

Statins, Inhibitors of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase, Function as Inhibitors of Cellular and Molecular Components Involved in Type I Interferon Production

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Objective. Statins, which are used as cholesterol-lowering agents, have pleiotropic immunomodulatory properties. Although beneficial effects of statins have been reported in autoimmune diseases, the mechanisms of these immunomodulatory effects are still poorly understood. Type I interferons (IFNs) and plasmacytoid dendritic cells (PDCs) represent key molecular and cellular pathogenic components in autoimmune diseases such as systemic lupus erythematosus (SLE). Therefore, PDCs may be a specific target of statins in therapeutic strategies against SLE. This study was undertaken to investigate the immunomodulatory mechanisms of statins that target the IFN response in PDCs.

Methods. We isolated human blood PDCs by flow cytometry and examined the effects of simvastatin and

pitavastatin on PDC activation, IFN α production, and intracellular signaling.

Results. Statins inhibited IFN α production profoundly and tumor necrosis factor α production modestly in human PDCs in response to Toll-like receptor ligands. The inhibitory effect on IFN α production was reversed by geranylgeranyl pyrophosphate and was mimicked by either geranylgeranyl transferase inhibitor or Rho kinase inhibitor, suggesting that statins exert their inhibitory actions through geranylgeranylated Rho inactivation. Statins inhibited the expression of phosphorylated p38 MAPK and Akt, and the inhibitory effect on the IFN response was through the prevention of nuclear translocation of IFN regulatory factor 7. In addition, statins had an inhibitory effect on both IFN α production by PDCs from SLE patients and SLE serum-induced IFN α production.

Conclusion. Our findings suggest a specific role of statins in controlling type I IFN production and a therapeutic potential in IFN-related autoimmune diseases such as SLE.

Statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, represent the most important cholesterol-lowering agents used to treat hypercholesterolemia. Recent clinical and experimental evidence indicates that statins have pleiotropic effects that include antiinflammatory and immunomodulatory properties, as shown by reduced rates of graft rejection in statin-treated patients after heart transplantation (1), beneficial effects in autoimmune encephalomyelitis or multiple sclerosis (2,3), decreased leukocyte recruitment and edema formation in animal models of acute inflammation (4), and delayed disease progression in the

Supported by a Grant-in-Aid for Scientific Research (21591289) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, a Grant-in-Aid from The Japan Medical Association, and the Takeda Science Foundation.

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Submitted for publication May 19, 2009; accepted in revised form March 19, 2010.

NZB \times NZW murine model of systemic lupus erythematosus (SLE) (5).

Several recent studies have elucidated some of the mechanisms by which statins exert immunomodulatory effects on the immune system beyond their cholesterol-lowering effects. Statins alter the functions of T cells as well as antigen-presenting cells (APCs) in terms of inhibited production of proinflammatory mediators (tumor necrosis factor α [TNF α], interleukin-1 β [IL-1 β], IL-6, IL-8, RANTES, monocyte chemoattractant protein 1, cyclooxygenase 2, and nitric oxide) (6,7), decreased expression of class II major histocompatibility complex, costimulatory molecules (8), and adhesion molecules, and suppression of APC-mediated Th1 differentiation (9). In addition, statins directly suppress secretion of the inflammatory cytokine IL-17 by Th17 cells (10).

Recent studies have revealed that most of these effects are mediated through inhibiting the synthesis of isoprenoid intermediates, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are important lipid attachments for the intracellular signaling molecules Ras and Rho small GTPases, in the mevalonate pathway (11) (Figure 1A). Currently, these GTPase families are of special interest because they transduce other signaling pathways and, in turn, mediate various pivotal cellular functions, such as cell shape, motility, survival, proliferation, and gene expression (12,13), and statins exert their immunomodulatory actions mainly through the inactivation of the GTPases. The biologic effects of Rho are mediated by its downstream effectors, Rho kinases (6), and indeed Rho kinase inhibitor has antiinflammatory functions similar to those of statins in suppressing the activity of rheumatoid arthritis (RA) independently of lowering lipid levels (14). In addition, statins have been shown in several experimental systems to present their pleiotropic effects by modulating phosphatidylinositol 3-kinase (PI 3-kinase) and MAPKs (7,15–17). It thus appears that the physiologic background behind the immunomodulatory actions of statins involves various mechanisms that intersect with a number of intracellular events.

In humans, myeloid dendritic cells (MDCs) and plasmacytoid DCs (PDCs) represent 2 major subsets of DCs. They play distinct roles in innate and adaptive immune responses through the expression of their specialized cytokines and molecules (18). In contrast to MDCs, which have the ability to produce large amounts of IL-12, PDCs have the ability to produce robust amounts of type I interferons (IFNs) upon viral infection

by the selective expression of Toll-like receptor 7 (TLR-7) and TLR-9, which recognize viral RNA and DNA, respectively (19,20). These findings suggest that PDCs are specialized in viral recognition and play a central role in innate antiviral immune responses. However, recent studies indicate that PDCs also play a pathogenic role in the development of autoimmune diseases such as SLE and psoriasis by their dysregulated production of type I IFNs (21–23).

A mounting body of evidence has unveiled the molecular event underlying the capability of PDCs to produce large amounts of type I IFNs. PDCs constitutively express high levels of IFN regulatory factor 7 (IRF-7) (24). Upon TLR activation, the nuclear translocation of IRF-7 is eventually induced through rapid interaction between myeloid differentiation factor 88 and IRF-7 by forming a supercomplex consisting of TNF receptor-associated factor 6, IL-1 receptor-associated kinase 4 (IRAK-4), and IRAK-1 (25–27). IKK α is also critically involved in the nuclear translocation of IRF-7 (28). Thus, these unique molecular mechanisms may equip PDCs with the specialized ability to act as professional type I IFN-producing cells.

Although there is evidence indicating that statins have antiinflammatory effects on macrophages or monocyte-derived DCs (29,30), there are no studies showing their effects on PDCs. Therefore, we focused on the effects of statins on human PDCs and especially on their ability to produce type I IFNs. Clarification of the functional and molecular bases of the effects of statins on human PDCs would provide a novel perspective for elucidating their immunomodulatory actions and, moreover, serve as the foundation for the development of therapeutic tools for the treatment of autoimmune disorders.

