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Inhibitory effects of fluvastatin on cytokine and chemokine production by peripheral blood mononuclear cells in patients with allergic asthma

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Summary

Background Statins have anti-inflammatory effects on immune cells.

Objective To investigate the immunomodulatory effects of fluvastatin on peripheral blood mononuclear cells (PBMCs) after allergen-specific and non-allergen-specific stimulation in patients with asthma and in healthy subjects.

Methods PBMCs from seven patients with asthma who showed elevated immunoglobulin (Ig)E to house dust mite were isolated and stimulated with *Dermatofagoides farinae*, purified protein derivative, and phytohaemagglutinin (PHA) in the presence or absence of fluvastatin. PBMCs from seven healthy subjects were stimulated with PHA. The effects of fluvastatin on cell proliferation and production of cytokines (interferon [IFN]- γ and interleukin [IL]-5) and chemokines (chemokine CXC motif, ligand [CXCL10], and CC chemokine ligand [CCL17]) were measured. Migration of T helper (Th)1 and Th2 cell lines was also investigated. The expression of CXCR3 and CCR4 was analysed with flow cytometry. Steroid-insensitive PBMCs induced by preculture with IL-2 and IL-4 were also evaluated. Some experiments were performed in the presence of mevalonic acid.

Results Fluvastatin inhibited the proliferation of PBMCs and decreased the production of IL-5, IFN- γ , CCL17, and CXCL10 after allergen-specific and non-allergen-specific stimulation; all these effects, except for decreased CXCL10 production, were partially reversed by mevalonic acid. Culture supernatants obtained in the presence of fluvastatin prevented the migration of Th1 and Th2 cell lines in a dose-dependent manner. In addition, CCR4 and CXCR3 expression on CD4⁺ T cells was not affected by the presence of fluvastatin. Fluvastatin inhibited the proliferative response of steroid-insensitive PBMCs to phytohaemagglutinin.

Conclusion Fluvastatin has inhibitory effects on cytokine and chemokine production, and thus might be used as a potential therapeutic agent in severe asthma.

Keywords asthma, cell proliferation, chemokine, chemotaxis cytokine, statin

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Introduction

Bronchial asthma is characterized by persistent airway inflammation induced by several types of inflammatory cells, including CD4⁺ T cells, mast cells, and eosinophils [1, 2]. Because airway inflammation is closely associated with airway hyper responsiveness, airway limitation, and asthma symptoms, inhaled corticosteroids are the first-line agents for the treatment of asthma [3]. Although, asthma is often well controlled with inhaled corticosteroids, some patients with severe asthma require additional oral corticosteroids. However, long-term treatment with oral corticosteroids is associated with such side-effects as adrenal suppression, growth suppression, and osteoporosis.

Moreover, asthma in some patients is both severe and steroid-resistant. Therefore, alternatives to oral corticosteroids are needed for such patients [4].

Statins, which inhibit 3-hydroxy-3-methyl glutaryl coenzyme A reductase, are used to treat hypercholesterolaemia. Moreover, statins have pleiotropic effects that are related to the inhibition of mevalonic acid and its isoprenoid intermediates, which are involved in normal cellular functions and signal transduction [5, 6]. Because statins affect expression of class II major histocompatibility complex (MHC II) and modulate macrophage, monocyte, and lymphocyte functions, they have also been implicated in anti-inflammatory effects [7–10]. An alternative mechanism by which statins might inhibit lymphocytes is by

reducing the density of cholesterol-rich membrane microdomains or rafts, thus limiting the function of receptors on membrane rafts [11]. Indeed, statins have been shown to have anti-inflammatory effects in a murine model of allergic asthma [12]. Although T helper (Th) 2 cells play an important role in asthma, the effects of statins on T cells and steroid-insensitive T cells remain unclear.

In the present study, we investigated the effects of fluvastatin on the proliferation of and cytokine and chemokine production by peripheral blood mononuclear cells (PBMCs) from patients with allergic asthma and healthy subjects after allergen-specific or non-allergen-specific stimulation. We also analysed the effects of fluvastatin on Th1 and Th2 cell migration and steroid-insensitive PBMCs.

Materials and methods

Reagents

The following reagents were used: fluvastatin (donated by Novartis Pharmaceuticals Co., Osaka, Japan), *Dermatophagoides farinae* (Der f; donated by Torii Pharmaceuticals Co., Ltd, Tokyo, Japan), purified protein derivative (PPD; Japan BCG LA, Tokyo, Japan), phytohaemagglutinin (PHA), and mevalonic acid (Sigma-Aldrich Co., St Louis, MO, USA).

