indicate the percentage of transcription factor-expressing $CD4^+$ cells. **b** The *Tbx21* and *Rorc* mRNA expression in $CD4^+$ T cells treated as in a. The data were normalized for the basal gene expression in vehicle-treated cells. The results of one of two representative experiments are shown

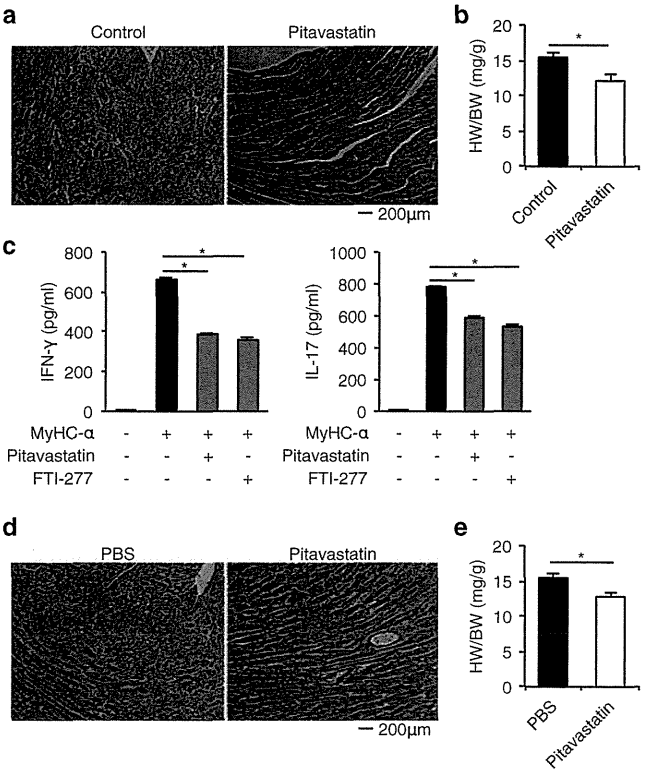

Fig. 6 Adoptive transfer of in vivo or in vitro pitavastatin-treated $CD4^+$ T cells prevents the induction of EAM in recipient mice. **a** and **b** 5×10^6 $CD4^+$ T cells were isolated from vehicle- or pitavastatin-treated EAM mice and restimulated with 5 μg/ml of MyHC-α in the presence of APCs for 2 d, and then transferred into SCID mice (n=5 per group). H&E-stained heart sections 10 d after transfer (**a**) and the heart-to-body weight ratios of the recipient mice (**b**). **c** $CD4^+$ T cells were isolated from EAM mice and treated with the indicated agents in the presence of APCs for 2 d in vitro. **d** and **e** 5×10^6 $CD4^+$ T cells were isolated from EAM mice and restimulated with MyHC-α and APCs in the presence or absence of pitavastatin for 2 d, and then transferred into SCID mice (n=5 per group). H&E-stained heart sections 10 d after transfer (**d**) and the heart-to-body weight ratios of the recipient mice (**e**). Data are expressed as the mean ± SEM from triplicate culture wells. The results of one of two representative experiments are shown. *P<0.05

these adoptive transfer studies provided evidence that pitavastatin treatment impaired the $CD4^+$ T-cell function that suppressed clinical autoimmune myocarditis.

Discussion

As a result of the HMG-CoA reductase inhibition, statins inhibit the synthesis of mevalonate pathway-derived isoprenoids (Fig. 4a). In the present study, we provide evidence that a blockade of this enzyme by pitavastatin inhibits Th1 and Th17 responses through the inhibition of the protein isoprenylation. Pitavastatin-treated T cells failed to differentiate into Th1 and Th17 cells, and this failure was rescued by adding mevalonate. In a mouse model of EAM, pitavastatin treatment ameliorated the pathophysiological severity of myocarditis associated with reduced Th1 and Th17 responses. Our results are important for understanding the anti-inflammatory effects of statins.

Myocarditis and subsequent dilated cardiomyopathy (DCM) are major causes of heart failure in young patients. The activation and differentiation of T cells play a critical role in the pathogenesis of both situations [31]. Although there are several reports on the effect of statins on T-cell cytokine secretion in EAE, there are few studies on EAM. Liu et al. reported that atorvastatin ameliorated EAM in rats, attributing it to a shift from Th1 to Th2 cytokine secretion [32]. Our study examined the effect of statins on the Th17 response in EAM, which has not been explored before. In the present study, we found that both in vivo and in vitro treatment with pitavastatin inhibited the Th1 and Th17 cytokine production from cardiac-specific $CD4^+$ T cells, which was associated with the disease severity. Thus, pitavastatin can directly modulate the T-cell function and reduce the pathogenicity of T cells confirmed by adoptive transfer experiments.