MATERIALS AND METHODS

Media and reagents. RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 ng/ml of streptomycin, and heat-inactivated 10% fetal bovine serum (FBS; BioSource International) was used for cell cultures. For cell stimulation, we used 5 hemagglutinating units/ml of ultraviolet-irradiated Sendai virus (Cantell strain), 5 μ M CpG-containing oligonucleotide (CpG ODN) 2216 (InvivoGen), 100 μ M loxoribine (InvivoGen), and 5 μ M CpG ODN C274 (Operon). Simvastatin (Calbiochem), pitavastatin (Kowa), HA1077 (Calbiochem), and mevalonate (Sigma) were dissolved in anhydrous ethanol. FPP (Calbiochem) and GGPP (Calbiochem) were dissolved in methanol. Ethanol was diluted in parallel to be used as vehicle control. SB202190, PD98059, U0126, FTI-277, geranylgeranyl transferase inhibitor 298 (GGTI-298), and LY294002 (all from Calbiochem) were dis-

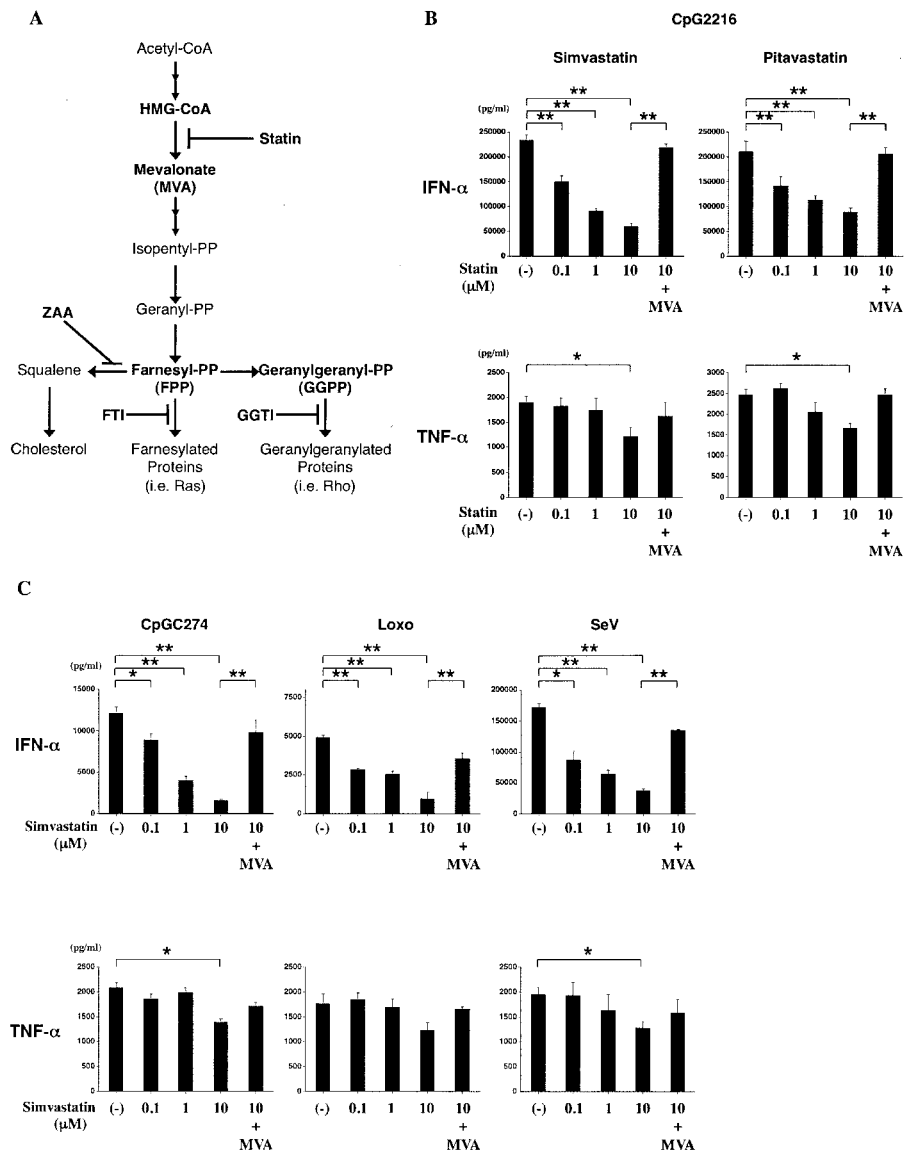


Figure 1. Inhibition of Toll-like receptor (TLR)-mediated interferon α ($IFN\alpha$) production in human plasmacytoid dendritic cells (PDCs) treated with statins. **A**, The mevalonate pathway, showing the sites of action of statins and other inhibitors. Statins inhibit the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate and thus inhibit the downstream synthesis of not only cholesterol, but also isoprenoid intermediates, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which regulate posttranslational modifications of the small GTPase Ras and Rho families. Zaragozic acid A (ZAA), farnesyl transferase inhibitor FTI-277, and geranylgeranyl transferase inhibitor GGTI-298 block the synthesis pathways that split off from FPP in the mevalonate pathway. **B**, Levels of $IFN\alpha$ and tumor necrosis factor α ($TNF\alpha$) in human PDCs that were cultured with the TLR-9 ligand CpG 2216 ($5\ \mu M$) in the absence or presence of different concentrations of simvastatin or pitavastatin. **C**, Levels of $IFN\alpha$ and $TNF\alpha$ in human PDCs that were cultured with CpG C274 ($5\ \mu M$), the TLR-7 ligand loxoribine (loxo; $100\ \mu M$), or Sendai virus (SeV; 5 multiplicities of infection) in the absence or presence of different concentrations of simvastatin. In some cultures, $100\ \mu M$ mevalonate was added. After 24 hours, the concentrations of $IFN\alpha$ and $TNF\alpha$ in the culture supernatants were measured by enzyme-linked immunosorbent assay. Bars in **B** and **C** show the mean and SEM ($n = 3$ independent donors per group). * = $P < 0.05$; ** = $P < 0.01$, by Student's paired t -test.

solved in DMSO. Zaragozic acid A (ZAA; Squalastatin) (Sigma) was dissolved in water. Ethanol, methanol, water, and DMSO were diluted in parallel to be used as vehicle control.

Cell isolation and culture. PDCs were isolated from peripheral blood mononuclear cells (PBMCs) from adult donors, as previously described (20). $CD123^{high}CD11c-$

lineage-CD4⁺ PDCs were isolated by FACS Aria (BD Biosciences) to reach >99% purity according to blood dendritic cell antigen 2 (BDCA-2) or BDCA-4 staining. Purified PDCs were cultured in flat-bottomed 96-well plates at 5×10^4 cells in 200 μ l of medium per well.

Analysis of PDCs. PDCs were stained with fluorescein isothiocyanate (FITC)-labeled CD86 (BD Biosciences) and then analyzed by FACSCalibur (BD Biosciences). The production of cytokines in the culture supernatants after 24 hours was determined by enzyme-linked immunosorbent assay (ELISA). TNF α was from R&D Systems, and IFN α was from PBL Biomedical. Intracellular cytokine staining in PDCs was performed after 8 hours of culture with different stimuli. Brefeldin A (10 μ g/ml; Sigma) was added during the last 2 hours. After stimulation, cells were fixed and permeabilized using the FIX and PERM kit (Caltag) and then stained with FITC-labeled anti-IFN α 2 monoclonal antibody (mAb; Chromaprobe), phycoerythrin (PE)-labeled anti-TNF α mAb (PBL Biomedical), and allophycocyanin-labeled anti-BDCA-4 mAb (Miltenyi Biotec). Dead cells were excluded on the basis of side and forward scatter characteristics. In the viability assay, cells were washed with PBS containing 2 mM EDTA, and viable cells were counted in triplicate with trypan blue exclusion of the dead cells. Viable cells were also evaluated as the annexin V-negative fraction using an annexin V-FITC kit (Bender MedSystems).