Subjects

Seven non-smoking patients (age range: 28 ± 3.4 years) with allergic asthma were selected on the basis of (a) positive cutaneous reactions to Der f extract, (b) high levels in the sera of immunoglobulin (Ig)E specific for house dust and Der f antigen, (c) baseline forced expiratory volume in 1 s greater than 70% of the predicted value, (d) increased bronchial responsiveness to histamine, and (e) no treatment other than rescue β_2 -agonists for at least 3 months. Seven healthy non-atopic subjects (age range: 26 ± 4.2 years) were also investigated. None of the subjects had respiratory infections in the previous 4 weeks. All subjects gave written informed consent before the study.

Cell preparation

PBMCs were isolated from heparinized whole blood of atopic and non-atopic subjects with density gradient centrifugation using a Lymphocyte Separation Solution (Nacalai Tesque Inc., Kyoto, Japan). The cells were washed and resuspended in RPMI-1640 supplemented with 10% v/v human AB serum, 100 U of penicillin, and 10 $\mu\text{g}/\text{mL}$ of streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Th cell lines

PBMCs were cultured with Der f (10 $\mu\text{g}/\text{mL}$) or PPD (5 $\mu\text{g}/\text{mL}$) allergens in RPMI-1640 supplemented with 10% v/v

human AB serum, penicillin (100 U), and streptomycin (10 $\mu\text{g}/\text{mL}$) in 24-well plates in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were stimulated every week in the presence of autologous PBMCs treated with mitomycin C (50 $\mu\text{g}/\text{mL}$) as antigen-presenting cells (APCs) and Der f (10 $\mu\text{g}/\text{mL}$) or PPD (5 $\mu\text{g}/\text{mL}$) and IL-2 (4 ng/mL). On day 28, T cell lines (5×10^4) were tested for specificity with a 3-day proliferation assay performed in 96-well plates supplemented with Der f (10 $\mu\text{g}/\text{mL}$) or PPD (5 $\mu\text{g}/\text{mL}$) allergens and mitomycin C-treated PBMCs (5×10^4) as APCs.

PBMC survival assay

Survival assays of PBMCs were performed for up to 7 days in the presence or absence of fluvastatin (0.1, 1, and 10 μM). The PBMCs were stained with trypan blue, and live cells were counted. Percentages of live cells were calculated by dividing the number of live cells by the total cell number. In some experiments, mevalonic acid (100 μM) was added to the culture medium 2 h before the cells were treated with fluvastatin.

Annexin V/propidium iodide staining

PBMCs treated with fluvastatin (0.1, 1.0, or 10 μM) for 1, 3, or 5 days were stained with annexin V conjugated to fluorescein and red-fluorescent PI nucleic acid-binding dye (Vybrant Apoptosis Assay Kit #3, Molecular Probes, Eugene, OR, USA). The cells were examined with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). In some experiments, mevalonic acid (100 μM) was added to the culture medium.

Proliferation assay

PBMCs (5×10^5 cells/well) were cultured with Der f (10 $\mu\text{g}/\text{mL}$) or PPD (5 $\mu\text{g}/\text{mL}$) allergens for 7 days and with PHA (1 $\mu\text{g}/\text{mL}$) for 3 days in 96-well plates immediately after purification. Fluvastatin (0.1, 1.0, and 10 μM) and mevalonic acid (100 μM) were added to the cell cultures. Cells were pulsed with 1 μCi of ^3H -methylthymidine (Amersham Corp., Arlington Heights, IL, USA) for the last 8 h of the culture period. The stimulation index (SI) was calculated by dividing the counts per million of allergen-stimulated and non-allergen-stimulated cultures by that of unstimulated cultures.

Cytokine and chemokine measurements

PBMCs (2×10^6 cells/mL) were cultured in 24-well culture plates and stimulated with Der f (10 $\mu\text{g}/\text{mL}$), PPD (5 $\mu\text{g}/\text{mL}$), or PHA (1 $\mu\text{g}/\text{mL}$) and treated with fluvastatin (0.1, 1.0, and 10 μM). In some experiments, mevalonic acid

(100 μ M) was added to the cell cultures 2 h before stimulation. Concentrations of IFN- γ and CC chemokine ligand 17 (CCL17) in the culture supernatants were measured after 48 h (PHA stimulation) and 72 h (PPD or Der f stimulation) with an enzyme-linked immunosorbent assay (ELISA) with a minimum detectable level of 7 pg/mL (R&D Systems, Minneapolis, MN, USA). Concentrations of IL-5 and CXC chemokine motif, ligand 10 (CXCL10), in the culture supernatants were measured after 48 h (PHA stimulation) and 72 h (Der f or PPD stimulation) with an ELISA (BD Biosciences Pharmingen, San Diego, CA, USA) sensitive to concentrations greater than 5 pg/mL. We chose these durations of stimulation because our preliminary experiments showed that they were optimal for the production of cytokines and chemokines from PBMCs.