EAM represents a $CD4^+$ T cell-mediated disease. Th1 cells were once considered the major pathogenic subset mediating

organ-specific autoimmune diseases [33, 34]. However, IFN-γ has recently been shown to be a down-regulatory cytokine, as evidenced by exacerbated myocarditis in IFN-γ receptor knockout (KO), IFN-γ KO, and T-bet KO mice [35–37]. On the other hand, Th17 cells have been implicated in the pathogenesis of various types of autoimmune diseases (reviewed in [38]); however, the genetic ablation of IL-17 had no significant impact on the incidence or severity of myocarditis [39]. These gene-ablated mice provided us with much important information, and we could not exclude the possibility that the inhibitory effect of pitavastatin on the IFN-γ production may weaken the immunosuppressive effect of pitavastatin. However, studies from gene ablation mice do not necessarily match the results of the real world. We have previously reported that the suppressor of cytokine signaling 1 (SOCS1) DNA administration ameliorated EAM, and SOCS1 DNA therapy suppressed both Th1 and Th17 cytokines from $CD4^+$ T cells [10]. Ohshima et al. also reported that systemic transplantation of allogenic fetal membrane-derived mesenchymal stem cells suppresses both Th1 and Th17 T-cell responses in EAM [40]. The question of the relative roles of Th1 cells versus Th17 cells on EAM is unresolved [39, 41], and autoimmune diseases have so far been considered to be associated with both Th1 and Th17 cells [39, 42]. Statins can inhibit both Th1 and Th17 responses and may be a useful therapy for autoimmune diseases in the clinical setting.

As shown in Fig. 2a, a larger amount of pitavastatin was needed to inhibit STAT3 phosphorylation than was needed to inhibit STAT4 phosphorylation. Kagami et al. showed similar results that simvastatin inhibited the differentiation of Th17 cells at the concentrations that did not inhibit the differentiation of Th1 cells [43]. These findings suggest that statins can more easily affect the Th17 differentiation than affect the Th1 differentiation.

Several studies have shown that statins can modulate the inflammatory milieu by altering chemokines and chemokine receptors, eventually inhibiting the leukocyte migration [44, 45]. We evaluated the chemokine concentrations in the heart and found decreased levels of chemokines, including CCL2, CCL3, CCL5, CCL17, CCL20 and CXCL10 (Fig. 1e). We could not evaluate whether these decreases were direct effects of pitavastatin or not in our system, and this may have contributed to the beneficial effects of pitavastatin in EAM.

There are several reports of the effects of statins on inducing Tregs in humans. Atorvastatin treatment of human PBMCs in vitro led to an induction of the transcription factor Foxp3, accompanied by an increase in the number of Tregs [46]. Simvastatin and pravastatin treatment in hyperlipidemic patients increased the number of Tregs in PBMCs [46]. Tang et al. showed that atorvastatin promoted the generation of Tregs from primary T cells of rheumatoid arthritis patients [47]. On the other hand, there are some conflicting data on the Treg induction by statins in mice. Consistent with our data (Fig. 2d), Mausner-Fainberg et al. did not find a statin-induced conversion of murine $CD4^+CD25^-Foxp3^-$ to Treg cells [46]. Mira et al. also demonstrated that no changes were noted in the Treg cell numbers in the spleen or draining lymph nodes of lovastatin-treated mice [48]. However, it has been reported that simvastatin increases the Treg differentiation in vitro [43, 49]. Thus, the question of the effect of statins on Tregs in mice is unresolved. It can be assumed that in murine models, the anti-inflammatory effects of pitavastatin are probably evident by inhibiting the Th1 and Th17 response rather than by the expansion of Tregs. Further studies are needed to elucidate the effect on the Treg induction in mice.