Detection of phospho-p38 MAPK, phospho-ERK-1/2, phospho-Akt, and phospho-NF- κ B p65. PDCs were stimulated for 90 minutes, and cells were immediately fixed and stained using PE-conjugated anti-phospho-Akt (pS473, pT308; BD Biosciences) and Alexa Fluor 488-conjugated anti-phospho-p38 MAPK (pS pT180/pY182; BD Biosciences), Alexa Fluor 488-conjugated anti-phospho-ERK-1/2 (pT202/pY204; BD Biosciences), or Alexa Fluor 488-conjugated anti-phospho-NF- κ B p65 (pS529; BD Biosciences) and analyzed using BD Phosflow according to the recommendations of the manufacturer (BD Biosciences). Cells were then analyzed by FACSCalibur.

Confocal microscopy. Cells were seeded onto glass slides by cytopsin, mounted, fixed with 2% paraformaldehyde and then permeabilized with 100% ice-cold methanol for 10 minutes at -20° C. Samples were labeled with rabbit polyclonal anti-human IRF-7 (H-246; Santa Cruz Biotechnology) and 4',6-diamidino-2-phenylindole (DAPI). Anti-rabbit IgG-Cy5 (Caltag) was used as a secondary antibody. Images were acquired using a confocal microscope (LSM 510 META; Carl Zeiss).

SLE PDCs and serum. PBMCs and sera were obtained from 3 patients with active SLE who had low complement levels prior to steroid therapy and satisfied 5 of the American College of Rheumatology criteria for the classification of SLE (31). All patients had anti-double-stranded DNA antibody. PDCs isolated from PBMCs obtained from SLE patients were cultured with CpG 2216 in flat-bottomed 96-well plates at 1×10^4 cells in 200 μ l of medium per well. In some experiments, PBMCs from healthy donors were stimulated with 20% SLE serum instead of 10% FBS. Preliminarily, we tested sera from 3 patients with SLE and selected the best serum for inducing IFN in healthy PBMCs (results not shown). Simvastatin (10 μ M), pitavastatin (10 μ M), or vehicle was added to these cultures.

In vivo assessment of cytokine production. C57BL/6 mice (purchased from Clea) were pretreated intraperitoneally with simvastatin (0.8 mg/animal), pitavastatin (0.2 mg/animal), or vehicle for 30 minutes, followed by intravenous injection of poly(U) (50 μ g/animal; Sigma) plus In Vivo JetPEI (Polyplus Transfection) according to the recommendations of the manufacturer. After 1 hour, serum IFN α levels were analyzed by ELISA (PBL Biomedical).

RESULTS

Inhibition of IFN α production in human PDCs treated with statins. To assess the immunomodulatory effects of statins on type I IFN production, we measured TLR-induced cytokine production by human PDCs in the presence and absence of simvastatin or pitavastatin. We found that IFN α production by PDCs in response to the TLR-9 ligand CpG 2216 was markedly inhibited by either simvastatin or pitavastatin in a dose-dependent manner (Figure 1B). Because statins inhibit the synthesis of mevalonate, the metabolite downstream of HMG-CoA (Figure 1A), mevalonate is the limiting step in the effect of HMG-CoA reductase. To investigate whether the inhibitory effects of statins are mediated by their actions as HMG-CoA reductase inhibitors, we added mevalonate to the PDC cultures along with the statins. We found that the suppression of IFN levels by statins was counteracted by the addition of mevalonate (Figure 1B). Similar inhibitory effects were obtained using CpG C274, the TLR-7 ligand loxoribine, and the Sendai virus (Figure 1C). In contrast to the strong suppression of IFN α , TNF α production by PDCs in response to these TLR ligands was modestly decreased by either simvastatin or pitavastatin (Figures 1B and C). These findings suggest that statins functioning as HMG-CoA reductase inhibitors had a more profound effect in inhibiting IFN α production than TNF α production in PDCs.

Lack of effect of statins on PDC survival or maturation. Statins have previously been shown to have cytotoxic effects at high concentrations (14). Therefore, to analyze whether statins at the concentration we used inhibited the production of IFN α by simply killing PDCs, we next evaluated the survival of PDCs in the presence of statins by trypan blue exclusion of dead cells or annexin V staining. (Data are available from the corresponding author upon request.) Although a very high concentration (100 μ M) of simvastatin actually killed PDCs, statin concentrations of 0.1–10 μ M did not induce cell death. To further confirm that statins inhibit IFN α production without killing PDCs, we analyzed intracellular IFN α expression in viable PDCs after exposure to CpG 2216. (Results are available from the