Chemotaxis assay

Th1 and Th2 cell lines (2×10^5 cells) were used and transmigrated through polyvinylpyrrolidone-free polycarbonate transwell culture inserts with a pore size of 5 μ m (Corning Costar Corp., Acton, MA, USA) for 6 h in a humidified atmosphere containing 5% CO₂ at 37 °C. Supernatants collected from the cytokine and chemokine experiments by using PBMCs from patients with asthma were added to the lower chambers in a final volume of 190 μ L. For blocking experiments, supernatants were pre-incubated with anti-human CCL17 (2 μ g/mL) and anti-CXCL10 (3 μ g/mL) monoclonal antibodies (R&D Systems), which can neutralize 0.01 μ g/mL of recombinant human CCL17 and 0.2 μ g/mL of recombinant human CXCL10, for 2 h at 4 °C. The cells that transmigrated into the lower chamber were recovered and counted with a haemocytometer.

Chemokine receptor analysis

PBMCs from patients with asthma were stimulated with Der f (10 μ g/mL) or PPD (5 μ g/mL) and treated with fluvastatin (0.1, 1.0, and 10 μ M). In some experiments, mevalonic acid (100 μ M) was added 2 h before stimulation. PBMCs were harvested and resuspended in PBS-Azide (phosphate-buffered saline containing 1% bovine serum albumin and 0.1% NaN₃) after 72 h, and incubated with a fluorescein isothiocyanate-conjugated antibody to CD4 (BD Biosciences Pharmingen) and phycoerythrin-conjugated antibodies to CCR4 (BD Biosciences Pharmingen) and CXCR3 (BD Biosciences Pharmingen). Cells were analysed with the FACSCalibur flow cytometer and CellQuest software (BD Biosciences Immunocytometry Systems).

Establishment of steroid-insensitive peripheral blood mononuclear cells in vitro

Steroid resistance was induced with a method reported previously [13]. Briefly, freshly isolated PBMCs from healthy

subjects or patients with asthma were cultured in the presence of IL-2 (50 U/mL) and IL-4 (50 U/mL) for 48 h. Cells were then washed, stimulated with PHA (1 μ g/mL) for an additional 72 h in the presence or absence of dexamethasone (10, 100, and 1000 nM) or fluvastatin (0.1, 1, and 10 μ M), and pulsed with 1 μ Ci of ³H-methylthymidine for the last 8 h of the culture period. In some experiments, cells were incubated with mevalonic acid (100 μ M) for 2 h and then stimulated.

Statistical analysis

We applied the Bonferroni correction for multiple comparisons. The significance of differences between groups was analysed with the Wilcoxon rank test. Data are expressed as mean $\pm$ standard error of mean (SEM), and differences with a probability value less than 0.05 were considered to be significant.

Results

Cell viability

Fluvastatin at a concentration of 1 μ M did not affect the viability of PBMCs obtained from patients with asthma after stimulation with Der f or PPD after 7 days of treatment (Der f: $76.7 \pm 2.1\%$ without fluvastatin and $74.5 \pm 3.0\%$ with 1 μ M fluvastatin, PPD: $79.7 \pm 1.5\%$ without fluvastatin and $76.9 \pm 1.2\%$ with 1 μ M fluvastatin) or with PHA after 3 days of treatment ($83.2 \pm 2.9\%$ without fluvastatin and $80.8 \pm 2.1\%$ with 1 μ M fluvastatin). Furthermore, 1 μ M fluvastatin did not induce apoptosis in PBMCs stimulated with Der f or PPD up to 5 days of treatment or with PHA up to 3 days of treatment, as analysed with annexin V and PI staining (Fig. 1). However, 10 μ M fluvastatin decreased the viability of Der f-stimulated PBMCs after 5 days ($91.0 \pm 0.9\%$ without fluvastatin to $73.6 \pm 4.0\%$ with 10 μ M fluvastatin, $P < 0.01$), of PPD-stimulated PBMCs after 3 days ($91.0 \pm 0.9\%$ without fluvastatin to $87.2 \pm 0.4\%$ with 10 μ M fluvastatin, $P < 0.01$), and of PHA-stimulated PBMCs after 3 days ($83.2 \pm 2.9\%$ without fluvastatin to $74.0 \pm 2.0\%$ with 10 μ M fluvastatin, $P < 0.01$). Fluvastatin at a concentration of 10 μ M also induced apoptosis in PBMCs stimulated with Der f up to 5 days of treatment ($P < 0.01$, Fig. 1a) and with PPD ($P < 0.05$, Fig. 1b) and PHA ($42.3 \pm 4.1\%$ without fluvastatin to $47.4 \pm 4.7\%$ with 10 μ M fluvastatin, $P < 0.05$) up to 3 days of treatment. Moreover, similar results were observed after PBMCs from healthy subjects were stimulated with PHA for 3 days ($P < 0.05$, Fig. 1c).