As a result of the HMG-CoA reductase inhibition, statins also inhibit the synthesis of downstream isoprenoids farnesyl-PP and geranylgeranyl-PP (Fig. 4a). The process of the isoprenylation of Ras/Rho family proteins is required for G protein activity. At least some of the pleiotropic benefits of statins that are independent of the cholesterol lowering effect are thought to involve interference with the normal synthesis of isoprenoids, thereby impairing the Ras/Rho G protein function (reviewed in ref. [50]). Dunn et al. showed that atorvastatin suppressed Th1 differentiation and caused a Th2 bias by inhibiting Ras farnesylation and RhoA geranylgeranylation [51]. Ras-ERK and RhoA-p38 signaling pathways are important in determination of Th1/Th2 fate [52–54]. In accordance with this evidence, our data have demonstrated that both inhibitors of farnesylation and geranylgeranylation prevented the Th1 differentiation (Fig. 4b and c). On the other hand, the Th17 differentiation was inhibited only by a farnesylation inhibitor (Fig. 4b and c). Recently, Rheb which belongs to the Ras family of G proteins [55] and downstream mammalian target of rapamycin (mTOR) signaling in T cells was shown to be necessary for Th1 and Th17 responses and the development of autoimmune disease [56]. Rheb-deficient T cells failed to differentiate into Th1 and Th17 cells, and mice with T cell-specific deletion of Rheb were resistant to the development of EAE [56]. Farnesylation of Rheb is necessary for its intracellular trafficking and subcellular localization to the plasma membrane and subsequent activation of the mTOR pathway [57]. Some in vitro studies have demonstrated an inhibitory effect of statin treatment on the farnesylation and membrane-association of Rheb [58, 59]. From our ongoing study, we have found that pitavastatin inhibited Rheb-mTOR signaling in a farnesylation-dependent manner (unpublished data). Collectively, these results suggest that pitavastatin prevents Th1 and Th17 differentiation by inhibiting the biosynthesis of farnesyl-PP, leading to reduced farnesylated Rheb and reduced downstream mTOR activity. Our data have demonstrated that both FTI-277 and GGTI-298 inhibited the Th1 differentiation, but Th17 differentiation was inhibited only by FTI-277 (Fig. 4). These results may have indicated that Th17 differentiation

is mainly regulated by farnesylated Rheb-mTOR signaling in our system, though Th1 differentiation is regulated not simply by farnesylated Rheb but also by other geranylgeranylated proteins. Further studies are needed to evaluate the mechanism.

In the present study, we have shown that both FTI-277 and GGTI-298 inhibited the Th1 differentiation, but the Th17 differentiation was inhibited mainly by FTI-277 (Fig. 4b and c). In contrast to our data, Kagami et al. reported that the inhibition of protein geranylgeranylation but not farnesylation is involved in the decreased differentiation of Th17 cells with simvastatin treatment [43]. There were some differences between their experiments and ours. They used a low-dose (5 μM) of FTI-277 and GGTI-298 in their experiments, whereas we used a relatively high amount of those reagents (20 μM). Therefore, we also checked the effects of low-dose FTI-277 and GGTI-298 on the Th differentiation and did not find any apparent effects on the Th differentiation (data not shown). Next, they used $CD4^+CD25^-$ T cells for the Th skewing experiments, whereas we used $CD4^+CD62L^+$ T cells. CD25 (IL-2 receptor α chain) has been used as a marker for Tregs as well as activated T cells [60]. On the other hand, CD62L (L-selectin) is an important T-cell homing receptor as well as a marker for T-cell development. Naive T cells are $CD62L^+$, and CD62L acts as a "homing receptor" for lymphocytes to enter secondary lymphoid tissues via high endothelial venules [61]. This difference may have influenced the results.

In conclusion, we have shown that pitavastatin inhibits $CD4^+$ T-cell proliferation and Th1/Th17 responses and ameliorates myocarditis in mice. Because oral statin administration is well tolerated, this treatment is a promising approach for the treatment of Th1- and Th17-mediated autoimmune diseases.

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Conflict of Interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Dynamics of cellular immune responses in the acute phase of dengue virus infection

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Abstract In this study, we examined the dynamics of cellular immune responses in the acute phase of dengue virus (DENV) infection in a marmoset model. Here, we found that DENV infection in marmosets greatly induced responses of CD4/CD8 central memory T and NKT cells. Interestingly, the strength of the immune response was greater in animals infected with a dengue fever strain than in those infected with a dengue hemorrhagic fever strain of DENV. In contrast, when animals were re-challenged with the same DENV strain used for primary infection, the neutralizing antibody induced appeared to play a critical role in sterilizing inhibition against viral replication, resulting in strong but delayed responses of CD4/CD8 central memory T and NKT cells. The results in this study may help to better understand the dynamics of cellular and humoral immune responses in the control of DENV infection.

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Introduction

Dengue virus (DENV) causes the most prevalent arthropod-borne viral infections in the world [29]. Infection with one of the four serotypes of DENV can lead to dengue fever (DF) and sometimes to fatal dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [12]. The serious diseases are more likely to develop after secondary infection with a serotype of DENV that is different from that of the primary infection. Infection with DENV induces a high-titered neutralizing antibody response that can provide long-term immunity to the homologous DENV serotype, while the effect of the antibody on the heterologous serotypes is transient [22]. On the other hand, enhanced pathogenicity after secondary DENV infection appears to be explained by antibody-dependent enhancement (ADE). Mouse and monkey experiments have shown that sub-neutralizing levels of DENV-specific antibodies actually enhance infection [1, 6, 11]. Thus, the development of an effective tetravalent dengue vaccine is considered to be an important public-health priority. Recently, several DENV vaccine candidates have undergone clinical trials, and most of them target the induction of neutralizing antibodies [20].

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