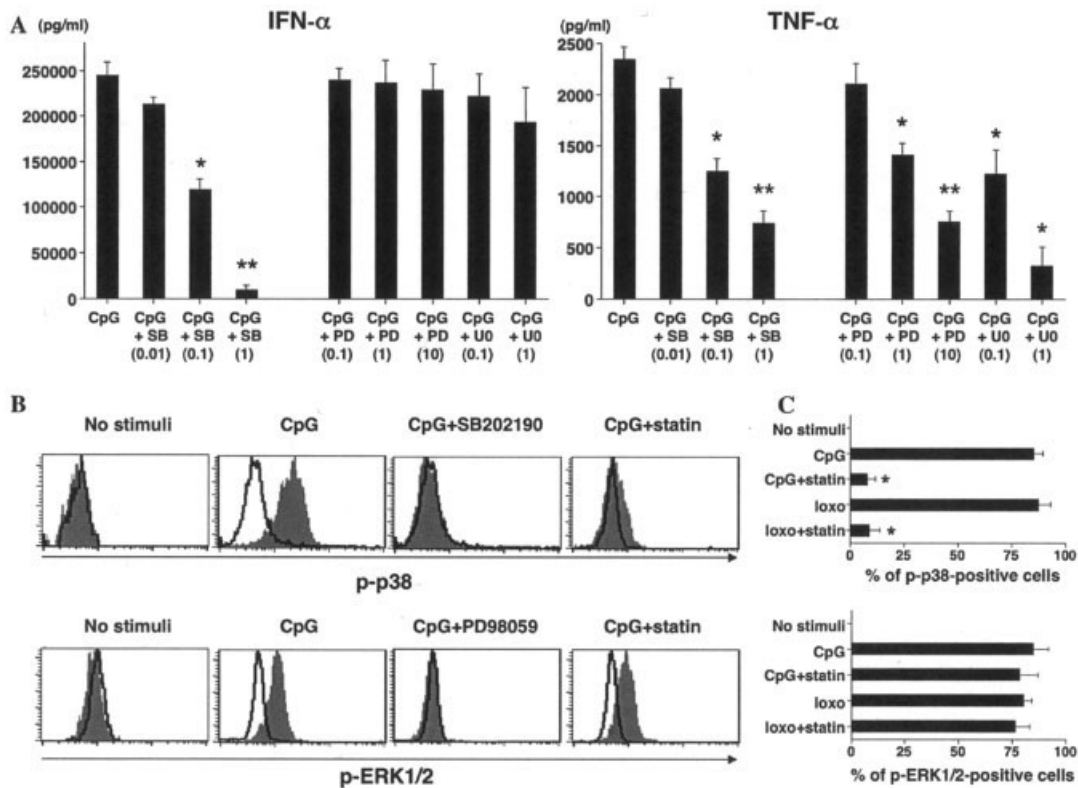


Figure 2. Inhibition of phosphorylation of p38 MAPK in the TLR-mediated IFN response in PDCs treated with statins. **A**, Levels of IFN α and TNF α in PDCs that were cultured with 5 μ M CpG 2216 in the absence or presence of different concentrations of the p38 MAPK inhibitor SB202190 (SB; 0.01–1 μ M), the MEK-1 inhibitor PD98059 (PD; 0.1–10 μ M), or U0126 (U0; 0.1 and 1 μ M). After 24 hours, the concentrations of IFN α and TNF α in the culture supernatants were measured by enzyme-linked immunosorbent assay. Bars show the mean and SEM (n = 3 independent donors per group). * = P < 0.05; ** = P < 0.01 versus CpG alone, by Student's paired t-test. **B**, Staining with anti-phospho-p38 MAPK monoclonal antibody (mAb; shaded areas in top panel), anti-phospho-ERK-1/2 mAb (shaded areas in bottom panel), and isotype-matched control (solid line) in freshly isolated PDCs or activated PDCs that were stimulated for 90 minutes with 5 μ M CpG 2216 or 100 μ M loxoribine in the absence or presence of 1 μ M SB202190, 10 μ M PD98059, or 10 μ M simvastatin. Similar results were observed in cells from at least 3 independent donors; the results of a representative experiment are shown. **C**, Percentages of cells treated as indicated that were positive for the indicated antibody. Bars show the mean and SEM (n = 3 independent donors per group). * = P < 0.05; ** = P < 0.01 versus CpG alone, by Student's paired t-test. See Figure 1 for other definitions.

corresponding author upon request.) Eight hours after activation by CpG, >25% of the PDCs produced IFN α ; however, the addition of 10 μ M simvastatin considerably reduced the percentage of PDCs that produced IFN α (to 8%). In contrast, the percentage of TNF α -producing cells was only slightly decreased by simvastatin. Thus, in this setting, 10 μ M simvastatin showed a strong inhibitory effect on the production of IFN α even in viable PDCs.

Next, we investigated the effect of statins on PDC maturation. Up to 10 μ M simvastatin did not influence the CD86 expression in PDCs that was induced in response to CpG or loxoribine. (Results are available

from the corresponding author upon request.) These findings suggest that, at the concentrations used in the present study (0.1–10 μ M), statins inhibit the pathway of IFN α induction but do not affect signaling related to viability and maturation of PDCs.

Inhibition of MAPK pathways in PDCs treated with statins. Recent evidence showing that CpG stimulation induces phosphorylation of p38 MAPK and that the inhibitor of p38 MAPK suppresses TLR-induced IFN α production by human PDCs (32) suggests that the MAPK pathway in PDCs is involved in the induction of type I IFNs. This, taken together with the results of several studies showing that statins exert pleiotropic

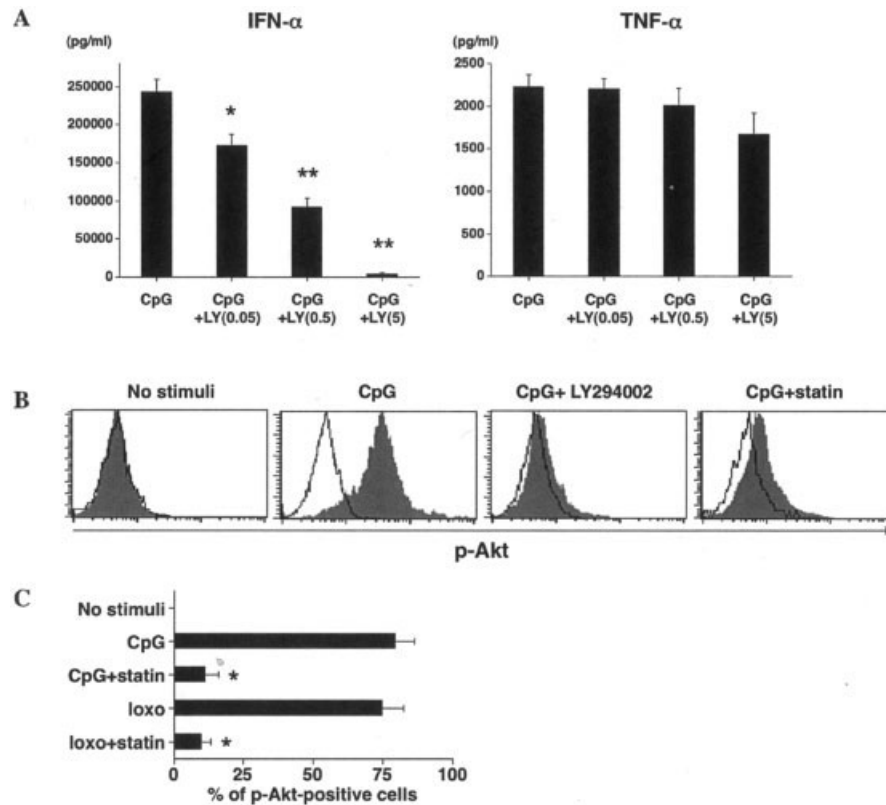


Figure 3. Inhibition of activation of phosphatidylinositol 3-kinase (PI 3-kinase) in the TLR-mediated IFN response in PDCs treated with statins. **A**, Levels of IFN α and TNF α in PDCs that were cultured with 5 μ M CpG 2216 in the absence or presence of different concentrations of the PI 3-kinase inhibitor LY294002 (LY; 0.05–5 μ M). After 24 hours, the concentrations of IFN α and TNF α in the culture supernatants were measured by enzyme-linked immunosorbent assay. Bars show the mean and SEM ($n = 3$ independent donors per group). * = $P < 0.05$; ** = $P < 0.01$ versus CpG alone, by Student's paired t -test. **B**, Staining with anti-phospho-Akt monoclonal antibody (mAb; shaded area) and isotype-matched control (solid line) in freshly isolated PDCs or activated PDCs that were stimulated for 90 minutes with 5 μ M CpG 2216 or 100 μ M loxoribine in the absence or presence of 5 μ M LY294002 or 10 μ M simvastatin. Similar results were observed in cells from at least 3 independent donors; the results of a representative experiment are shown. **C**, Percentages of cells treated as indicated that were positive for anti-phospho-Akt mAb. Bars show the mean and SEM ($n = 3$ independent donors per group). * = $P < 0.05$; ** = $P < 0.01$ versus CpG alone, by Student's paired t -test. See Figure 1 for other definitions.