Fluvastatin inhibits peripheral blood mononuclear cell proliferation

Treatment with fluvastatin inhibited, in a dose-dependent manner, proliferation of PBMCs from patients with

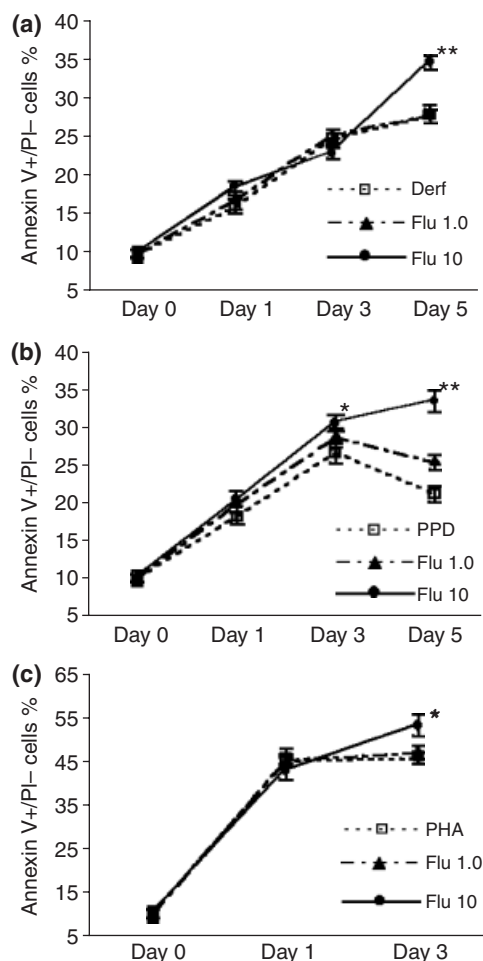


Fig. 1. Effect of fluvastatin on apoptosis. Effect of fluvastatin on annexin V and PI staining in peripheral blood mononuclear cells (PBMCs) from patients with allergic asthma after stimulation with Der f (a), and purified protein derivative (PPD) (b), or in PBMCs from healthy subjects after stimulation with phytohemagglutinin (PHA) (c). Data represent the mean $\pm$ SEM of seven independent samples. * $P < 0.05$ vs. PPD or PHA, ** $P < 0.01$ vs. Der f or PPD.

asthma stimulated with Der f (Flu 1 μ M: $P < 0.05$, Flu 10 μ M: $P < 0.01$, Fig. 2a) or PPD (Flu 1 μ M: $P < 0.05$, Flu 10 μ M: $P < 0.01$, Fig. 2b). Furthermore, fluvastatin-inhibited proliferative responses of PBMCs from patients with asthma to PHA (SI: PHA 27.6 ± 4.2 , Flu 1 μ M 21.3 ± 4.7 ; $P < 0.05$, Flu 10 μ M 4.3 ± 1.4 ; $P < 0.01$). Similarly, fluvastatin-inhibited proliferative responses of PBMCs from healthy subjects to PHA (Flu 1 μ M: $P < 0.05$, Flu 10 μ M: $P < 0.01$, Fig. 2c), and the decrease in cell proliferation induced by fluvastatin (1, 10 μ M) was significantly reversed by mevalonic acid (Fig. 2).

Fluvastatin decreases cytokine production

Treatment with fluvastatin inhibited, in a dose-dependent manner, production of both IL-5 (Flu 1 μ M: $P < 0.01$, Flu 10 μ M: $P < 0.01$, Fig. 3a) and IFN- γ (Flu 1 μ M: $P < 0.05$,

Flu 10 μ M: $P < 0.01$, Fig. 3c) by PBMCs from patients with asthma after stimulation with Der f or PPD. This inhibition by fluvastatin of IL-5 and IFN- γ production after stimulation with Der f or PPD was significantly reversed by mevalonic acid ($P < 0.01$, Figs 3a and c). In addition, production of both IL-5 (PHA 112.7 ± 22.3 , Flu 1 μ M 46.7 ± 11.7 ; $P < 0.05$, Flu 10 μ M 9.1 ± 4.6 ; $P < 0.01$) and IFN- γ (PHA 237.9 ± 46.7 , Flu 1 μ M 115.1 ± 12.9 ; $P < 0.05$, Flu 10 μ M 27.1 ± 8.9 ; $P < 0.01$) by PBMCs after stimulation with PHA was significantly inhibited. Similarly, fluvastatin dose-dependently inhibited production of IL-5 (Flu 1 μ M: $P < 0.05$, Flu 10 μ M: $P < 0.01$, Fig. 3b) and IFN- γ (Flu 1 μ M: $P < 0.05$, Flu 10 μ M: $P < 0.01$, Fig. 3d) by PBMCs from healthy subjects after stimulation with PHA. The addition of mevalonic acid resulted in the significant recovery of both IL-5 and IFN- γ production by PBMCs stimulated with PHA (Figs 3b and d).