effects by regulating the MAPK pathway, including p38 and MEK/ERK, through various types of cells (7,15), prompted us to assess whether statins target MAPK pathways in the inhibition of IFN production by PDCs. First, we investigated whether p38 MAPK inhibitor or MEK inhibitor prevents type I IFN induction from PDCs, as the statins do. We found that the p38 MAPK inhibitor SB202190, but not the MEK-1 inhibitors PD98059 or U0126, significantly inhibited IFN α production by PDCs in response to CpG 2216 in a dose-dependent manner (Figure 2A). All 3 of these inhibitors significantly inhibited TNF α production (Figure 2A).

Next, to assess whether statins alter the activities of MAPKs in PDCs upon activation, we analyzed the expression of phospho-p38 MAPK and phospho-ERK-1/2 by flow cytometry (Figure 2B). Phospho-p38 MAPK and phospho-ERK-1/2 were not detected in unstimulated PDCs but were induced after CpG stimulation for 90 minutes. We found that simvastatin inhibited CpG-induced phospho-p38 MAPK expression but had no effect on phospho-ERK-1/2 expression (Figure 2B). Similar results were obtained in PDCs stimulated with the TLR-7 ligand loxoribine and in PDCs stimulated with CpG 2216 (Figure 2C). Similar results were obtained with pitavastatin (data not shown). Our results

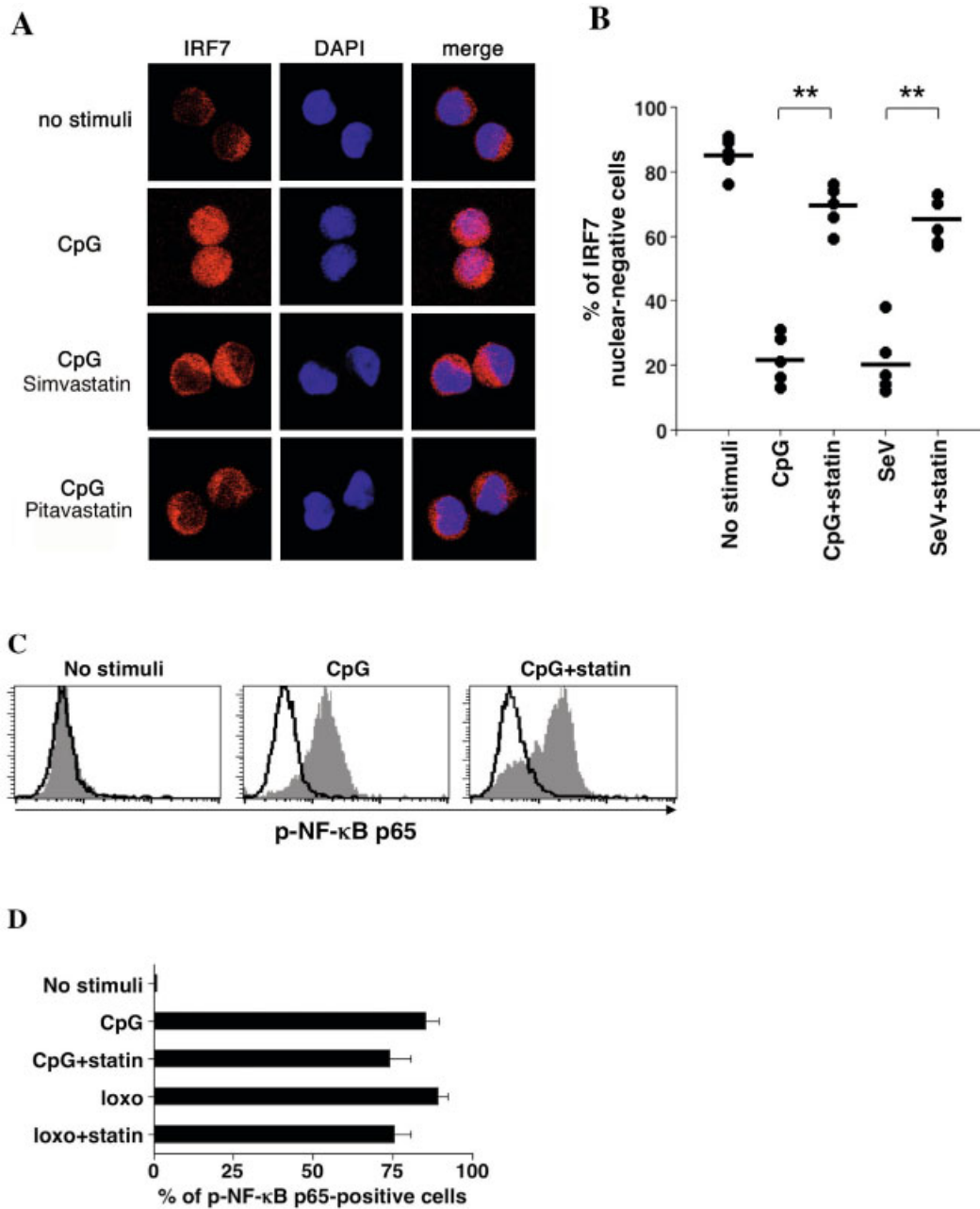


Figure 4. Inhibition of IFN regulatory factor 7 (IRF-7) nuclear translocation, but not NF-κB phosphorylation, in TLR-mediated activation in PDCs treated with statins. **A**, Staining of freshly isolated PDCs or activated PDCs that were stimulated for 3 hours with CpG alone or with CpG and 10 μM simvastatin or pitavastatin. Cells were visualized by immunofluorescence staining with IRF-7 antibody (red) and nuclei staining with 4',6-diamidino-2-phenylindole (DAPI; blue). Similar results were observed in cells from 5 independent donors. **B**, Percentages of cells on the slide that were negative for nuclear IRF-7 expression (per 50 cells from each donor). Cells were regarded as negative when the expression level of IRF-7 in the cytoplasm was higher than, and distinguishable from, that in the nucleus, as shown by DAPI staining. Circles represent individual donor samples; horizontal lines indicate the mean. **C**, Staining with anti-phospho-NF-κB p65 monoclonal antibody (mAb; shaded area) and control (solid line) in freshly isolated PDCs or activated PDCs that were stimulated for 90 minutes. Similar results were observed in cells from at least 3 independent donors. **D**, Percentages of cells treated as indicated that were positive for anti-phospho-NF-κB p65 mAb. Bars show the mean and SEM (n = 3 independent donors per group). Simvastatin was used in **B–D**. ** = P < 0.01 by Student's paired t-test. See Figure 1 for other definitions.

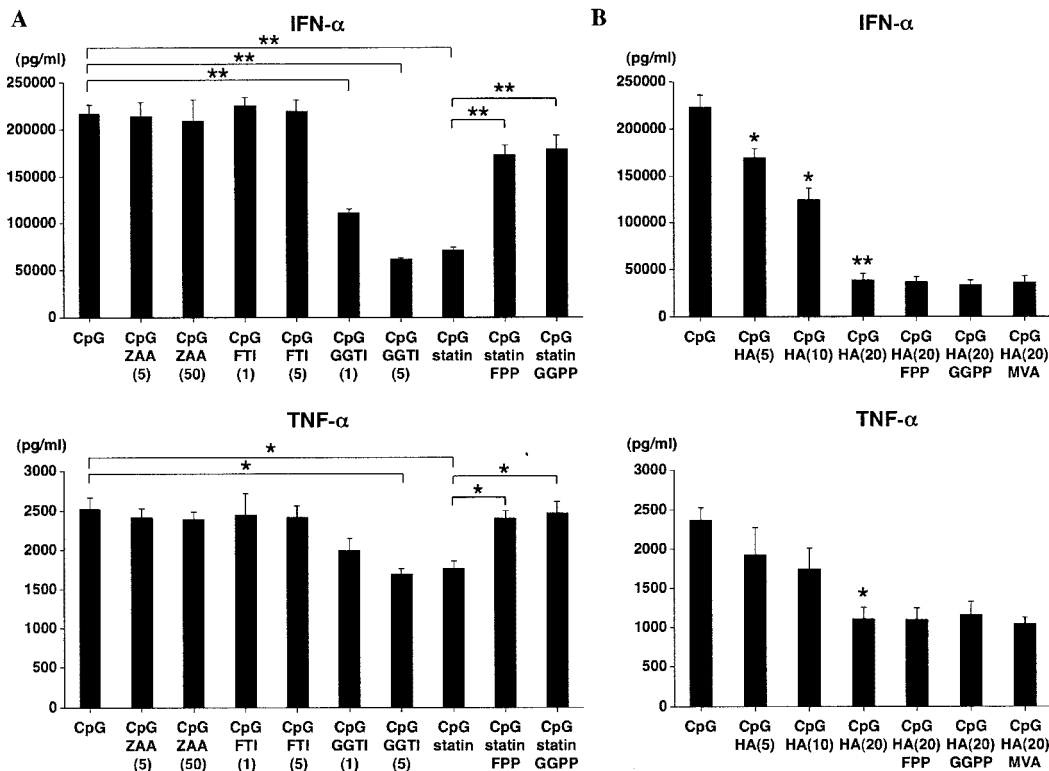


Figure 5. Inhibition of TLR-mediated IFN production in PDCs treated with GGTI or Rho kinase inhibitor, but not in PDCs, treated with ZAA or FTI. **A**, Levels of IFN α and TNF α in PDCs that were cultured with 5 μ M CpG 2216 in the absence or presence of different concentrations of ZAA, FTI-277, or GGTI-298 or 10 μ M simvastatin. In some cultures, 10 μ M FPP or 10 μ M GGPP was added. **B**, Levels of IFN α and TNF α in PDCs that were cultured with 5 μ M CpG 2216 in the absence or presence of different concentrations of Rho kinase inhibitor HA1077 (HA). In some cultures, 10 μ M FPP, 10 μ M GGPP, or 100 μ M mevalonate was added. After 24 hours, the concentrations of IFN α and TNF α in the culture supernatants were measured by enzyme-linked immunosorbent assay. Bars show the mean and SEM (n = 3 independent donors per group). * = $P < 0.05$; ** = $P < 0.01$ versus CpG alone, by Student's paired t -test. See Figure 1 for other definitions.

suggest that statins suppress the activity of p38 MAPK, but not ERK, in the inhibition of type I IFN production.

Inhibition of the PI 3-kinase pathway in PDCs treated with statins. Although statins have been shown to modulate PI 3-kinase activity in several cell types (6,16,33–39), no published study has addressed PI 3-kinase activity in DC subsets. PI 3-kinase is critically involved in many biologic events, including TLR-induced responses. Because a recent study demonstrated that PI 3-kinase plays a critical role in the induction of type I IFN production by human PDCs (40), we assessed whether statins inhibit the PI 3-kinase pathway in human PDCs upon signaling through TLR by measurement of phospho-Akt, a downstream target of PI 3-kinase. Like the statins, the PI 3-kinase inhibitor LY294002 significantly inhibited production of IFN α but not TNF α by PDCs in response to CpG 2216 in a dose-dependent

manner (Figure 3A), as previously described (40). In addition, phosphorylation of Akt was induced in CpG-stimulated PDCs, and this induction was blocked by LY294002 (Figure 3B). We found that the CpG-induced expression of phospho-Akt was repressed by treatment with simvastatin (Figure 3B) and pitavastatin (results not shown). Similarly, an inhibitory effect of simvastatin on phospho-Akt expression was observed in response to loxoribine (Figure 3C). Thus, these findings suggest that statins inhibit type I IFN production by PDCs through regulating the PI 3-kinase pathway upon activation of the TLR-induced response.

Inhibition of nuclear translocation of IRF-7 in PDCs treated with statins. The essential step in the production of type I IFN by PDCs in response to TLR ligands has been shown to be the selective nuclear translocation of the constitutive expression of IRF-7

(24). To assess whether statins modulate this process, we next examined by confocal microscopy the nuclear translocation of IRF-7 in PDCs in the presence or absence of statins (Figure 4A). IRF-7 was constitutively expressed and localized in the cytoplasmic area of unstimulated PDCs. After 3 hours of stimulation with CpG, IRF-7 was detected in the nucleus, as shown by colocalization with DAPI nuclear staining, indicating the nuclear translocation of IRF-7. We found that this colocalization of IRF-7 and DAPI stainings was inhibited by the presence of either simvastatin or pitavastatin (Figure 4A). Thus, the statins helped retain IRF-7 in the cytoplasm, suggesting an inhibitory effect on IRF-7 nuclear translocation in PDCs. To show this finding quantitatively, we counted the cells without nuclear IRF-7 expression in PDCs on the slide. The frequency of cells without IRF-7 nuclear translocation was significantly augmented by simvastatin in PDCs stimulated with either CpG or Sendai virus (Figure 4B).

DC maturation and the production of most inflammatory cytokines have been shown to take place primarily through NF- κ B activation (41). Unlike IRF-7 activation, TLR-induced NF- κ B phosphorylation was

only slightly blocked by simvastatin (Figures 4C and D) and pitavastatin (results not shown). Our findings indicate that the preferential inhibition of type I IFN production by statins is due to its strong inhibitory effect on the nuclear translocation of IRF-7.