Fluvastatin suppresses chemokine production

Stimulation of PBMCs from patients with asthma by Der f or PHA increased CCL17 production (Der f: $P < 0.01$, Fig. 4a, PHA: 147.4 ± 32.4 to 433.5 ± 48.1 , $P < 0.01$), and treatment with fluvastatin decreased CCL17 production in a dose-dependent manner (Der f: Flu 1 μ M; $P < 0.05$, Flu 10 μ M; $P < 0.01$, Fig. 4a, PHA: Flu 1 μ M 250.3 ± 9.3 ; $P < 0.05$, Flu 10 μ M; 154.3 ± 8.6 , $P < 0.01$). The addition of mevalonic acid resulted in a partial recovery of CCL17 production by PBMCs stimulated with Der f (Fig. 4a) or PHA (Flu 1 μ M 314.6 ± 21.2 ; $P < 0.05$, Flu 10 μ M; 226.5 ± 19.7 , $P < 0.05$). Stimulation with PPD ($P < 0.01$, Fig. 4c) or PHA (288.3 ± 38.9 to 2173.0 ± 296.8 , $P < 0.01$) led to increases in CXCL10 production by PBMCs, but these increases were inhibited by treatment with fluvastatin (PPD: Flu 1 μ M; $P < 0.05$, Flu 10 μ M; $P < 0.05$, Fig. 4c, PHA: Flu 1 μ M 1680.4 ± 258.9 ; $P < 0.05$, Flu 10 μ M 865.3 ± 193.6 ; $P < 0.01$). Similarly, production of CCL17 and CXCL10 from PBMCs of healthy subjects with PHA was significantly inhibited by fluvastatin (Figs 4b and d). However, addition of mevalonic acid did not result in the recovery of CXCL10 production by PBMCs stimulated with either PPD or PHA (Figs 4c and d) from patients with asthma or from healthy subjects.

Fluvastatin inhibits migration of T helper 1 and T helper 2 cell lines

After investigating the effect of fluvastatin on chemokine production, we examined whether fluvastatin affects the migration of Th1 and Th2 cell lines. In the chemotaxis assays, we used a PPD-specific Th1 cell line, which was confirmed to be composed of Th1 cells by stimulation with PPD or Der f, and found that these cells responded only to PPD and produced IFN- γ but not IL-5. Similarly, Der-f-specific Th2 cell lines were also confirmed to be

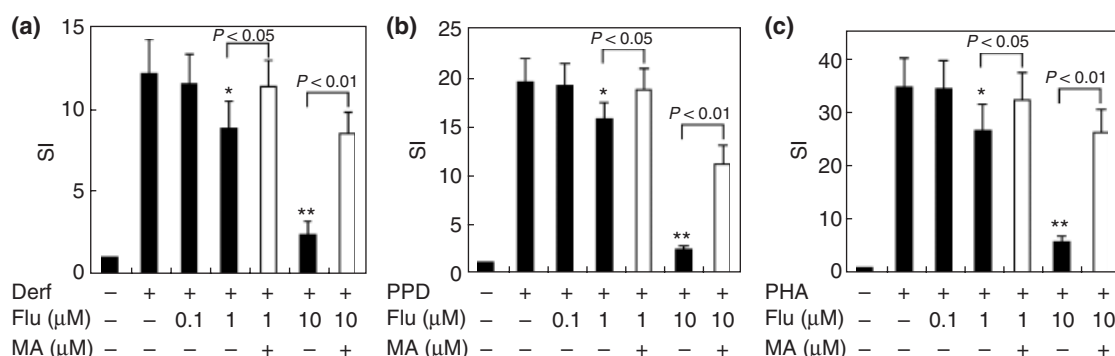


Fig. 2. Effect of fluvastatin on cell proliferation. Effect of fluvastatin on proliferative responses of peripheral blood mononuclear cells (PBMCs) from patients with allergic asthma after stimulation with Der f (a) and purified protein derivative (PPD) (b), or of PBMCs from healthy subjects after stimulation with phytohemagglutinin (PHA) (c). Results are mean $\pm$ SEM of seven independent samples. Flu, fluvastatin; MA, mevalonic acid; * P < 0.05 vs. Der f, PPD or PHA; ** P < 0.01 vs. Der f, PPD or PHA.

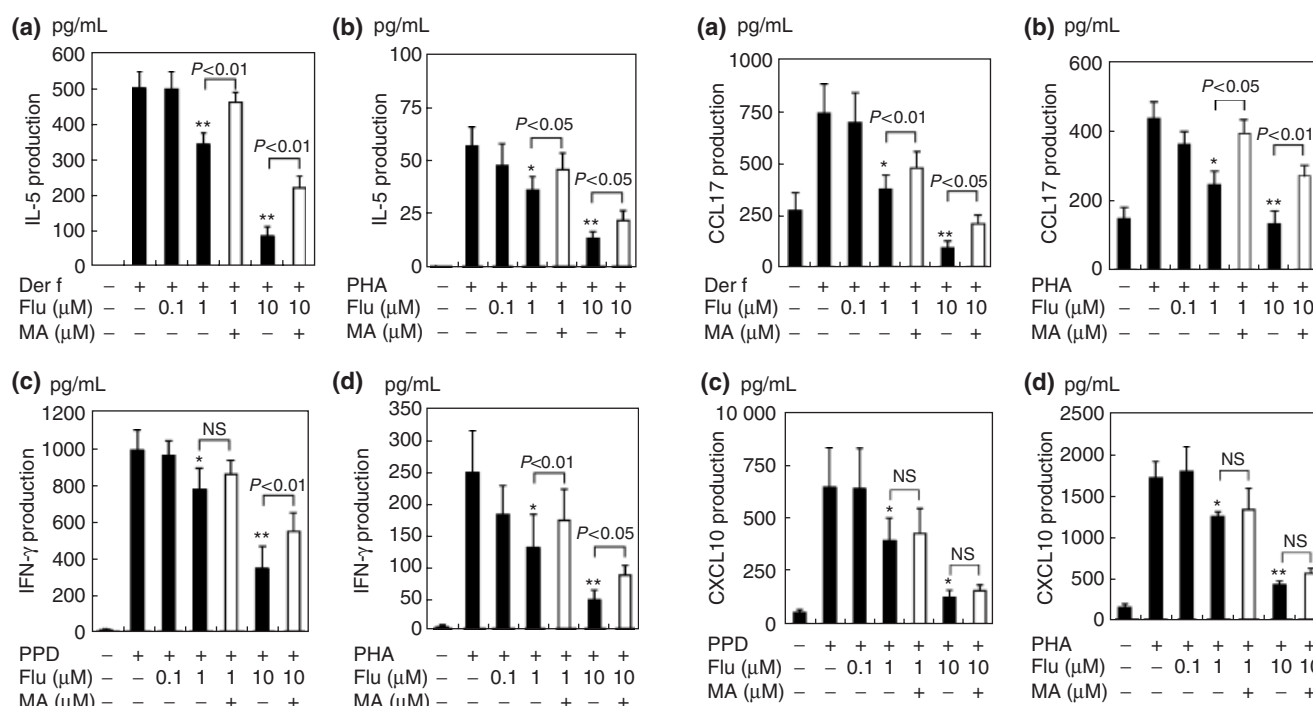


Fig. 3. Effect of fluvastatin on cytokine production. Effect of fluvastatin on cytokine production by peripheral blood mononuclear cells (PBMCs) from patients with allergic asthma after stimulation with Der f (a) and purified protein derivative (PPD) (c), or by PBMCs from healthy subjects after stimulation with phytohemagglutinin (PHA) (b and d). Results are mean $\pm$ SEM of seven independent samples. Flu, fluvastatin; MA, mevalonic acid; * P < 0.05 vs. Der f, PPD or PHA; ** P < 0.01 vs. Der f, PPD or PHA.

Fig. 4. Effect of fluvastatin on chemokine production. Effect of fluvastatin on chemokine production by peripheral blood mononuclear cells (PBMCs) from patients with allergic asthma after stimulation with Der f (a) and purified protein derivative (PPD) (c) or by PBMCs from healthy subjects after stimulation with phytohemagglutinin (PHA) (b and d). Data are mean $\pm$ SEM of seven independent samples. Flu, fluvastatin; MA, mevalonic acid; * P < 0.05 vs. Der f, PPD or PHA; ** P < 0.01 vs. Der f, PPD or PHA.

composed of Th2 cells. Fluvastatin inhibited the migration of both Th1 and Th2 cell lines in a dose-dependent manner (Figs 5a and b).

Fluvastatin has no effect on expression of CCR4 and CXCR3

Because we found that fluvastatin inhibited the migration of Th1 and Th2 cell lines, we next investigated whether

expression of chemokine receptors in these cell lines is decreased by fluvastatin. Expression of CCR4, the receptor of CCL17, did not change significantly before and after 3 days of stimulation with Der f ($6.8 \pm 0.7\%$ on day 0 and $4.7 \pm 0.9\%$ on day 3). Similarly, expression of CXCR3, the receptor of CXCL10, did not change before and after 3 days of stimulation with PPD ($6.9 \pm 0.8\%$ on day 0 and $5.1 \pm 2.0\%$ on day 3). Moreover, the presence of

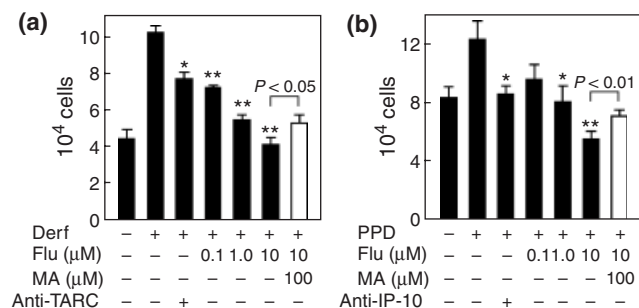


Fig. 5. Effect of fluvastatin on cell migration. Effect of fluvastatin on T helper (Th)2 (a) and Th1 (b) cell line migration. Results are mean $\pm$ SEM of seven independent samples. Flu, fluvastatin; MA, mevalonic acid; * $P < 0.05$ vs. Der f or purified protein derivative (PPD); ** $P < 0.01$ vs. Der f or PPD.