Suppression of IFN α production in PDCs through inhibition of geranylgeranyl transferase or Rho kinase. Statins can inhibit the synthesis of isoprenoids (FPP and GGPP) and the resultant Ras and Rho GTPases, which are responsible for the pleiotropic effects of statins (11,14,42). FPP is a bifurcate point in the mevalonate pathway; the Ras family is farnesylated and the Rho family is geranylgeranylated via GGPP, the synthesis of which splits off from the process of cholesterol biosynthesis (Figure 1A). To assess the targets of statins in inhibiting IFN responses in the mevalonate pathway in PDCs, we compared the effects of the squalene synthetase inhibitor ZAA, the farnesyl transferase inhibitor FTI-277, the geranylgeranyl transferase inhibitor GGTI-298, and the Rho kinase inhibitor HA1077 with that of simvastatin in cytokine production from PDCs in response to CpG.

We found that although ZAA and FTI-277 did

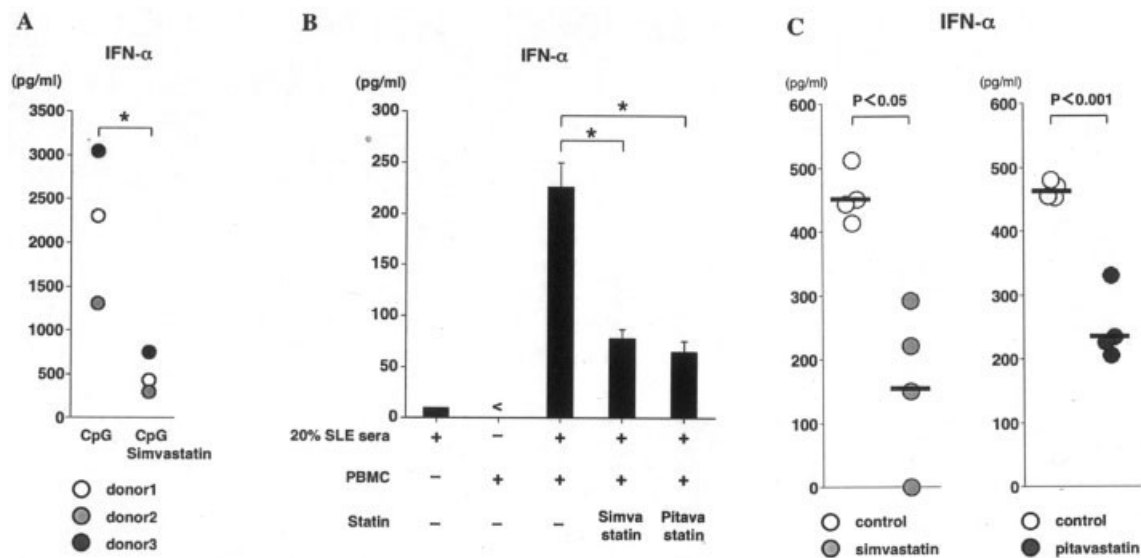


Figure 6. Prevention of systemic lupus erythematosus (SLE)-mediated IFN α production in vitro and reduction in serum IFN α levels in vivo by statins. **A**, Levels of IFN α in PDCs from 3 patients with SLE. PDCs were isolated, and 1×10^4 cells were cultured in 200 μ l of medium per well with 5 μ M CpG 2216 in the absence or presence of 10 μ M simvastatin for 24 hours. **B**, Levels of IFN α in healthy human peripheral blood mononuclear cells (PBMCs) that were preincubated for 15 minutes with 10 μ M simvastatin, 10 μ M pitavastatin, or vehicle in serum-free RPMI. Serum from an SLE patient (at a final concentration of 20% [v/v]) was then added. After 24 hours, the concentrations of IFN α in the culture supernatants were measured by enzyme-linked immunosorbent assay. Bars show the mean and SEM (n = 4 independent donors per group). * = $P < 0.05$, by Student's paired *t*-test. **C**, IFN α levels in C57BL/6 mice that were pretreated intraperitoneally with simvastatin (0.8 mg/animal), pitavastatin (0.2 mg/animal), or vehicle for 30 minutes, followed by intravenous injection of 50 μ g/animal of poly(U). Serum IFN α was measured 1 hour after poly(U) injection. Bars show the mean (n = 4 independent experiments). See Figure 1 for other definitions.

not inhibit cytokine production by PDCs, GGTI-298 mimicked the effect of simvastatin on PDCs in its inhibition of IFN α and TNF α production (Figure 5A). In addition, the inhibitory effect of simvastatin was reversed by the addition of GGPP or FPP. Like statins, HA1077 significantly inhibited IFN α production in a dose-dependent manner and moderately inhibited TNF α production (Figure 5B). However, the suppressive activity of HA1077 was not affected by the addition of the isoprenoid intermediates (FPP and GGPP). No negative effects on PDC survival or maturation were observed at the concentrations of ZAA, FTI-277, GGTI-298, and HA1077 used (data not shown). These findings indicate that statins exert a cholesterol-independent inhibitory effect on IFN responses in PDCs through geranylgeranylated Rho and its effector Rho kinases.

Inhibition of pathogen-conditioned IFN production by statins. Serum immune complexes (ICs) consisting of autoantibodies and self DNA in SLE induce type I IFN production by blood PDCs through TLR-9, causing the development of the autoimmune process (43). Thus, the PDCs and serum represent the pathogenic components in SLE. To investigate the possibility of statin therapy inhibiting type I IFN production in SLE, we designed an additional experiment using PDCs and serum from patients with SLE. Because blood PDCs in SLE are continuously triggered by serum ICs, the numbers of circulating PDCs are decreased and their function is defective (44,45). Indeed, we found a low IFN response to CpG in PDCs from SLE patients (Figure 6A). Even in pathogenic PDCs, simvastatin inhibited PDC-derived IFN α production.

We next confirmed the observation that serum from SLE patients promoted IFN production by healthy PBMCs. Both simvastatin and pitavastatin exerted an inhibitory effect on SLE serum-induced IFN production by PBMCs (Figure 6B). These data suggest that statins function as inhibitors of type I IFN production under the pathophysiologic condition of SLE.

Inhibition of in vivo IFN production by statins. Finally, to explore the therapeutic potential of statins, we evaluated the in vivo effect of statins on the IFN response in mice. We analyzed the serum IFN level 1 hour after injection of poly(U) into C57BL/6 mice that had been treated with statins or left untreated (Figure 6C). Injecting mice with poly(U) rapidly increased the serum IFN level. Pretreatment with either simvastatin or pitavastatin prevented any increase in serum IFN levels. Based on our data, treatment with statins has the potential to attenuate increased IFN levels in vivo.