fluvastatin had no effect on the expression of CCR4 on CD4⁺T cells after stimulation with Der f ($4.7 \pm 0.9\%$ without fluvastatin and $4.9 \pm 0.8\%$ with 10 μ M fluvastatin) or CXCR3 on CD4⁺ T cells after stimulation with PPD ($5.1 \pm 2.0\%$ without fluvastatin and $5.0 \pm 1.6\%$ with 10 μ M fluvastatin).

Inhibition of steroid-insensitive T cells by fluvastatin

Because fluvastatin inhibited proliferative responses and production of cytokines and chemokines by PBMCs from patients with allergic asthma and healthy subjects, we also studied the effects of fluvastatin on steroid-insensitive PBMCs. Although the proliferative response of steroid-sensitive PBMCs from healthy subjects was inhibited by both dexamethasone and fluvastatin, the proliferative response of steroid-insensitive PBMCs from healthy subjects precultured with both IL-2 and IL-4 was decreased by dexamethasone ($P < 0.05$, Fig. 6a), but was inhibited by fluvastatin (Fig. 6b). This inhibitory effect of fluvastatin on steroid-insensitive PBMCs was partially reversed by the addition of mevalonic acid (Fig. 6b). Similar results were also obtained using PBMCs from patients with asthma (Flu 1 μ M: untreated PBMCs $-24.6 \pm 7.5\%$ vs. steroid-insensitive PBMCs $-25.1 \pm 5.4\%$, Flu 10 μ M: untreated PBMCs $-85.9 \pm 4.6\%$ vs. steroid-insensitive PBMCs $-80.2 \pm 6.7\%$).

Discussion

Statins have pleiotropic effects on immune cells [5, 6], decrease the incidence of major rejection, and increase long-term survival rates in patients undergoing pulmonary, cardiac, or renal transplantation [14–16]. Although statins have immunoregulatory effects, whether they affect Th1- and Th2-type responses and steroid-sensitive and steroid-insensitive T cells remains unclear. In the present study, we found that fluvastatin inhibited the proliferation of PBMCs stimulated with allergens and

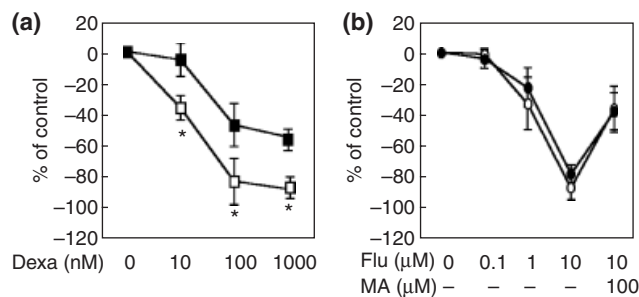


Fig. 6. Effect of fluvastatin on steroid-insensitive peripheral blood mononuclear cells (PBMCs) established from healthy subjects. Effects of fluvastatin (right) and dexamethasone (left) on proliferation of steroid-insensitive PBMCs after stimulation with phytohemagglutinin. Horizontal bars indicate the percent changes in proliferative response compared with control values. Open squares and circles: steroid-sensitive PBMCs; closed squares and circles: steroid-insensitive PBMCs. Results are mean $\pm$ SEM of seven independent samples. Flu, fluvastatin; MA, mevalonic acid; Dexa, dexamethasone; * $P < 0.05$ vs. control.

PHA and the production by these PBMCs of Th1- and Th2-type cytokines and chemokines. We also found that fluvastatin, but not dexamethasone, inhibited the proliferation of steroid-insensitive T cells.

We found that fluvastatin at concentrations of 1 μ M or greater inhibited the proliferation of PBMCs activated by both Th1- and Th2-type allergens and their production of IL-5 and IFN- γ . The well-documented inhibitory effects of statins in atherogenesis suggest that fluvastatin may have affected both T cells and monocytes [17]. Results of a previous study also suggest that statins inhibit T cell activation by decreasing the expression of MHC II on monocytes induced by IFN- γ [7]. However, fluvastatin does not affect the expression of MHC I and II on dendritic cells and B lymphocytes [7].

Although fluvastatin might affect the function of APCs, our present findings suggest that fluvastatin directly inhibits T cells, because their proliferative responses and production of cytokines and chemokines were significantly inhibited after stimulation with PHA, which directly activates T cells through CD2. In a murine model of collagen-induced arthritis, simvastatin inhibited the proliferation of anti-CD3 antibody-stimulated PBMCs and their production of IFN- γ [18]. Furthermore, fluvastatin inhibits the proliferative response of T cells to cross-linking of CD3 [9]. Therefore, fluvastatin has direct inhibitory effects on T cells.