DISCUSSION

Although the beneficial effects of statins in ameliorating SLE disease activity have been shown in humans (46) and in a murine model of the disease (5), the cellular and molecular targets of statins in SLE remain elusive. Recently, evidence has been mounting indicating an intimate link between PDCs and autoimmune diseases such as SLE or psoriasis (21–23). Taken together with findings indicating the importance of type I IFNs in the development of autoimmunity (47,48), this evidence seems to indicate that PDCs, through their production of large quantities of type I IFNs, play a critical role in the pathogenesis of SLE. Therefore, control of dysregulated type I IFN production by PDCs may constitute a treatment strategy for SLE. Our study demonstrated the ability of statins to inhibit type I IFN production by human PDCs and showed that this inhibitory activity involves different molecular pathways, p38 MAPK, PI 3-kinase/Akt, and nuclear translocation of IRF-7. Based on the results of previous studies, either p38 MAPK or PI 3-kinase/Akt appears to be essential components of the transduction pathway leading to IFN synthesis in PDCs (32,40). This finding, taken together with the results of the present study indicating that statins suppress the expression of both phospho-p38 MAPK and phospho-Akt in activated PDCs, indicates that the inhibitory effect of statins on IFN synthesis could be attributed to the suppressive function of both kinase pathways.

It has been demonstrated that the molecular mechanism underlying the immunomodulatory effect of statins is mostly through the inhibition of Rho/Rho kinases (6). Also, our results showing that GGPP reversed the effects of statins and that both GGTI-298 and Rho kinase inhibitor mimicked the inhibitory function of statins suggest that statins inhibit type I IFN production in PDCs by targeting geranylgeranylated Rho and the Rho kinase pathway. Additionally, our data imply a molecular link between the inhibitory effect of statins and the intracellular pathways p38 MAPK and Rho/Rho kinases. Indeed, statins have been shown to regulate p38 MAPK through the inhibition of Rho activation in a variety of cell types (6,7,9,15,17,49). Although both phosphorylation of p38 MAPK and ERK-1/2 were induced in PDCs by stimulation with CpG (Figure 2B) (50), only p38 MAPK inhibitor (and not MEK/ERK inhibitor) suppressed IFN α production (Figure 2A) (36), indicating a selective involvement of p38 MAPK in type I IFN production in PDCs. These findings, taken together with our results showing that statins blocked

the TLR-induced phosphorylation of p38 MAPK, suggest that inhibition of p38 MAPK by statins contributes to at least some of the inhibitory effect on IFN response in PDCs. However, considering the observation that statins had no effect on TLR-induced ERK-1/2 phosphorylation in PDCs, it seems that there is no involvement of ERK signaling in the underlying mechanism of the inhibitory effect of statins on IFN α production. Ras proteins have been strongly implicated in ERK signaling (15,49). In addition, our finding that the inhibition of Ras farnesylation by farnesyl transferase inhibitor (FTI-277) did not affect IFN α production by PDCs further indicates that the Ras/ERK pathway does not play a significant role in IFN synthesis in PDCs.

Several previous studies have documented the effect of statins in activating the PI 3-kinase/Akt pathway in some cell types (6,33,34). This may not be the case in PDCs, since statins inhibited the PI 3-kinase/Akt pathway in response to TLR ligands. This finding is consistent with recent studies showing that statins suppress PI 3-kinase/Akt activity in human monocytes (35), endothelial cells (16,36,37), osteoblasts (38), and neuroblasts (39). Thus, our findings suggest that statins may intersect with the p38 MAPK and PI 3-kinase pathways in the induction of type I IFNs in PDCs, possibly through Rho/Rho kinase inactivation.

Type I IFN production is differentially regulated from the production of other inflammatory cytokines by specific intracellular events under TLR stimuli. A late molecular switch responsible for type I IFN synthesis in PDCs is the nuclear translocation of IRF-7 (24). Our findings indicate that statins function as an inhibitor of IRF-7 nuclear translocation. Recent studies have revealed that IRF-7 nuclear translocation upon signaling through TLR-9 is dependent on PI 3-kinase/Akt (40) but is independent of p38 MAPK (32). Therefore, the molecular mechanism by which statins block the nuclear translocation of IRF-7 may be their ability to inhibit PI 3-kinase/Akt activity. Based on our results, the activation of IRF-7 appears to be independent of the NF- κ B activation pathway, which links to cell maturation and TNF α secretion. Consistent with our findings, the differential regulation of the nuclear translocation of phosphorylated IRF-7 and NF- κ B in PDCs has been verified in independent studies using IKK α -deficient mice (28) and PI 3-kinase inhibitor in human PDCs (40).

In the present study, we further showed that although PI 3-kinase inhibitor did not inhibit TNF α production by PDCs, which was consistent with the findings of a previous study (40), p38 MAPK inhibitor inhibited not only IFN α production, but also TNF α

production. In support of our finding, there is evidence that the induction of the inflammatory chemokines CXCL10 and CCL3 by human PDCs is suppressed by p38 MAPK inhibitor (32). Thus, we speculate that the inhibition of both p38 MAPK and PI 3-kinase/Akt pathways by statins contributes to the strong suppression of type I IFN production in PDCs and that the inhibition of p38 MAPK contributes to the modest suppression of TNF α production in PDCs.

In SLE, PDCs and serum consisting of self DNA and autoantibody complexes are the pathogenic cellular and humoral factors, respectively. We have also identified a specialized role of statins, in that they have inhibitory effects on such pathogen-conditioned IFN α production. Moreover, statins functioned as a repressor of the IFN response in vivo. On the basis of our results, we believe that statins have the potential to attenuate the IFN environment, even under the pathophysiologic conditions of SLE, by regulating PDC functions. Statins have recently been shown to inhibit IL-17 secretion in human CD4⁺ T cells (10), which are involved in autoimmune responses in RA, chronic colitis, and multiple sclerosis. However, there is no direct evidence that IL-17 is pathogenic in SLE. Thus, our findings provide new evidence that statins directly inhibit the pathogenic molecular and cellular components of SLE.

In conclusion, we have identified a novel immunomodulatory mechanism in the efficacy of statins, which preferentially target the cellular and molecular pathways involved in IFN production. We have shown that statins have suppressive effects on the multiple signal transduction pathways that mediate the IFN response in PDCs. Thus, our data provide the foundation for potential therapeutic approaches to IFN-mediated diseases, such as SLE and possibly psoriasis.

ACKNOWLEDGMENTS

The authors thank Ms Mihoko Inoue and Ms Hitomi Yoshimura for manuscript preparation.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ito had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Amuro, Ito, Miyamoto, Sugimoto, Torii, Son, Nakamichi, Yamazaki, Hoshino, Kaisho, Ozaki, Inaba, Amakawa, Fukuhara.

Acquisition of data. Amuro, Ito, Miyamoto, Sugimoto, Torii, Son, Nakamichi, Yamazaki, Hoshino, Kaisho, Ozaki, Inaba, Amakawa, Fukuhara.

Analysis and interpretation of data. Amuro, Ito, Miyamoto, Sugimoto, Torii, Son, Nakamichi, Yamazaki, Hoshino, Kaisho, Ozaki, Inaba, Amakawa, Fukuhara.

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