We recently reported that fluvastatin (10 μ M) induces apoptosis in resting CD4⁺ T cells and activates CD4⁺ T cells with anti-CD3 antibodies. Activities of caspases -8, -9, and -3, cytochrome c release, and the expression of Bax/Bcl-2 ratio are increased in CD4⁺ T cells by treatment with fluvastatin (10 μ M). Therefore, fluvastatin at 10 μ M induces apoptosis in T cells [19]. In the present study, we found that apoptosis was not induced after treatment of PBMCs with 1 μ M fluvastatin. However,

proliferative responses and production of IL-5 and IFN- γ were significantly inhibited by this concentration of fluvastatin. These results suggest that the inhibitory effects on cytokine and chemokine production might simply reflect reduced proliferation or metabolic activity rather than an increase in the apoptosis of T cells.

Chemokines and their receptors participate in Th1- and Th2-mediated diseases [20]. The effect of statins on chemokines involved in allergic inflammation has not been definitively established. Statins down-regulate the chemokine network in patients with coronary artery disease and inhibit production of CCL2 by PBMCs stimulated with lipopolysaccharide and *Streptococcus hemolyticus* [21, 22]. Although fluvastatin-inhibited the production of CCL17 and CXCL10 in the present study, it had no effect on the expression of CCR4 or CXCR3.

Fluvastatin at concentrations greater than 1 μ M inhibited the migration of Th2 cell lines more strongly than did treatment with an anti-CCL17 antibody, suggesting that fluvastatin might inhibit other mediators that recruit Th2 cells, such as CCL22, which binds to the same receptor as CCL17. Moreover, our present results suggest that CCL17 is not the only chemokine that induces Th2 cell migration. Similarly, the inhibition of Th1 cell line migration by 10 μ M fluvastatin inhibited the migration of Th1 cell lines more strongly than did treatment with an anti-CXCL10 antibody, suggesting that other chemokines for Th1 cells might also be inhibited by fluvastatin.

The results of our study have shown that the mevalonate pathway plays several roles in cell proliferation and in cytokine (IL-5 and IFN- γ) and CCL17 production, because addition of mevalonic acid partially reversed the inhibitory effects of fluvastatin. Products of mevalonic acid are required for post-translational modification of small GTP-binding proteins essential for cellular processes [23]. However, CXCL10 production was not affected by mevalonic acid, a finding that suggests that the mechanism of CXCL10 production is independent of mevalonic acid or its intermediate products. The transcriptional regulation of CXCL10, which includes both calcineurin-dependent and calcineurin-independent pathways, is complex and remains poorly understood [24]. Although mevalonic acid reverses the statin-induced decrease in vascular cell adhesion molecule-1 expression on human endothelial cells, it does not reverse the decrease in CD40 expression [25]. Thus, production of cytokines and chemokines by PBMCs may or may not depend on the intermediate products of mevalonic acid.

In an *in vivo* murine model of allergic asthma, simvastatin demonstrated anti-inflammatory effects by suppressing Th2 cytokine secretion [11]. Our *in vitro* study has shown that 1 μ M fluvastatin decreases T cell proliferation, Th1 and Th2 cytokine and chemokine secretion, and cell line migration without affecting the viability of PBMCs. The peak serum concentration of fluvastatin after a single

60-mg oral dose is 1.76 μ M in healthy male subjects [26]. Therefore, such an oral dose might be sufficient to inhibit PBMCs in humans.

As the prevalence of asthma increases, so does the need for new or alternative therapeutic agents. Special attention should be paid to patients with severe asthma, in which airway inflammation persists despite treatment with oral corticosteroids [27]. Analysis of bronchoalveolar lavage fluid also indicates that the Th2 cytokines IL-4 and IL-5 are produced despite treatment with oral corticosteroids in steroid-insensitive asthma [28]. Furthermore, targeting both Th1 and Th2 responses seems appropriate, because both responses coexist in asthma [29]. We have reported that corticosteroids and lidocaine inhibit proliferative responses in steroid-sensitive T cells but not in steroid-insensitive T cells [30]. In the present study, we found that fluvastatin inhibits the proliferative responses of steroid-insensitive T cells induced by incubation with IL-2 and IL-4. To our knowledge, fluvastatin is the only agent, other than immunosuppressants such as cyclosporine, that inhibits the proliferative responses of steroid-insensitive T cells *in vitro*.

In conclusion, this study has demonstrated the immunomodulatory effects of fluvastatin in human T cells obtained from patients with asthma and from healthy subjects through effects on T cell activation and migration. In addition, fluvastatin has inhibitory effects on steroid-insensitive T cells. Therefore, statins might be used as potential therapeutic agents in severe asthma.

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